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Mitochondrial Tumor Suppressor

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14. ABSTRACT

The incidence of human malignancies increases significantly with age, suggesting a mechanistic connection between aging (longevity) and carcinogenesis Breast malignancies that develop with increasing age very likely represent a spectrum of cancers arising from different types of hereditary and spontaneous tumors. Murine models for hereditary breast cancer in younger women have been established by altering the expression of BRCA genes. In contrast, there are no murine models for the spontaneous breast cancers that are more common in older women. The work supported by this DOD idea award has allows us to not only validate the first ER+ in vivo murine model for breast cancers but is has also allowed the discovery of several potential molecular targets that may potential be used for either chemoprevention or as anticancer agents.

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INTRODUCTION:

The overarching goal of this proposal is to determine if the mitochondrial sirtuin *SIRT3* is a tumor suppressor gene (TSG) that may be used to: (1) establish a murine model to investigate the mechanisms of carcinogenesis in ER/PR-positive mammary tumors; and (2) determine if SIRT3 may serve as a new molecular biomarker that correlates with clinically and pathologically significant outcomes including response to therapy, local tumor control, disease free survival, and overall survival. Preliminary data from our laboratory demonstrated that: (1) *Sirt3* knockout mice exhibit decreased mitochondrial integrity and are genomically unstable when exposed to genotoxic agents; (2) $Sirt3^{-/-}$ MEFs transformed by either Myc or Ras have aberrant intracellular metabolism including increases in glycolysis, superoxide levels, and chromosomal abnormalities; (3) MnSOD prevents immortalization of $Sirt3^{-/-}$ MEFs by a single oncogene; (4) *Sirt3* knockout mice develop ER/PR-positive mammary tumors; and (6) SIRT3 expression is decreased in human breast tumors. Based on these results we initially proposed a hypothesis that longevity genes impact the process of carcinogenesis via the maintenance of mitochondrial integrity and oxidative metabolism. Specifically, loss of sirtuin expression could result in mitochondrial damage and a phenotype permissive for mammary tumors. In this regard, we proposed to: **(i)** Identify SIRT3 mitochondrial deacetylation targets and determine if these targets are regulated by extracellular stimuli known to activate sirtuin function (resveratrol). These targets will subsequently be knocked down (siRNA) to determine if there is a mechanistic connection between the increase in superoxide and stress-induced genomic instability observed in $Sirt3^{-/-}$ cells. **(ii)** 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. **(iii)** Determine if loss of SIRT3 protein in ER/PR-positive and negative breast samples correlates with clinically significant endpoints including tumor control, disease free survival, and overall survival.

The final year of the work was supervised by Dr. Michael Freeman at Vanderbilt Medical School.

Publications

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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).

I. Sirt3-Mediated Deacetylation of Evolutionarily Conserved Lysine 68 and 122 Regulates MnSOD Activity in Response to Stress. (Tao et al., 2010, Molecular Cell, See Appendix A).

The work proposed in task 1 has been completed.. The first protein that we identified was MnSOD that appeared to have altered acetylation as well as the function activity in cells lacking Sirt3. Specifically, livers of $Sirt3^{-/-}$ mice contained hyperacetylated MnSOD as well as exhibited decreased MnSOD activity, but not immunoreactive protein, relative to wild-type livers. Re-introduction of wild-type, but not deacetylation null Sirt3, into $Sirt3^{-/-}$ MEFs deacetylated lysine and restored MnSOD activity. Site-directed mutagenesis of MnSOD lysine 122 to an arginine, mimicking deacetylation (lenti-$MnSOD^{K122-R}$), increased MnSOD activity when expressed in $MnSOD^{-/-}$ MEFs, suggesting acetylation directly regulates function. Furthermore, infection of $Sirt3^{-/-}$ MEFs with lenti-$MnSOD^{K122-R}$ inhibited in vitro immortalization by an oncogene (Ras), inhibited IR-induced genomic instability, and decreased mitochondrial superoxide. This work was subsequently published last December in *Molecular Cell* (Tao et al., 2010)[1]. Finally, lysine specific

antibodies were made to these two lysine and these two antibodies will be used in task 3 to identify potential molecular targets for chemoprevention and molecular biomarkers that may be used to develop personalized treatment protocols. Thus, this work showed for the first time that there may be a connection between loss of *Sirt3* and the aberrant regulation of reactive oxygen species levels in the mitochondria that may play a role, at least in part, in the mechanism by which the mice lacking *Sirt3* develop ER+ tumors as well as develop spontaneous genomic instability.

II. OSCP Contains an Evolutionarily Conserved Lysine that is Deacetylated by Sirt3 that Directs ATP Synthase Activity. (Pennington et al., 2011, Submitted).

Genetic deletion of *Sirt3* in vitro and in vivo decreases mitochondrial ATP levels suggesting a mechanistic connection to aberrant energy homeostasis and preferential use of glycolysis by the mammary tumors that develops in the *Sirt3* knockout mice. As such, we investigated if the aberrant energy production in mice lacking Sirt3 may play a role in why these mice develop mammary tumors. ATP synthase is made up of multiple proteins and it is primarily responsible for the production of ATP and since the mice lacking *Sirt3* have decreased ATP levels and increased glycolysis it seemed logical to propose that the dys-regulation of ATP synthase may play a role in the ER+ tumor permissive phenotype observed in these mice.

OSCP sits atop the ATP synthase (F1Fo ATPase) that uses chemiosmotic energy across the inner mitochondrial membrane to convert ADP and orthophosphate to ATP[2-4]. We have shown that OSCP contains at least one nutrient status-reversible acetyl-lysine that is evolutionarily conserved in multiple species. In this regard, mass spectrometry demonstrated that lysine 139 is hyperacetylated in *Sirt3* knockout livers, as compared to the control, wild-type mice. Re-introduction of wild-type, but not deacetylation null *Sirt3*, into Sirt3$^{-/-}$ MEFs deacetylated OSCP and increased mitochondrial ATP levels. IP experiments showed a physical interaction between OSCP and Sirt3. A series of in vitro and in vivo analysis demonstrated that OSCP lysine 139 is deacetylated by Sirt3. Finally, OSCP lysine 139 was mutated to arginine, mimicking deacetylation (CMV-OSCP^{K139-R}) and expression of CMV-OSCP^{K139-R} increased mitochondrial ATP levels demonstrating that Sirt3 post-translational acetyl signaling contributes to mitochondrial energy homeostasis by deacetylating OSCP. Finally, a lysine specific antibody was made to this lysine and this antibody will be used in task 3 to identify potential molecular targets and/or biomarkers. These results not only identify an additional Sirt3 target but also for the first time showed that the production of ATP in the mitochondrial is regulated by a mechanism involving the post-translation modification of a reversible acetyl lysine. This work has recently been submitted and is in review at *PNAS* and the manuscript has been included in appendix B.

SIRT3 deacetylates several potential reversible acetyl-lysines in ATP synthase alpha and beta.
Since OSCP appeared to have a reversible acetyl-lysine it seems reasonable to propose that the other major proteins in the ATP synthase complex, including ATP synthase alpha and beta, might also have Sirt3-dependent reversible acetyl-lysine(s). To address this question a series of in vitro deacetylation assays were preformed. Affinity purified ATP synthase was obtained from bovine mitochondria with a GST-tagged inhibitor protein (I1-60) that was obtained (a kind gift from J.E. Walker, MRC, unpublished). Subunits from two 50 μg samples were resolved by SDS-PAGE and immunoblotted with an anti-acetyl-lysine antibody (Fig. 1, left panel lane 1) or stained with Coomassie (lane 2). These results suggest that in addition to OSCP, ATP synthase proteins alpha and beta may also contain specific reversible acetyl-lysines. These samples were subsequently treated with PCAF, which is an acetyl transferase previously shown to acetylate potential sirtuin targets, separated, and immunoblotted with an anti-acetyl antibody (Fig. 1, middle panel, lane 1 vs.

2). Finally, the PCAF-treated ATP synthase samples from lane 2, middle panel were mixed with recombinant SIRT3 without and with NAD for an in vitro deacetylation assay, similar to the experiments above (Fig. 1, right panel, lane 1 vs. 2). In these blots the ATP synthase alpha and beta proteins run at similar locations, and based on the differences in band intensity, it seems reasonable to assume that these two proteins contain multiple reversible acetyl-lysines (Fig. 1, right panel, lane 1 vs. 2). Finally, the samples in Fig. 1, right panel, lanes 5 and 6, were isolated and analyzed by mass spectrometry (that was done in Dr. Liebler's Proteinomics group) and these results identified 10 alpha and 5 beta *in vitro* reversible acetyl-lysines (Supporting Data, pages 1 and 2). When these results were combined with published results that identified mitochondrial reversible acetyl-lysines after CR or fasting (12 hrs) (Supporting Data) we determined that ATP synthase alpha contains 7 reversibly acetylated lysines that appear in all three data sets and 3 that appear in two data sets. In addition, beta contains 3 reversible lysines that appear in two data sets. These combined findings strongly suggest that the ATP synthase alpha and beta, which are critical to the generation of mitochondrial ATP, may also be SIRT3 deacetylation targets that respond to stress or changes in cellular nutrient status. We are in the process of determining if these lysines are function in the regulation of ATP synthase activity and when this is done antibodies will also be made against the specific lysine as outlined above. Thus, this work has shown for the first time that ATP production via Sirt3 acetylation of proteins within the ATP synthase complex is regulated by Sirt3 and may play a role, at least in part, in the tumor permissive phenotype observed in mice lacking *Sirt3*. This work is not ready for publication yet but we anticipate that this work in will submitted sometime in the spring of 2012. In conclusion, the work outlined above has made very good progress in addressing the research proposed in task 1.

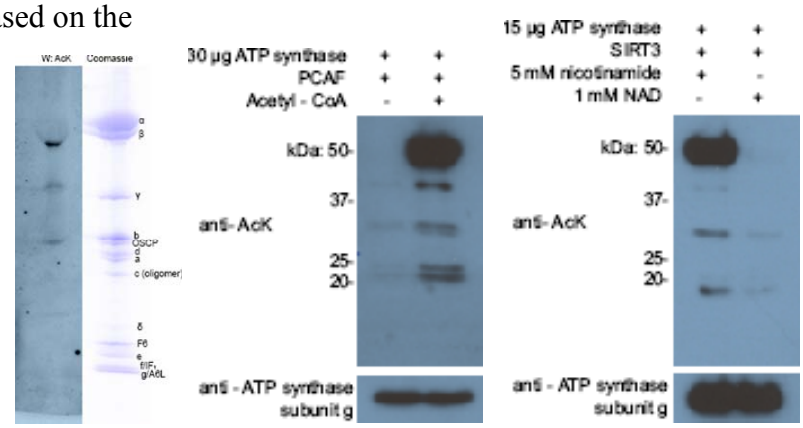

Figure 1. The ATP Synthase complex contains multiple Sirt3-dependent reversible acetyl-lysines. 50 mg samples of highly purified bovine F1Fo ATP synthase were separated and immunoblotted with an anti-acetyl antibody (left panels). These samples were mixed with PCAF to acetylated lysines (middle panels). Finally, an in vitro Sirt3 deacetylation assay was done as describe above and samples were separated and immunoblotted with an anti-acetyl antibody (right panel).

III. OSCP Contains an Evolutionarily Conserved Lysine that is Deacetylated by Sirt3 that Directs ATP Synthase Activity. (Pennington et al., 2011, Submitted).

Genetic deletion of Sirt3 decreases mitochondrial ATP levels suggesting a mechanistic connection to energy homeostasis. ATP synthase uses chemiosmotic energy across the inner mitochondrial membrane to convert ADP and orthophosphate to generate ATP. In vitro and in vivo experiments demonstrated that ATP synthase F1-proteins, alpha, beta, gamma, and OSCP bind to SIRT3 and contain multiple SIRT3 specific reversible acetyl-lysines that are evolutionarily conserved. OSCP was further investigated since it only appeared to have on deacetylation site and of in vitro and in vivo analysis by mass spectrometry demonstrated that lysine 139 is a nutrient-sensitive SIRT3-dependent deacetylation target directing, at least in part, ATP production. Mice lacking Sirt3 exhibited an exercise deficient phenotype, decreased ATP levels, and hyperacetylation of ATP-synthase F1-proteins. Finally, antibodies to SIRT3 deacetylation targets, including Ac-OSCP139,

showed hyperacetylation with strenuous exercise, caloric restriction, and aging suggesting that acetyl signaling contributes to mitochondrial energy homeostasis by deacetylating ATP synthase.

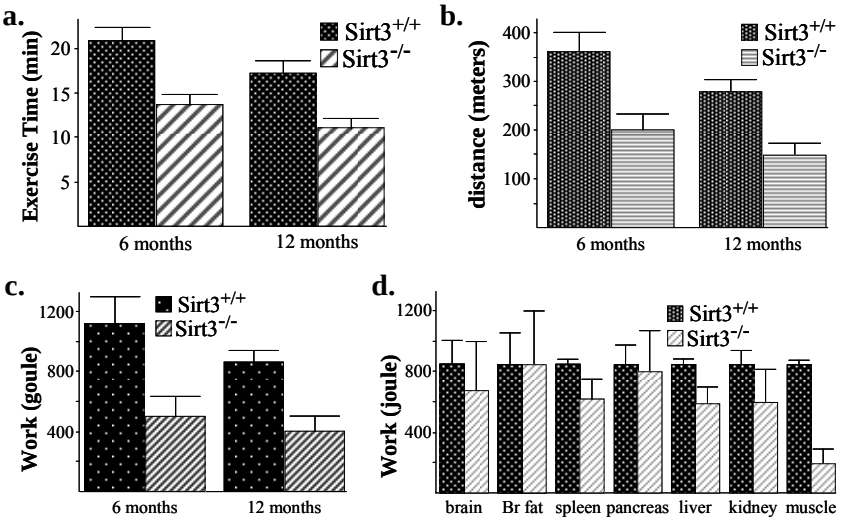

Figure 2. SIRT3$^{-/-}$ mice exhibit decreased muscle endurance. (a, b, c) After acclimatization, mice were run on a treadmill with increasing speed and slope to exhaustion. Time **(a)**, distance **(b)** and work **(c)** were calculated from the individual performances. Bars represent mean values and error bars represent standard error. * $p<0.05$ between WT and Sirt3$^{-/-}$ mice. **(d)** Several tissues were harvested immediately after sacrificing the mice at the end of the treadmill experiment. Tissues were lysed and ATP levels were measured using a bioluminescence assay for quantitative determination of ATP.

SIRT3$^{-/-}$ mice exhibit decreased muscle endurance

Previously we showed that acetylation of OSCP affects, at least in part, the efficiency of the ATP synthase enzyme to provide energy for the cell through the synthesis of adenosine triphosphate. Taking under consideration that acetylation of OSCP was detected in skeletal muscle of Sirt3-/- mice; we wanted to determine the role of this phenomenon under energetically demanding conditions. For this purpose, we challenged both WT and Sirt3-deficient mice with involuntary physical exercise testing muscle endurance by putting them to run on treadmill to exhaustion. After acclimatization, mice were run with a protocol using increasing slope and speed until they were unable to run on the treadmill despite electric shocks. Both 6 and 12 months old control animals ran roughly 7.2 and 5.34 min longer than the knockout mice, an average of 20.8 min for the controls versus 13.68 min for the knockout 6 months old mice and 17.3 min for the control versus 11.96 min for the 12 months old knockout mice (Fig. 2). The difference in running distance was even bigger because of the running protocol with increasing speed (Fig. 2). Whereas Sirt3$^{-/-}$ mice covered an average distance of 200.6 and 166.2 meters (numbers refer to 6 and 12 months old animals, respectively), control mice ran 358.9 and 277.1 meters on the average (a difference of 44% and 40%). Finally, total work performed by control animals was also higher than that of knockout (Fig. 2). Control mice used energy equivalent to 1109J (6 months old) and 847J (12 months old) whereas the average work of Sirt3-deficient mice came to 499J and 427J (a difference of 55% and 49%, respectively). In summary, all the parameters obtained from the treadmill running experiment describing muscle endurance were significantly lower for Sirt3$^{-/-}$ mice compared to control mice. Because the Sirt3 mutant mice used in these experiments were whole body knockout mice we wanted to exclude that the observed phenotypic differences could reflect additional defects in heart function. Under both basal and stressful conditions, such as beta-adrenergic stimulation or reperfusion after ischemia where rapid increase in energy demand is needed, no difference in heart function was found between Sirt3 WT and mutant mice.

Interestingly, in accordance with the decreased muscle endurance in the Sirt3$^{-/-}$ mice, a significant decrease in ATP levels was observed in skeletal muscle compared to other tissues (Fig.2), as well as increased acetylated levels of OSCP were detected by using our anti-OSCPAc-K139 antibody when skeletal muscle was isolated immediately after treadmill (Fig. 2). More specific, OSCP was deacetylated in the wild-type mice under exercise (Fig. 2, lanes 1-2 versus 3-4) suggesting that SIRT3-mediated deacetylation of OSCP enhances ATP synthase enzymatic activity in order to help

meet energy demand of the muscle. In contrast, no difference was observed in the mice lacking Sirt3 (lanes 5-6 versus 7-8) that as expected found to contain hyper-acetylated OSCP. Same pattern was observed when acetylated levels of Mn-SOD were checked (Fig. 2), indicating that acetylation of

OSCP follows changes in SIRT3 activity and OSCP is a specific SIRT3 target in the mitochonrion. Thus, these results clearly show that OSCP lysine 139 is a reversible Sirt3 deacetylation target that is deacetylated under stress.

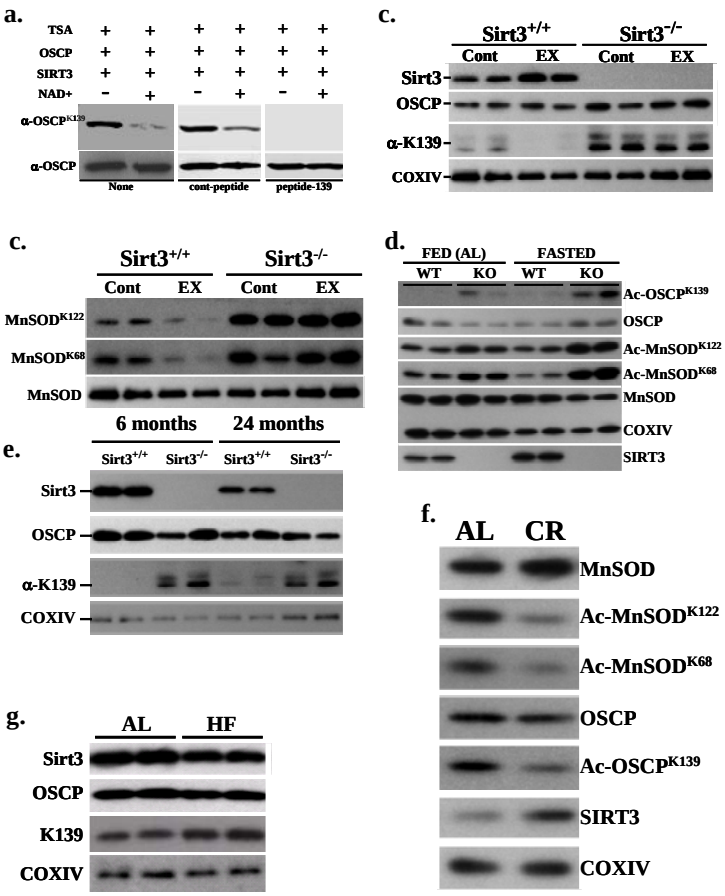

Figure 3. OSCP is a physiological relevant reversible acetyl lysine under cell stress. **(a)** Validation of an OSCP lysine 139 anti-acetyl antibody. A flag-tagged OSCP expression vector was transfected into HEK 293T cells that contained TSA (1 μM) and after 48 hours Flag-OSCP was IPed with an anti-Flag antibody (Sigma, Inc). The samples were subsequently washed, and incubated with purified SIRT3 protein (BioMol, Inc.) without (lane 1 or with (lane 2) NAD. After 2 hrs mixtures were immunoblotted with an anti-OSCP acetyl 139 lysine antibody (Epitomics, Inc, - The Rabbit Monoclonal Antibody Company, Burlingame, CA, produced for our laboratory). Identical experiments were done with the 13 amino acid lysine 122 acetylated peptide (Ac-peptide) or the control non-acetylated lysine 139 peptide (Cont-peptide). **(b)** Skeletal muscle from isogenic *Sirt3*$^{+/+}$ and *Sirt3*$^{-/-}$ mice both before exercise (cont) and immediately after exercise (EX) were harvested and mitochondrial extracts were isolated, separated, and subsequently blotted with antibodies to Sirt3, OSCP, Ac-OSCP^{K139}, and COXIV. **(c)** Same samples as in (b) were blotted with antibodies to Ac-MnSOD^{K122}, Ac-MnSOD^{K68} and MnSOD. **(d)** OSCP contains a CR dependent, reversible acetyl lysine. Livers from isogenic, two-month-old age-matched Sirt3$^{+/+}$ and Sirt3$^{-/-}$ mice placed on a CR diet for 12 weeks were harvested and mitochondrial extracts were isolated, separated, and subsequently blotted with antibodies to MnSOD, Ac-MnSOD^{K122}, Ac-MnSOD^{K68}, OSCP, Ac-OSCP^{K139}, Sirt3, and COXIV. **(e)** Livers from isogenic, three-month-old age-matched Sirt3$^{+/+}$ mice fasted for 48 hours were harvested and mitochondrial extracts were isolated, separated, and subsequently blotted with antibodies outlined above. **(f)** Livers from isogenic, six- and 24-month-old age-matched Sirt3$^{+/+}$ and Sirt3$^{-/-}$ mice were harvested and mitochondrial extracts were isolated, separated, and subsequently blotted with antibodies to Sirt3, OSCP, Ac-OSCP^{K139}, Sirt3, and COXIV. **(g)** Wild-type mice were placed on a high fat diet as previously shown (Kendrick et al., 2011), livers were processes as described above, and stained with antibodies to Sirt3, OSCP, Ac-OSCP^{K139}and COXIV.

MnSOD gene expression is decreased in human breast tumors - We have previously shown that there is a subgroup of human tumors that express decreased levels of SIRT3 as shown by IHC, RT-PCR, and genomic analysis (Appendix 1). Since it is proposed that the dys-regulation of MnSOD plays a role, at least in some significant part, in the ER+ tumor permissive phenotype observed in the Sirt3$^{-/-}$ mice, it seemed reasonable to propose that a decrease in *MnSOD* expression would also be observed in previously published genomic studies. To address this we used the University of Michigan Oncomine data repository that uses an this analysis showed that *MnSOD* expression is decreased in human breast tumor samples (Figs. 2a-d) as well as three other data sets (data not show) that ranged in statistical significance from P equals $3.2x10^{7}$ to 0.007. In addition, a decrease in MnSOD was also associated with a poorly differentiated tumor type (data not shown). These

results demonstrate that there may be a subgroup of breast tumors where the dys-regulation of MnSOD is an early event in mammary carcinogenesis and as such, suggests that superoxide may be a potential molecular target.

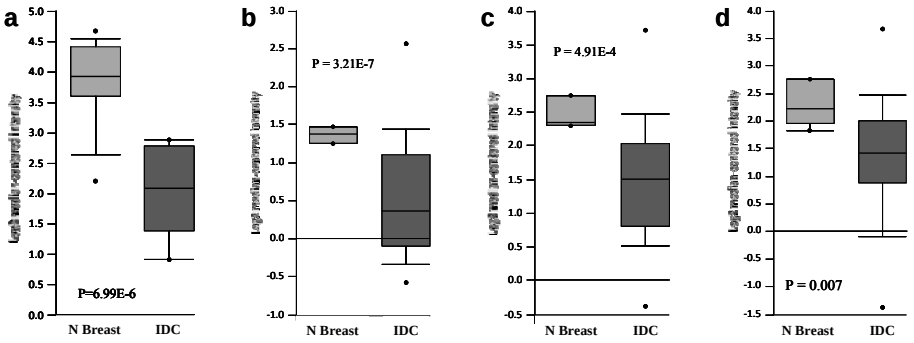

Figure 2. Genomic analysis of *MnSOD* expression in human breast **tumors. (a-d)** *MnSOD* gene (mRNA) expression is decreased human breast tumor samples. The Oncomine cancer University of Michigan microarray data-base (http://-www.oncomine.org) profiles with a sophisticated analysis engine for data-mining to identify was used to determine MnSOD expression in normal (N Breast) vs. invasive ductal Carcinoma (IDC) breast malignancies.

MnSOD contain two reversible acetyl-lysines that are deacetylated by Caloric Restriction (CR) - The results above suggest that then may be a subgroup of human breast malignancies due to dysregulation of MnSOD however, to begin to determine if this may be correct, it was necessary validate that our acetyl-lysine MnSOD antibodies recognized legitimate, physiologically nutrient sensitive reversible lysines. We[17], and other[21,22], have previously shown that the acetylated status of MnSOD lysines 68 and 122 directs MnSOD activity. As such, wild-type C57B mice were placed on a CR diet, previously shown to activate Sirt3, and after 12 weeks livers were harvested and blotted with antibodies that recognized the acetylated form of MnSOD lysines 68 and 122. These results showed that CR significant decreased the acetylated of MnSOD K68/122 while the cellular protein levels of MnSOD remained unchanged (Fig. 3). In addition, other forms of nutrient stress, including time restricted fasting (tRF for 24 hours) and exercise also resulted in the deacetylation of MnSOD lysines 68 and 122 (data not shown, see appendix 4). The results of these experiments suggest increased acetylation of MnSOD inhibits its superoxide deacetylation activity and suggests, but does not yet scientifically demonstrate, that there is a subset of human breast tumors with acetylated MnSOD. As such, it is proposed that these lysine specific, anti-acetyl antibodies can be used to identify molecular biomarkers.

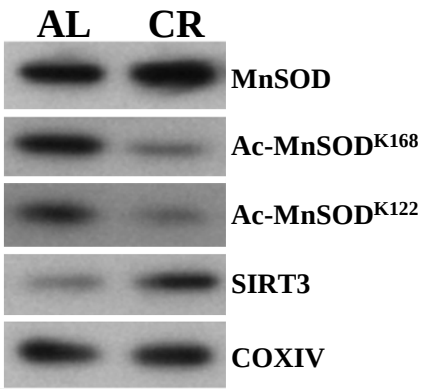

Figure 3. MnSOD, OSCP, and IDH contain physiologically relevant reversible acetyl-lysine after CR. Livers from isogenic, 6-month-old age-matched wild-type mice placed on a CR diet for 12 weeks were harvested and mitochondrial extracts were isolated. Samples were subsequently separated and blotted with antibodies to MnSOD and COXIV as controls as well as antibodies made in collaboration with Epitomics, Inc., - The Rabbit Monoclonal Antibody Company, Burlingame, CA, included lysine specific acetyl anti-Ac-MnSOD^{K68} and anti-Ac-MnSODK122 antibodies. A representative immunoreactive western blot is shown.

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).

I. The research proposed in task 2 are taking the longest since these experiments are *in vivo* chemoprevention work and will take most of, and perhaps all of the two years under this proposal. In addition, these experiments were not slated to start until month seven and as such,

this work has only just begun. However, I have included the proposed experiments and the mice housing, breeding, and generation protocol / schema that is nearly completed. These experiments are ongoing and will be completed some time in this next year. Some funds are being requested to pay for the mouse housing costs for roughly one year for roughly 40 cages of mice.

II. For the proposed experiments in Aim 1, a total of 130 female *SIRT3* knockout mice are required for each of the two groups that will contain 65 mice each (control and resveratrol). An outline is provided showing how we will generate roughly 65 female mice (Fig. 2). The number will be slight greater than 65 to adjust for decreased liter sizes and other issues so as the desired number of 65 mice will be obtained. This breeding scheme will be done in a very similar manner to generate the number of mice necessary for the control group. These mice are all genetically identical, originating from the same group of 2 to 4 females that are continuously maintained in a barrier facility in the Medical Center North (MCN) mouse facility at Vanderbilt.

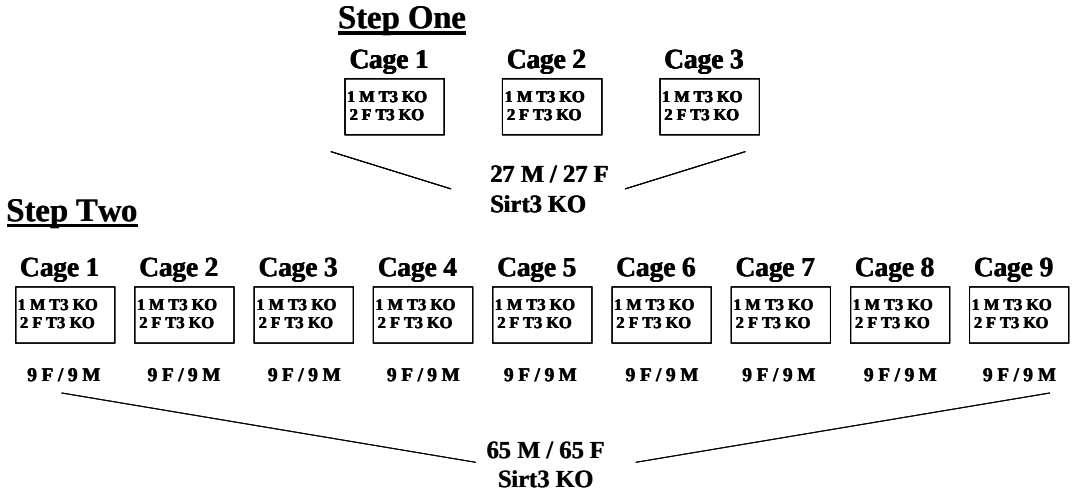

Figure 2. Generation of SIRT3 KO (shown) and wild-type mice (not shown) for task 2.

For the resveratrol experiments wild-type and *Sirt3* knockout mice will be treated with resveratrol (AKSci, Cat. No. 60512A) supplemented drinking water daily (7.5 mg/mL) while the control group will be treated with carrier drinking water (DMSO 0.015%) daily. The drinking water will be kept away from light and changed every 3 days, and the mice will be maintained on resveratrol treatment until tumor formation or death. This regimen has previously been used to decrease tumors in mice.

These experiments have been somewhat delayed due to are relocation to Vanderbilt from the NCI however, they were recently started. Generation of the mice required for the work in task 2 is straightforward but somewhat more complex than the mice above. Step 1 involves the generation of the MnSOD-Sirt3$^{-/-}$ mice that this will be done by crossing the transgenic MnSOD mice with the Sirt3 knockout mice and then these mice will be back crosses to obtain the MnSOD-Sirt3$^{-/-}$ mice. To obtain roughly 9 male mice, one cage will be set up that contains 2 females and 1 male. From this round of breeding 8 MnSOD-Sirt3$^{-/-}$ males and 16 SIRT3$^{-/-}$ females will be mated as shown in Figure 3. Since both the MnSOD$^{+/+}$-Sirt3$^{-/-}$ males and Sirt3$^{-/-}$ females are

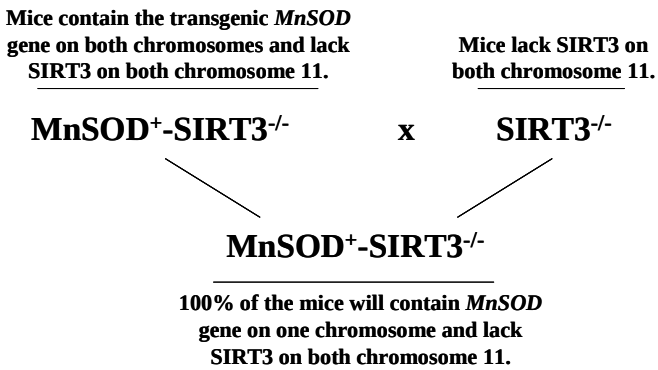

Figure 3. Generation of the MnSOD^{+}-SIRT3$^{-/-}$ Mice

homogenous for loss of *Sirt3*, all these offspring will be $Sirt3^{-/-}$. In addition, since all the male mice contain the *MnSOD* gene on both chromosomes, the offspring will only receive one copy of the exogenous *MnSOD* gene (Fig. 3). As such, after 10 weeks, 16 $Sirt3^{-/-}$ females and 8 MnSOD-$Sirt3^{-/-}$ males will be used to set up 8 cages of 2 females and 1 male. This should result in roughly 65 females and 65 males that contain one copy of the exogenous *MnSOD* gene that also lack both copies of *Sirt3* ($MnSOD^{+}$-$Sirt3^{-/-}$), of which 65 will be used for the experiments proposed in Aim 2. This should take roughly 6 months. The *Sirt3* knockout mice that will act as controls will be generated as described above (Figure 2). Finally, an additional 5 mice will be added to each group to account for mice that may become infect or sick and these mice will assure that the number of mice that can be evaluated will remain at or above 65 mice per group.

II. Statistical Considerations: The primary objective of several aspects of this grant proposal is to compare the tumor development between study groups. The Vanderbilt Power and Sample Size Estimation software and the Radiation Oncology network station was used to estimate the sample size required for our proposed experiments with resveratrol (task 2). The sample size calculations are based on a two-sample chi-square test of proportions without a continuity correction. At 30 months, the incidence of ductal mammary tumors is estimated to be 60%. This number (60%) is based on our results that are already published (Kim et al., 2010, *Cancer Cell*) as well as an additional 20 mice in each arm that was just completed at the NCI. If CR decreases the tumor incidence to 35%, the number of mice needed in each arm is roughly 65 control *SIRT3* knockout mice and 66 *SIRT3* knockout mice placed on a CR diet, which will give 80% power using a Type I error of 0.05. These proposed experiments are on going and it is still too early to make any determinations at this time..

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).

In this task we proposed to conduct H & E stained of human samples or slides that will be reviewed to assess the diagnosis and grade, in a blinded manner with no prior knowledge of IHC results. In addition, we will determine clinicopathologic variables including age, tumor grade, stage, mitotic count, and ER and PR status and these will be assesses by response to therapy, local tumor control, disease free survival, and overall survival. These endpoints will be determined via Kruskal-Wallace non-parametric analysis of variance will be done to determine how marker expression varies with grade. The log-rank test will be used to evaluate the statistical significance of disease-free survival by the Kaplan–Meier method for univariate analysis. Finally, log-plots will be used to estimate survival function to test the proportional hazards assumption. The Wald test will be used for Cox proportional hazards regression analysis, and data will first be tested to ensure they meet the assumptions for using the Cox test.

This work is on going and has been slightly delayed to accommodate the new antibodies were validated and are discussed in the "statement of work – task 1 section" that may be molecular biomarkers. In addition, new preliminary data from our laboratory and others, has demonstrated that mice lacking *Sirt3* exhibit increased ROS, including superoxide levels (O_2^{-}), due to: (1) the aberrant acetylation of oxidative phosphorylation complex I-III proteins; and (2) the aberrant regulation of MnSOD ROS scavenging activity (Tao et al, 2010, *Molecular Cell*, Appendix A) that may be early steps in carcinogenesis. In addition, we and others have also shown that cells lacking *Sirt3* also exhibit increased HIF-1α as well as the expression of HIF-1α-dependent pro-proliferative proteins resulting in cellular metabolic reprogramming (Warburg effect).

I. Sirt3 directs cellular metabolic reprogramming that mirrors the Warburg effect

Tumor cells exhibit reprogramming of cellular metabolism involving an increase in glucose consumption described by Otto Warburg 60 years ago and referred to as the "Warburg effect." Interestingly, any underlying cellular processes linking aberrant mitochondrial metabolism and increased ROS to the Warburg effect have, until recently, remained unclear. In this regard, we have shown that Sirt3$^{-/-}$ cells exhibit increased glucose uptake that is preferentially used to make ATP as a result of what appears to be the aberrant acetylation and decreased activity of ETC complex III[5,6]. It has been previously suggested that mitochondrial ROS can induce HIF-1α activity[7-9], and cells lacking Sirt3 exhibit increased ROS. Thus, is there a connection between Sirt3, HIF-1α, and changes in metabolic reprogramming? To address this issue we showed that Sirt3$^{-/-}$ MEFs exhibit increased HIF-1α protein levels (Fig. 5a, lane 1 vs. 4) as well as HIF-1α dependent gene expression, as shown by co-transfection assays using an HRE luciferase reporter (Fig. 4b, bar 1 vs. 2). In addition, exposure to NAC, which scavenges ROS, prevents the increase in HIF-1α levels (Figs 4a, lane 4 vs. 5) and HIF-1α dependent gene expression (Fig. 4b, bar 2 vs. 4).

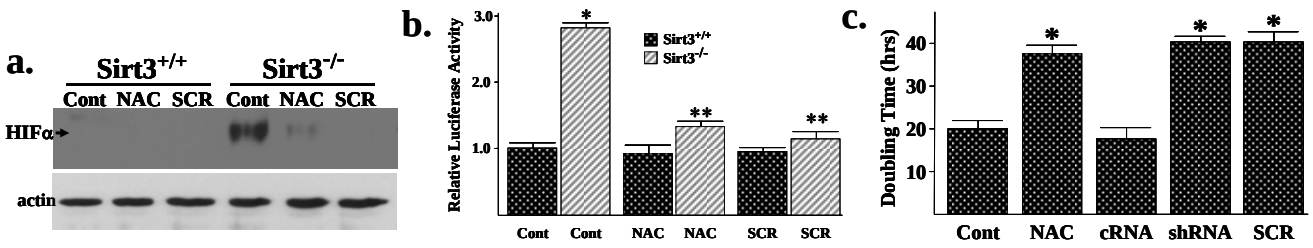

Figure 4. Loss of Sirt3 increases HIFα protein levels and gene expression via a ROS-dependent mechanism. (a) Cell extracts from Sirt3$^{+/+}$ and Sirt3$^{-/-}$ MEFs, with and without exposure to either NAC or stigmatellin (SCR), were blotted with an anti-HIF-1α antibody. **(b)** MEFs were co-transfected with p3x-HRE-luciferase with or without NAC or stigmatellin and 40 hours afterwards luciferase levels were determined. **(c)** MTCLT3$^{-/-}$ Cells were exposed to NAC, control shRNA, HIF-1α shRNA, or stigmatellin and cell doubling times were determined. All the experiments for this figure were done in triplicate. * indicates $P < 0.05$ by t-test.

As such, we now have scientific data demonstrating that cells lacking *Sirt3* exhibit decreased ETC complex III activity that may play a critical role, at least in part, in the elevated cellular ROS levels. Stigmatellin is a chemical agent that binds to complex III, inhibits electron transfer, and prevents generation of ROS[5]. Exposure of Sirt3$^{-/-}$ MEFs to stigmatellin decreased HIF-1α levels (Fig. 4a, lane 4 vs. 6) and HIF-1α gene expression (Fig. 4b, bar 2 vs. 6). In addition, tissue culture cells were also treated with either NAC or stigmatellin and these results clearly show that both agents decreased the growth rate of mammary tumor cells lacking *Sirt3* (Fig. 4c, lane 1 vs. 2 and 5). Finally, cells were treated with either control shRNA or HIF-1α shRNA and decreased HIF-1α protein levels were observed (data not shown) as well as an increase in the tumor cell doubling time (Fig. 4c, lane 3 vs 4). Decreases in several other *in vitro* transformation properties including growth in soft agar, foci formation, and tumor formation, were seen in nude mice (data not shown).

Thus, we propose a model where an increase in ROS and HIF-1α results in a phenotype favoring both genomic mutations (Fig. 5. blue arrow) and proliferation (purple arrow) that are established early driving events for breast tumorigenesis. Thus, it is hypothesized that ROS and/or HIF-1α may be molecular

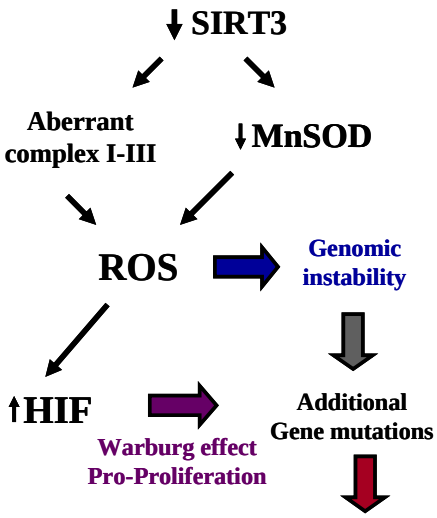

Figure 5. Cancer Model

targets for therapeutic intervention. As such, we propose that mice lacking Sirt3 are a novel in vivo model in which to investigate the well-established connection between decreased SIRT3 levels and ROS, HIF-1α, the Warburg effect, and breast malignancies. We would propose that agents thought to scavenge or decrease cellular ROS (O_2^-) and/or inhibit HIF-1α activity will prevent tumors in Sirt3 knockout mice. If this is true, there is a subgroup of human breast cancers with decreased SIRT3 expression or enzymatic activity for which these agents may be chemopreventive or cytotoxic on their own and/or synergize in combination with chemo- or radiation therapy. This work is being investigated in the laboratory falls under task number 3.

Over the last 10 years it has been suggested that there our four major subgroups of breast malignancies (See Prevalence Table) as determined by genetic biomarkers. Our preliminary data suggests that the tumors observed in the mice lacking *Sirt3* may be genetically similar to the luminal B cancer or more likely a subgroup of this classification that comprises roughly 15% of all breast cancers[10-12]. As such, a critical aspect of our research proposal as outlined in task 3 is the identification of a subgroup of human breast cancer tumors where loss of *SIRT3* is an early driving or initiating event in carcinogenesis. Thus, the relationship of SIRT3 expression to clinicopathologic parameters—age, tumor grade, stage, mitotic count, ER and PR status, as well as response to therapy, local tumor control, disease free survival, and overall survival is being determined with a series of biomarkers that are discussed above. These include the new antibodies that are discussed above ($OSCP^{K139}$, $MnSOD^{K68}$, and $MnSOD^{K122}$) as well as HIF-α, and the markers shown in the table above. With the recent validation of these antibodies as well as the identification of HIF-α, we have been staining both commercial human breast cancer arrays as well as the human samples in the Vanderbilt Breast Cancer SPORE sample archive. Thus, we hope to identify a subgroup of human breast cancer tumors where loss of SIRT3 is an early driving or initiating event in carcinogenesis that has a proteomic signature that also includes increased ROS and HIF-1a that is likely to be a subgroup of "Luminal B" breast malignancies. Thus, these experiments are intended to begin the scientific process of a more extensive biological characterization of the proposed *Sirt3* knockout mouse and its potential use as a model of the subtype of mammary tumors that are observed in older women. This work is on going and it is likely that a manuscript containing these results should be ready to send out some time in the summer of 2012.

Subtype	Tumor Biomarkers	Prevalence
Luminal A	ER+ and/or PR+, HER2- low Ki67	42 – 59%
Luminal B	ER+ and/or PR+, HER2+, or HER2- high Ki67	6 – 19%
TN/basal-like	ER-, PR-HER2-CK5/6+, or HER1+	15 – 20%
HER2+	ER-, PR-, HER2+	7 – 12%

II. There is a subgroup of breast tumor expressing hyper-acetylated MnSOD

The connection between MnSOD expression and breast carcinogenesis has been published by others[23,24] as such, we determined if there is a subgroup of human breast tumors that contain hyper-acetylated MnSOD as well as increased superoxide levels. To address this idea it was determined if there are breast tumor samples that will staining positively with our two anti MnSOD-Ac-K68 and MnSOD-Ac-K122 antibodies to identify breast tumor samples containing hyper- acetylated MnSOD suggesting decreased SIRT3 and MnSOD activity. As such, 10 ER+ breast tumor were stained with Sirt3, anti-acetyl-$MnSOD^{K122}$,or -$MnSOD^{K68}$, merged and several samples exhibited a decreased in Sirt3 immuno-reactive protein with a corresponding increase in anti-acetyl-MnSOD staining (Fig. 4a) that is representative of 4 of the 10 ER+ samples (data not shown) examined by IHC staining. This was also seen when immunoreactive staining was down with the anti-acetyl-$MnSOD^{K68}$ antibody

(Fig. 4b) and with and anti-SIRT3 antibody (data not shown, Appendix 3). These results suggest that these MnSOD antibodies work in human samples and also suggest that there may be a subgroup of ER+ human breast tumors that exhibit hyper-acetylation of MnSOD and by extension a decrease in enzymatic detoxification activity and increased intracellular superoxide levels.

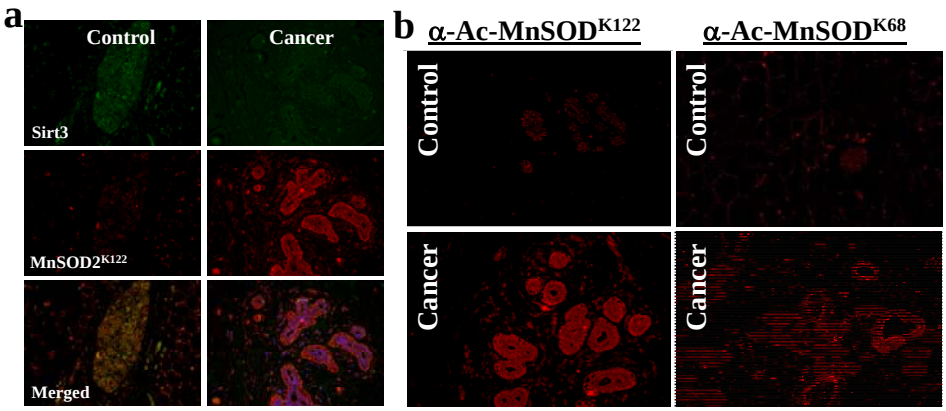

Figure 4. A subgroup of breast tumors exhibiting increased IHC staining with the anti-MnSOD-Ac-K68 / 122 antibodies. (a) Two consecutive slices of normal breast as well as breast tumor were stained with an anti-Sirt3 (data not shown) or the anti-MnSOD-Ac- antibodies and the IHC images were merged. **(b)** TMA's of normal breast as well as breast tumors samples were processed and IHC staining was done with an anti-Ac-MnSOD^{K122} or anti-Ac-MnSOD^{K68} antibody.

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 a publically available breast cancer expression database[25,26]. In this study breast cancer patient samples were subdivided 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 any potential relationship to relapse free survival. Remarkably, low SIRT3 expression is found to be associated with significantly reduced survival in Luminal A (ER+ and/or PR+, HER2/neu-)(Fig. 5a), Luminal B (ER+ and/or PR+, HER2/ neu+ (Fig. 5b), HER2+ (Fig. 5c). Thus, the experiments outlined above are a proof-in-principle that theses anti-acetyl-lysine antibodies, as well as decreased SIRT3 expression, may be used as potential molecular biomarkers that may identify a specific subgroup of ER+ breast cancer patients that may benefit for agents that reduce and/or scavenge superoxide.

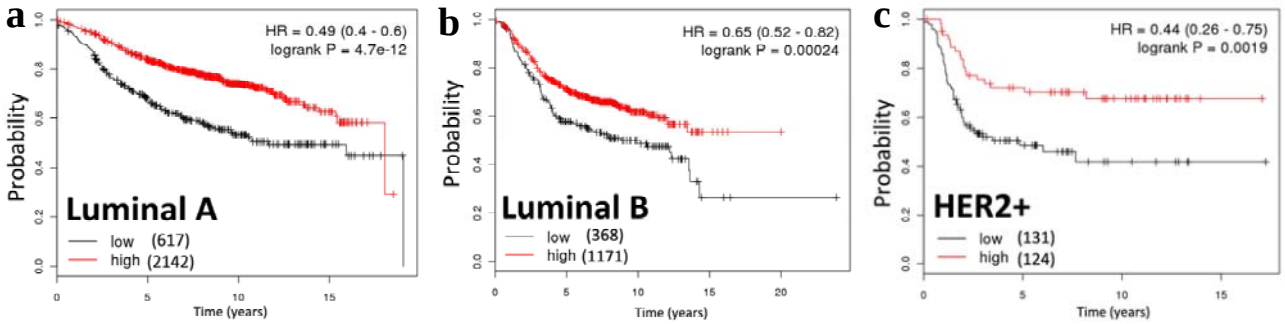

Figure 5. Analysis of KM Plotter database shows that low expression or SIRT3 is associated with poor outcome (relapse free survival) in subtypes of breast cancer. The numbers of samples in each group are indicated in parentheses, and the hazard ratios (HR) and log rank p values are shown. Data is only shown for Luminal A-B and Her2+ subgroups.

KEY RESEARCH ACCOMPLISHMENTS:

1. The identification of MnSOD as a legitimate Sirt3 deacetylation target that for the first time shows that post-translation modifications of mitochondrial proteins plays a role in mitochondrial detoxification. This work was published in *Molecular Cell*.
2. Sirt3 is activated in vivo by 36 hours of fasting showing for the first time that Sirt3 is a nutrient distress protein.
3. Sirt3 is the first mitochondrial tumor suppressor protein that may play a role in the development of luminal B breast malignancies.
4. Sirt3 regulates the ATP synthase complex and the aberrant regulation of ATP synthase may play a role, at least in part, in breast cancers that are similar to luminal B human breast malignancies.
5. Tumors lacking Sirt3 may require the dysregulation of HIF-1α suggesting that there may be a sub-group of human tumors that may response to HIF-1α inhibitors in both chemoprevention as potentially as anti-cancer agents.
6. The identification of MnSOD function as both a molecular mechanism in breast cancers as well as the suggestion that agents that activate MnSOD or scavenge superoxide may be agents in chemo-prevention. In addition, these agents may also be anticancer in luminal B breast tumors.

REPORTABLE OUTCOMES:

Animal models supported by this award:

1. The Sirt3 knockout mouse supported by this award is now available to other researchers who wish to work on ER+ murine breast cancer models.

Training supported by this award

1. Ozkan Ozden, Ph.D. (2010 - present). Post Doctoral Fellow.
2. Seong Park, Ph.D. (2010 – present). Post Doctoral Fellow.

CONCLUSION:

The incidence of human malignancies increases significantly with age, suggesting a mechanistic connection between aging (longevity) and carcinogenesis. Breast cancer has a very specific age-related incidence that increases until menopause and then increases more slowly until the mid 60's, when a second significant increase in incidence is observed. Breast malignancies that develop over time very likely represent a spectrum of cancers arising from different types of hereditary and spontaneous tumors. Murine models for hereditary breast cancer in younger women have been established by altering the expression of BRCA genes, and these mice develop triple negative (ER/PR negative, her2neu) tumors. In contrast, there are no murine models for the spontaneous breast cancers that are more common in older women. The work supported by this DOD idea award has allows us to not only validate the first ER+ in vivo murine model for breast cancers but is has also allowed the discovery of several potential molecular targets that may potential be used for either chemoprevention or as anticancer agents. We have also developed three antibodies that along with the human SIRT3 antibody that will allow us to determine potential sub-groups of women with breast cancer that may benefit from specific anticancer therapies. In addition, these antibodies will allow the further sub-classification of women with breast malignancies that other researcher may find helpful in their own research into the mechanisms of both breast carcinogenesis as well as the potential development of anticancer agents.

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