

AD _____

Award Number: W81XWH-10-1-0662

TITLE: SIRT3 Is a Mitochondrial Tumor Suppressor and Genetic Loss Results in a Murine Model for ER/PR-Positive Mammary Tumors Connecting Metabolism and Carcinogenesis

PRINCIPAL INVESTIGATOR: Sarki Abdulkadir

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203-6876

REPORT DATE: September 2013

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE September 2013		2. REPORT TYPE Final		3. DATES COVERED 1 September 2010 - 31 August 2013	
4. TITLE AND SUBTITLE SIRT3 Is a Mitochondrial Tumor Suppressor and Genetic Loss Results in a Murine Model for ER/PR-Positive Mammary Tumors Connecting Metabolism and Carcinogenesis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0662	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sarki Abdulkadir E-Mail: sarki.abdulkadir@vanderbilt.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37203-6876				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 The goals of this synergistic project are to establish the mitochondria localized sirtuin protein SIRT3 as a tumor suppressor in breast cancer and to define its role as a molecular link between aging and breast cancer. Sirt3 knockout mice develop ER/PR-positive breast tumors later in life. These ER/PR-positive tumors are histologically similar to breast tumors common in older women. SIRT3 protein expression is significantly reduced in human breast cancer epithelium. Breast cancer patients in the lowest quartile of SIRT3 expression had a significantly shorter relapse free survival [Hazard ratio = 0.53 (0.47-0.61), log rank p=0]. Notably, low SIRT3 expression was associated with reduced relapse-free survival in all breast cancer subtypes analyzed, including ER+, ER-, HER2+, luminal A, luminal B and basal subtypes (Hazard ratios =0.44 to 0.65; log rank p=0 to 0.0019). These results highlight the importance of the SIRT3 as a TSP in breast cancer and suggest that SIRT3 may be a potential molecular biomarker to identify high risk patients across all molecular subtypes of breast cancer.					
15. SUBJECT TERMS Breast cancer, SIRT3, acetylation, aging, biomarkers					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	3
BODY	3-5
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusion.....	5
References.....	5

INTRODUCTION

The mammalian *Sirtuin* genes are homologs of the yeast *Saccharomyces cerevisiae* *Sir2* gene that is implicated in the regulation of longevity (Haigis et al., 2012). There are seven Sirtuin proteins, with SIRT1, SIRT6, and SIRT7 localized in the nucleus, SIRT2 localized in the cytoplasm and SIRT3, SIRT4, and SIRT5 localized in the mitochondria (Finkel et al., 2009). The mitochondrial deacetylase SIRT3 is thought to act on numerous substrates to regulate several processes including fat and amino-acid metabolism as well as electron transport (Huang et al., 2010).

SIRT3 has been proposed to function in maintaining mitochondrial integrity and to serve as a bona fide tumor suppressor (Finley et al., 2011; Kim et al., 2010). We have shown that *Sirt3*^{-/-} MEFs expressing only Myc or Ras are able to grow in soft agar and form tumors in nude mice, unlike wild type MEFs which require the expression of both oncogenes (Kim et al., 2010). Furthermore, *Sirt3* knockout mice develop estrogen receptor and progesterone receptor (ER/PR) positive breast cancers (Kim et al., 2010).

One third of female *Sirt3* knockout mice developed mammary gland tumors by 24 months. These tumors were well-differentiated, ER/PR+ tumors similar to the tumors commonly seen in breast malignancies in older women. Our analysis of 992 human breast cancer samples from human tumor mRNA expression databases showed a significant reduction in SIRT3 mRNA in breast cancers compared to benign tissue as well as an association with grade (Kim et al., 2010). We have further examined SIRT3 expression by immunohistochemistry (IHC) of human breast cancer samples and examined the correlation between SIRT3 and patient outcome in a large collection of human breast cancer samples.

BODY

Statement of Work - Task 1 - Identify Sirt3 mitochondrial deacetylation targets and determine if these targets are regulated by extracellular stimuli known to activate sirtuin function (e.g., resveratrol). These targets will subsequently be knocked down (with siRNA) to determine if there is a mechanistic connection between the increase in superoxide and the stress-induced genomic instability observed in SIRT3^{-/-} cells (months 1-18).

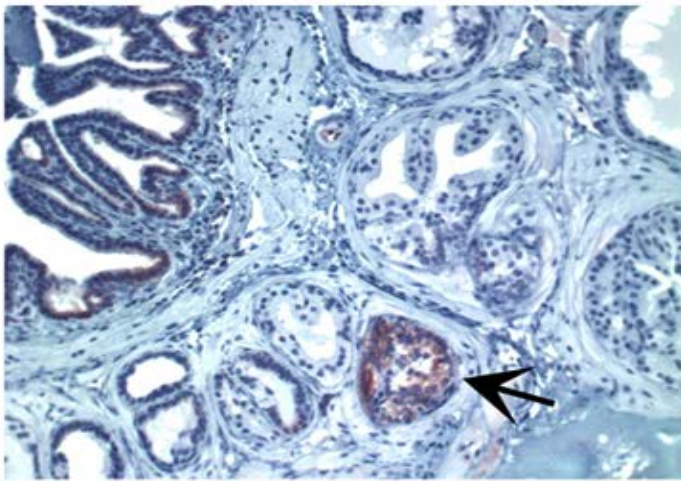
Results: The results for Task 1 have been detailed in the report for W81XWH-10-1-0661, which is the companion grant for W81XWH-10-1-0662 in this synergistic award mechanism. These studies have identified Manganese Superoxide Dismutase (MnSOD) and OSCP as SIRT3 targets. The specific lysine residues in these proteins targeted by Sirt3 have been identified and new antibodies have been developed. These include the OSCP acetylated K139 and MnSOD acetylated K68 antibodies.

Statement of Work - Task 2 - Determine if exposure to resveratrol or overexpression of a MnSOD gene will prevent increases in ROS in MEFs and/or decrease the development of mammary tumors in Sirt3 knockout mice and transformation in SIRT3^{-/-} MEFs (months 7-24).

Results: The details for this Task are presented in the report for W81XWH-10-1-0661. W81XWH-10-1-0662 has established the histological and immunohistochemical protocols for analyzing mammary tumors and expression of Sirt3 and acetylated Sirt3 substrates in tissues, including OSCP acetylated on K139 and MnSOD acetylated on K68 (Figure 1).

Figure 2. Validation of the OSCP acetylated K139 and MnSOD acetylated K68 antibodies. Sirt3 knockout mouse prostate sections were stained with the indicated antibodies and positive glands showing cytoplasmic staining detected by Nova red staining (arrows). Negative glands on the same sections serve as internal negative control. No primary antibody controls shows no staining (not shown).

OSCP acetylated K139



MnSOD acetylated K68

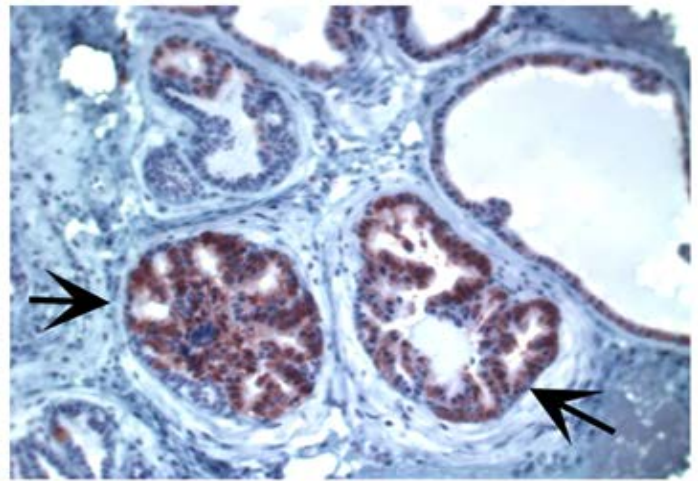


Figure 1. Validation of the OSCP acetylated K139 and MnSOD acetylated K68 antibodies. Sirt3 knockout mouse prostate sections were stained with the indicated antibodies and positive glands showing cytoplasmic staining detected by Nova red staining (arrows). Negative glands on the same sections serve as internal negative control. No primary antibody controls shows no staining (not shown).

Statement of Work - Task 3 - Determine if loss of SIRT3 ductal protein in ER/PR-positive and -negative breast samples from the Vanderbilt Breast Spore correlates with clinically significant outcomes including response to therapy, local tumor control, disease free survival, and overall survival (months 1-24).

SIRT3 expression is reduced in breast ductal carcinoma in situ (DCIS) and invasive breast cancer

We evaluated SIRT3 expression in normal and breast carcinomas by IHC (Figure 2). SIRT3 expression was consistently lower in neoplastic tumor cells compared to normal breast epithelial cells. Small clusters of breast cells were scored as low/absent (negative or no staining), weak/focal (1-10% positive), intermediate (11-50% positive) and strong (>50%) positive cytoplasmic depending on the percentage of positive cells and intensity of staining. The overall average score expression in normal breast duct epithelium was significantly higher (Figure 3), as compared to that in invasive/in situ lesions (1.8 vs. 0.5, $p < 0.001$). For example, while only 23% normal breast tissue has low/absent SIRT3 expression, 72% of in situ breast lesions and 74% of invasive lesions were negative for the expression of SIRT3 (Figure 4). There was no statistically significant difference in SIRT3 IHC expression across tumor grades in invasive carcinomas. All metastatic carcinomas in lymph nodes ($n=9$) have corresponding SIRT3 staining with that of primary breast tumor except 2 cases with weak/focal staining in the primary tumor and negative staining in the matched lymph node metastasis.

SIRT3 protein expression relation to breast cancer hormone receptor status

We also examined SIRT3 expression in relation to estrogen receptor (ER) and progesterone receptor (PR) status. SIRT3 loss occurs with a higher frequency in ER negative (ER-) and PR negative (PR-) tumors. SIRT3 loss is seen in 83% of ER- tumors compared to 62% of ER+ tumors ($p=0.002$). Similarly SIRT3 is lost in 82% of PR- breast cancers compared to 68% of PR+ cases ($p=0.04$). Analysis of the expression of SIRT3 according to hormone receptors and HER2 status of breast cancers revealed that 75% of triple negative cases (ER-, PR- and HER2-) show loss of SIRT3 expression which was comparable to 80% cases with at least one positive marker that show loss of SIRT3.

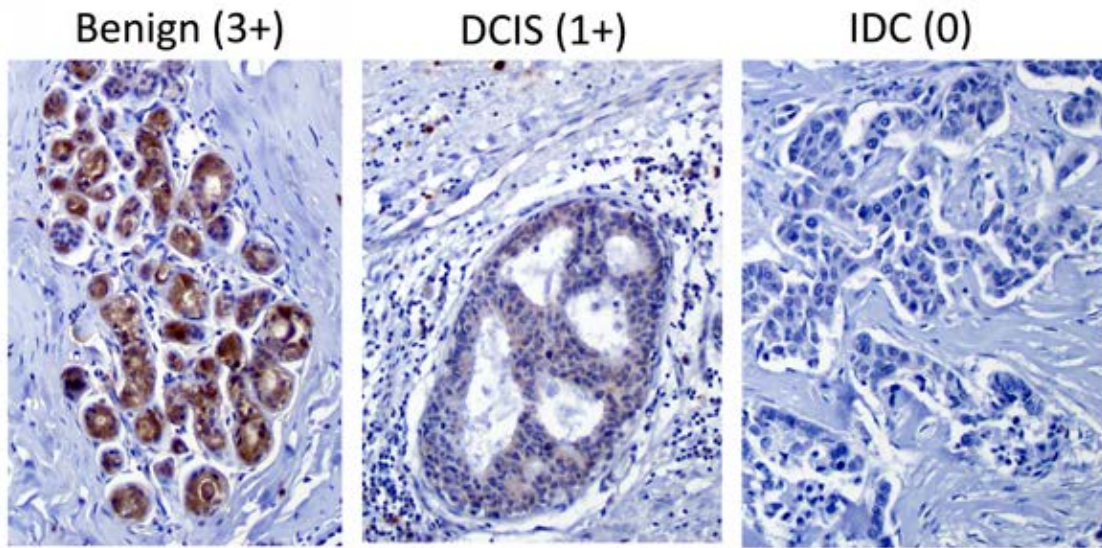


Figure 2: *SIRT3* expression in normal, ductal carcinoma in situ (DCIS) and invasive duct carcinoma of the breast (IDC). *SIRT3* is strongly expressed (3+) in benign breast ducts compared to low expression (1+) expression in DCIS and negative (0) in IDC. Original magnifications, 200X.

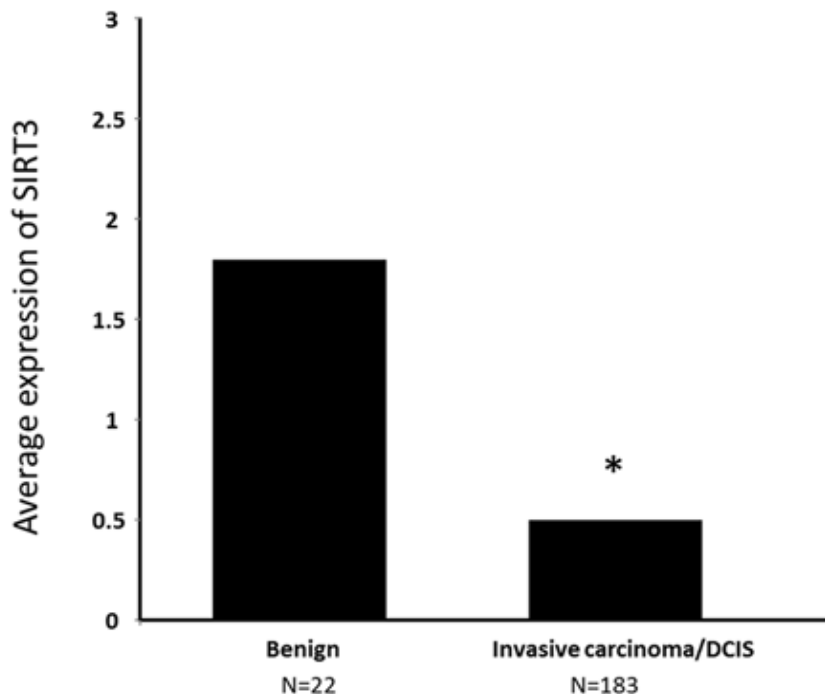


Figure 3: Average score expression of *SIRT3* by IHC in normal and invasive/in situ breast carcinomas. Notice more than three-fold expression of *SIRT3* in normal compared to breast carcinomas. * $p < 0.001$.

Decreased *SIRT3* is associated with poor outcome in multiple subtypes of breast cancer

To determine a potential association of low *SIRT3* expression with patient outcome, we interrogated data in a publically available breast cancer expression hub, KM Plotter. We subdivided samples based on *SIRT3* expression, with those in the lowest quartile in one group (low *SIRT3*) and the rest in another group (high *SIRT3*) and tested for the relationship to relapse free survival. Remarkably, low *SIRT3* expression is found to be associated with significantly reduced survival in all breast cancers, as well as in ER+, ER-, HER2+, Luminal A (ER+ and/or PR+, HER2/neu-), Luminal B (ER+ and/or PR+, HER2/ neu+) or basal-like type (ER-, PR-, HER2/neu-, CK 5/6+, and/or EGFR+) breast cancers (Figure 5).

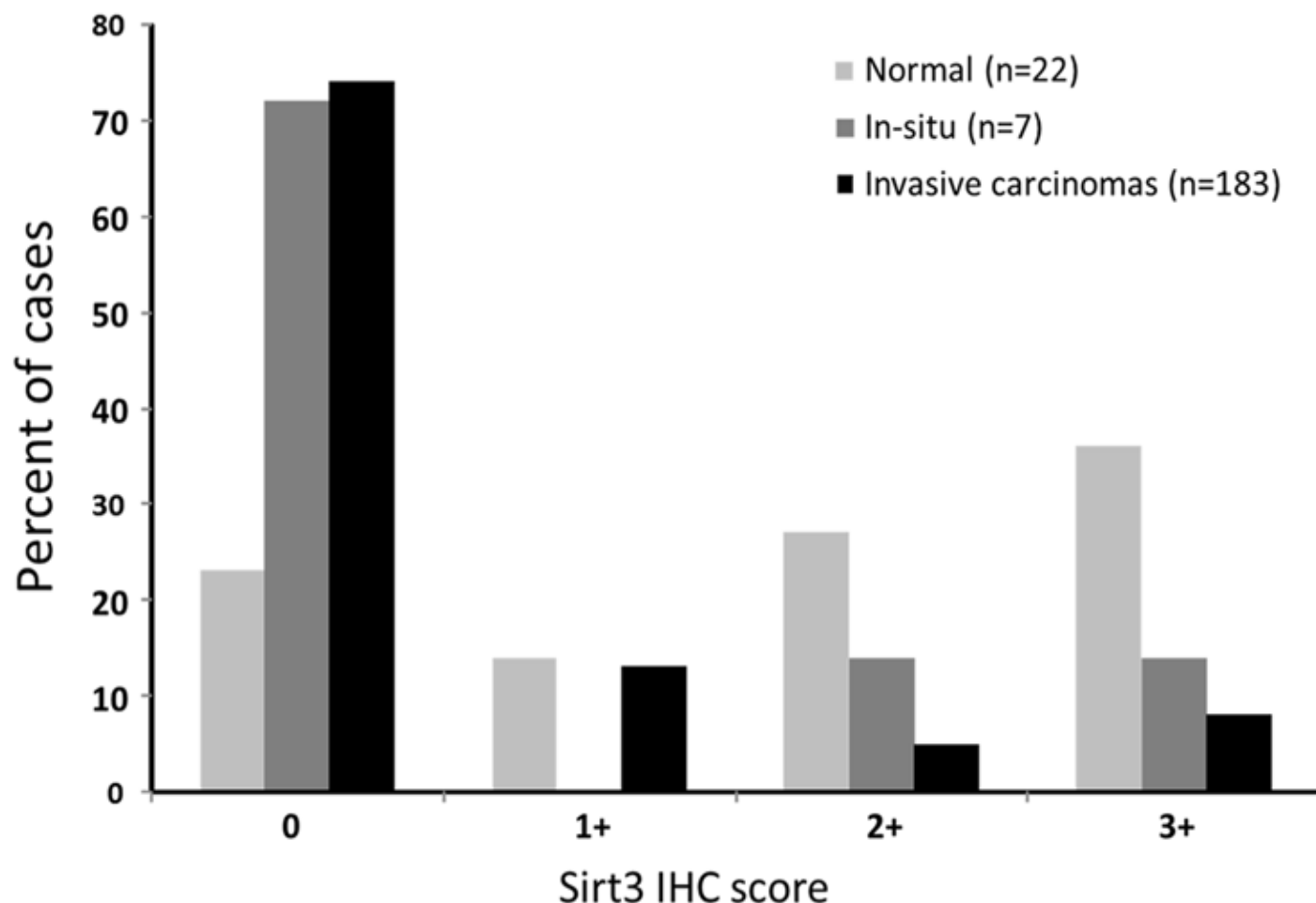


Figure 4: Expression of SIRT3 by IHC in normal, in situ and invasive breast carcinomas shows evidence for reduced SIRT3 expression in in-situ and invasive carcinoma compared to normal glands.

We have also demonstrated for the first time that low SIRT3 expression is associated with poor outcome in multiple subtypes of breast cancer. Remarkably, low SIRT3 expression is found to be associated with reduced survival in all breast cancers, as well as in ER+, ER-, HER2+, Luminal A, Luminal B or basal type breast cancers. Taken together, this study underscores the importance of *SIRT3* as a potential tumor suppressor gene in breast cancer. Additionally, the study highlights a potential role of SIRT3 as a biomarker to assist in identifying high risk patients across all molecular subtypes of breast cancer. More studies are needed to identify the role of SIRT3 and other sirtuin genes and understand the mechanisms involved in this process.

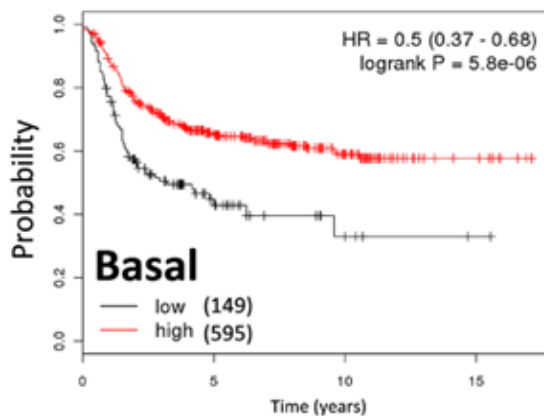
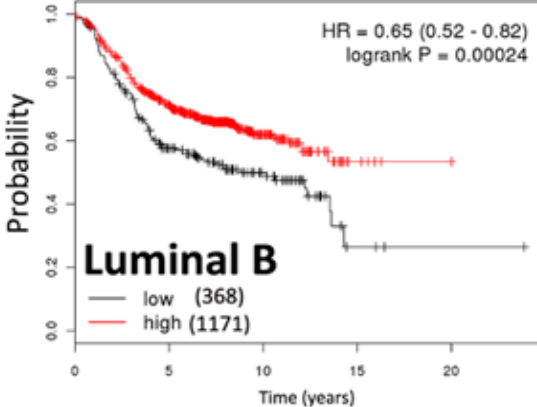
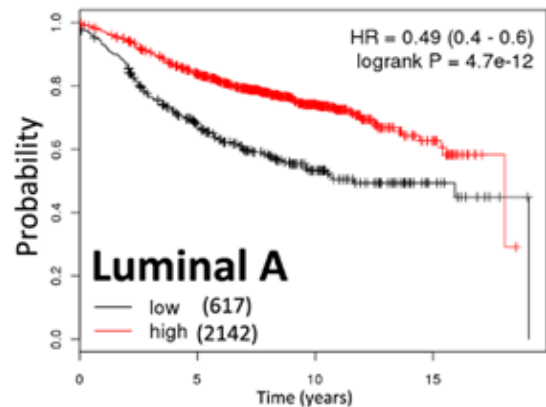
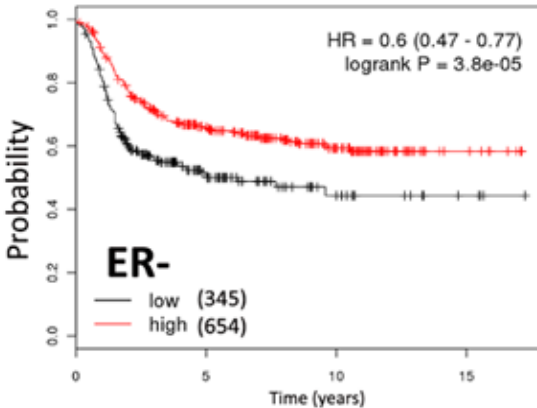
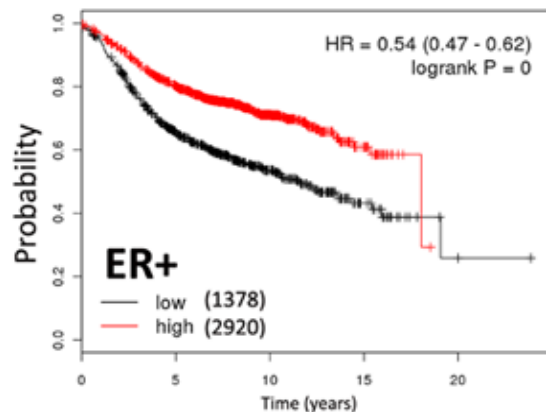
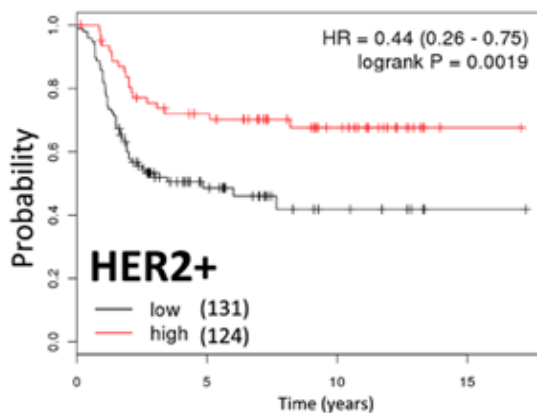
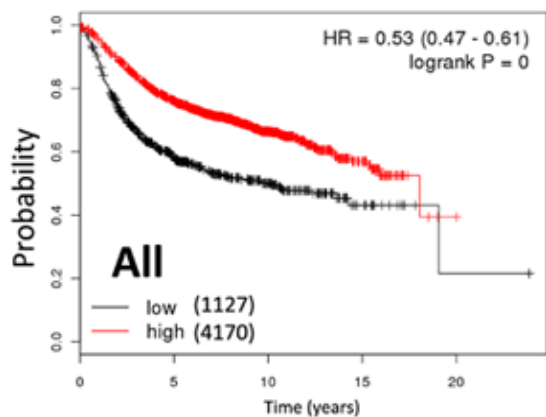


Figure 5: Analysis of breast cancer data by Kaplan-Meier Plotter shows that low SIRT3 expression is significantly associated with poor outcome (relapse free survival) in all types of breast cancer examined. The numbers of samples in each group are indicated in parentheses, and the hazard ratios (HR) and log rank p values are shown.

KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstration of loss of SIRT3 expression in human breast ductal carcinoma in situ and invasive breast cancer.
2. Showing that breast cancer patients with low SIRT3 expression had a significantly shorter relapse free survival
3. Demonstrating that low SIRT3 expression was associated with reduced relapse-free survival in all breast cancer subtypes analyzed, including ER+, ER-, HER2+, luminal A, luminal B and basal subtypes.
4. Validation of antibodies for SIRT3 lysine substrates in MnSOD and OSCP in immunohistochemistry using formalin-fixed paraffin embedded samples.

REPORTABLE OUTCOMES:

1. Antibodies for SIRT3 lysine substrates in MnSOD and OSCP suitable for immunohistochemistry
2. Manuscript in review: Decreased SIRT3 Expression is a Potential Molecular Biomarker Associated with Poor Outcome in Breast Cancer. Mohamed Mokhtar Desouki , Irina Doubinskaia , David Gius , Sarki A. Abdulkadir

CONCLUSION:

Aging has long been recognized as a risk factor for breast cancer incidence, but the molecular basis for this association is not understood. Our studies suggest that SIRT3 may provide a molecular link between breast cancer and aging. This synergistic DOD idea award has enabled the development of a Sirt3 knockout mouse model of aging-related receptor-positive breast cancers. In addition, new potential biomarkers for breast cancer are being developed. These studies provide important new insights into breast tumorigenesis as well as provide possible new therapeutic and prognostic targets for human breast cancer.

REFERENCES

- Finkel, T., Deng, C.X., and Mostoslavsky, R. (2009). Recent progress in the biology and physiology of sirtuins. *Nature* 460, 587-591.
- Finley, L.W., Carracedo, A., Lee, J., Souza, A., Egia, A., Zhang, J., Teruya-Feldstein, J., Moreira, P.I., Cardoso, S.M., Clish, C.B., *et al.* (2011). SIRT3 opposes reprogramming of cancer cell metabolism through HIF1alpha destabilization. *Cancer Cell* 19, 416-428.
- Haigis, M.C., Deng, C.X., Finley, L.W., Kim, H.S., and Gius, D. (2012). SIRT3 is a mitochondrial tumor suppressor: a scientific tale that connects aberrant cellular ROS, the Warburg effect, and carcinogenesis. *Cancer Res* 72, 2468-2472.
- Huang, J.Y., Hirschey, M.D., Shimazu, T., Ho, L., and Verdin, E. (2010). Mitochondrial sirtuins. *Biochim Biophys Acta* 1804, 1645-1651.
- Kim, H.S., Patel, K., Muldoon-Jacobs, K., Bisht, K.S., Aykin-Burns, N., Pennington, J.D., van der Meer, R., Nguyen, P., Savage, J., Owens, K.M., *et al.* (2010). SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. *Cancer Cell* 17, 41-52.