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TITLE: NOXA Loss as a Major Mechanism of Intrinsic Resistance to Targeted Therapies in Breast Cancer

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CONTRACTING ORGANIZATION: Virginia Commonwealth University, Richmond, VA

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<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> We continued to make significant and timely progress with a focus on ER+/MCL-1 inhibitor combinations and the histological studies. Our grant hypothesis was that MCL-1 inhibition can sensitize HER2 inhibitors in HER2 amplified breast cancer and ER inhibitors in ER+ breast cancer. Highlights of the past 12 months include further support of the use of ER inhibitors in combination with MCL-1 inhibitors and demonstrating a causal connection with NOXA in this sensitivity, consistent with our hypothesis. We observed numerically lower levels of NOXA and MCL-1 expression in post-treatment (chemo/endocrine) ER+/HER2- breast cancers compared to therapy-naïve tumors, as well as higher expression of NOXA in poorly differentiated compared to well/moderately differentiated ER+/HER2 breast cancers. Moreover, in univariate but not in multivariable analysis, NOXA-high and MCL1-low ER+/HER2- breast cancers were associated with shorter recurrence free survival. We are re-submitting the newest peer-review manuscript from this project, entitled "Adaptive resistance to ER inhibition is overcome by MCL-1 inhibition" in after receiving initial editorial feedback, and anticipate acceptance in the next 3 months.						
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## 1. Introduction

*HER2*-amplified breast cancers and Estrogen receptor (ER) positive breast cancers are susceptible to *HER2* inhibitors and ER inhibitors, respectively. However, treatment with these targeted agents elicit transient responses, and ways to sensitize these cancers further with the addition of rationally implemented targeted therapies continues to be the subject of intense research. In this grant, we have posited that low expression of the endogenous MCL-1 inhibitor, NOXA, in *HER2*-amplified breast cancers causes 1) resistance to *HER2* inhibitors through MCL-1 activity and 2) susceptible to combination therapy with MCL-1 inhibitors. The mechanism is through suppression of ER-mediated loss of NOXA transcription, which is mediated by miRNA4728, a coamplified gene with *HER2* in these cancers. In addition, by way of a overlapping mechanism, in ER+ breast cancers, treatment with ER inhibitors leads to loss of NOXA transcription. In both cases, addition of MCL-1 inhibitors –either MCL-1 BH3 mimetics or CDK inhibitors that block MCL-1 transcription-- can induce cell death.

**2. Keywords:** *MCL1*, targeted therapy, apoptosis, resistance, NOXA

## 3. Accomplishments

### Major Task 1

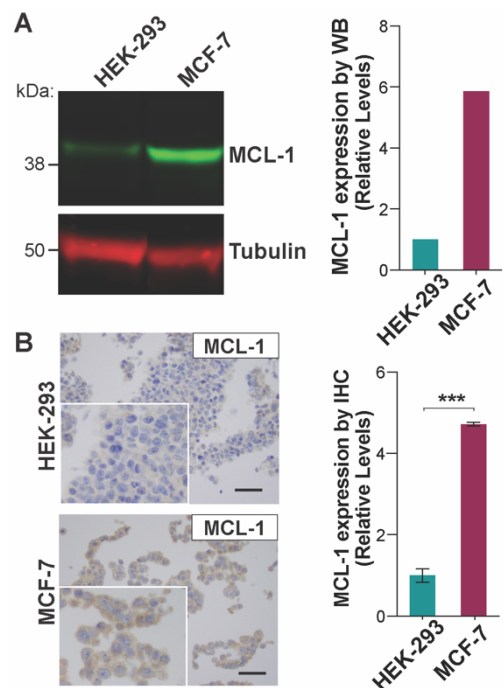
Characterize the miRNA4728/ER/NOXA axis in *HER2*-amplified breast cancers and its role in intrinsic resistance to *HER2*

Ongoing subtasks below:

Subtask 2: In collaboration with Dr. Edi Brogi, (Director of Breast Pathology, MSKCC), and Dr. Mikhail Dozmorov (Department of Biostatistics, VCU), we will evaluate 180 samples of clinically annotated *HER2*+ breast cancer specimens collected at MSKCC for *HER2* levels, NOXA levels, and MCL-1 levels by immunohistochemistry.

### Immunohistochemical Detection of MCL-1

To establish a robust IHC assay for the assessment of MCL-1 expression, we assessed the performance of different commercially available antibodies against MCL-1. Based on the high and low MCL-1 mRNA expression levels of in MCF-7 (normalized protein-coding transcripts per million, nTPM=160.3) and HEK293 cells (nTPM)=68.3), respectively (PMID: 31857451), formalin-fixed paraffin embedded (FFPE) cell pellets were used as controls. As an orthogonal validation, we conducted the assessment of MCL-1 protein expression in HEK293 and MCF-7 cell lysates by western blot (MCL1 antibody, clone D2W9E; #94294, Cell Signaling Technology, Danvers, MA). In agreement with the gene expression levels, we observed higher MCL-1 protein levels in MCF7 cells compared to HEK293 cells (Fig. 1A). Using these FFPE cell pellets, as well as a panel of 10 normal tissues, we proceeded to assess the suitability of various monoclonal antibodies for the immunohistochemical detection of MCL-1, as well as their optimization, including the modification of titration steps. The MCL-1 (clone RC13) antibody (Santa Cruz Biotechnology, Dallas, Texas; dilution 1:100) displayed expression in normal tissues compatible with the reported expression MCL-1 (PMID: 31857451), such as high expression in testis and low in lung (data not shown). Upon IHC analysis of the FFPE cell pellets with this clone, we observed statistically significantly higher MCL-1 protein expression levels in MCF-7 cells compared to HEK293 cells ( $P=2.9 \times 10^{-5}$ , student's t-test; Fig. 1B), in agreement with our observations by western blot. Taken together, we have established a robust assay for detection of MCL-1 by IHC, which was used for the assessment in tissues samples.

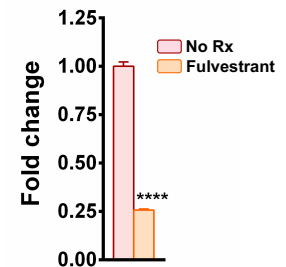


**Figure 1. Optimization of MCL-1 detection by immunohistochemistry.** (A) MCL-1 protein expression in HEK-293 and MCF-7 cells by western blot and (B) MCL-1 protein expression by IHC using the clone RC13 MCL-1 antibody and the optimized protocol.

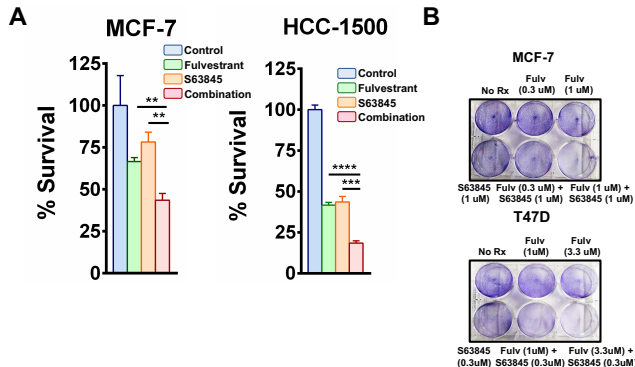
We have now obtained HPRO approval and MSK IRB approval for our plans for testing breast cancers using this assay. We have further searched among an initial cohort of over 600 samples; regrettably, however, the vast majority of tissue blocks related to the initial cohort have been exhausted. We subsequently searched for additional samples under our protocol and identified a cohort of over

400 samples and verified adequate remaining tumor in the majority of these. We have now begun cutting unstained slides of this and anticipate results in 4-6 months.

### PMAIP1 mRNA



**Figure 2. RNA expression of PMAIP1 (NOXA) is decreased following ER inhibitor therapy.** qPCR normalized to B-actin in the HCC-1500 ER+ breast cancer cells.



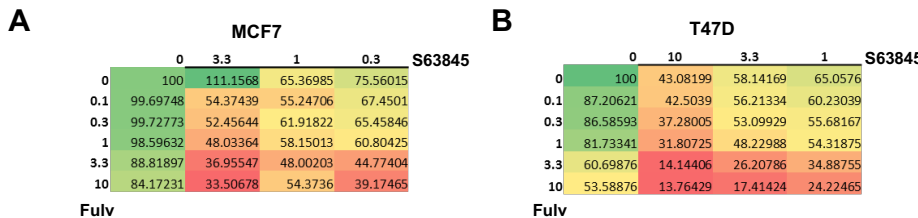
**Figure 3. Fulvestrant and S63845 combine to eliminate ER+ breast cancer cells.** (A) cell-titer glo assay (72h) following treatment with fulvestrant, S63845 or the combination. (B) Crystal violet medium-term assays (about 5-7d) evaluating fulvestrant, S63845 or the combination across the indicated doses.

### Major Task 2: Characterize the role of the ER-NOXA axis in response to anti-estrogens in ER+ positive breast cancer in vitro and in clinical specimens.

Subtask 1: In the Faber and Scaltriti (now Reis-Filho) laboratory, we will determine how the MCL-1 inhibitor S63845 is sensitizing to ER inhibitors

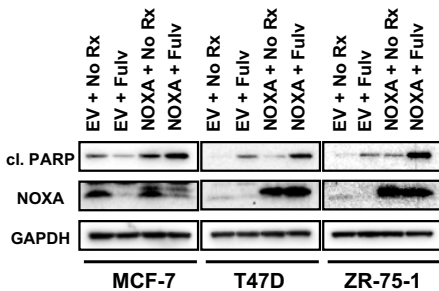
We have previously reported over the first two reports that the combination of ER inhibitor fulvestrant and MCL-1 inhibitor S63845 result in enhanced cell death which centers on the decrease of NOXA following ER inhibition with ER inhibitors.

We confirmed that this decrease of NOXA is at the RNA level, where it would be expected (Fig. 2). We demonstrate now further evidence in cell culture models that fulvestrant and S63845 have substantial activity in ER+ breast cancers (Fig. 3). We next asked using a dose-matrix whether there was synergistic activity



**Figure 4. Bliss sum synergies of ER inhibitor fulvestrant (fulv) plus MCL-1 inhibitor (S63845) across ER+ breast cancer models MCF7 and T47D.** Please note: bright orange and red is synergy, yellow to light orange is additive.

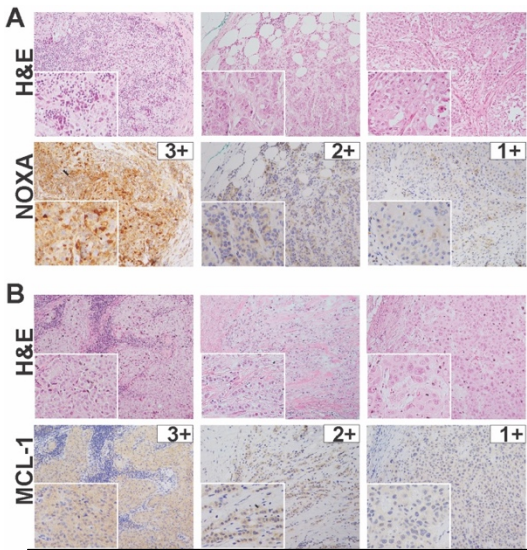
of the combination as determined by bliss-sum analysis. Indeed, we found synergy across a number of concentrations of both drugs (Fig. 4), confirming the exciting activity of this novel combination. Lastly, re-expression of NOXA is sufficient to sensitize to ER inhibition, demonstrating a causal relationship across ER+ breast cancer cell lines (Fig. 5).



**Figure 5. NOXA expression confers sensitivity to Fulvestrant.** Exogenous NOXA was expressed using lentiviral vectors in three ER+ breast cancer cell lines, and cleaved PARP, a measure of cell death, is demonstrated.

United Kingdom, dilution 1:2000) monoclonal antibodies for 30 minutes. The BOND Polymer Refine Detection kit was used as secondary reagent (Leica Biosystems, Wetzlar, Germany). Evaluation of MCL-1 and NOXA expression was conducted by a board-certified pathologist using the H-score, a semi-quantitative scoring

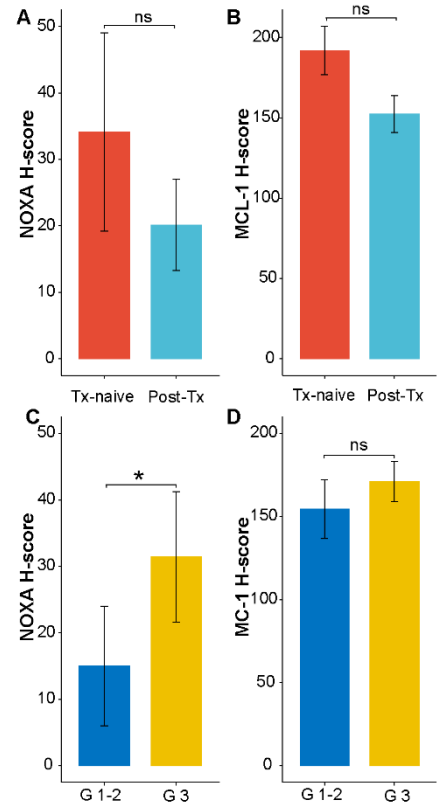
Subtask 2: In collaboration with the Reis-Filho team and Dr. Mikhail Dozmorov (Department of Biostatistics, VCU), we analyzed the expression NOXA and MCL-1 in 52 ER+/HER2- breast cancer samples, which included 17 (33%) and 35 (67%) treatment-naïve and post-treatment samples, respectively, and correlated it with patient outcomes. Immunohistochemical analysis was performed using a Bond-3 automated stainer platform (Leica Biosystems, Wetzlar, Germany). In brief, following antigen retrieval (ER2) for 30 minutes, tissue sections were incubated with the anti-MCL-1 (clone RC13; Santa Cruz Biotechnology, Dallas, Texas; dilution 1:100) or with anti-NOXA (clone 114C307; Abcam, Cambridge,



**Figure 6. NOXA and MCL-1 expression in ER+/HER2- breast cancer.** Representative H&E micrographs and micrographs of (A) NOXA and (B) MCL-1 protein expression as assessed by immunohistochemistry in ER+/HER2- breast cancers.

system, which captures the intensity and proportion of cells at each staining intensity level (Fig 6A-B), using the following formula: H score = [1x(%cells 1+) + 2x (%cells 2+) + 3x (%cells 3+)].

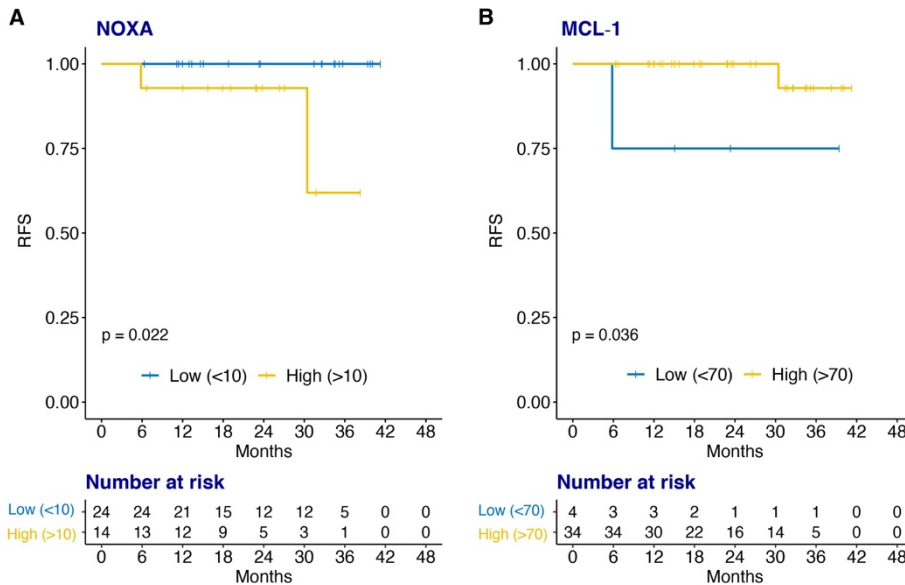
Although not statistically significant, we observed a numerically lower NOXA H-score (34.1 vs 20.1,  $P=0.8$ ; Fig. 7A) following treatment, consistent with the hypothesis. We are further analyzing these data to determine if pre and post treatment samples from the same patient will yield consistently decreased staining scores, as we would predict. In addition, MCL-1 expression in post-treatment samples ( $n=35$ ) compared to treatment-naïve ( $n=17$ ) ER+/HER2- breast cancer samples (H-score, 191.7 vs 152.2,  $P=0.06$ ; Fig. 7B) was lower. Notably, we observed a higher expression of NOXA in poorly differentiated tumors (histologic grade 3) compared to well/moderately differentiated ER+/HER2- breast cancers (31.4 vs 15.0,  $P=0.04$ ; Fig 7C). No statistically significant difference was observed for MCL-1 expression according to grade (Fig 7D).



grade 3) compared to well/moderately differentiated ER+/HER2- breast cancers (31.4 vs 15.0,  $P=0.04$ ; Fig 7C). No statistically significant difference was observed for MCL-1 expression according to grade (Fig 7D).

The maximally selected rank statistics method identified a value of 10 and 70 H-index as the cut-off levels of NOXA and MCL1, respectively, which were associated with the most significant differences in relapse-free survival (RFS) Kaplan–Meier curves. Based on these cut-offs, 24 (63%) breast cancers were classified as NOXA-low (H-index<10), and 14 (37%) breast cancers as NOXA1-high (H-index>10),

**Fig 7. NOXA and MCL-1 expression by sample type and pathologic characteristics.** (A-B) Expression of NOXA (A) and MCL1 (B) in therapy-naïve vs post-treatment primary ER+/HER2- breast cancers. (C-D) NOXA (C) and MCL1 (D) expression in primary ER+/HER2- breast cancers according to histologic grade. \*,  $P<0.05$ ; ns, not significant; Mann-Whitney  $U$  test



**Fig 8. Relapse-free survival (RFS) according to NOXA and MCL-1 expression in primary ER+/HER2- breast cancer.** Kaplan-Meier curves showing RFS according to NOXA (A) and MCL1 (B) expression, using H-score cut-offs identified through maximally selected rank statistics method.  $P$  values were determined by log-rank test.

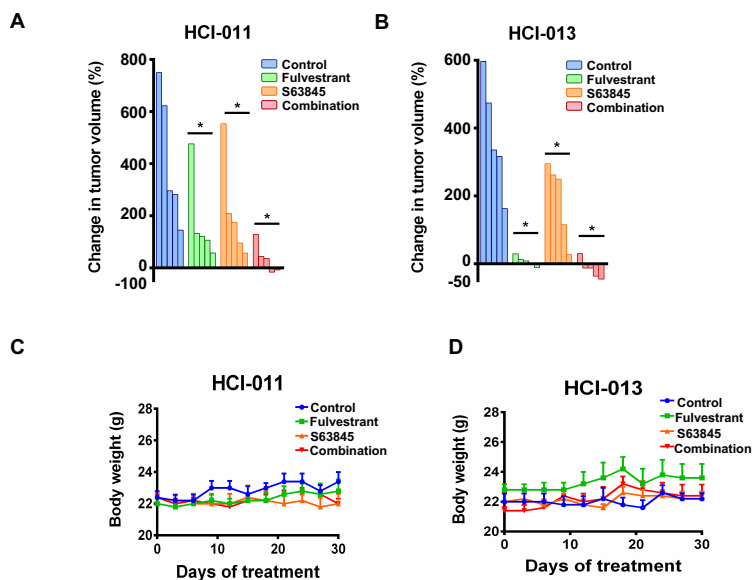
whereas 4 (11%) breast cancers were classified as MCL-1-low (H-index<70) and 34 (89%) patients as MCL-1-high (H-index>70). We observed that patients with NOXA-high ER+/HER2- breast cancer had a significantly lower relapse-free survival (RFS) compared to patients with NOXA-low tumors ( $P=0.02$ ; log rank test; Fig. 8A), whereas low MCL-1 expression was associated with a longer RFS ( $P=0.03$ , log rank test; Fig. 8B). These differences, however, were found not to be

statistically significant in multivariable Cox proportional hazard models including age, stage and grade. The

analysis of NOXA1 and MCL-1 expression in a separate cohort of ER+ breast cancers with OncotypeDx scores, RFS and OS survival available is currently being performed, and will help clarify whether these observations can be validated in a subsequent cohort.

### Major Task3: Assess the efficacy of dual HER2 and MCL-1 inhibition in diverse *HER2* amplified breast PDX models and dual ER and MCL-1 inhibition in diverse ER+ breast PDX models.

We have now added analyses of PD in these studies as well as increased statistical rigor to determine the efficacy of the combination in two ER+ breast cancer PDX models. In addition, we have evaluated toxicity. As demonstrated below (Fig. 9), we see statistical differences in efficacy, without statistically different weight differences, demonstrating a potential therapeutic window of the combination.



**Figure 9. Efficacy and tolerability of fulvestrant/S63845 in ER+ breast cancer.** (A and B) ER+ breast cancer PDX models treated with fulvestrant, S63845 or the combination for ~30d. Tumor volume change is shown. Student t test's demonstrating differences between cohorts (asterisk represents p value <0.05) comparing control to single-agents or, for the combination, to the single-agents. (C and D) Mouse weights per cohort are shown. No statistical differences demonstrated throughout the study between the different cohorts.

#### 4. Impact

In all, our data now demonstrate convincingly that the addition of an MCL-1 inhibitor, which a number are in clinical testing, sensitizes both HER2 amplified breast cancers and ER+ breast cancers to HER2 inhibitors and ER inhibitors, respectively. When ER is downregulated either as a result of miRNA4728 amplification in HER2 amplified breast cancers, or ER+ breast cancers following ER inhibitor, NOXA is loss and MCL-1 becomes unhinged and interferes with a cell death response. The next step is to move these findings into clinical testing. We are focusing on the ER+/MCL-1 inhibitor combinations first and have already had discussions with AstraZeneca about these concepts. We are hopeful to move this forward in the next 12 months.

#### 5. Changes/Problems

n/a

#### 6. Products

### Adaptive resistance to ER inhibition is overcome by targeting MCL-1

Konstantinos V. Floros<sup>a</sup>, Sheeba Jacob<sup>a</sup>, Bin Hu<sup>b</sup>, Madhavi Puchalapalli<sup>b</sup>, Mohammad A. Alzubi<sup>b,c</sup>, Sosipatros A. Boikos<sup>d</sup>, Edi Brogi<sup>e</sup>, Sarat Chandarlapaty<sup>e</sup>, Jennifer E. Koblinski<sup>b</sup>, J. Chuck Harrell<sup>b,c,f</sup>, Maurizio Scaltriti<sup>e,g,h</sup>, and Anthony C. Faber<sup>a</sup>

#### 7. Participants & Other Collaborating Organizations

The SOW has been faithfully followed for the contributions of VCU and MSKCC

The following individuals have worked on the grant at VCU:

- 1) Name: Anthony Faber  
Project Role: Lead PI  
Nearest person month worked: 2  
Dr. Faber oversees the everyday experimentation in the laboratory related to the proposal
- 2) Name: Sheeba Jacobs  
Project role: Postdoctoral Fellow

Nearest person month worked: 9

Dr. Jacobs participates in all aims at VCU as a scientist in the laboratory

3) Jennifer Ramachandran (Koblinski)

Project role: co-I

Nearest person month worked: 1

Dr. Ramachandran assists in all mouse-related work and pathology at VCU

4) Mikhail Dozmorov

Project role: co-I

Nearest person month worked: 1

Dr. Dozmorov assists in all statistical matters for this grant

The following individuals have worked on the grant at MSK:

1) Jorge Reis-Filho

Project Role: Partnering PI

Nearest person month worked: 2

Dr. Reis-Filho oversees all day-to-day experimentation in the laboratory related to this proposal and oversees the design and data analysis.

Funding Support: This award (W81XWH-18-1-0562)

Sarat Chandarlapaty

Project Role: Co-Investigator

Nearest person month worked: 1

Dr. Chandarlapaty directs the efforts in his laboratory on developing model systems, collects human samples from breast cancer patients treated at MSKCC and assesses the benefit of different therapeutic strategies.

Funding Support: This award (W81XWH-18-1-0562)

Edi Brogi

Project Role: Co-Investigator

Nearest person month worked: 1

Dr. Brogi analyzes tissue samples from breast cancer patients undergoing treatment and evaluates the purity of cancer tissues and performs immunohistochemistry assays.

Funding Support: This award (W81XWH-18-1-0562)

Yanyan Cai

Project Role: Research Scholar

Nearest person month worked: 4

Dr. Cai, a postdoctoral scholar, leads all lab experiments, coordinates with genomics core and was responsible for animal work.

Funding Support: This award (W81XWH-18-1-0562)

Shirin Issa Bhaloo

Project Role: Research Associate

Nearest person month worked: 3

Dr. Issa Bhaloo is a Research Associate in the Reis-Filho Lab who assists with all aspects of this proposal, including tissue culture, cloning, biochemical assays, maintenance of patient-derived models needed for in vivo studies and sample preparation for sequencing.

Funding Support: This award (W81XWH-18-1-0562)

Mahsa Vahdatinia

Project Role: Research Associate

Nearest person month worked: 3

Dr. Issa Vahdatinia is a Research Associate in the Reis-Filho Lab who assists with all aspects of this proposal, including tissue culture, cloning, biochemical assays, maintenance of patient-derived models needed for in vivo studies and sample preparation for sequencing.

Funding Support: This award (W81XWH-18-1-0562)

**8. *Special Reporting and Requirements***

N/A