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PRINCIPAL INVESTIGATOR: Dr. MeghaShyam Kavuri, PhD

CONTRACTING ORGANIZATION: Baylor College of Medicine

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal investigates the role of aberrations (overexpression and somatic mutations) in the collagen receptor DDR1 (Discoidin Domain Receptor 1) in driving endocrine resistance, metastasis, and poor prognosis in estrogen receptor positive breast cancer.

15. SUBJECT TERMS

DDR1 aberrations (amplification and mutations), endocrine treatment resistance, oncogenic signaling, Tumor growth, multi-organ metastasis, ER+ breast cancer.

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1. INTRODUCTION

Despite the success of endocrine therapies that block estrogen receptor (ER) function in ER+ breast cancer, one-third of women will develop resistance. The causes of endocrine resistance in ER+ breast cancer are poorly understood. To identify the genes that are overexpressed in ER+ breast cancer and are associated with relapse and with poor outcome, we interrogated two independent data sets of ER+ breast cancers and found 20% overexpression of the Discoidin domain receptor (DDR1), strongly correlated with increased risk of metastasis and death. Our proposed work will identify associated molecular factors by using cell lines, animal models, and patient core biopsies. Additionally, we investigated the genes that can be found mutated in breast cancer cells as compared to normal cells, by sequencing over 625 breast tumor DNAs from patients who were treated by standard-of-care hormone therapy. They were followed for over ten years, and we then identified mutations in the DDR1 gene as the mutations most strongly associated with increased risk of cancer death out of all the genes examined in our study. Our preliminary data thus strongly suggest that DDR1 gene mutations induce resistance to standard-of-care endocrine therapy and increase cell migration, perhaps explaining why mutations in DDR1 were so lethal in our sequenced patient cohort — almost every patient with a DDR1 mutation had died. There are now no drugs that specifically target the lethal DDR1 aberrations, so our proposed work will also generate novel functional DDR1-specific monoclonal antibodies. Additionally, we aim to reposition drugs that are in common clinical use in other settings to target downstream DDR1 mutant induced cell growth. Finally, this study will

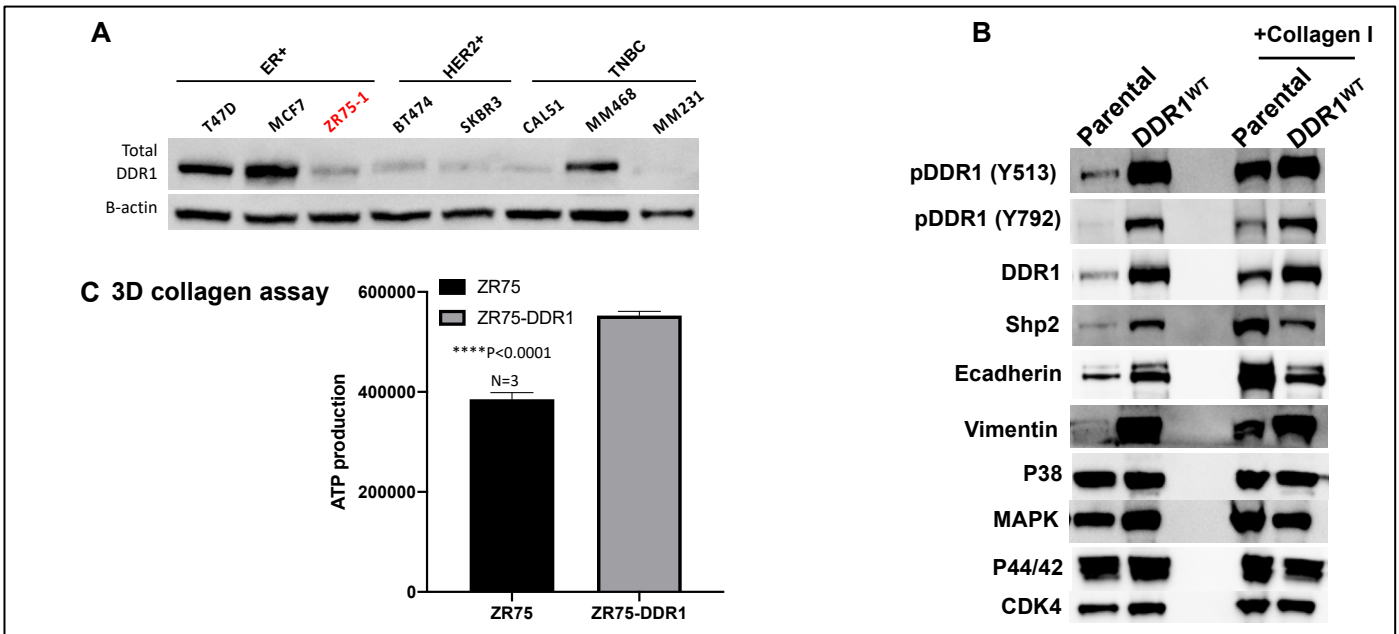

Figure 1. Endogenous expression of DDR1 in breast cancer cell lines. DDR1 overexpression leads to increased cell growth and oncogenic signaling. (A). ER+, HER2+ve, and TNBC cell lines were harvested, and protein lysates were subjected to immunoblot analysis for total DDR1 endogenous expression. (B). ZR-75 parental cells or cells expressing DDR1 WT were grown in low estrogen conditions in the presence or absence of collagen I treatment for 24 hrs at 1ug/ml and protein lysates were harvested. DDR1 downstream signaling proteins were assessed by immunoblot analysis. (C). ZR-75 parental cells or cells expressing DDR1 WT were grown in low-estrogen conditions. The cells were grown in collagen coated plates for 6 days and assayed for cell growth using Cell Titer GLO. Data is representative of three independent experiments and values are expressed as mean ±SEM. **** $p < 0.0001$.

investigate the prevalence of DDR1 aberrations (amplification and mutations) in metastatic ER+ breast cancer, who progressed upon standard-of-care endocrine therapy using metastatic breast cancer data sets.

2. KEYWORDS

DDR1 aberrations (amplification and mutations), endocrine treatment resistance, oncogenic signaling, Tumor growth, multi-organ metastasis, ER+ breast cancer.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Major goals of the project were to (a) Determine the effect of DDR1 overexpression on metastasis in ER+ breast cancer. (b). Determine the functional impact of DDR1 somatic mutations on tumor growth in ER+ models. (c). Establish a strategy to effectively treat DDR1 overexpressing and mutant breast cancer.

What was accomplished under these goals?

Major Task. 1 and 2/ Sub task. 1.

(1). DDR1 overexpression is highly correlated with metastasis and poor patient outcome in ER+ breast cancer, and ER+ ILC and IDC mBC patients harboring DDR1 amplification induce bone metastasis and early relapse. In this study we investigated the impact of DDR1 overexpression at the RNA level on metastasis in different

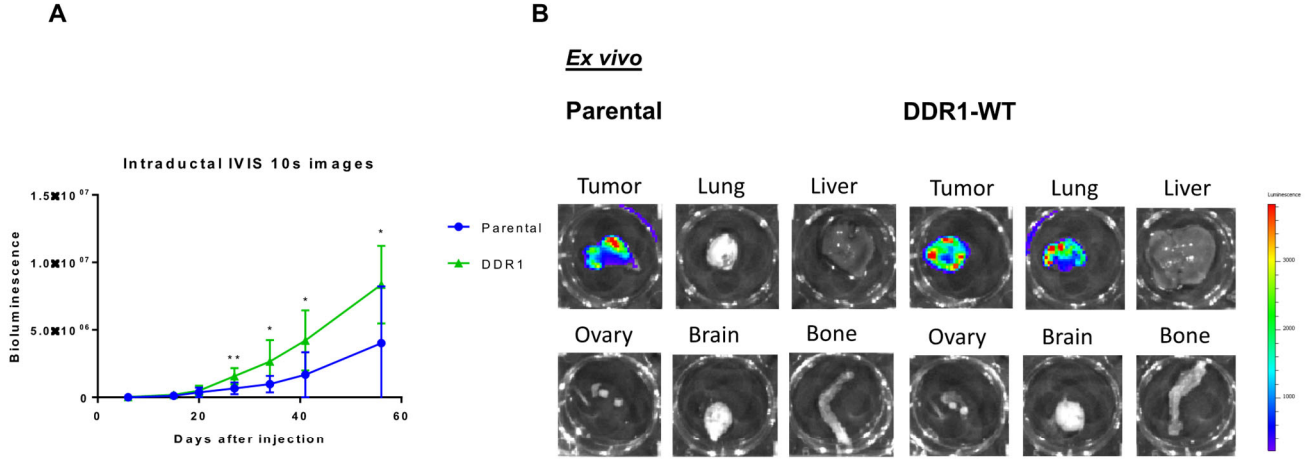

Figure 2. DDR1 WT overexpression promote orthotopic tumor growth and lung metastasis. (A). ZR-75 luciferase expressing parental cells or cells expressing DDR1 WT were engrafted into mice mammary ducts and generate Mammary INtraDuctal (MIND) xenografts). Tumor growth was monitored by bioluminescence imaging (BLI). Each data point represents the mean tumor volume in mm^3 ± SEM, n=3-5. *p=0.04. (B). Representative bioluminescence ex vivo images of parental or DDR1 WT mice.

publicly available data sets (Kao et al, 2011 (A) and Gyorffy et al, 2010) and found that DDR1 expression was increased in high grade and advanced stage ER+ tumors compared to low grade and early-stage tumors (Fig. 3A). Additionally, we show that overexpression is correlated with poor survival (Fig. 3B). In order to study the prevalence of DDR1 amplification in ER+ IDC and ILC mBC, we searched ER+ metastatic breast cancer studies and found that 12.5% of invasive ductal ER+ mBC and 28% of invasive lobular ER+ mBC had amplification of a collagen-binding receptor, Discoidin domain receptor (DDR1), as compared to ER+ primary breast cancers (Fig. 3C). Additionally, ER+ patients with DDR1 amplification showed bone metastasis, resulting in frequent early metastatic relapses and death (Fig. 3D). These findings suggest that DDR1 amplification is a novel therapeutic target in a subset of ER+ IDC and ILC mBC.

(2). Investigate the effect of DDR1 overexpression on oncogenic signaling, *In vitro* cell growth, tumor growth, and metastasis. To investigate the endogenous expression of DDR1 across all subtypes of breast cancer cell lines, we harvested ER+ cells (MCF7, T47D, and ZR-75), HER2+ ve cells (BT474 and SKBR3), and TNBC cells (CAL51, MDA-MB-468 and MDA-MB-231) protein lysates and subjected to immunoblot analysis and total DDR1 endogenous protein expression was analyzed. We show that DDR1 endogenous protein expression is relatively low in ZR-75 cells as compared to MCF7 or T47D cells (Fig. 1A). Therefore, we choose ZR-75 cells for our subsequent

ectopic expression of DDR1 WT (Aim. 1) and DDR1 mutations (Aim. 2 or Aim. 3) to investigate the effect on oncogenic signaling, therapeutic response, tumor growth, and metastasis.

(a). Effect of DDR1 overexpression on oncogenic signaling and *in vitro* cell growth. To investigate the effect of DDR1 WT overexpression, we ectopically overexpressed DDR1 WT in ZR-75 cells and analyzed the DDR1 oncogenic signaling. We show that increased pDDR1 (Y513 and Y792), total Shp2, E-cadherin, and vimentin in DDR1 overexpressed cells as compared to parental cells in the presence or absence of collagen I treatment (Fig. 1B). Next, we tested the effect of DDR1 overexpression on *in vitro* cell growth and we show that DDR1 WT overexpression induced significant increased cell growth as compared to parental cells (Fig. 1C), suggesting that DDR1 WT overexpression alone can induce increased oncogenic signaling and *in vitro* cell growth.

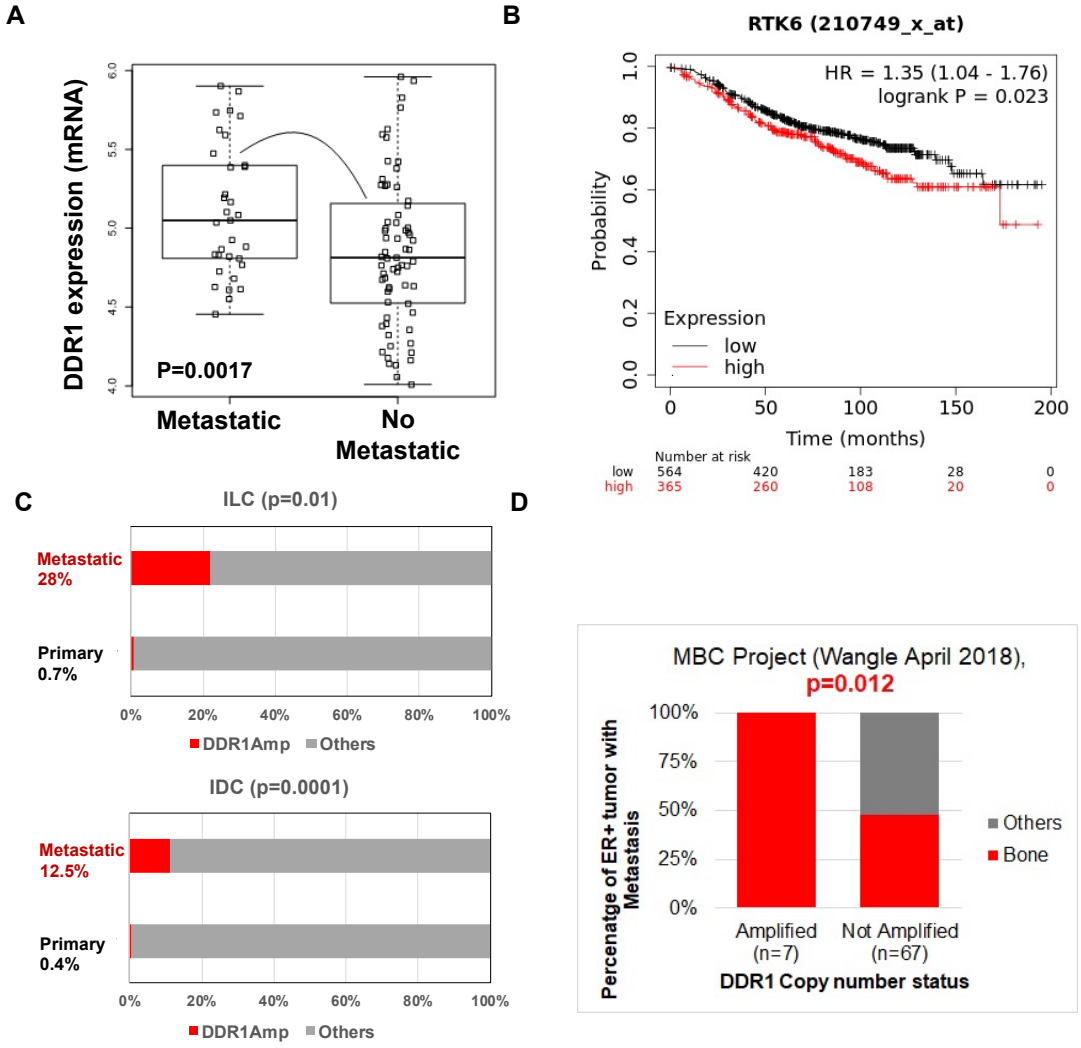

Figure 3. DDR1 overexpression is highly correlated with metastasis and poor patient outcome. ER+ invasive lobular breast carcinoma (ILC) and ER+ invasive ductal breast carcinoma (IDC) metastatic breast cancers (mBCs) harboring DDR1 amplification induce bone metastasis. Two independent sequencing data sets, Kao et al, 2011 (A) and Gyorffy et al, 2010 (B) of ER+ breast cancer patients' RNA sequencing data (A) and Affymetrix based microarrays and their overall survival information upon endocrine therapy were analyzed using KM-plotter. **Hazard ratio** (HR) = 1.35, p = 0.023 (B). (C). DDR1 amplification of primary and metastatic ER+ invasive lobular breast carcinoma (ILC) and invasive ductal breast carcinoma (IDC) copy number variation (CNV) gain data (ER+ mBC of the Wagle data set and the ER+ primary Metabric data set[2]) were analyzed. (D). CNV gain of metastatic DDR1 amplified ER+ patients and their metastatic sites were analyzed using Wagle ER+ mBC patient cohort.

(b). Effect of DDR1 overexpression on tumor growth and metastasis. To test the effect of DDR1WT overexpression on tumor growth and metastasis, we engrafted luciferase expressing ZR-75 parental cells or cells ectopically expressing DDR1 WT into mice mammary ducts using MIND (Mouse INtraDuctal) xenografts. The tumors were grown in the absence of estrogen supplementation and monitored intraductal tumor growth by bioluminescence imaging (BLI). The DDR1 WT cells bearing mice showed increased tumor growth as compared to parental cells bearing mice (Fig. 2A). Further, we harvested primary tumors and corresponding lungs, ovaries, liver, brain, and bones and performed BL imaging. We show that DDR1 WT bearing primary tumors and corresponding lung metastases are positive for BL signal (Fig. 2B), suggesting that DDR1 WT overexpression may promote tumor growth and

metastasis.

Conclusion of Major Task. 1 and 2/ Sub task. 1. Completed

Major Task. 1 and sub task. 2

(1). Proteomic screens of naturally harboring ER+ DDR1 overexpressed patient derived xenografts (PDXs). To search naturally occurring ER+ DDR1 overexpressed (single copy gain) patient derived xenografts (PDXs), we performed proteomic analysis of 55 ER+ Baylor College of Medicine (BCM) PDX models and identified 4 ER+ PDX models (BCM 15034, 15057, 15100, 5097) and harboring high DDR1 protein expression by Immunohistochemistry (IHC) (Fig. 10A) and immunoblot analysis (Fig. 4A). In order to sstudy the kinome reprogramming in response to DDR1 overexpression, we harvested tumors of DDR1 overexpressed PDXs (BCM 15034, 15057, 15100) performed proteomic analysis. The DDR1 overexpressed PDXs showed increased phospho and total protein expression of top 10 novel candidates (Fig. 14). Further, we tested the effect of DDR1 overexpression on DDR1 signaling axis. We show that increased pDDR1, pStat3, pSrc and pRb, cyclin D1 activation by immunoblot analysis (Fig. 4A), suggest that DDR1 overexpression induce constitutive oncogenic signaling and may be critical for DDR1 overexpression induced tumor growth and metastasis. In the proposed sub task 2, we identified DDR1 overexpressed driven novel kinase candidates using naturally occurring ER+ patient derived xenografts (PDXs) harboring DDR1 overexpression by proteomic profiling (Fig. 14). Our future studies will validate the increased expression of top 10 novel candidates using DDR1 overexpressed PDXs (BCM 15034, 15057, 15100) by immunoblot analysis and immunohistochemistry (IHC).

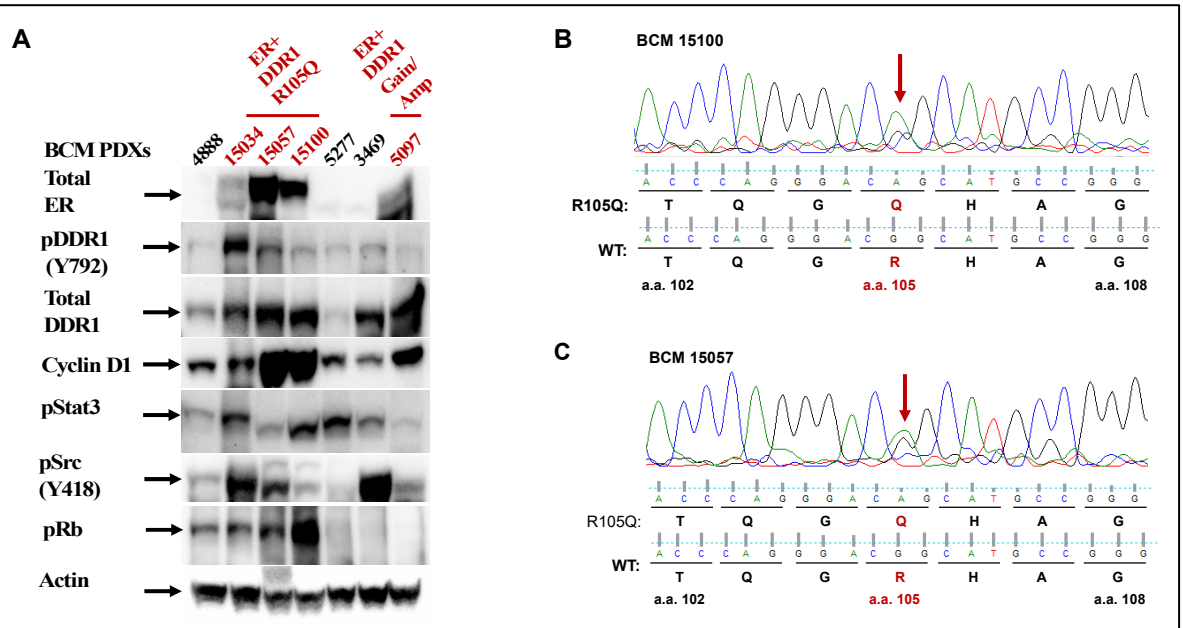

Figure 4. ER+ DDR1 overexpression (BCM 4888), DDR1 single copy gain (BCM 5097) and R105Q PDX models (BCM 15034, 15057 and 15100) signaling and R105Q validation using Sanger sequencing. Indicated PDX tumor lysates were subjected to immunoblot analysis, and DDR1 autophosphorylation and downstream signaling proteins were measured (A). BCM 4888 harbors DDR1 overexpression; BCM 5097 harbors a single copy gain of DDR1; BCM 15034, 15057 and 15100 harbor R105Q; BCM 5277 is DDR1 WT; and BCM 3469 harboring a single copy gain of DDR1 TNBC PDXs served as control. (B-C). Sanger sequencing of ER+ BCM 15057 and 15100 tumors. The arrows identify the DDR1 R105Q mutation.

Conclusion of Major Task. 1 / Sub task. 2. Completed

Major Task. 3 and sub task. 1. Tumor growth and metastasis.

(1). DDR1 mutations induce tumor growth and metastasis using the intraductal route. To study the effect of DDR1 mutations on tumor growth in their natural microenvironment, we injected 80,000 ZR-75-luciferase parental cells or cells expressing DDR1 WT, T102S, and R686W, engrafted into mammary fat pads by the intraductal route. They were grown in the absence of estrogen supplementation and monitored for tumor growth using IVIS imaging. The T102S and R686W cells showed increased tumor growth as compared to parental or WT cells in the absence of

estrogen (Fig. 8). These findings suggest that DDR1 T102S and R686W promote intraductal tumor growth in an

A

Sample id	DDR1 mutation	Evolution status	MET site location	ER	PR	HER2	Exon location	ED therapy
H_KU-444-1226776	R93Q	NA	NA	+	-	-	Exon. 3	Yes
MBC-114	T102S	Acquired	NA	+	+	-	Exon. 3	NA
H_LV-3245-1316073	R105Q	NA	NA	+	-	-	Exon. 3	Yes
TCGA-A8-A07U-01	E618Q	NA	NA	-	+	-	Exon. 12	NA
H_LV-3705-1316159	L626V	NA	NA	+	-	-	Exon. 13	Yes
MBC-372	R686W	Acquired	Bone	+	+	-	Exon. 14	Yes
MBC-W4F	A783S	Acquired	Lymph node	+	-	-	Exon. 15	NA
H_KU-1153-1153	R770W	NA	NA	+	-	-	Exon. 15	Yes

B

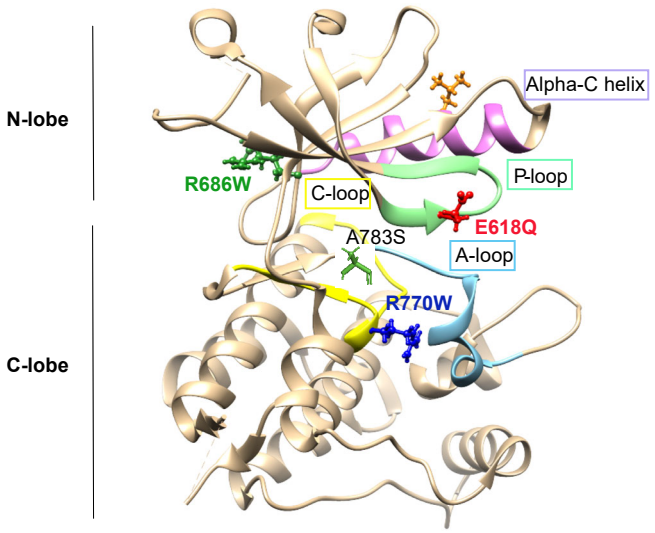

Figure 5. DDR1 mutations identified in ER+ breast cancer and their protein structure visualization. DDR1 mutations were selected from multiple ER+ sequencing studies (Table 1) based on their location in the indicated functional exons. Metastatic relapse site information on ER+ DDR1 somatic mutant patients and the DDR1 mutant patient's endocrine therapy (ED) treatment history is indicated (A). (B). DDR1 kinase domain structure showing the location of somatic mutation.

estrogen-independent manner.

(2). DDR1 mutations induce organ specific metastasis using the tail vein route. In order to study the metastatic potential of DDR1 mutations, we injected 1x10^6 ZR-75 cells expressing luciferase DDR1 WT or R105Q, and T102S, into immune-compromised mice by the tail vein route in the absence of estrogen supplementation. We monitored secondary organ metastasis using IVIS imaging, and 9 weeks after inoculation, when mice became moribund, they were euthanized by cervical dislocation. The lungs, liver, ovaries, fallopian tubes, brain, bones, and fat tissue were collected, and ex vivo imaged using IVIS immediately. Additionally, harvested organs were fixed in

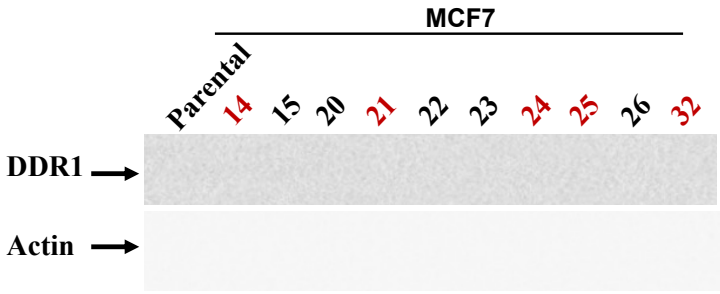

Figure 6. Generating MCF7 DDR1 CRISPR knockout cells. The protein lysates were harvested and subjected to western blot analysis. The total DDR1 protein expression was validated.

formalin and subjected to H&E and IHC staining. We observed increased secondary organ metastatic spread as indicated by bioluminescence signals. Our ex vivo IVIS imaging analysis revealed that parental, DDR1 WT, and all mutants developed lung lesions as indicated by bioluminescence signals (Fig. 7A-B). One out of 4 DDR1 WT mice

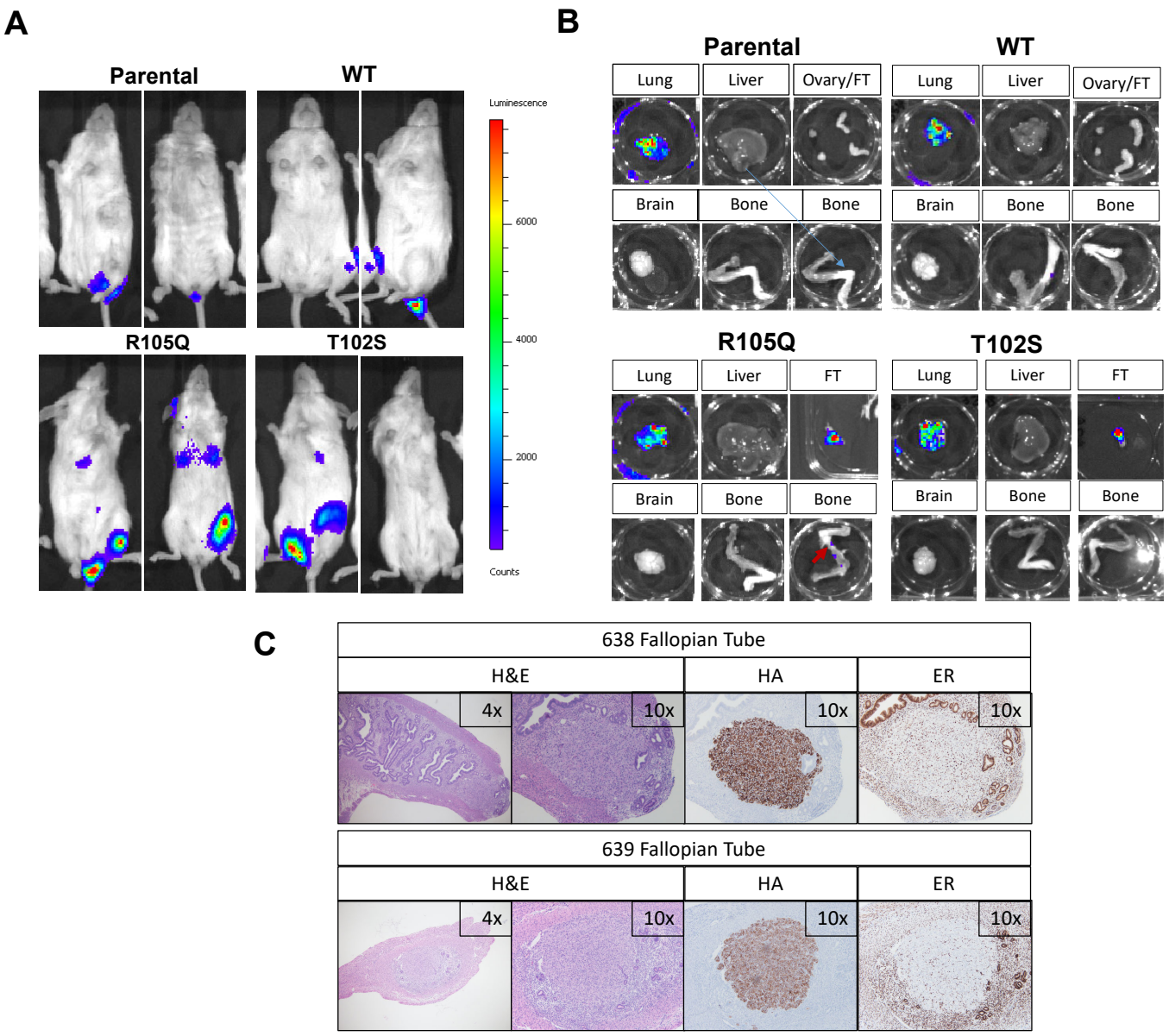

Figure 7. DDR1 mutations induce metastasis. (A-B). 1x10^6 ZR-75 parental cells expressing luciferase as well as WT or indicated mutants were injected by the tail vein route and monitored for metastatic spread. After 9 weeks mice were sacrificed and bioluminescence images were taken. (n=4 mice/arm). (B). Mice from A were sacrificed and all indicated organs were harvested. *Ex vivo* bioluminescent images of lungs, liver, bone, brain, and FT-fallopian tubes were taken. The R105Q bioluminescence image (BL) signal of bone micro metastasis is indicated by an arrow. (C). The two images of DDR1-T102S-HA are representative of 4 mice per each arm. Sections from (B) were fixed in formalin, and hematoxylin and eosin (H&E) staining was performed. Immunohistochemistry (IHC) for HA and ER antibodies was performed for two independent DDR1-T102S sections (638 and 639).

developed fallopian tube lesions. Additionally, R105Q mice developed bone lesions (2/4 mice) and fallopian tube lesions (1/4 mice); T102S mice developed fallopian tube lesions (1/4 mice) (Fig. 7B). Further, our H&E and IHC staining of T102S fallopian tube sections confirmed macro and micro secondary metastases evident by the presence of T102S cells detected using HA and ER antibodies (Fig. 7C). These results revealed that DDR1 mutations induce

bone and fallopian tube secondary metastasis in ER+ breast cancer.

Conclusion. Completed

Major Task. 3 and sub task. 2

(1). To test if DDR1 mutations induce SRC1 and CDK4/6 axis using naturally occurring ER+ DDR1 mutant PDX models, we harvested tumors of 3 ER+ DDR1 R105Q PDXs (BCM 15034, 15057, and 15100). The protein lysates were subjected to immunoblot analysis and analyzed pDDR1, pSRC and downstream substrates of CDK4/6, cyclin D1 and pRb. We show that BCM 15057 and 15100 that harbor both DDR1 R105Q and DDR1 overexpression induce constitutive DDR1 autophosphorylation, phospho Src, and phospho Shp2 as compared to BCM 4888 and 3469. Additionally, we show that DDR1 endogenous expression in BCM 4888, 15057, and 15100 is similar to BCM 3469, which is a TNBC DDR1-amplified model. Moreover, we confirmed the ER+ expression in BCM 15057 and 15100 (Fig. 4A). Next, to test if DDR1 mutations alone induced SRC1 and CDK4/6 axis is critical for tumor growth and metastasis we generated MCF7 CRISPR-knock-In (KI) DDR1 mutant cell lines (DDR1 R105Q, T102S, R686W, and A783S) and sequence verified (Fig. 13A-D). Our future work will test if acquired resistance DDR1 mutations alone induced SRC1 and CDk4/6 axis causal role on tumor growth using CRISPR-KI MCF7 DDR1 mutant cells.

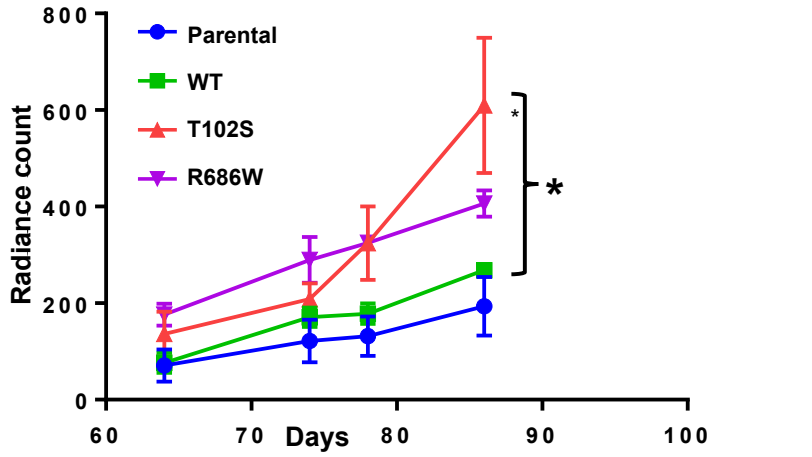

Figure 8. DDR1 T102S and R686W promote tumor growth. 80,000 ZR-75 cells expressing the indicated DDR1 variant were engrafted into female NSG mice in the absence of estrogen supplementation. Intraductal tumor growth was monitored based on bioluminescence imaging. (WT *versus* T102S * p = 0.02).

Conclusion. Partially completed

Major Task. 4

(1). To identify druggable candidates in response to DDR1 mutations we generated physiologically relevant 4 CRISPR-KI MCF7 DDR mutant (R105Q, T102S, R686W, and A783S cells and sequence verified (Fig. 13A-D). Further, we performed proteomic screens of ER+ DDR1 mutant PDX models to identify novel druggable candidates (Fig. 14 and Fig. 4A) and our future studies will validate novel druggable candidates' activation and its effect on cell growth using MCF7 CRISPR-KI 4 MCF7 DDR1 mutant cell lines. Our future work will perform CRISPR screens using MCF7-CRISPR-KI MCF7 DDR1 mutant cell lines and identify druggable candidates that overlap with novel candidates identified from proteomic screens in this study.

Conclusion. Partially completed

Major Task. 5

(1). DDR1 mutations are enriched in exons 3 and 12-15, using multiple ER+ sequencing studies. In order to search for mutations in the functional exons of DDR1, we searched four ER+ sequencing patient data sets. The four ER+ sequencing studies included the Tam and MA12 series (Griffith. et. al, 2017), unpublished metastatic breast cancer (mBC) sequencing data sets (Dr. Nik Wagle, DFCI, Harvard Medical School), and unpublished ATAC ER+ sequencing studies (Dr Matthew Ellis, BCM). We identified three ECD mutations, R93Q, T102S, and R105Q, in exon 3, which is in the collagen (ligand) binding domain. T102S is a resistance mutation acquired upon endocrine

therapy. Additionally, we identified a P-loop mutation, E618Q, in exon 12, and L626V in exon 13 in close proximity to the P-loop. R686W are in exon within the αC-helix and in close proximity to the C-loop; the metastatic site of R686W is bone. R770W and A783S are in exon 15 in close proximity to the A-loop (Fig. 5A-B). These findings suggest that DDR1 mutations are enriched in functional exons 3 and 12-15 and could be likely drivers of endocrine therapy resistance and metastasis.

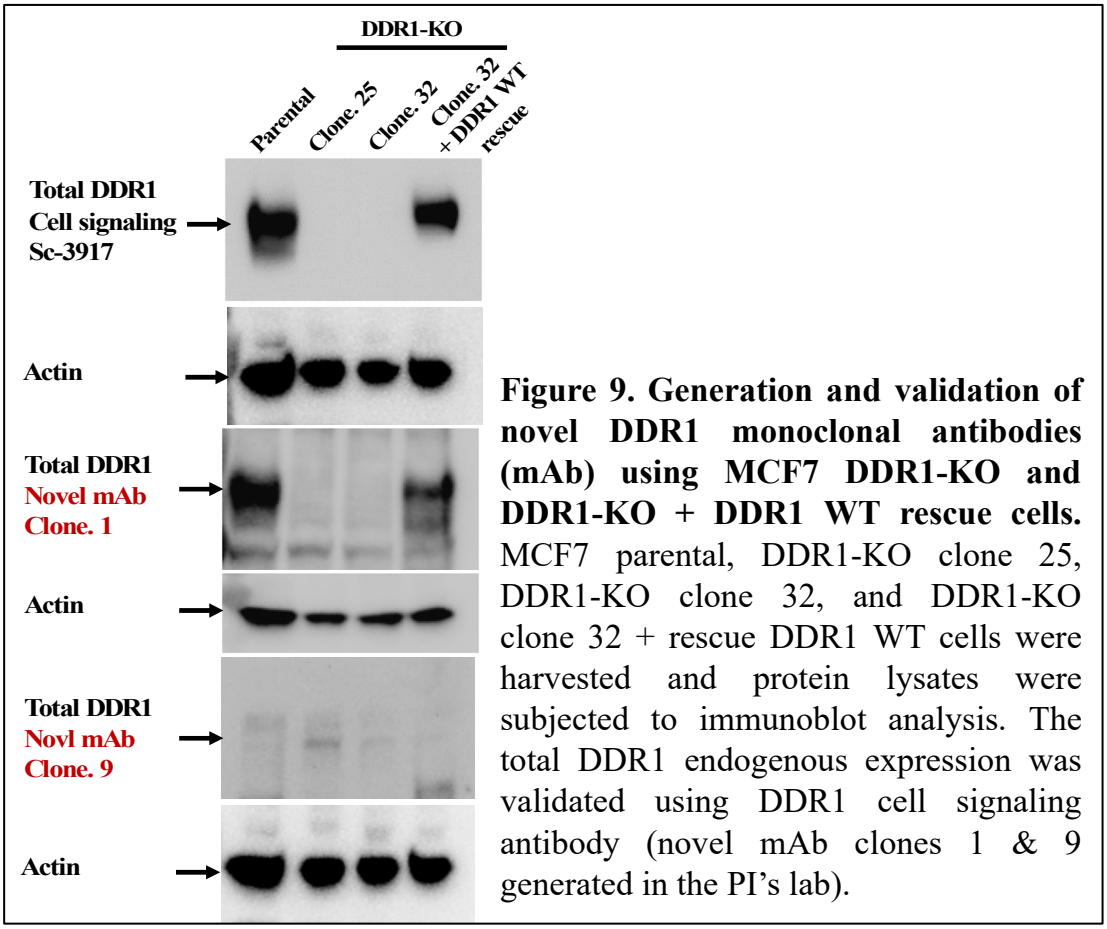

Figure 9. Generation and validation of novel DDR1 monoclonal antibodies (mAb) using MCF7 DDR1-KO and DDR1-KO + DDR1 WT rescue cells. MCF7 parental, DDR1-KO clone 25, DDR1-KO clone 32, and DDR1-KO clone 32 + rescue DDR1 WT cells were harvested and protein lysates were subjected to immunoblot analysis. The total DDR1 endogenous expression was validated using DDR1 cell signaling antibody (novel mAb clones 1 & 9 generated in the PI's lab).

(2). ER+ DDR1 amplification/gain and ER+ DDR1 R105Q PDX model validation and DDR1 signaling axis. In order to search for ER+ DDR1 mutant PDX models, we performed Sanger sequencing of BCM ER+ PDX models. We identified two ER+ PDX models harboring DDR1 gain and four ER+ PDX models harboring R105Q, BCM 15100, BCM 15057 (Fig. 4B&C), BCM 15030, and BCM 15034 (sequence verified and data not shown). Using existing RNA sequencing and Whole Exome Sequencing (WES), we identified BCM 4888 and 5097, harboring DDR1 copy number gain (single copy) and DDR1 overexpression at the RNA level. To test the effect of naturally harboring DDR1 gain or DDR1 R105Q, we harvested BCM 4888 (DDR1 gain), BCM 15057, BCM 15100 (DDR1 R105Q), BCM 5277 (ER+ control), and BCM 3469 (TNBC-DDR1 amplified model, served as positive control) tumors, and cell lysates were analyzed for DDR1 signaling. We show that BCM 15057 and 15100 induce constitutive DDR1 autophosphorylation, phospho Src, and phospho Shp2 as compared to BCM 4888 and 3469. Additionally, we show that DDR1 endogenous expression in BCM 4888, 15057, and 15100 is similar to BCM 3469, which is a TNBC DDR1-amplified model. Moreover, we confirmed the ER+ expression in BCM 15057 and 15100 (Fig. 4A). These findings suggest that ER+ BCM 15057 and 15100 harbor DDR1 R105Q and induce constitutive DDR1 signaling in ER+ breast cancer.

Conclusion. Completed

Major Task. 6

(1). Generation and characterization of affinity purified DDR1 monoclonal antibody (mAb) clone. 1. The recombinantly purified DDR1 ECD protein (His tag-DDR1 397aa). was injected into 5 BALB/c mice and after the third booster mice were euthanized. The sera were collected and tested for specificity using ELISA. Further, hybridoma clones were prepared and validated using ELISA. Next, the top 2 hybridoma supernatants were affinity-purified and tested for DD1 endogenous expression using MCF7 parental, DDR1 CRISPR-KO clone 25, clone 32,

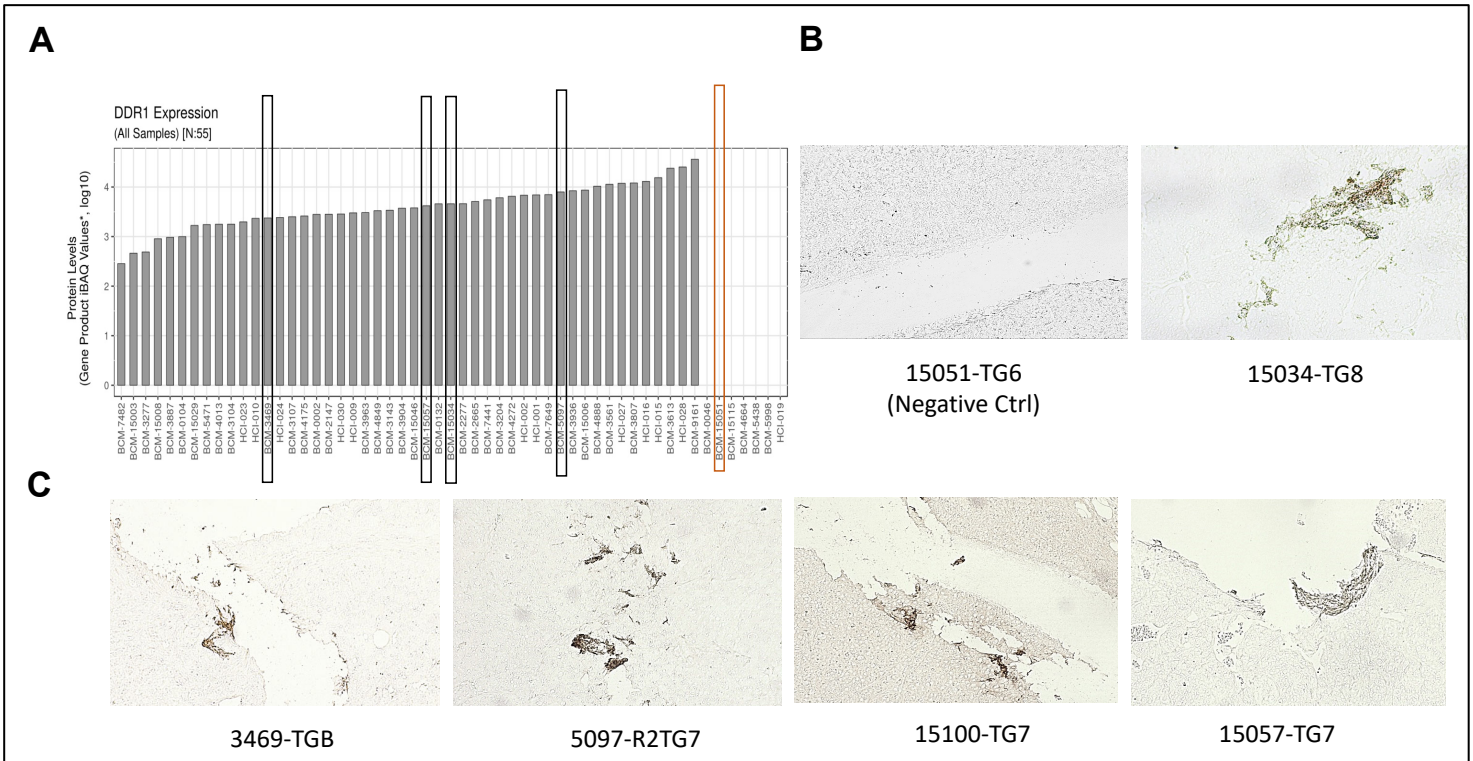

Figure. 10. Identification of ER+ DDR1 overexpressed patient derived xenografts (PDXs) and DDR1 protein expression status of ER+ DDR1 overexpressed PDXs. (A). The relative protein abundance of total DDR1 expression of 55 ER+ PDX tumors quantified by mass spectrometry. (B-C). The 5 selected ER+ DDR1 overexpressed PDX tumors (15034, 3469, 5097, and 15100) or ER+ DDR1 and 1 selected ER+ DDR1 low expressed PDX tumor (15051 and served as a negative control) were harvested and immunohistochemistry were performed using novel DDR1 monoclonal antibodies generated in PI's lab (clone. 1, shown in fig. 8).

and DDR1-CRISPR-KO clone 32 + rescue WT, using WB analysis. We show that affinity purified clone. 1 specifically recognizes a specific band at 120KD, similar to commercial DDR1 antibody as expected. Interestingly, we could not observe any DDR1 specific band in two independent DDR1-KO clones (clone 25 and 32) (Fig. 9). In addition, we validated the specificity of DDR mAb (clone. 1) generated in this study using ER+ DDR1 overexpressed PDX tumors by immunohistochemistry (IHC). We show the modest positive staining of DDR1 expression in these tumors (Fig. 10B-C), suggesting that novel DDR1 mAb generated in this study could be used for immunoblot and IHC on ER+ DDR1 overexpressed patient tumors.

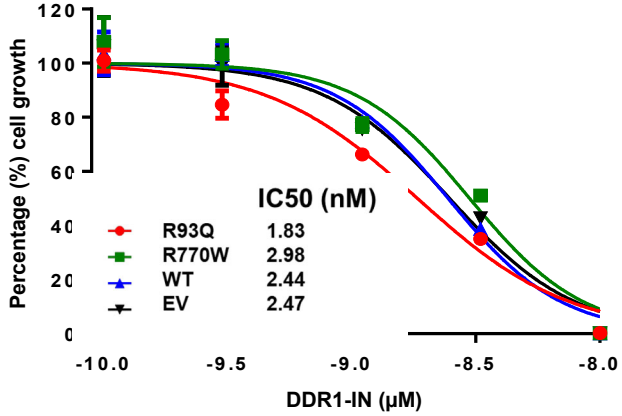

Figure 11. DDR1 WT or mutations are sensitive to a selective inhibitor of DDR1, DDR1-IN. ZR-75 parental, WT, R93Q, or R770W cells were grown under low estrogen conditions for 5 days and seeded in 96 well plates. Subsequently, cells were treated in the presence or absence of increased concentrations of DDR1-IN. Cell growth was measured using CellTiter-GLO.

Conclusion. Completed

Major Task. 7

(1). DDR1-IN inhibits cell growth of DDR1 mutant ER+ cells. To test the effect of a selective inhibitor of DDR1, DDR1-IN, on cell growth, ZR-75 cells expressing empty vector (EV), WT, R93Q, or R770W were grown in low estrogen conditions for 5 days. Subsequently, cells were grown in 96-well plates in the presence or absence of DDR1-IN for 7 days. Cell growth was assayed using cell titer GLO. We show that DDR1-IN is highly active in EV, WT,

R93Q, or R770, with IC50 values ranging from 1.8 to 2.8nM concentration (Fig. 11), suggesting that DDR1-IN can be repurposed for treating ER+ DDR1 WT or mutant tumors.

(2). DDR1 mutations are highly sensitive to palbociclib but moderately sensitive to higher concentrations of sitravatinib. We seeded parental, DDR1 WT, and L626V cells on 96-well plates and treated cells in the presence or absence of sitravatinib in a dose dependent manner (20µM to 10 nM) for 6 days. Subsequently assayed for cell growth. We observed parental, WT, and L626V cells showed resistant to sitravatinib alone below 2µM concentration. At higher concentration (2.2µM) we see growth inhibition of parental, WT, and L626V cells (Fig. 12B). Next, we seeded parental, DDR1 WT, and L626V cells on 96-well plates and treated cells in the presence or absence of palbociclib in a dose dependent manner (10µM to 4nM) for 6 days and subsequently assayed for cell growth. We observed that parental cells, cells expressing WT, R93Q, and L626V are highly sensitive to palbociclib at lower concentrations with IC50 values ranging from 0.10-0.11 µM (Fig. 12A), suggesting that DDR1 mutations are highly sensitive to palbociclib.

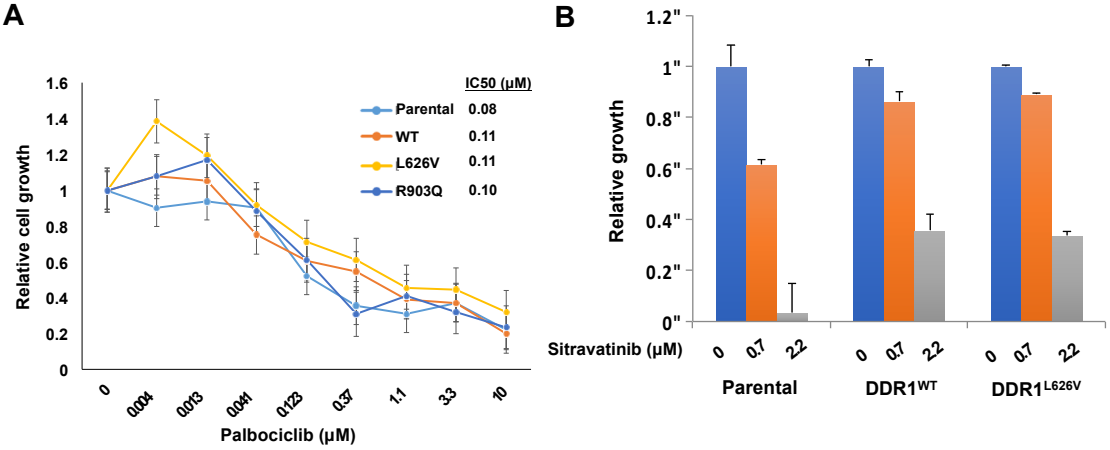

Figure. 12. DDR1 mutations are highly sensitive to palbociclib but moderately sensitive to higher concentration of sitravatinib. Indicated cells were seeded in 96 well plates and subsequently these cells were treated in the presence or absence of increased concentrations of palbociclib (A) or Sitravatinib (B).

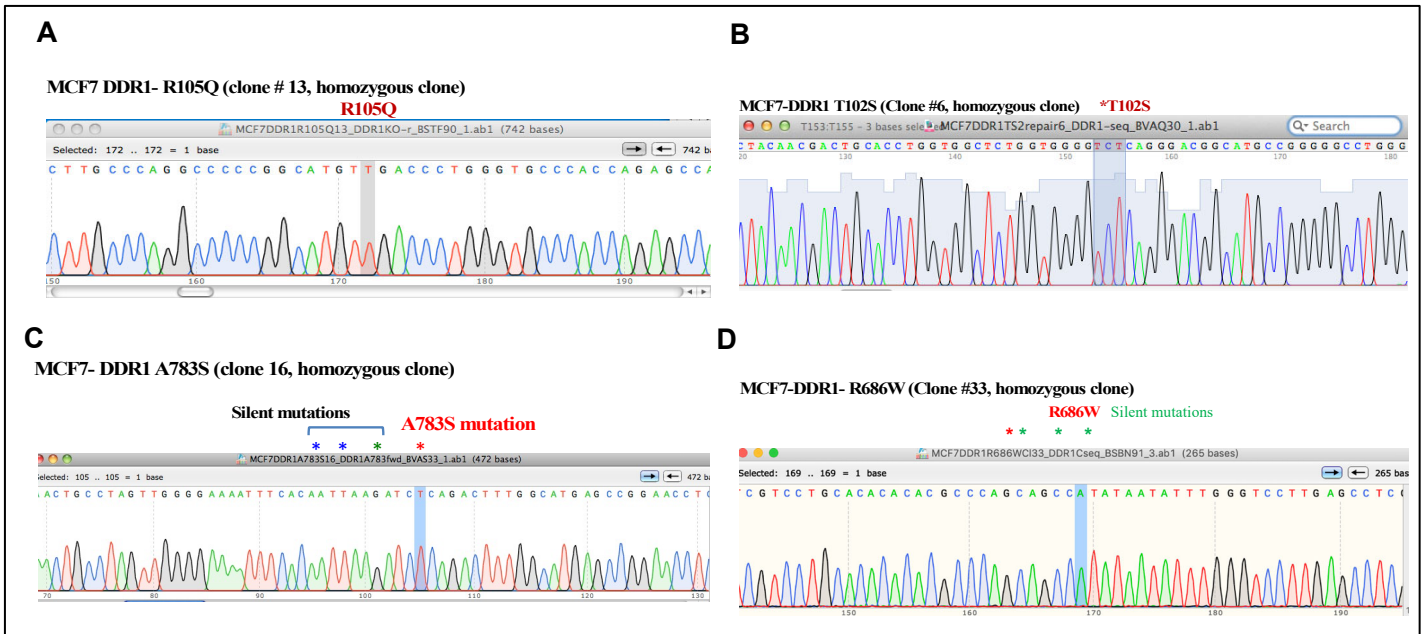

Figure. 13. Generation of MCF7 CRISPR knock-In (KI) DDR1 mutant cell lines. Chromatograms of the MCF7 CRISPR KI (A). DDR1 R105Q cells clone 13 and sequenced with reverse primer appears to be homozygous (insertion from CCG to TTG or (B). DDR1 T102S cells clone 16 and sequenced with reverse primer appears to be homozygous (insertion from GGT to AGA) or (C). DDR1 A783S cells clone 16 and sequenced with reverse primer appears to be homozygous (insertion from G to A) or (D). DDR1 R686W cells clone 33 sequenced with reverse primer appears to be homozygous (insertion from G to T).

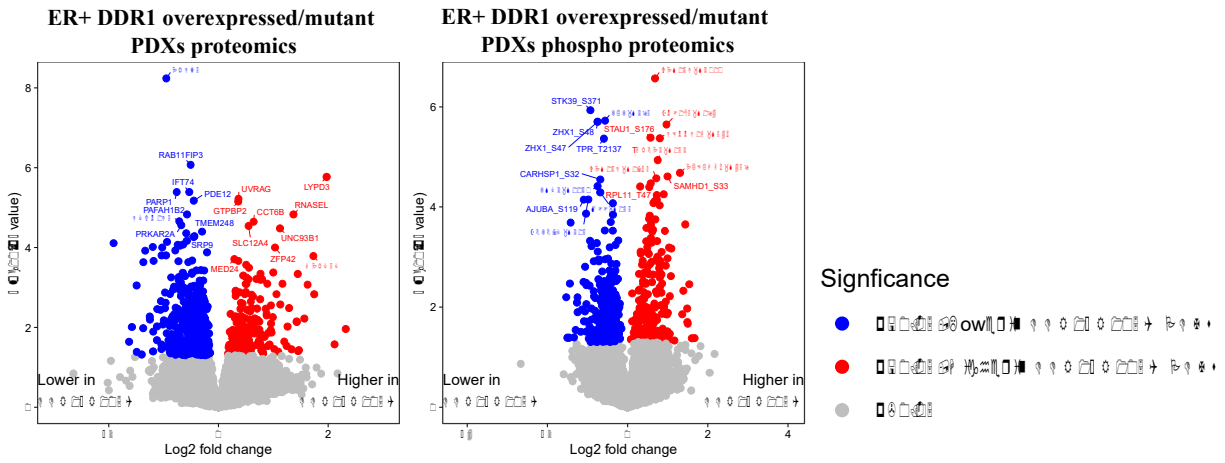

Figure. 14. Proteomic analysis of DDR1 overexpressed/mutant PDX tumors. Differential protein and phosphosite abundance in ER+ PDXs harboring concurring DDR1 overexpression and DDR1 R105Q. 3 ER+ PDXs with concurring DDR1 overexpression and DDR1 R105Q were compared to 19 ER+ PDXs with DDR1-WT by t-test using TMT proteomics and phosphoproteomics data generated from tumors. The top 10 upregulated and downregulated proteins and phosphosites are annotated.

Conclusion. In this major task, we identified DDR1 selective inhibitor, DDR1-IN shown to be more effective as compared to downstream signaling of DDR1 including CDK4/6 inhibitors or sitravatinib Our future studies will test the *in vivo* effect of DDR1 mutations using naturally harboring ER+ DDR1 mutant PDX models and CRISPR-KI DDR1 mutant cell lines models generated in this study.

Status. Partially completed.

Overall, in years 1-3, we have accomplished all the major goals and completed significant amounts of work towards the outstanding major tasks as demonstrated in the SOW shown below. The PI's lab (Dr. Kavuri) generated two peer-reviewed publication (Punturi et al, 2021 and Kalra. R. et. al, Cancer Research, 2022 accepted) and have a invited review in revision (Elli. L and Kavuri. SM, review in Cancer Drug Resist, 2022 (revision) and, co-authored manuscript and (Karunanithi S, Oncogene, 2021). Detailed list of publications generated during the grant period are included below in the publication section. Our partially completed experiments will be completed and written up as a manuscript for peer-reviewed publication.

STATEMENT OF WORK

Specific Aim 1: Determine the effect of DDR1 overexpression on metastasis in ER+ in vivo models	**Timeline**	**Completion**
Major Task 1: Generation of DDR1 WT intraductal xenograft models and identify/validate adaptive kinases that are associate with metastasis	Months	
ACURO protocol approval will be completed. No animal work tasks will be initiateed untill I have ACURO approval month 3. HRPO approval	1-3	
Subtask 1: Generation of DDR1 WT intraductal xenograft models Injecting MCF7 and ZR75 (luminal B cell line) cells lentivirally-expressing GFP and luciferase parental cells/DDR1 WT ectopically expressed cells into mammary gland of female SCID/Beige mice. Sample size: 48 mice (12 mice per arm/ 4 arms). Experimental arms: MCF7 parental, MCF7 DDR1 WT, ZR-75 parental, ZR-75, DDR1-WT Metastatic spread and growth will be measured weekly using bioluminescence imaging. Tumors will be harvested, frozen, and used it for subtask 2 Metastatic organs (lungs, liver, and bone) will be collected and fixed in formalin for paraffin embedding and immunohistochemistry (IHC) for DDR1 expression.	6-12	Completed
Subtask 2: To perform proteomic screens on harvested metastatic tumors and matched primary tumors iTRAQ and MIB/MS-based MS on DDR1 tumors and validate top 3 candidates status on DDR1 overexpressing core biopsies from UBC and TAM series by IHC	12-24	Completed
Milestone #1: Corresponding author manuscript on the metastasis underpinnings of DDR1 overexpression in ER+ breast cancer.	24	
Major Task 2: To test the effect of mutant p53 on DDR1-WT induced growth and metastatic potential in T47D (TP53 mutant) cell line.	Months	
Generating T47D - GFP/firefly luciferase-DDR1WT/TP53-KO and DDR1WT/TP53-KO cells reconstituted with either P53 WT or mutant lentivirally. To test the above recombinant cells on 2D and 3D growth, cell migration, tumor growth. Sample size: 60mice (12 mice per arm/ 5 arms). Experimental arms: T47D parental, T47D DDR1-WT, T47D-DDR1/TP53-KO, T47D-DDR1-WT/TP53-KO + TP53-WT rescue, T47D-DDR1-WT/TP53-KO + TP53-mut rescue	24-36	Completed
Milestone #1: Corresponding author manuscript on the impact of mutant P53 on DDR1 overexpression in ER+ breast cancer.	24-36	
Specific Aim 2: Determine the functional impact of DDR1 activating mutations in vitro and in vivo ER+ models.		

Major Task 3: Characterize the effect of DDR1 mutations on tumor growth and endocrine therapy resistance.	Months	
Sub task 1: To test the effect of DDR1 mutations on 2D and 3D growth, cell migration, and endocrine therapy resistance using ER+ cells (MCF7 and ZR-75) To test the effect of activating DDR1 mutations on tumor growth (using SCID/Beige mice strain) and sensitivity to endocrine agents (fulvestrant, tamoxifen) <u>Tumor growth experimental arms</u>: MCF7 parental, MCF7 DDR1-WT, MCF7 DDR1 mut-1, DDR1 mut-2, DDR-mut-3; ZR-75 parental, ZR-75 DDR1-WT, ZR-75 DDR1 mut-1, ZR-75-DDR1 mut-2, ZR-75-DDR1-mut-3. Mouse cost: 150mice (15 mice per arm/10 arms). <u>Drug treatment experimental arms</u>: Vehicle, fulvestrant, tamoxifen - MCF7 parental, MCF7 DDR1-WT, MCF7 DDR1 top mut 1. Total 135 mice (15 mice/3 cell lines x 3 treatment arms = 9 total)	3-12	Completed
Subtask 2: To test the impact of SRC1 and CDK4 on endocrine therapy resistance and tumor growth using DDR1 or DDR1 top highly activating mutant cells Generating stable knockdowns of SRC1 and CDK4 in parental, DDR1 WT, and DDR1 mutant cells Test the effect of SRC1 and CDK4-KO on fulvestrant and tamoxifen sensitivity 2D and tumor growth (using SCID/Beige mice strain) Generation of xenograft models: 9 arms x 12 mice arm = total 108 mice	12-18	Partially completed
  <u>*Experimental arms*</u>: MCF7 parental, parental-SRC1-KO, parental CDK4-KO; DDR1 WT, DDR1 WT-SRC1-KO, DDR1 WT-CDK4-KO; DDR1 mut1, DDR1 mut-SRC1-KO, DDR1 mut-CDK4-KO		
  Milestone #2: Corresponding author manuscript on the impact of DDR1 mutations and targeting DDR1 activating mutations in ER+ breast cancer	12	
Major Task 4: To profile CRISPR-mediated genetic screens in response to activating DDR1 mutations	Months	
Sub task 1: To profile genome wide CISPR screens in response to 2 highly activating DDR1 mutations. Transfection of genome wide CRISPR guide RNA (gRNA) in parental, DDR1 WT, DDR1 MUT cells Selection with puromycin, isolation of barcoded genomic DNA for NGS, and analysis of depleted barcoded sgRNA Validate top 2 candidates that are significantly depleted in DDR1 mutant cells over DDR1 WT cells on 2D and 3D growth and endocrine therapy response	12-24	Partially completed

Major Task 5: To study the prevalence of DDR1 activating mutations in relapsed ER+ breast cancer patients progressing on endocrine therapy using ctDNA approach	Months	
HRPO protocol approval will be completed. No human use tasks will be initiateed untill I have ACURO approval month 3	1-3	
Sub task 1: Collection of 110 ER+ breast cancer patients samples at the time of their relapse	6-36	Completed
Extraction of ctDNA from these patients and perform targeted specific sequencing for DDR1 gene to identify DDR1 mutations		
<u>Specific Aim 3</u>: Establish a strategy to effectively treat DDR1 overexpressing and activating mutant breast cancers		
Major Task 6: To generate novel monoclonal antibodies against the ECD domain of DDR1	Months	
Sub task 1: Cloning ECD domain of DDR1 into baculovirus vectors Recombinant expression, purification using baculovirus system	1-12	Completed
Injecting purifying DDR1-ECD purifying protein into 5 BALB/c mice Collecting serum from the mice and test the recombinant serum specificity for DDR1 expression by WB, ELISA and sensitivity to growth inhibition using MCF7 parental cells (express low levels endogenous DDR1), MCF7-DDR1-KO cells (negative control), MCF7-DDR1-WT cells (positive control) Successfully tested mice, isolate spleen cells form the mice expressing recombinant DDR1-ECD and fuse with myeloma cells for generating hybridomas cells	12-24	Completed
Purifying recombinant hybridomas for generating DDR1-specific monoclonal antibodies	24-36	Completed
Major Task 7:To test the effect of drugs targeting sitravatinib, palbociclib, Abemaciclib, and bufalin on 2D, 3D, and tumor growth using DDR1 WT and mutant models		
Sub task 1: Sitravatinib, fulvestrant, sitravatinib fulvestrant sensitivity using MCF7, ZR-75, and T47D DDR1 WT and mutants in vitro cell lines, MCF7 DDR1 WT and top mutant-1 cell line xenograft models (using SCID/Beige mice) Drug treatments of sitravatinib, palbociclib, fulvestrant, sitravatinib+ fulvestrant, palbociclib + fulvestrant using MCF7 parental, MCF7 DDR1 WT, MCF7-DDR1 mut-1. Experimental arms: 216 mice (12 mice per arm x 3 cell lines x 6 arm)	1-12	Partially completed
* <u>Animal experiments will be performed upon approval of animal protocol from 3rd month</u>. *Milestone #3*: Corresponding author manuscript on the impact of DDR1 mutations and targeting DDR1 activating mutations in ER+ breast cancer	12	

Milestone #4: Submission of RO1 on "Targeting DDR1 aberrations in colorectal and bladder cancers"	30	Dr. Kavuri

The milestone tasks proposed by Dr. Kavuri were completed or ongoing to be completed shortly. The major task. 3/sub task. 2, major task. 4 and the major task. 7, the work is ongoing. These delays did not impact for establishing DDR1 aberrations as novel therapeutic targets in ER+ breast cancer.

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

The work during the grant period was presented at the SABCS at a Spotlights session in 2020 and at the GRASP poster highlight session in 2021. During the grant period, Dr. Kavuri published two peer reviewed publications (Punturi. N. et. al, 2021, Nature communications, PMID. and Kalra. R. et. al, 2022, Cancer Research, 2022, accepted). In addition, the paper we published in Nature Communications on this project was also highlighted by the Nature Publishing Group on their website and was reported on by several news channels. In addition, the Cancer research publication that got accepted will in press by the end of June, 2022. During three years of funding, Dr. Kavuri co-hosted BCM Breast center retreat and chaired a session on the "tumor microenvironment". Dr. Kavuri was invited to present his lab work about Allele specific therapeutic response and metastasis of HER2 mutations in ER+ breast cancer at SABCS, 2021 (https://aacrjournals.org/cancerres/article/82/4_Supplement/P2-13-24/680822). My lab member, Vaishnavi Deverakonda, who is a co-author in this manuscript secured a PhD position at Cedar-Sinai Samule Oschin Comprehensive Cancer Institute, Los Angles, CA.

How were the results disseminated to communities of interest?

During the funding period, Dr. Kavuri was invited to present his lab work at patient advocacy group, Guiding Researchers & Advocates To Scientific Partnerships (GRASP), 2021.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We have demonstrated that 12.5% of ER+ IDC patients harboring DDR1 amplification and 28% of ER+ ILC patients harboring DDR1 amplification. Using proteomic profiling and genome sequencing, we identified several naturally harboring ER+ DDR1 overexpressed/gain (1 copy) and DDR1 mutant patient derived xenografts (PDX). Using proteomic profiling of ER+ DDR1 overexpressed/gain and DDR1 mutant PDX models we identified and validated driver oncogenic signaling pathways. In this study, we have generated novel DDR1-specific monoclonal antibodies (mAb) and validated the specificity of DDR1 mAb using ER+ PDX tumors and MCF7-CRISPR-KO DDR1 cell line models. Our ongoing future studies will establish DDR1 aberrations as novel therapeutic targets in ER+ breast cancer and establish the novel DDR1 mAb preclinical efficacy using ER+ PDXs naturally harboring DDR1 overexpression/gain and DDR1 mutations. More importantly, these studies will lay the foundation for clinical evaluation of DDR1 aberrations in ER+ metastatic breast cancer.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Metastatic breast cancer patients harboring DDR1 aberrations (DDR1 overexpression and DDR1 mutations) in ER+ disease might now be candidates for DDR1-IN inhibitor therapy. An ongoing collaboration aims to conduct a proof-of-concept clinical trial to test this therapeutic hypothesis.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change.

None.

Actual or anticipated problems or delays and actions or plans to resolve them.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. PRODUCTS

Publications, conference papers, and presentations.

Publications:

1. Punturi, N., Seker, S., Devarakonda, V., Mazumder, A., Kalra, R., Chen, C.H., Li, S., Primeau, T., Ellis, M.J., **Kavuri, S.M**., Haricharan, S. "Mismatch repair deficiency predicts response to HER2 blockade in HER2- negative breast cancer." (**Corresponding author, *Nature Communications* (2021**).
2. Poziotinib inhibits HER2 mutant-driven therapeutic resistance and multi-organ metastasis, Rashi Kalra, Ching Hui Chen, Junkai Wang, Ahmad Bin Salam, Lacey E. Dobrolecki, Alaina Lewis, and Christina Sallas, Carolina Gutierrez, Richard Bryce, Clayton C. Yates, Alshad S. Lalani, Meenakshi Anurag, Balasubramanyam Karanam, Matthew. J. Ellis, and, **Shyam. M. Kavuri***. (**Corresponding author, "Cancer Research", 2022**, **accepted**).
3. Karunanithi S, Liu R, Hou Y, Gonzalez G, Oldford N, Roe AJ, Idipilly N, Gupta K, Amara CS, Putluri S, Lee GK, Valentin-Goyco J, Stetson L, Moreton SA, Putluri V, **Kavuri SM**, Sauntharajah Y, de Lima M, Tochtrop GP, Putluri N, Wald DN. Thioredoxin reductase is a major regulator of metabolism in leukemia cells. **Oncogene. 2021** Aug;40(33):5236-5246. doi: 10.1038/s41388-021-01924-0. Epub 2021 Jul 8. PubMed PMID: 34239044; PubMed Central PMCID: PMC8380733.
4. Epigenetic loss of AOX1 expression via EZH2 leads to metabolic deregulations and promotes bladder cancer progression., Vantaku V, Putluri V, Bader DA, Maity S, Ma J, Arnold JM, Rajapakshe K, Donepudi SR, von Rundstedt FC, Devarakonda V, Dubrulle J, Karanam B, McGuire SE, Stossi F, Jain AK, Coarfa C, Cao Q, Sikora AG, Villanueva H, **Kavuri SM**, Lotan Y, Sreekumar A, Putluri N. Oncogene. 2019 Aug 5, PMID: 31383940, Epub ahead of print. (URL: https://www.ncbi.nlm.nih.gov/pubmed/31383940).

5. Eli. L and **Kavuri. SM**, Acquired resistance to neratinib in HER2-mutant metastatic breast cancer, (**Corresponding author, Cancer Drug Resist, Invited Review, 2022, Accepted)**.

Website(s) or other Internet site(s).

Nothing to Report.

Technologies or techniques.

Nothing to Report.

Inventions, patent applications, and/or licenses.

Nothing to report

Other products.

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Meghashyam kavuri, Partnering Principal Investigator – 4.5 Calendar months
Chen Ching Hui, Research Associate – 5.0 person months
Rashi Kalra, Postdoctoral Associate – 5.6 Calendar months

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.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

9. APPENDICES

Nothing to Report.