Manganese Superoxide Dismutase (MnSOD):
A Driver of Metabolism Critical for Breast Cancer Progression

BY

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THESIS

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This dissertation is dedicated to my father and mother.
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LIST OF ABBREVIATIONS

MnSOD/SOD2 - manganese superoxide dismutase

ROS - reactive oxygen species

H$_2$O$_2$ - hydrogen peroxide

Cav-1 - caveolin-1

Nrf-2 - nuclear factor (erythroid-derived 2)-like 2

Keap1 - Kelch-like ECH associated protein 1

AMPK - AMP-activated kinase

PFK2 - phosphofructokinase 2

LDH - lactate dehydrogenase

GPx1 - glutathione peroxidase

OXPHOS - oxidative phosphorylation

OCR - oxygen consumption rate

ECAR - extracellular acidification rate

TMX - tamoxifen

IDC - invasive ductal carcinoma
SUMMARY

Manganese superoxide dismutase (MnSOD) is a critical mitochondrial resident enzyme responsible for the conversion of the mild oxidant superoxide anion (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$), a highly potent and freely diffusible reactive oxygen species (ROS). The current study identifies MnSOD overexpression as both necessary and sufficient to promote glycolytic metabolism requisite for malignant transformation and tumor progression in breast cancer. This novel pro-tumorigenic role of MnSOD is dependent on its product, mtH$_2$O$_2$, which acts as a primary signaling molecule resulting in the activation of AMP-activated kinase (AMPK) to initiate the glycolytic shift. We further show that MnSOD overexpression in breast cancer is due to enhanced Nrf-2 transcriptional activity as a consequence of the loss of Caveolin-1 (Cav-1), and describe novel biomarkers which may be critical in patient risk stratification as well as the assessment of therapies targeted towards highly glycolytic tumors.
I. INTRODUCTION

A. Background

i. Cancer metabolism and oncogenesis

The molecular underpinnings of metabolic reprogramming that promote tumor initiation and progression have been recently realized as critical in the etiology of lethal breast cancer. It has been observed that mitochondrial regulation of cellular events related to metabolism (Warburg 1956, Mullen, Wheaton et al. 2012, Woo, Green et al. 2012) as well as related to pro-oncogenic processes, such as de-differentiation (Tormos, Anso et al. 2011) and survival (Brunelle, Santore et al. 2004, Majewski, Nogueira et al. 2004), may be essential in the prevention and treatment of breast cancer (Bell, Klimova et al. 2008). One central cellular process that is well exploited by progressing and aggressive tumors to utilize glucose is known as aerobic glycolysis (Warburg Effect) for the generation of macromolecules requisite for rapid proliferation and survival [see (Gatenby and Gillies 2004) for review]. This metabolically driven adaptive response is accompanied by localized acidosis, an additional measure of growth advantage exploited to ensure stable clonal expansion of highly deregulated and adapted tumor cells. The implication of these events to the promotion of heterogenous neoplasia possessing a subset of cells with the capacity to initiate tumors and ultimately metastasize is not well described; however, the notion that tumorigenic cells exploit glycolytic pathways presents an attractive target for the development of novel diagnostic tools and therapeutic compounds required to circumvent the severity and lethality of the disease. The identification of how mitochondria, and its key constituents, may drive tumor development will be critical in isolating and exploiting these mechanisms for the benefit of improving clinical diagnosis and selective targeted therapy.
ii. Human breast cancer and metabolism

Breast cancer is a disease whose prevalence impacts 1 in 8 women, and nearly a quarter of a million women are estimated to be diagnosed with some form of breast cancer (ACS 2015). Advanced stage and aggressive breast cancer will result in 40,000 deaths this year (ACS 2015). Invasive ductal carcinoma is the predominant histological phenotype of the disease, making up 80% of total cases, and is characterized by the hyperplastic growth and dedifferentiation of ductal epithelial cells (ACS 2015, NCI 2015). These aberrant growths are removed upon diagnosis; however, an estimated 6-10% of patients present advanced stage or metastatic disease (NCI 2015), and up to 20% of patients present a basal-like triple negative subtype (ACS 2015), a molecular subtype that is highly resistant to conventional chemotherapeutic targeting that often results in recurrent and lethal disease (Hudis and Gianni 2011, Pogoda, Niwinska et al. 2013). Like several other forms of cancer, a striking molecular phenotype and microenvironment adopted by aggressive and metastatic mammary tumors involves predominantly glycolytic metabolism to bolster proliferation and survival (Hennipman, van Oirschot et al. 1988, Gatenby and Gillies 2004). While the core of developed tumors tend to become hypoxic, well vascularized peripheral tumor cells utilize this metabolism even in the presence of oxygen, a phenomenon known as the Warburg Effect (Warburg 1956, Gatenby and Gillies 2004, Vander Heiden, Cantley et al. 2009).

iii. The Warburg Effect and the role of mitochondria in tumor progression

The identification that tumor cells in vitro would preferentially utilize glycolysis was first proposed by Dr. Otto Warburg (Warburg 1956) [also see (Racker and Spector 1981) for review], in which tumor cells had apparent affinity for this type of metabolism in spite of plentiful oxygen. Differentiated somatic cells will engage glycolytic metabolism under oxygen deprivation, such as
anoxia or hypoxia, in order to maintain ATP levels to compensate for loss of oxidative phosphorylation (Gatenby and Gillies 2004). Under normal conditions, the rates of glycolysis are limited as allosteric regulation by ATP inhibits the activity of several key glycolytic enzymes, including AMP-activated kinase (AMPK) (Hardie, Ross et al. 2012) and phosphofructokinases (PFK1 and PFK2)(Dobson, Yamamoto et al. 1986, Berg JM 2002). During oxygen deprivation in normal cells, ATP stores are rapidly lost as a result of inhibited mitochondrial respiration, thus enabling glucose utilization in order to restore energy balances to sustain ATP-dependent cellular functions(Berg JM 2002, Gatenby and Gillies 2004, Wilson and Hay 2011). In contrast, it has been observed that tumor cells will utilize glycolysis independent of the levels of O₂ and abundance of ATP (Conley, Kushmerick et al. 1998). Exhaustive interrogation of this phenomenon elucidated that while normal cells under hypoxia would use glycolysis to maintain ATP synthesis required for survival, tumor cells under normoxia would utilize glycolytic metabolism in order to generate macromolecules to promote biosynthetic pathways required for rapid proliferation (Gatenby and Gillies 2004, Chiarugi, Dolle et al. 2012). Notably, glycolytic metabolism is often preferentially used in invasive and metastatic tumors (Hennipman, van Oirschot et al. 1988, Gatenby and Gawlinski 1996, Younes, Lechago et al. 1996, Gatenby and Gillies 2004), and is associated with poor prognosis (Gatenby and Gillies 2004). In addition to facilitating growth and proliferation (DeBerardinis, Lum et al. 2008), aerobic glycolysis regulates other important adaptive processes in cancer progression, including the induction of pluripotency (Folmes, Nelson et al. 2011), epithelial-to-mesenchymal transition (EMT) (Dong, Yuan et al. 2013), metastasis (Younes, Lechago et al. 1996), and drug resistance (Xu, Pelicano et al. 2005). Thus, an understanding of how and why glycolysis occurs is central to the development of therapeutics to prevent tumor progression. Contrary to the original concept that glycolysis in tumor cells is a direct result of
dysfunctional mitochondria, recent evidence indicates that in many cases tumor cells do possess mitochondria capable of oxidative phosphorylation (Lunt and Vander Heiden 2011). This metabolic flexibility allows for tumor cells to utilize glycolytic pathways in order to reroute metabolic substrates required for synthesis of proteins and lipids while simultaneously maintaining mitochondrial respiration to provide ATP to meet energy requirements for cell growth. The exact mechanisms by which glycolysis and mitochondrial respiration can be concurrently used by tumor cells is still poorly defined; however, it is clear that several oncogenes contribute to this unique metabolism acquired by aggressive cancers (Gordan, Thompson et al. 2007, Qing, Skuli et al. 2010, Zheng 2012, Semenza 2013). In the current project, we propose that manganese superoxide dismutase (MnSOD) acts as a tumor promoter in breast cancer through signaling by its product, mtH₂O₂, resulting in aberrant redox signaling that bolsters glycolytic metabolism to promote tumor progression.

iv. Redox signaling and oxidative stress in cancer

Recent evidence suggest that redox signaling in cancer may play several roles throughout the disease, effecting tumor initiation, growth and metastasis (Forristal, Wright et al.). Cancer cells have been shown to generate high levels of ROS such as superoxide and hydrogen peroxide, which may both bolster tumor progression (Liou and Storz 2010). The observation that MnSOD is often expressed at high levels in aggressive cancers (Cobbs, Levi et al. 1996, Kahlos, Anttila et al. 1998, Quiros, Sainz et al. 2009, Sgambato, Camerini et al. 2009), suggest its potential impact on cancer development is likely through its product, hydrogen peroxide (H₂O₂). H₂O₂ and other ROS have been demonstrated as a potent signaling molecules, as protein oxidation on key residues (e.g., cysteine, methionine), although reversible depending on the extent of oxidation, can result in
altered or depleted function of proteins that could impact a variety of pathways (Bonini, Consolaro et al. 2014). The oxidation of susceptible residues can inhibit enzymatic function of proteins by interfering with substrate binding or other protein:protein interactions necessary for catalytic activity, and can also lead to complete degradation (Bonini, Consolaro et al. 2014). Further, ROS accumulation has been demonstrated to cause the stabilization and enhanced signaling of HIF-1α, a transcription factor that upregulates several glycolytic enzymes, such as glucose transporter (GLUT1), hexokinase (HK), phosphofructokinase (PFK1 and PFK2) and lactate dehydrogenase (LDH) (Chandel, Maltepe et al. 1998, Chandel, McClintock et al. 2000, Bell and Chandel 2007, Krohn, Link et al. 2008), thereby inducing glycolysis in response to redox imbalances caused by inadequate clearance of H₂O₂. Together, these findings supported the concept that MnSOD may be central to the enhancement of H₂O₂-dependent signaling that enhance glycolytic metabolism, thus bolstering tumor progression and aggressiveness.

v. **Manganese superoxide dismutase (MnSOD): Function, regulation and the controversy about its roles in cancer**

Several superoxide dismutase enzymes exist in the cellular milieu, including the cytosolic SOD (SOD1), the mitochondrial SOD (MnSOD/SOD2), and the extracellular matrix SOD (SOD3) (Weisiger and Fridovich 1973, Weisiger and Fridovich 1973, McCord and Fridovich 1978, Zelko, Mariani et al. 2002). Manganese superoxide dismutase (MnSOD/SOD2) is a key mitochondrial protein involved in the dismutation of superoxide anion (O₂⁻) to hydrogen peroxide (H₂O₂) (Weisiger and Fridovich 1973, McCord and Fridovich 1978, Sevilla, Lopez-Gorge et al. 1980). This conversion results in the alleviation of the mild oxidant superoxide to the nonpolar, diffusible strong oxidant hydrogen peroxide (McCord and Fridovich 1978). The generation of O₂⁻ occurs
due to incomplete reduction of $O_2$ during oxidative phosphorylation at the electron transport chain (Fridovich 1978, Barja 1999, Brand, Affourtit et al. 2004, Drose and Brandt 2008), in which several of the enzymatic complexes yield $O_2^{-}$ formation during the transport of electrons towards ATP synthase, the final enzyme that utilizes this electron flux to produce ATP at relatively low cost from cellular pools of NADH and other rate limiting molecules (Beal 2005). The elimination of $H_2O_2$ that results from MnSOD dismutation of $O_2^{-}$ is typically resolved in the mitochondria by glutathione peroxidases (Mills 1957) and in the peroxisome by catalase (Chelikani, Fita et al. 2004). Interestingly, the balance between MnSOD expression and the expression of peroxidases (e.g., GPx1, catalase) is largely lost in progressing tumors, as MnSOD:GPx1 or MnSOD:catalase ratios has been shown to increase in progressive lung, colon and prostate cancer tissue (Miar, Hevia et al. 2015), indicating a loss of compensation in addition to the overexpression of MnSOD result in aberrant signaling that may promote tumor progression. This tight regulation of oxygen/reduction [redox] status is becoming increasingly important in the appreciation of the impact of mitochondrial bioenergetics on deleterious clonal expansion [e.g., fixation of premalignant or malignant cells; (Gasparre, Hervouet et al. 2008, Wallace 2012)] and endow \textit{de novo} tumor cells with the capacity to invade local tissue (Suh, Arnold et al. 1999, Porporato, Payen et al. 2014) or to extravasate and metastasize (Goh, Enns et al. 2011, Porporato, Payen et al. 2014).

In parallel to a predominant utilization of glycolytic metabolism (Gatenby and Gillies 2004), advanced grades and stages of breast cancer tend to have enhanced expression of MnSOD(Tsanou, Ioachim et al. 2004, Kumar, Loo et al. 2014). It is clear that the impediment of routes to remove its product, $H_2O_2$, are necessary for the function of MnSOD to proceed from a tumor suppressive to a tumor promoting role in breast cancer (Miar, Hevia et al. 2015). Indeed, $H_2O_2$ signaling has been associated with a myriad of mechanisms that endow tumor cells with the
capacity for invasion (Suh, Arnold et al. 1999, Porporato, Payen et al. 2014) and metastasis (Goh, Enns et al. 2011, Porporato, Payen et al. 2014). Therefore, it had become critical to understand how the mitochondrial enzyme MnSOD may regulate the tumorigenicity and metastatic potential of de novo and progressing tumor cells.

The interrogation of the role of MnSOD to cancer progression has yielded inconclusive results. Although its enzymatic function as an antioxidant would indicate its role as a tumor suppressor, it has been shown to be involved in both suppressing and promoting tumor progression, as described below. Here, we examine the integral role of MnSOD, contexts in which it can function as either anti- or pro-tumorigenic, and suggest other levels of regulation that are still poorly understood which may elucidate mechanisms to exploit in order to prevent oncogenic properties of the enzyme.

vii. Context dependent dualistic role of MnSOD as either a tumor suppressor or promoter

MnSOD has historically been considered as a strict anti-oxidant enzyme responsible for the alleviation of potentially harmful superoxide anion \( \text{O}_2^- \). Under normal conditions, MnSOD dismutates \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \), which is then further reduced by GPx1 or other peroxidases (e.g., thioredoxin, peroxiredoxin) to \( \text{H}_2\text{O} \) and \( \text{O}_2 \), essentially removing the oxidative insult within the mitochondrial matrix (Miriyala, Spasojevic et al. 2012, Miar, Hevia et al. 2015). In line with this, complete ablation of MnSOD results in neonatal fatality due to excessive and irreversible oxidative damage (Li, Huang et al. 1995), indicating its critical role in cellular redox homeostasis. Furthermore, the diminished expression of MnSOD has been associated with promotion of tumor progression in several studies, to a certain extent. For example, overexpression of enzymatically
active MnSOD was shown to suppress tumor cell proliferation and survival in several contexts (Weydert, Roling et al. 2003, Ough, Lewis et al. 2004, Oberley 2005, Zhang, Smith et al. 2006, Echiburu-Chau, Roy et al. 2011). Intriguingly, indicators of tumor aggressiveness other than proliferative capacity are not as well described in regards to this potential tumor suppressive role of MnSOD, although some observations did indicate that MnSOD could reduce tumorigenicity (Church, Grant et al. 1993, Stclair 1996) and metastasis (Stclair, Wan et al. 1997) in certain contexts. Despite this compelling body of literature suggesting potential benefit to essential MnSOD expression, it is impossible to disregard mounting evidence that the overexpression of MnSOD occurs in aggressive breast (Tsanou, Ioachim et al. 2004, Kumar, Loo et al. 2014), prostate (Miar, Hevia et al. 2015), colon (Miar, Hevia et al. 2015) and lung (Svensk, Soini et al. 2004, Miar, Hevia et al. 2015) patient cancer tissue. Indeed, we had confirmed these observations that MnSOD overexpression may occur in a variety of contexts, such as advanced grade breast, colon and prostate cancers [see (Hart 2015), and further]. The notion that the product of MnSOD and other SODs [e.g., H2O2] may relate to tumorigenesis and metastasis is becoming more critically analyzed (Lopez-Lazaro 2007, Lisanti, Martinez-Outschoorn et al. 2011), and suggest that the cellular redox environment may drive critical processes involved in fixation of oncogenic mutations and cellular reprogramming necessary for tumor development. The current project confirms that enhancement of MnSOD is required for tumor progression, and delineates its central role in metabolic regulation necessary for this process.

viii. **Post-translational and genetic regulation of MnSOD in breast cancer**

Interrogating multiple facets of functional regulation of proteins has elucidated increasingly complex levels of regulation in a variety of mechanisms and critical cellular systems.
Interestingly, while there has been some analysis of the post translational regulation of MnSOD by acetylation on several key sites (Chen, Zhang et al. 2011), it remains unclear whether this level of regulation is sufficient to regulate the anti- or pro-tumorigenic capacities of the enzyme. It has been demonstrated that the deacetylation of the lysine residue 68 (K68) on MnSOD reduced the enzymatic activity of the enzyme by nearly 60% (Chen, Zhang et al. 2011). Intriguingly, the oxidant sensitive mitochondrial deacetylase, Sirt3, had been shown to directly enhance MnSOD enzymatic activity in response to ROS (Chen, Zhang et al. 2011); however, Sirt3 has been shown by several groups to be repressed in several types of cancer (Finley, Carracedo et al. 2011, Yang, Fu et al. 2014, Zhu, Yan et al. 2014). Given that the acetylation status of MnSOD is both transient and not sufficient to fully modulate its enzymatic activity(Chen, Zhang et al. 2011), it is clear that other regulatory processes may act in concert to direct the actions of MnSOD in the mitochondria. Overall, while this and other levels of regulation likely contribute to the role of MnSOD in tumor progression, there is currently insufficient understanding of how post translational modification may influence the enzyme in either an anti- or pro-tumorigenic function.

Similarly, the genetic variation of MnSOD has been somewhat described in nested case-control studies, suggesting a potential allelic variation of amino acid valine to alanine in its mitochondrial targeting sequence (Val16Ala) that has been observed to occur in some breast cancer patients (Ambrosone, Freudenheim et al. 1999). This variation is suggested to play a role in the trafficking and possibly the enzymatic activity of the enzyme (Shimoda-Matsubayashi, Matsumine et al. 1996). Patients with the homozygous alanine allele were found in multiple studies to not be at increased risk of breast cancer (Tamimi, Hankinson et al. 2004, Silva, Cabral et al. 2006), it several studies of this polymorphism led to inconclusive results as certain populations of men were at significantly higher risk of early onset prostate cancer (Arsova-Sarafinovska,
Matevska et al. 2008) while there was no significant impact of the allelic variation alone in other cohorts (Mikhak, Hunter et al. 2008); however, in the latter study it was observed that patients homozygous for the alanine allele with low antioxidant intake (e.g., lycopene), suggesting that the functional regulation of MnSOD along with systemic antioxidant balance may impact cellular redox status that effects risk of developing cancer. Interestingly, the variation of a compensatory mitochondrial enzyme responsible for detoxification of mtH$_2$O$_2$ resulting from MnSOD, glutathione peroxidase (GPx1), has also been associated with a non-significant enhancement of risk in a subset of individuals who possess several GCG repeats (Knight, Onay et al. 2004). While GPx1 activity was determined to be suppressed in erythrocytes of patients with prostate cancer, a polymorphism in the GPx1 (Pro198Leu) did not impact its enzymatic activity or modulate prostate cancer risk (Erdem, Eken et al. 2012), and similarly had no apparent impact on breast cancer risk (Cox, Hankinson et al. 2004, Hu, Zhou et al. 2010). Thus, these genetic variations in two key mitochondrial proteins responsible for redox homeostasis were not independently relevant to the risk assessment of patients with breast cancer; however, patients who possessed both “at-risk” alleles (e.g., MnSOD Val16Ala and GPx1 Pro198Leu) showed a nearly two fold increased risk of developing the disease (Cox, Tamimi et al. 2006). Taken together, these studies suggest that tight regulation and complex interactions of mitochondrial enzymes involved in redox homeostasis may be a central to tumor development. While the understanding of how these genetic variations truly affect cellular redox status mechanistically are entirely unclear, in addition to a well defined etiologic occurrence, these levels of modification and their impact on MnSOD function suggest that even moderate alterations in the rate and type of ROS occurring in the mitochondria may play a distinctively critical role in the suppression or promotion of tumor progression.
The current study focuses on the expression of MnSOD and the resulting egression of its product H₂O₂ from the mitochondria. While the aforementioned levels of complexity are likely critical in its contribution to tumor development and aggression, an exhaustive analysis of these potential contributing factors is beyond the scope of the current project and require further study.

ix. **Transcriptional upregulation of MnSOD may be enhanced by caveolin-1 (Cav-1) loss**

To understand the capacity for tumor cells to upregulate MnSOD to an extent which could provide molecular and metabolic reprogramming to bolster tumor progression, it became clear that interrogating the regulation of its expression would provide an additional level of complexity necessary to exploit for drug and biomarker development. Three transcription factors have been well appreciated to enhance MnSOD expression via interaction with its antioxidant response element (ARE) in its promoter region, including NFκB (Schulze-Osthoff, Los et al. 1995, Darville, Ho et al. 2000, Chow, Yen et al. 2002), FOXO3a (Kops, Dansen et al. 2002) and Nrf-2 (McDonald, Kim et al. 2010). It was hypothesized that the enhanced oxidative environment caused by acutely heightened MnSOD activity may promote a progressive positive feedback loop of chronic MnSOD overexpression; therefore, we chose to analyze the oxidant-sensitive nuclear factor (erythroid-derived 2)-like 2 (Nrf-2). Notably, it had been shown that a regulatory complex of Nrf-2 and its negative regulator, Kelch-like ECH associated protein 1 (Keap1), could be enhanced by the binding of an additional integral plasma membrane protein, Caveolin-1 (Cav-1), to inhibit Nrf-2 transcriptional activity (Volonte, Liu et al. 2013). Cav-1 is a key constituent of caveolae, forming lipid raft associated vesicles in cholesterol-rich portions of plasma membrane that are responsible for the endo- and trans- cytosis of molecules (Rothberg, Heuser et al. 1992, Lisanti, Scherer et al.
It had been demonstrated that Cav-1 may be lost in certain compartments of heterogenous breast tumors, most specifically in the stromal compartments (Pavlides, Tsirigos et al. 2010). Although it was suggested by this group that little change occurred in epithelial tissue expression of Cav-1 in patient samples or that epithelial Cav-1 expression had clinical relevance (Pavlides, Tsirigos et al. 2010), extensive analysis of several tissue micro-arrays during the current study indicated that Cav-1 expression was indeed highest in healthy mammary epithelium and severely repressed in advanced breast cancer epithelial tissue. Combined with evidence suggesting the overexpression of Nrf-2 in breast cancer (Hayes and McMahon 2009), it became clear that the relationship between Cav-1 and Nrf-2 was potentially critical in the regulation of MnSOD, and ultimately the mitochondrial redox homeostasis that was suspected to be instrumental to metabolic reprogramming and tumor progression. For these reasons, we chose to interrogate whether and how this potential relationship between Cav-1 and Nrf-2 may relate to the aberrant overexpression of MnSOD in breast cancer.

B. Statement of hypothesis

In the current studies, we have tested the hypotheses that: 1) MnSOD overexpression in breast cancer promotes an AMPK-dependent pathway that enhances the Warburg effect as an adaptive response to facilitate tumor progression; and that, 2) regulation of MnSOD may prevent the exploitation of this pathway that would otherwise lead to highly lethal molecular phenotypes. As glycolysis is a well appreciated hallmark of the endowment of tumors to proceed to malignancy and lethality (Gatenby and Gillies 2004), uncovering novel therapeutic targets and biomarkers related to these potential therapeutics is essential in our understanding of the progression and treatment of the disease. Mitochondrial regulation of metabolism and its relationship to oncogenic
transformation and metastatic potential are becoming recognized as indispensable in the understanding of tumor development. Despite this, the critical understanding of the prototypic antioxidant MnSOD has led to an impasse in resolving how this key mitochondrial enzyme may play a dualistic role in cancer progression, fulfilling a role as either a tumor suppressor or promoter strictly dependent on the tumor milieu. Here we propose that the overexpression of MnSOD, concurrent with the lack of compensatory mechanisms to remove its product, leads to the enhancement of glycolytic metabolism that bolsters the progression of breast cancer.

C. Significance

This current study identifies the deregulation of mitochondrial redox status as a critical priming factor for the selection of highly aggressive tumor cells. The evidence presented here indicates a novel role of a critical "antioxidant" in establishing a novel niche in which tumor cells can utilize both oxidative and glycolytic metabolism, enhancing a molecular plasticity whose growth advantage is critical in the aggressive advancement of the disease. Further, this project elucidates several therapeutic targets for the development of novel specialized compounds, and describes biomarkers with the potential for determining the efficacy of such targeted therapies.
II. METHODS

*Cell culture-* MCF-7 cells constitutively expressing an empty vector (neo) or Ile$^{58}$MnSOD on the pcDNA3 vector (Mn1, Mn44, Mn11 and Mn28) were generated by Dr. Larry Oberley, University of Iowa (Zhang, Yan et al. 1999). MCF-7 cells expressing AMPKα1 shRNA or scrambled RNA (scrRNA) were obtained from Dr. Kevin P. Claffey, University of Connecticut Health Center. The cells were cultured in either RPMI 1640 medium or DMEM medium (Invitrogen, Grand Island, NY), respectively, supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1% Antibiotic-Antimycotic (Invitrogen) and selection was maintained with G418 (200mg/L) or puromycin (1mg/L), respectively (Sigma Aldrich, St. Louis, MO). MCF-7 cells stably expressing Cav-1(c-myc) were generated by Dr. Ayesha Shajahan (Shajahan, Wang et al. 2007), Georgetown University, and maintained in MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). MDA-MB231 cells were cultured in DMEM:F12 supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin (Invitrogen). MCF10A(Er/Src) cells were a generous gift from Dr. Kevin Struhl, Harvard University, and grown in DMEM:F12 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin insulin (10mg/mL), human growth factor (20ng/mL), and hydrocortisone (500μg/mL). All cell lines were grown under 5% CO2 atmosphere at 37°C. Treatments with exogenous H$_2$O$_2$ (Sigma) were performed in serum-free medium for 20 min before replenishment with preconditioned medium. Treatments with Compound C, an AMPK inhibitor (Sigma) were performed in RPMI 1640 for 24 hours, then in serum free media for 24 hours.
**Mito-Catalase Transfection** - Mitochondrial targeted catalase (Ad5-CMV-MT-catalase) was obtained from Dr. J. Andres Melendez (State University of New York at Albany). MCF-7 cells were grown to 80% confluence in a 6-well plate in growth media with 10% FBS, and the mt-catalase adenovirus (Rodriguez, Carrico et al. 2000) was added to treatment wells in Opti-MEM without serum and allowed to incorporate for 24 hours. Media was then changed to fresh RPMI 1640 with 10% serum and then cells were incubated for an additional 48 hours. Cells were washed with PBS and protein lysates were collected for Western blot (see further).

**MnSOD/AMPK/Nrf-2 silencing** - Cells were grown to 80% confluence in a 6-well plate in growth media with 10% FBS. MnSOD, AMPK, or Nrf-2 and scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, TX) and transfected into cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. After 24 hours, media was replaced with fresh growth media for an additional 36-48 hours for sufficient knock down and collected for analysis by Western blot. For functional assays (e.g., glycolysis), at 24 hours post transfection, cells were seeded to 96 well plates and sub-cultured for an additional 36-48 hours prior to functional measurements.

**Nrf-2 luciferase reporter assay** - Cells were grown to 80% confluence in 6 well dishes, and then co-transfected with pNLFl-NRF-2[CMV/neo] and pKeap1 according to manufacturer’s protocol (Promega, Madison, WI). Transfection was performed using Continuum Transfection Reagent (Gemini Bio-Products, Sacramento, CA) and incubated for 16 hours. Cells were then seeded to 96-well white-walled plates and grown for an additional 48 hours. Nrf-2 stability was determined using Nano-Glo Luciferase Assay System (Promega), and luminescence was measured on a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). Relative
luminescent units (RLU) were then normalized to protein concentration as measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

**Western Blot Analysis**- Protein was analyzed by electrophoresis on 4-12% Bis-Tris gels followed by transfer to nitrocellulose membranes. The membranes were blocked in 5% milk/PBS for 1.5 hours at room temperature, washed with TBS-T three times for 5 minutes, and then incubated with primary antibody [rabbit anti-MnSOD, 1:1000 (Abcam, Cambridge, MA), rabbit anti-β-actin, 1:1000 (Cell Signaling Technology, Danvers, MA), rabbit anti-vinculin, 1:1000 (Cell Signaling Technology), rabbit anti-AMPKα1, 1:1000 (Abcam), rabbit anti-AMPKα1 (piThr172), 1:1000 (Abcam), mouse anti-LDH, 1:1000 (Santa Cruz), rabbit anti-catalase, 1:1000 (Abcam), rabbit anti-Caveolin-1, 1:1000 (Abcam), mouse anti-Caveolin-1, 1:1000 (BD Biotechnologies, Franklin Lakes, NJ), rabbit anti-Nrf-2, 1:500 (Santa Cruz), mouse anti-Keap1, 1:500 (Santa Cruz), mouse anti-GFP, 1:500 (Santa Cruz)] in TBS-T overnight at 4°C. After 3 washes, the IRDye secondary antibody for rabbit/mouse (LI-COR Biotechnology, Lincoln, NE) was incubated at 1:10,000 for 120 min. Membranes were washed three times for 10 min each and then analyzed by infrared detection using Odyssey Imaging System (LI-COR).

**Immunoprecipitation of Keap1/Nrf-2/Cav-1 complex**- The Cav-1/Keap1/Nrf-2 complex was co-immunoprecipitated from MCF-7 cells with ectopic stable expression of Cav-1. The direct method of immunoprecipitation was used, in which magnetic beads [Dynabeads M-280 anti-mouse or anti-rabbit IgG (Thermo Fisher Scientific)] were incubated with primary antibody [2µg of anti-rabbit Nrf-2 (Santa Cruz) or anti-mouse Keap1 (Abcam)] per sample) for 6h at 4°C under constant agitation. Unbound primary antibody was then removed by washing three times. Cell lysates were added to the Dynabead/ Antibody solution following treatment (400µM H₂O₂, 1h), and incubated at 4°C overnight under agitation. Samples were washed twice with PBS,
resuspended in 1X Laemmli sample buffer (BioRad, Hercules, CA) and then analyzed by Western blot.

*Human patient sample analysis by tissue micro-array*- Tissue micro-arrays Brca1503c and TMA-1005 used in this project were obtained from US Biomax (Rockville, MD) and Protein Biotechnologies (Ramona, CA), respectively. Slides were processed as described below in immunofluorescent microscopy. Relative fluorescent intensity (RFU) was measured using ImageJ, and RFU values were correlated with clinical histological and molecular subtypes. Representative images were selected by clearest association with the mean RFU within each category.

*Immunofluorescent microscopy*- Antigen retrieval was performed using Antigen Unmasking Solution (a 10mM Sodium Citrate buffer, Vector Laboratories, Burlingame, CA) and pressure cooked at 20 psi for 5 min in a Decloaking Chamber electric pressure cooker (Biocare Medical, Walnut Creek, CA). Slides were blocked with 5% bovine serum albumin (BSA) for 45 min and then incubated with primary antibody overnight [goat anti-MnSOD, 1:100 (Santa Cruz), rabbit anti-AMPKα1 (pThr172), 1:100 (Abcam), mouse anti-LDH, 1:100 (Santa Cruz), mouse anti-Caveolin-1, 1:100 (BD Biotechnologies), rabbit anti-Nrf-2, 1:500 (Santa Cruz)] in 1% BSA in 1% TBS-T at 4°C in a humid chamber. After rinsing in TBS-T, samples were incubated with AlexaFluor-568 anti-rabbit secondary antibody, AlexFluor-488 anti-mouse secondary antibody and AlexaFluor-647 anti-goat secondary antibody (Invitrogen) at 1:200 for 2 hours at room temperature, in a dark humid chamber. Slides were then incubated with 50μM DAPI (Invitrogen) for 30 min, mounted with FluoroMount Aqueous Solution (Sigma) and imaged on an Apotome microscope (Zeiss, Jena, Germany).

*Confocal microscopy*- Cells were plated onto MatTek 1.5mm glass-bottomed culture dishes (MatTek Corporation, Ashland, MA) and allowed to grow to 80% confluence. After
treatments were performed, confocal dishes were washed twice with PBS and then fixed with 4% paraformaldehyde for 15 min. After several washes, cells were permeabilized using 100% ice cold methanol for 15 min. Dishes were again washed with PBS three times for 3 min each, blocked using 5% BSA in 1% TBS-T for 45 min, washed (3 times for 3 min each) and then incubated with primary antibody [goat anti-MnSOD (Santa Cruz), rabbit anti-AMPKα1 (pThr172) (Abcam), mouse anti-LDH (Santa Cruz), mouse anti-Caveolin-1 (BD Biotechnologies), rabbit anti-Nrf-2 (Santa Cruz)] at 1:100 in 1% BSA in 1% TBS-T overnight at 4°C in a humid chamber. Secondary antibody (Alexafluor-488, -568 and -647, as mentioned above) was then incubated at 1:200 in 1% BSA in 1% TBS-T for 2 hours at room temperature in a dark humid chamber. Confocal plates were then incubated with DAPI (50 μM) for 30 min with agitation in the dark, washed three times for 3 min each, and then left in 2mL PBS during analysis. Images were recorded using a LSM510UV confocal microscope (Zeiss). Relative fluorescent intensity was measured using ImageJ. Representative images were selected by clearest association with the mean RFU within each group.

**Quantitation of Relative Fluorescent Units:** Relative fluorescent units (RFU) were determined using corrected total cell fluorescence which was calculated as follows: Integrated density of selection – (area of selection x mean background integrated density). Measurements were recorded using ImageJ. Three background samples were taken per selection to assure proper calibration per image. Statistical analysis was performed as described below.

**Amplex Red Assay:** H2O2 production was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen/Life Sciences). Cells were grown to 80% confluence and incubated with the reaction buffer for 30 min at 37°C. Supernatant [Amplex Red reaction mix] was then moved to a black-walled 96-well plate and fluorescence was read at 560Ex/590Em on a
SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA) and normalized to protein content as determined using bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific, Rockford, IL).

**ATP Assay**- Cells were grown in a white-walled96-well plate to 80% confluence. Cells were transferred to glucose free media and analyzed for ATP production using the Mitochondrial Tox-Glo Assay Kit (Promega, Madison, WI) according to the manufacturer’s protocol. ATP production was determined by luminescence measurement on a SpectraMax M5 spectrophotometer (Molecular Devices) and normalized to protein concentration determined by BCA protein assay reagent (Thermo Fisher Scientific).

**Glycolysis Assay**- Cells were grown to 80% confluence in a 96-well plate. Cells were switched to serum free media for 6 hours, then analyzed for L-Lactate production as a measure of glycolytic activity using the Glycolysis Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI). Lactate concentration in media was measured by absorbance at 480nm on a SpectraMax M5 spectrophotometer (Molecular Devices) and normalized to protein concentration determined by BCA protein assay reagent (Thermo Fisher Scientific).

**JC-1 Assay**- Cells were grown to 80% confluence in MatTek confocal dishes (MatTek Corporation). Cells were incubated in 5μM JC-1 for 20 minutes at 37°C, washed twice in PBS, and then immediately imaged on the LSM510UV microscope (Zeiss). Relative fluorescent intensity was measured using ImageJ.

**Mito-roGFP Oxidation Assay**- Cells were infected with adenoviral vector expressing mito-roGFP (at 100Pfu per cell), incubated for 6 hours at 37°C, and then media was replaced with standard growth medium and incubated overnight at 37°C. 1 x 10^5 cells were plated on MatTek
glass-bottom dishes (MatTek Corporation) in 2 mL of media overnight, and then treated with CMXROS prior to imaging (see below).

**CMXROS Assay**- Mitochondrial potential was measured using the MitoTracker Red CMXRos reagent (Thermo Fisher Scientific). Following mito-roGFP transfection, cells were washed twice with PBS, and then incubated with CMXRos (1µg/mL) for 15 min at 37°C. Cells were washed twice with PBS, and left in 2mL PBS during imaging using LSM510UV microscope (Zeiss).

**Extracellular Flow Analysis**- Cells were plated and grown in Seahorse Bioscience (North Billerica, MA) custom cell culture microplates to a uniform monolayer at ~80% confluence. Cells were then washed and transferred to bicarbonate free assay medium for 1 hour, and then analyzed on the Seahorse Extracellular Flux (XFe24) Analyzer using either the Mitochondria Stress Test Kit or Glycolysis Assay Kit. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were recorded at intervals according to the manufacturer’s protocol. OCR indicates mitochondrial oxidative phosphorylation, and ECAR is predominately the result of lactate produced by glycolysis.

**Soft agar assay**- 1 x 10^5 cells were seeded using 0.4% soft agar in normal growth medium on 0.8% soft agar media (2X DMEM, 20% FBS, 1% Pen/Strep) in 12-well dishes. Colonies were allowed to grow for 4 weeks prior to staining using Trypan Blue (Invitrogen) and were assessed using EVOS Cell Imaging Systems (Life Technologies). The threshold for scoring as a colony was kept to no fewer than 5 cells within one cluster.

**Epidemiological statistics**- Caveolin-1 and MnSOD mRNA expression were obtained from the Oncomine® database (Compendia Bioscience, Ann Arbor, MI) using the Curtis-Breast (Curtis, Shah et al. 2012) and Sorlie-Breast (Sorlie, Perou et al. 2001) datasets. Clinical status and Kaplan-
Meier estimates were derived using Microsoft Excel (Microsoft, Redmond, WA) and confirmed using SPSS Statistics 20 (IBM, Armonk, NY).

*Statistical analysis*- Statistical analyses were performed with GraphPad InStat by using one-way ANOVA with Student-Newman-Keuls comparison, 2-way Contingency Table and Chi-Square Tests. A value of $P<0.05$ was considered significant and a value of $P<0.01$ was considered highly significant.
III. RESULTS


A. Overview

The main objective of the current project was to determine the role of enhanced MnSOD expression, such as observed in cancer, in the energetic and metabolic perturbations that accompany tumor progression. Results are presented that demonstrate that the stable elevation of MnSOD expression in breast cancer is sufficient to promote the activation of glycolysis. Using a number of complementary techniques we also showed that MnSOD is necessary for the glycolytic switch and that this is dependent on AMP-activated kinase (AMPK). We found that the activation of glycolysis through AMPK requires H$_2$O$_2$ that is generated in mitochondria as a result of MnSOD upregulation. Hence, our results indicated that the metabolic switch operated by the MnSOD/mtH$_2$O$_2$/AMPK axis is important for cancer cell survival, and perhaps, is an important component of the Warburg effect. Having found the enhanced MnSOD expression is important, we next sought to determine how upregulation of MnSOD expression occurs in breast cancer. It was found using a model of induced malignant transformation that Cav-1 loss precedes the upregulation of MnSOD through increased stability of its upstream nuclear transcription factor, nuclear factor (erythroid–derived 2)-like 2 (Nrf-2). Further, we demonstrate that Cav-1 directly binds Nrf-2 and markedly reduces its stability in a ternary complex with Kelch-like ECH
associated protein 1 (Keap1) that likely facilitates Nrf-2 degradation. By this mechanism Cav-1 represses MnSOD expression reflecting in the restriction of AMPK activation by H$_2$O$_2$ and glycolytic metabolism. Together, our data unraveled a novel pathway contributing to the Warburg effect, its relationship with the plasma membrane protein Cav-1, and the potential role of this pathway for tumor cell survival through metabolic reprogramming.
B. MnSOD is highly overexpressed in breast cancer and is associated with expression of glycolytic enzymes.

To assess the expression of MnSOD in breast cancer, patient-derived epithelial cell lines of increasing stage of luminal breast cancer were used. Immunofluorescent staining of MnSOD increased in a stage dependent manner, having the highest expression in cells derived from a patient with histological stage IIIB (T4N2M0; Fig. 1A). This finding was consistent with staining of patient tissue biopsies assessed by tissue micro-array, in which MnSOD was similarly increased in a stage dependent manner (Fig. 1B). Interestingly, it was observed that MnSOD expression was associated with increased LDH expression, a marker of glycolysis associated with poor prognosis when expressed at higher levels in tumors (Fig. 1A) (Dawson, Goodfriend et al. 1964, Ma, Zhang et al. 2013). This association was consistent with an increase in the activation of the master metabolic regulator, AMPK (Winder and Hardie 1999, Wu, Neilson et al. 2007), as indicated by phosphorylation of its active site threonine 172 (Thr172) in both patient-derived cell lines (Fig. 1A) as well as strongly positively correlated with MnSOD in patient tissue (Fig. 1C). These findings indicated that MnSOD expression may be critical in the utilization of glycolytic metabolism in advanced breast cancer.
Figure 1 - MnSOD is highly overexpressed in breast cancer and is associated with expression of glycolytic enzymes. (A) Representative images of MnSOD (red), AMPK T172 (green) and LDH (purple) protein expression in HMEC and patient-derived cells. (B) Quantification of relative fluorescent units (RFU) of MnSOD protein expression in patients with invasive ductal carcinoma obtained by tissue micro array (TMA-Br1503c, Biomax). (C) Correlation plot showing a positive correlation in the RFU values for MnSOD and AMPK Thr172 obtained from (B). (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
C. **MnSOD overexpression enhances mtH$_2$O$_2$ and promotes mitochondrial oxidative stress.**

To determine the impact of MnSOD overexpression on mitochondrial function, isogenic cell lines expressing increasing levels of MnSOD were obtained from Dr. L.W. Oberley (University of Iowa). MCF-7 cells had been transfected with pcDNA3-Ile58-MnSOD and stably selected using G418 (Zhang, Yan et al. 1999). These cells expressed from 2 fold (Mn1) up to 20 fold (Mn28) of MnSOD compared to the control MCF-7 cell line (neo; Fig. 2A). Concomitant enhancement in steady state levels of H$_2$O$_2$ were observed in cells expressing progressively increasing levels of MnSOD (Fig. 2B). To determine that this excess H$_2$O$_2$ production was occurring in the mitochondria of MnSOD overexpressing cells, a mitochondrial targeted roGFP sensor (mito-roGFP) was transfected into neo, Mn44 and Mn11 cells. Mito-roGFP fluorescence due to its oxidation in the mitochondria indicated an increase in mitochondrial oxidative stress in a MnSOD-dependent manner, and this enhanced oxidative state was associated with the reduction in mitochondrial membrane potential as determined by CMXROS (Fig. 2C). This loss in membrane potential resulting from overexpression of MnSOD was confirmed using JC-1, a cationic probe that accumulates in the mitochondria (Reers, Smiley et al. 1995), which demonstrated an overall repression of mitochondrial electrochemical potential (Fig. 2D). Together, these data indicated that MnSOD overexpression in breast cancer cells induces mitochondrial oxidative stress and dysfunction, suggesting a possible role for MnSOD in defining metabolic regulation in progressing tumors.
Figure 2 - MnSOD overexpression enhances mtH$_2$O$_2$ and promotes mitochondrial oxidative stress.

(A) Representative images of MnSOD (red) in isogenic MCF-7 cell lines with stable transfection of increasing levels of MnSOD. (B) Steady state H$_2$O$_2$ levels in MCF-7 cells expressing increasing amounts of MnSOD, as measured by Amplex Red assay. (C) Oxidative stress was determined using mito-roGFP (green) and mitochondrial potential by CMX-ROS (red) in MCF-7 cells with basal (neo), intermediate (Mn44) and high (Mn11) levels of MnSOD. (D) Quantification of mitochondrial polarization was determined using JC-1. JC-1 aggregates (oligomer) indicate polar mitochondria while accumulation of JC-1 in the monomeric form indicate depolarization of mitochondria. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
D. Role of MnSOD in regulating cellular bioenergetics.

The reduction in mitochondrial polarization due to MnSOD overexpression (Figure 2) indicated that these cells may be unable to maintain an electron gradient in the mitochondria, which would thereby directly impact mitochondrial respiration (Perry, Norman et al. 2011), and relate to metabolic alterations in these cells. In order to address this possibility, MCF-7 cells overexpressing MnSOD were assessed using the extracellular flux analyzer (Seahorse Bioscience), which measures oxygen consumption rate (OCR, a measure of mitochondrial respiration due to oxygen utilization by the electron transport chain) parallel to extracellular acidification (ECAR, a result of lactate production produced during glycolytic activity) in real time. The Seahorse electron flux assay revealed that MCF-7 cells expressing high levels of MnSOD had a reduction in oxidative phosphorylation (OXPHOS) as determined by a loss in OCR parallel to an increase in ECAR, suggesting a switch from oxidative to predominantly glycolytic metabolism (Fig. 3A). Interestingly, while cells expressing high amounts of MnSOD (Mn11) had a complete ablation of OXPHOS, cells with intermediate levels of MnSOD (Mn44) maintained some basal and maximal respiration, albeit still markedly reduced from the control (neo) cells (Fig. 3B). Notably, this ~30% reduction in oxidative phosphorylation was overcompensated by a 3-fold increase in L-lactate production, a direct measure of glycolysis (Fig. 3C). These data indicated that MnSOD endows tumor cells to maintain an extent of mitochondrial respiration in addition to marked increase of glycolytic metabolism that foster rapid tumor growth, possibly by contributing biosynthetic precursors needed to support active proliferation. Importantly, silencing of MnSOD using siRNA suppressed glycolysis only in cells that expressed the high levels of MnSOD, most notably in Mn11 cells. These results confirmed that the levels of MnSOD are determinant of the type of energetic metabolism cancer cells display, whether predominantly aerobic or glycolytic (Fig. 3A).
Figure 3 - Role of MnSOD in regulating cellular bioenergetics. (A) Mitochondrial respiration and glycolytic metabolism were simultaneously measured in MCF-7 cells by using Seahorse electron flux assay. Oxygen consumption rate (OCR) indicates oxidative phosphorylation while extracellular acidification rate (ECAR) is an indirect result of glycolysis due to lactate production. (B) OCR in neo, Mn44 and Mn11 showing a MnSOD-dependent inhibition of OXPHOS. (C) Direct measurement of L-lactate indicated an increase in glycolytic metabolism in cells overexpressing MnSOD. (D) Measurement of L-lactate production of MCF-7 cells with high expression of MnSOD (Mn44, Mn11) was normalized to control (neo), showing a reduction in glycolysis due to silencing of MnSOD only in cells with the highest expression (Mn11). Absolute L-lactate levels were determined by normalizing to a L-lactate standard curve. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
E. **MnSOD-derived mtH\textsubscript{2}O\textsubscript{2} promotes AMPK activation and glycolysis.**

Having found that the levels of MnSOD regulate the glycolysis, we next sought to determine the mechanism by which MnSOD promotes glycolysis by focusing on its regulation of key glycolytic enzymes. AMP-activated kinase (AMPK) is a key metabolic regulator activated by nutrient deprivation and mitochondrial dysfunction, which promotes anti-apoptotic and energy sparing pathways (Winder and Hardie 1999, Wu, Neilson et al. 2007, Bonini and Gantner 2013). In the cell lines and tissue studied, MnSOD expression had a strong positive correlation with the activation of AMPK in human patient tissue (Fig. 1A and 1C) as assessed by phosphorylation of its active site threonine 172 (Thr172). AMPK phosphorylation was also significantly increased in a MnSOD-dependent manner in MCF-7 cells (Fig. 4A). This activation of AMPK was directly associated with the enhanced enzymatic activity of its downstream target, phosphofructokinase 2 (PFK2), one of the rate limiting enzymes in glycolysis (Berg JM 2002) (Fig. 4B). Previous studies indicated that AMPK is activated by H\textsubscript{2}O\textsubscript{2} (Horie, Ono et al. 2008, Irrcher, Ljubicic et al. 2009), the product of MnSOD catalytic activity. Hence, it was hypothesized that MnSOD positively regulates glycolysis through enhancing H\textsubscript{2}O\textsubscript{2} production by mitochondria. To test this possibility, MCF-7 cells expressing increasingly high levels of MnSOD were treated with a mitochondria-targeted catalase, a highly efficient enzyme responsible for quenching H\textsubscript{2}O\textsubscript{2}. Quenching of MnSOD-derived mtH\textsubscript{2}O\textsubscript{2} with mt-catalase attenuated the activation of AMPK in cells overexpressing MnSOD, but had little effect on control MCF-7 cells (neo) as well as MCF-7 cells with only ~ 2-fold enhanced MnSOD (Mn1; Fig. 4C). This inhibition of AMPK activation was consistent with an overall suppression of glycolytic rate in these cells, as indicated by a reduction in L-lactate production (Fig. 4D).
Figure 4 - MnSOD-derived mtH2O2 promotes AMPK activation and glycolysis. (A) Western blot showing increasing amounts of AMPK T172 in a MnSOD-dependent manner. (B) Phosphofructokinase activity was determined using Phosphofructokinase (PFK) Activity Colorimetric Assay Kit (Biovision). (C) AMPK activation was determined by AUC quantification of Western blot, taking AMPK T172 relative to total amounts of AMPK. Mitochondria-targeted catalase (mt-catalase) reduced AMPK\textsuperscript{T172}/AMPK\textsuperscript{total} ratio only in cells expressing intermediate to high levels of MnSOD. (D) Effects of mt-catalase on glycolytic activity in MCF-7 cells overexpressing MnSOD was determined using L-lactate production. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
F. **AMPK is critical in the MnSOD-dependent metabolic switch.**

We further explored the role of AMPK in MnSOD overexpressing cells displaying a predominantly glycolytic metabolism. Inhibition of AMPK with dorsomorphin (Compound C) resulted in suppressed glycolysis as determined by a marked reduction in L-lactate production in cells with high MnSOD expression (Mn11). As expected, the effect of AMPK inhibition in control MCF-7 cells was negligible confirming that cells operate independently from the MnSOD/H₂O₂/AMPK axis (neo; Fig. 5A). To confirm the observation that AMPK inhibition neutralizes the effects of MnSOD overexpression on the metabolism, a genetic approach was used. Ectopic expression of MnSOD in AMPK-competent cells (Fig. 5B) recapitulated the increase in glycolysis observed in cells stably expressing high MnSOD levels. In contrast, overexpression of MnSOD in AMPK-null cells did not affect mitochondrial respiration (Fig. 5C) similar to what was observed previously (Fig. 3A). Together these results established that AMPK is required for the metabolic reprogramming resulting from MnSOD overexpression in breast cancer cells.
Figure 5 - AMPK is critical in the MnSOD-dependent metabolic shift. (A) Measurement of glycolytic metabolism in MCF-7 cells with overexpression of MnSOD was performed using Compound C, a selective inhibitor of AMPK. Compound C (25µM, 24 h) inhibited glycolytic metabolism in cells expressing intermediate (Mn44) and high (Mn11) levels of MnSOD, but not in control MCF-7 cells (neo). (B) MCF-7 cells with stable knockdown of AMPK using siRNA were transfected with MnSOD-GFP, and analyzed by Western blot at 48 h. (C) MCF-7 cells transfected with MnSOD-GFP on either an AMPK-null or AMPK-competent background were analyzed by electron flux assay (Seahorse). MnSOD led to predominantly glycolytic metabolism in AMPK-competent cells, but had no effect on cells with depleted AMPK. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
G. Highly aggressive tumorigenic cells are dependent on MnSOD-driven glycolytic metabolism.

To test whether MnSOD was necessary for glycolytic metabolism in highly aggressive breast cancer cell lines, we analyzed MDA-MB231 cells, which are breast cancer epithelial cells notable for their highly glycolytic metabolism in vitro and aggressive metastatic capacity in vivo (Gatenby and Gillies 2004, Iorns, Drews-Elger et al. 2012). We determined by Western blot that MDA-MB231 cells express levels of MnSOD comparable to that of the Mn11 cell line (Fig. 6A). Transient silencing of MnSOD in MDA-MB231 using siRNA (Fig. 6B) led to remarkable reduction in L-lactate production indicating an inhibition in glycolytic metabolism (Fig. 6C). The relationship between MnSOD and extent of glycolysis observed in two phenotypically unique cell lines indicated that MnSOD may be essential for the survival of transformed cells. To test this hypothesis, we assessed MnSOD in MCF10A(Er/Src) cells, which undergo v-Src-driven transformation within 48-72 hours when induced by tamoxifen (TMX), and produce a highly tumorigenic phenotype (Iliopoulos, Hirsch et al. 2009). MnSOD upregulation occurred as soon as 72 hours after TMX exposure in parallel with the enhanced activation of AMPK (Fig. 7A). To determine if this MnSOD-dependent pathway is necessary for transformed cell survival, silencing of MnSOD prior to the induction of transformation led to cell death (~ 70-75%) (Fig. 7B and 7C, respectively). These findings indicate that transforming tumor cells are reliant upon MnSOD expression to sustain their characteristic glycolytic metabolism, and that impeding this metabolic pathway may be useful in selective targeting of glycolytic tumors as a potential therapeutic strategy.
Figure 6 – Suppression of MnSOD in MDA-MB231 cells inhibits glycolysis. (A) MnSOD expression between neo, Mn11 and MDA-MB231 cell lines. (B) Silencing of MnSOD in MDA-MB231 was confirmed by Western blot at 72h. (C) Silencing MnSOD in MDA-MB231 cells represses glycolysis as determined by L-lactate production. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
Figure 7–Upregulation of MnSOD is critical for malignant transformation in MCF10A(Er/Src).

(A) MnSOD expression and AMPK activation were determined by Western blot in MCF10A(Er/Src) cells undergoing malignant transformation using tamoxifen (TMX, 1µM). Lysates were collected at the times indicated. (B) Western blot showing silencing efficiency of MnSOD in MCF10A(Er/Src) cells prior to transformation. (C) Cell death (%) was determined using Trypan blue exclusion. Cells were treated with scrambled (scrRNA) or MnSOD-silencing (siRNA) prior to 48h TMX induction. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
H. Caveolin-1 (Cav-1) is inversely expressed with Nrf-2 in human breast cancer.

Having established a link between elevated MnSOD expression and the promotion of glycolysis, we next sought to determine the mechanism of MnSOD upregulation in breast cancer. Several transcription factors had been shown to regulate expression of MnSOD, including NFκB, FOXO3a and Nrf-2 (Miao and St Clair 2009). Given that transformation is often accompanied by increased oxidant production and Nrf-2 is activated by ROS, we hypothesized that MnSOD expression may be enhanced through increased transcriptional activity of Nrf-2. It had recently been shown that the complex formation of Nrf-2 and its negative regulator, Keap1, could be stabilized by Caveolin-1 (Cav-1) (Li, Liu et al. 2012, Kansanen, Kuosmanen et al. 2013, Volonte, Liu et al. 2013). Immunofluorescent staining of patient tissue samples indicated an inverse relationship between Cav-1 and Nrf-2 expression in epithelium of patients with invasive ductal carcinoma in comparison to control healthy subjects (Fig. 8A). Cav-1 expression is strongly repressed in ductal epithelium of high grade tumors (II-III), which is associated with markedly increased expression of Nrf-2 in these patients (Fig. 8B). Together, this indicated that Cav-1 loss throughout the progression of the disease may be related to enhanced Nrf-2 activity and the expression of MnSOD.
Figure 8 - Caveolin-1 (Cav-1) is inversely expressed with Nrf-2 in human breast cancer. (A) Representative images of Cav-1 (green) and Nrf-2 (red) protein expression in patients with invasive ductal carcinoma obtained by tissue micro-array (TMA-1005, Protein Biotechnologies). (B) Quantification of patients stratified by tumor grade for Cav-1 (left) and Nrf-2 (right) expression. RFU was determined as described in methods. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
I. Loss of Cav-1 during malignant transformation is associated with Nrf-2 transcriptional upregulation of MnSOD and the downstream AMPK-driven glycolytic shift.

To determine the expression of Cav-1 throughout tumor development, the MCF10A(Er/Src) model of malignant transformation was used. MCF10A(Er/Src) cells exhibited a rapid reduction of Cav-1 expression early during transformation (24h), which was followed by a marked suppression within 48-72h (Fig. 9A). The overall reduction in Cav-1 oligomers, the functional form of Cav-1 in caveolar microdomains (Lisanti, Scherer et al. 1994, Lisanti, Scherer et al. 1994), at 48h directly preceded the upregulation of MnSOD (Fig. 8A) and AMPK activation (Fig. 7A). Similarly, this repression of functional Cav-1(Sargiacomo, Scherer et al. 1995), as indicated by the loss of oligomeric Cav-1, was associated with enhanced expression and nuclear localization of Nrf-2 at 48h and was sustained well after transformation had occurred (Fig. 9B-D). Although the nuclear localization of Nrf-2 is implicit with its transcriptional activity (Theodore, Kawai et al. 2008, Kim, Kim et al. 2012), enhancement in Nrf-2 stability was confirmed using a luciferase-based reporter assay (Fig. 9E), indicating that Cav-1 loss early during transformation may bolster the transcription of MnSOD via Nrf-2. Interestingly, MCF10A(Er/Src) cells did not demonstrate an enhancement of glycolytic activity prior to upregulation of MnSOD or activation of AMPK at 24h; however, at the sustained increases in MnSOD and AMPK phosphorylation following transformation [96 – 120h], a marked increase in glycolytic metabolism was observed (Fig. 9F). Combined with the finding that depletion of MnSOD in transformed MCF10A(Er/Src) cells led to cell death [Fig. 7C], these data suggest that MnSOD is required to sustain the glycolytic shift necessary for developing tumors, and this overexpression of MnSOD may be in part due to loss of Cav-1.
Figure 9 - Loss of Cav-1 during malignant transformation is associated with Nrf-2 transcriptional upregulation of MnSOD and the downstream AMPK-driven glycolytic shift. (A) Representative Western blot showing loss of Cav-1 early during transformation (24-48h) followed by upregulation of MnSOD (72h). (B) Nrf-2 nuclear localization throughout malignant transformation of MCF10A(Er/Src). Nrf-2 total expression and nuclear:total ratio are quantified in panels (C) and (D), respectively. (E) Nrf-2 stability was measured as an indirect indication of Nrf-2 transcriptional activity, as determined using Nrf-2 luciferase reporter. (F) Seahorse electron flux analysis of glycolytic metabolism throughout transformation indicate increases during and following transformation. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
J. Cav-1 expression in MCF-7 cells inhibits MnSOD-dependent glycolytic metabolism by attenuating Nrf-2 activity.

The observation that Cav-1 loss may precede MnSOD upregulation and the glycolytic shift prompted the hypothesis that Cav-1 may indirectly impede MnSOD expression through interaction and regulation of its upstream transcription factor, Nrf-2. To interrogate if Cav-1 expression prevents MnSOD overexpression, Cav-1 (wild-type, myc-tagged) was ectopically expressed in MCF-7 cells, which typically lack detectable Cav-1 (Shajahan, Wang et al. 2007). Expression of Cav-1 in these cells caused a marked reduction of Nrf-2 expression and nuclear localization that was associated with a repression of MnSOD (Fig. 10A). To confirm that Cav-1 may directly bind Nrf-2, co-immunoprecipitation of Cav-1 from either Cav-1 null or competent MCF-7 cells demonstrated that Cav-1 binds to both Nrf-2 as well as its negative regulator, Keap1, and further that the binding of Cav-1 to this complex was sensitive to oxidative stress (Fig. 10B). Consistent with the inhibition of Nrf-2 expression observed, Cav-1 expression in MCF-7 cells suppressed Nrf-2 stability, indicating a reduction of Nrf-2 transcriptional activity (Fig. 9C) (Theodore, Kawai et al. 2008). Importantly, either silencing of Nrf-2 using siRNA or ectopic expression of Cav-1 reduced MnSOD mRNA to a comparable extent (Fig. 10D). Reduction of MnSOD resulting from Cav-1 expression was associated with an overall decrease in H$_2$O$_2$ production (Fig. 11A) and reduction in AMPK activation (Fig. 11B). Further, Cav-1 expression in these cells restored oxidative phosphorylation while dampening glycolysis (Fig. 11C and 11D, respectively), effectively reversing the metabolic repurposing observed from MnSOD overexpression (Fig. 3). Taken together, this data strengthened the notion that Cav-1 loss in tumor progression may promote Nrf-2 transcriptional upregulation of MnSOD, thus promoting the glycolytic shift.
Figure 10 - Cav-1 expression in MCF-7 cells represses MnSOD through inhibition of Nrf-2 transcriptional activity. (A) Representative confocal images of MCF-7 cells with ectopic expression of Cav-1 (green, c-myc tagged wild-type Cav-1), indicating a loss of MnSOD (red) and Nrf-2 (purple) expression. (B) Western blot showing co-immunoprecipitation of Cav-1/Nrf-2/Keap1 complex by either Keap1 or Nrf-2 pull down. Binding of either Nrf-2 or Keap1 to Cav-1 was lessened by exogenous H₂O₂ exposure. (C) Nrf-2 stability was measured using Nrf-2 luciferase reporter (Promega) in MCF-7 cells expressing empty vector or Cav-1. (D) MnSOD mRNA expression was measured by qPCR in MCF-7 cells transfected with either Nrf-2 siRNA or Cav-1 (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
Figure 11 - Cav-1 expression in MCF-7 cells inhibits MnSOD-dependent glycolytic metabolism.

(A) Measurement of H$_2$O$_2$ using Amplex Red shows a reduction of steady-state H$_2$O$_2$ by Cav-1 expression. Signal was quenched by concurrent incubation with catalase (100U/mL). (B) Representative Western blot showing a marked reduction in Nrf-2 and MnSOD expression, as well as AMPK activation, in response to Cav-1 expression in MCF-7 cells. (C) Oxidative phosphorylation was measured using Seahorse electron flux assay. (D) Glycolytic rate of MCF-7 cells expressing either empty vector or Cav-1 were assessed indirectly by ECAR during Seahorse analysis. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
K. Reconstitution of MnSOD in Cav-1-competent MCF-7 cells promotes mitochondrial dysfunction and glycolytic metabolism.

To examine whether the impact of Cav-1 overexpression was dependent on its observed reduction of MnSOD expression, MnSOD was ectopically expressed by adenoviral infection in Cav-1-null and Cav-1-competent MCF-7 cells. The repression of H$_2$O$_2$ by Cav-1 expression was restored by MnSOD overexpression to levels consistent with the Cav-1-null MCF-7 cells (Fig. 12A and 12B). Consistently, MnSOD expression in either Cav-1-null or Cav-1-competent cells enhanced glycolytic rate and capacity (Fig. 12C). Together, this data supports that MnSOD-derived H$_2$O$_2$ impacts the glycolytic shift independent of Cav-1 expression, and supports the notion that Cav-1 may repress glycolytic metabolism through inhibition of MnSOD expression via reduction of Nrf-2 stability (Fig 11 and 12).
Figure 12 - Reconstitution of MnSOD in Cav-1-competent MCF-7 cells promotes mitochondrial dysfunction and glycolytic metabolism. (A) Representative Western blot showing infection efficiency of the empty vector (+(av)EV) or MnSOD (+(av)MnSOD) into MCF-7 cells either control(MCF-7) or Cav-1 expressing cells (MCF-7(Cav-1)). (B) Measurement of H₂O₂ production using Amplex Red indicates enhanced H₂O₂ in MCF-7 stably expressing empty vector or Cav-1. (C) Seahorse electron flux assay indicates enhanced glycolysis in both EV and Cav-1-competent MCF-7 cells transfected with MnSOD. (One-way ANOVA with post-hoc two-sided t-test, ** = p < 0.005, *** = p < 0.001).
Clinical Implications: Cav-1 and MnSOD expression indicate risk in patients with invasive ductal carcinoma.

Evaluation of clinical patient mRNA derived from previously reported studies (Sorlie, Perou et al. 2001, Curtis, Shah et al. 2012) revealed that the effects of the MnSOD/H$_2$O$_2$/AMPK pathway may be relevant in assessing patient prognosis and indicate therapeutic strategies targeted towards glycolytic tumors. The expression of Cav-1 and MnSOD was found to be inversely associated, as Cav-1 expression was decreased (and MnSOD expression enhanced) in patients with invasive ductal carcinoma (Fig. 13A and 13D). Interestingly, Cav-1 expression was reduced in a grade and stage dependent manner (Fig. 13B and 13C, respectively), consistent with the observed progressive increases in Nrf-2 (Fig. 8) and MnSOD expression (Fig. 1). Stratification of this cohort by either decreasing expression of Cav-1 or increasing levels of MnSOD indicated that low Cav-1 or high MnSOD expression were independently associated with mortality in patients with invasive ductal carcinoma (Fig. 13E and 13F, respectively). In certain histological subtypes of invasive breast cancer, Cav-1 and MnSOD mRNA were strongly negatively correlated (Fig. 13G).

While there was a clear association between Cav-1 and MnSOD expression levels on mortality, in order to relate to our mechanistic findings it was critical to determine if the interaction between these genes was relevant to the severity of the disease. Stratification of patients in this cohort by the lowest quartile (quartile 1) of Cav-1 (Cav-1$^{low}$) paired with the highest quartile (quartile 4) of MnSOD (MnSOD$^{high}$) conferred risk of mortality (odds ratio, OR = 1.576, 95% CI 1.076 – 2.307, p < 0.05) and increased incidence of aggressive disease (OR = 2.099, 95% CI 1.321 – 3.333, p < 0.005), as shown in Table 1. In contrast, stratifying this cohort by the highest quartile of Cav-1 (Cav-1$^{high}$) with the lowest quartile of MnSOD (MnSOD$^{low}$) conferred a two-fold protection from mortality (OR = 0.545, 95% CI 0.354 – 0.839, p < 0.01) and could successfully discriminate
between healthy control patients and those with invasive ductal carcinoma (OR = 0.203, 95% CI 0.127 – 0.324, p < 0.01) (Table 2). Together, these data indicated the relationship between Cav-1 and MnSOD may be useful as a novel biomarker to improve diagnostic and prognostic criteria in patients with invasive ductal carcinoma.

While promising, it was still clear that the current biomarkers derived from this interaction were far from impacting clinical decision making. In order to enhance the predictive power of this biomarker, in addition to Cav-1 and MnSOD, we selected genes primarily involved in the maintenance of redox homeostasis, AMPK activity and glycolysis that were likely to be effected by the proposed MnSOD/H$_2$O$_2$/AMPK pathway. Genes that had a moderate to high correlation (approximately Pearson’s R = 0.5 or -0.5, +/- 0.1) were selected and included in a gene expression profile, CM-GEP (Cav-1/MnSOD Gene Expression Profile). The final gene expression profile included Cav-1, MnSOD, catalase, methionine sulfoxide reductase (MSRA), pyruvate kinase 2 (PKM2) and survivin. The CM-GEP markedly enhanced the ability of the Cav-1$^{\text{high}}$/MnSOD$^{\text{low}}$ biomarker (Table 3) to discriminate between healthy control subjects and those with breast carcinoma (OR = 0.008, 95% CI 0.004 – 0.015, p < 0.001). Further, the CM-GEP phenotype conferred protection from advanced grade (Grade 3, OR = 0.084, 95% CI 0.040 – 0.195, p < 0.001) and aggressive disease (Basal triple-negative phenotype, OR = 0.052, 95% CI 0.007 – 0.374, p < 0.001), and further had a decreased incidence of dying from the disease within 5 years (OR = 0.030, 95% CI 0.004 – 0.215, p < 0.001). Taken together, these data indicate that development of a novel biomarker based on the mechanistic findings reported in these studies may enhance prognostic criteria and risk assessment of patients with invasive ductal carcinoma.
Nested case-control studies were performed on mRNA expression data obtained from previously reported studies by Sorlie (Sorlie, Perou et al. 2001) and Curtis (Curtis, Shah et al. 2012). Cav-1 and MnSOD mRNA expression is expressed as log2 median centered ratio. (A) Cav-1 mRNA expression between controls and patients with invasive ductal carcinoma (N = 116). (B) Cav-1 mRNA expression stratified by grade (N = 116). (C) Cav-1 mRNA expression stratified by tumor size (N = 116). (D) MnSOD expression (N = 116). (E) 5-year survival (%) of Cav-1 expression (Log2 Median Centered Ratio) (p < 0.01). (F) 5-year survival (%) of MnSOD expression (Log2 Median Centered Ratio) (p < 0.01). (G) Scatter plot of Cav-1 vs. MnSOD expression (R = -0.51, P < 0.001).

**Figure 13 - Cav-1 and MnSOD expression indicate risk in patients with invasive ductal carcinoma.**
(D) MnSOD mRNA expression between controls and all cases with IDC (N = 116). (E) Cohort is stratified using Cav-1 mRNA expression set as the continuous variable (N = 116). Comparison between highest and lowest Cav-1 mRNA expression was assessed by Pearson’s Chi-Square Test.

(F) Cohort is stratified using MnSOD mRNA expression set as the continuous variable (N = 116). Comparison between lowest and highest MnSOD mRNA expression was determined by Pearson’s Chi-Square Test. (G) Correlation plot of Cav-1 and MnSOD expression indicated a moderate negative correlation in patients with aggressive IDC (e.g., medullary carcinoma, N = 172). (One-way ANOVA with post-hoc two-sided t-test, *** = p < 0.001; Student’s two-sided t-test, *** = p 0.001; ).
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M. Cav-1 inhibits MnSOD-dependent glycolytic metabolism via suppression of Nrf-2.

Together our findings indicate that Cav-1 loss occurs early during malignant transformation and precedes the stabilization of Nrf-2 and the resulting high expression of MnSOD. Constitutively high MnSOD thereby promotes glycolytic metabolism to enhance tumor cell survival and development. Thus, Cav-1 may pose a barrier to the glycolytic shift by suppression of Nrf-2 upregulation of MnSOD and reduction of mtH₂O₂, thereby inhibiting downstream signaling events that enhance glycolysis (Fig. 14).

![Figure 14 - Cav-1 inhibits MnSOD-dependent glycolytic metabolism via suppression of Nrf-2.](image)
IV. DISCUSSION

Unraveling the multifaceted process of tumor development has indicated glycolysis as a critical adaptive response that bolsters cancer cell survival and growth. Recently, MnSOD and its product H$_2$O$_2$ have been shown to regulate oxygen consumption and glucose uptake during the progression from quiescent to proliferative states in mouse embryonic fibroblasts (Guo, Chen et al. 2013), and have also been shown to affect cellular function in a variety of other contexts (Saccani, Pantano et al. 2001, Bernard, Monte et al. 2002, Vrailas-Mortimer, del Rivero et al. 2011, Chetboun, Abitbol et al. 2012, Marosi, Bori et al. 2012, Zarse, Schmeisser et al. 2012, Kang, Wijesinghe et al. 2013). It has also been demonstrated that enhanced steady-state levels of H$_2$O$_2$ could sufficiently induce glycolysis (Deng, Liang et al. 2014), as well as suppress mitochondrial respiration (Reddi and Culotta 2013). However, the role of MnSOD in pathogenesis and progression of cancer has motivated heated debates over many years with multiple studies indicating tumor suppressive or tumor promoting activities. The notion that MnSOD can have either beneficial or deleterious impacts on tumor development is interesting per se, and its involvement appears to be related to specific contexts regarding the counterpoint of balanced redox states. The data presented in the current study elucidate a novel pro-tumorigenic role of MnSOD, as we demonstrate the dependence of aggressive tumorigenic cells on AMPK-dependent glycolytic metabolism driven by H$_2$O$_2$ derived from constitutively high overexpression of MnSOD (Fig. 3-5). These studies also support the idea that the redox balance is critical to determine phenotypic and functional cellular outcomes as scavenging of H$_2$O$_2$ suppresses the effects of MnSOD upregulation. Together, our main findings indicate that markedly high sustained steady-state levels of H$_2$O$_2$ contribute to the ability of cancer cells to grow and survive in anaerobic conditions at the expense of metabolic flexibility, and the MnSOD/mtH$_2$O$_2$/AMPK signaling axis could be
exploited in therapeutic strategies, such as small molecules to inhibit H$_2$O$_2$ production or signaling, could eliminate MnSOD-dependent glycolytic tumor cells while sparing normal glycolysis-dependent processes in healthy somatic cells.

While it is clear that MnSOD is required to maintain redox homeostasis under normal conditions through the prevention of O$_2^-$ accumulation (McCord and Fridovich 1978), it is becoming appreciated that MnSOD and its product H$_2$O$_2$ need to be highly regulated to prevent signaling events that support tumor growth (Lopez-Lazaro 2007, Hempel, Carrico et al. 2011, Lisanti, Martinez-Outshoorn et al. 2011, Chen, Wu et al. 2013). In support of this notion, it was observed that constitutive high overexpression of MnSOD could suppress mitochondrial respiration while enhancing glycolytic metabolism; however, expression of MnSOD to two fold over the control MCF-7 cells reduced overall glycolytic metabolism (Fig. 3). This low-to-moderate increase in MnSOD was not observed to occur in human breast cancer, as patients with invasive ductal carcinoma tended to have several fold increased expression over healthy controls (Fig. 1). Cancer cells with MnSOD expression more representative of these clinical observations severely impacted mitochondrial bioenergetics and resulted in the enhancement of glycolysis through excess H$_2$O$_2$ production and the activation of AMPK (Fig. 2-4). Similarly, sustained overexpression of MnSOD late during malignant transformation resulted in accumulation of H$_2$O$_2$, and this persistent upregulation led to the enhancement of metabolic reprogramming to support tumor cell survival (Fig. 7 and 9). Repression of mtH$_2$O$_2$ or inhibition of AMPK attenuated glycolytic metabolism in cells dependent on MnSOD-driven metabolism while having no effect on cells with low levels of MnSOD, indicating that cells highly overexpressing MnSOD became reliant on mtH$_2$O$_2$/AMPK signaling in order to sustain metabolic processes required for growth (Fig. 4 and 5). Interruption of this MnSOD-dependent pathway resulted in overall inhibition of
glycolysis in aggressive and metastatic cells derived from advanced stage cancer (e.g., MDA-MB231, see Fig. 6). Moreover, this pathway was shown to be enhanced later stages of malignant transformation (Fig. 7A), and importantly was indispensable for transformed epithelial cell survival, as suppression of MnSOD resulted in marked cell death of transforming MCF10A(Er/Src) cells (Fig. 7C).

Hydrogen peroxide emanating from the mitochondria (mtH$_2$O$_2$) was identified as the signaling molecule responsible for the impact of MnSOD on AMPK activation and the resulting glycolytic shift. The ectopic expression of a mitochondria-targeted catalase (mt-catalase), which rapidly quenches H$_2$O$_2$, suppressed glycolytic metabolism only in MnSOD-overexpressing cells, confirming that mtH$_2$O$_2$ resulting from MnSOD upregulation was required for the glycolytic shift (Fig. 4). Given that MnSOD expression and AMPK activation were highest in advanced stage breast cancer (Fig. 1), these findings suggest that the MnSOD/mtH$_2$O$_2$/AMPK axis may be crucial to the aggressive progression of the disease.

This pathway may constitute a metabolic phenotype that endows tumor cells with the capacity to activate glycolysis in parallel to maintaining mitochondrial respiration. This concept is supported by the observation that MCF-7 cells with high expression of MnSOD (Mn11) exhibited close to no ability to consume oxygen, while cells with intermediate expression of MnSOD (Mn44) did maintain basal and maximal respiration, albeit reduced compared to normal MCF-7 cells, in combination with having markedly enhanced glycolysis (Fig. 3). This data suggests that there may be a continuum of MnSOD expression in which certain degrees of overexpression can enable tumor cells to utilize both mitochondrial respiration as well as enhanced glycolytic metabolism, whereas MnSOD levels exceeding a certain threshold may result in predominant reliance on glycolysis that could be targeted therapeutically. This unique potential metabolic flexibility allows
for both the generation of ATP for the myriad of energy-dependent signaling processes, as well as the utilization of metabolic substrates required for bolstering biosynthetic pathways.

The delineation of how MnSOD overexpression could promote tumor survival through bolstering glycolysis led to the critical question of how MnSOD could become constitutively overexpressed, as inhibiting the activation of this pathway may be a crucial strategy in the development of novel therapeutic strategies to selectively eliminate glycolytic tumors. As tumor initiation and progression are closely associated with enhanced ROS production (Ames, Shigenaga et al. 1993, Dizdaroglu, Jaruga et al. 2002, Waris and Ahsan 2006, Liou and Storz 2010), the oxidant-sensitive transcription factor upstream of MnSOD, Nrf-2, was likely involved in the prolonged transcriptional upregulation of MnSOD. Consistent with previous reports (Hayes and McMahon 2009, Kansanen, Kuosmanen et al. 2013), Nrf-2 expression was markedly increased in advanced stage breast cancer, which closely mimicked the progressive increases in MnSOD (Fig. 1 and Fig. 8). We found that Nrf-2 was inversely associated with the expression of Cav-1, a protein recently identified to bind and repress Nrf-2 (Volonte, Liu et al. 2013). This relationship was particularly obvious in ductal epithelium, an observation contrary to previous reports (Pavlides, Tsirigos et al. 2010), and was most pronounced in advanced and aggressive stages (Fig. 8). In in vitro studies, the loss of Cav-1 during malignant transformation preceded the stabilization and nuclear localization of Nrf-2, as well as the concomitant increases in MnSOD expression, steady state H₂O₂, activation of AMPK and glycolytic metabolism, indicating that Cav-1 loss may bolster MnSOD-driven glycolysis through enhanced stabilization of Nrf-2. In contrast, the expression of Cav-1 in MCF-7 cells reduced steady state H₂O₂, which was attributed to the reduction in Nrf-2 stability and the resulting MnSOD expression, and consistently reduced AMPK activation and glycolytic metabolism (Fig. 11). Together, these findings delineated a novel mechanism by which
Cav-1 poses a barrier to glycolytic metabolism by prevention of Nrf-2 transcriptional upregulation of MnSOD. Further, we confirmed that Cav-1 could bind to Nrf-2 as shown previously (Li, Liu et al. 2012), and that the stability of this Cav-1/Nrf-2/Keap1 complex was reduced during exposure to exogenous H$_2$O$_2$, supporting the notion that persistent activation of Nrf-2 due to elevations in ROS may sustain enhancement of MnSOD expression that is sufficient to promote glycolysis. Interestingly, use of the caveolin-1 scaffold domain peptide (CSD) did not notably impact MnSOD expression or AMPK activation (own observations), suggesting that either an alternate binding motif or functional full-length caveolin-1 are required for its inhibitory effects on Nrf-2.

Importantly, the effects of Cav-1 on H$_2$O$_2$ and glycolysis were overcome by reconstitution of these cells with MnSOD, confirming that MnSOD expression is necessary for metabolic reprogramming independent of the other potential effects resulting from Cav-1 expression.

Analysis of both protein and mRNA expression in human patient samples indicated that loss of Cav-1 occurs early during the progression of breast cancer, a finding that was analyzed by the MCF10A(Er/Src) model of malignant transformation (Fig. 13 and Fig. 9, respectively). Consistent with this model system in which Cav-1 loss preceded MnSOD overexpression, patients with advanced stages of breast cancer were observed to have enhanced mRNA expression of MnSOD, supporting the mechanistic observations that Cav-1 repression of Nrf-2 transcriptional upregulation of MnSOD is critical in posing a barrier for adaptive responses of progressing tumors to utilize glycolysis. Further analysis of clinical samples revealed that the relationship between Cav-1 and MnSOD could indicate the severity of the disease. Patients with the most polar expression of either (e.g., lowest quartile of Cav-1 paired with highest quartile of MnSOD) were observed to be at significantly higher risk of lethal molecular subtypes such as triple-negative breast cancer, and at higher risk of dying from the disease within 5 years following initial treatment.
In line with data demonstrating that Cav-1 loss enables MnSOD-dependent glycolysis in vitro (Fig. 8-10), the Cav-1\textsuperscript{low}/MnSOD\textsuperscript{high} phenotype may indicate a molecular signature of aggressive tumors reliant on glycolytic metabolism. Additionally, examining this molecular profile has the potential to distinguish if novel compounds to inhibit $\mathrm{H}_2\mathrm{O}_2$ or AMPK signaling would be effective in patients with glycolytic tumors.

The opposing molecular signature of Cav-1\textsuperscript{high}/MnSOD\textsuperscript{low} suggested a protection from mortality, as well as ability to discriminate between patients with invasive ductal carcinoma from controls (Table 2), which may be useful in reclassification of patients from high to low risk if used in combination with other diagnostic criteria. The further development of this molecular signature was enhanced by assessing genes related to the proposed Cav-1/MnSOD/mtH\textsubscript{2}O\textsubscript{2}/AMPK pathway. Incorporation of genes related to the maintenance of ROS and oxidative damage (e.g., catalase, MSRA (Chelikani, Fita et al. 2004, Moskovitz 2005)), AMPK activity (survivin (Liu, Liang et al. 2010)) and glycolysis (PKM2 (Luo and Semenza 2012, Yang, Xie et al. 2014)) into the Cav-1\textsuperscript{high}/MnSOD\textsuperscript{low} molecular profile tremendously enhanced its predictive power in the ability to discriminate between healthy patients and those with invasive ductal carcinoma, increasing the probability of being categorized as healthy over 100-fold over the original biomarker (Table 3).

While many gene expression profiles being developed focus on highly correlated genes that are highly ranked in the change in their expression, development of the CM-GEP suggests that utilization of genes involved in closely related biological events can vastly improve predictive probability of a biomarker set. The current molecular profile describes the relationship between these genes in a more dynamic context relevant to the biological system in which the disease is occurring, and therefore may be more useful in determining therapeutic strategy. It is important to note that the total number of patients in the CM-GEP who were categorized as either basal
phenotype or dead of the disease within 5 years only reached 1% of the CM-GEP group; therefore, external validation on a new patient cohort would be required to determine the reliability of the CM-GEP in discrimination between patients with lethal triple-negative breast cancer. Overall, use of the CM-GEP profile supports the notion that the articulation of a pathway (e.g., the MnSOD/mtH_{2}O_{2}/AMPK axis) and development of a complementary gene expression profile will allow for determination on whether a patient may benefit from a specific therapeutic strategy focused on tumors that rely on MnSOD-driven glycolysis. Further prospective studies examining the relationship between these genes in the CM-GEP, and perhaps others involved in the MnSOD/mtH_{2}O_{2}/AMPK pathway, may result in a refined biomarker suitable for clinical application.

Overall, the current project has elucidated a novel pathway by which MnSOD overexpression leads to the activation of AMPK via sustained mtH_{2}O_{2} production, thereby leading to the glycolytic shift that enhances tumor progression. We have further identified a mechanism by which Cav-1 loss during tumor development facilitates the upregulation of MnSOD by unleashing Nrf-2 activity (Fig. 14). This study provides both mechanistic and epidemiologic evidence indicating that the loss of Cav-1 followed by the gain of MnSOD promotes breast cancer progression and thus negatively impacts patient outcome. Together, the Cav-1/MnSOD/AMPK axis may act as a primary regulator of the Warburg effect in breast cancer, and the relationship between Cav-1 and MnSOD expression may be a specific molecular signature of glycolytic tumors. Thus, the interplay between Cav-1 loss, MnSOD upregulation and AMPK activation may be a crucial part of the multifaceted processes that confer tumor progression; however, the complex network of interactions between MnSOD and other critical enzymes involved in redox regulation
merit further investigation to determine how this pathway fully contributes to pathogenesis and progression of human breast cancer.
V. FUTURE DIRECTIONS

Low Cav-1 mRNA expression in the presence of elevated MnSOD conferred risk of lethal disease (likely attributed to resistance, recurrence and/or metastasis), no direct measurements of metastatic lesions were performed in the course of this study. A continuation of this project to identify the interaction between Cav-1 and MnSOD, as well as the resulting pathway, in resistant, recurrent and metastatic disease may elucidate whether and how this relationship impacts a variety of processes, beyond metabolic regulation, that confer aggressive cancer and may lead to lethality of the disease.

Similarly, 37 genes related to the Cav-1/MnSOD/mtH2O2/AMPK pathway were excluded from the CM-GEP, primarily due to poor correlation. More refined biostatistics and informatics approaches may be able to identify potentially useful relationships, as these genes largely act dynamically to regulate various concerted signaling processes. Further, it would be interesting to identify if the opposing molecular signature to the CM-GEP (e.g., based on the Cav-1 low/MnSOD high phenotype) could be predictive for identifying high risk patients and further act as an independent biomarker of highly glycolytic tumors.

While the current project focused on the impact of MnSOD expression and its regulation by Cav-1, there was also preliminary evidence that suggested that Cav-1 may also bind MnSOD directly. Under certain contexts, Cav-1 and MnSOD bind similar chaperones (e.g., HSP70 and HSP90 (Brouet, Sonveaux et al. 2001, Candas and Li 2014)), and further Cav-1 has been suggested to translocate to the mitochondria ((Fridolfsson, Kawaraguchi et al. 2012); own observations). It may be important to assess whether part of the regulation of MnSOD by Cav-1 is through direct complex formation with other scaffolding proteins. This could be critical in light of the fact that some preliminary evidence suggests that MnSOD may translocate to the nucleus under ROS
(H$_2$O$_2$) accumulation, and this translocation was prevented by Cav-1 expression in luminal breast cancer epithelial cells (MCF-7).

The exact nature of what signaling events are promoted by nuclear translocation of MnSOD are still very unclear; however, it is likely that this may be important in some of the different processes engaged during acute and chronic cellular responses to oxidative damage. Nuclear MnSOD was not observed to occur in healthy patient controls, nor in cell culture without H$_2$O$_2$ treatment or genetic manipulation. In clinical tissue samples, MnSOD nuclear localization was highest in ductal invasive carcinoma in situ (DCIS) and in stage IIIb invasive ductal carcinoma (~40%), suggesting that there are two points along tumor progression in which nuclear-MnSOD signaling may be critical in adaptations essential for priming tumors initially for invasion and again for metastasis. Combined with the observations that MnSOD can stabilize HIF2α, a transcription factor that promotes reprogramming to facilitate invasion and metastasis by enhancing factors involved in epithelial-to-mesenchymal transition (EMT), and that MnSOD enhances the occurrence of luminal progenitor and so-called tumor initiating cells within cell populations in a HIF2α-dependent manner, it is quite clear that this alternate role of MnSOD may be one of the key events that transitions a tumor cell from dormancy to highly invasive and metastatic states through enabling cells to undergo EMT. Evaluating the functional role of MnSOD in the nucleus by through in vitro studies (e.g., ChIP) may reveal a novel mechanism by which MnSOD facilitates tumor progression.

It is likely that the Cav-1 also contributes to the regulation of this potential novel function of MnSOD. As mentioned above, it was observed that Cav-1 can limit the nuclear localization of MnSOD caused by exogenous H$_2$O$_2$. In line with our current findings on possible acetylation-dependent MnSOD migration, Cav-1 may also reduce the acetylation of MnSOD in addition to its
overall expression. Further, Cav-1 expression favored the tetrameric (enzymatically functional) form of MnSOD, suggesting it preserves MnSOD in a functional state in the mitochondria. This additional modulation of MnSOD may be important in determining under what conditions and to what extent MnSOD can activate pro-tumorigenic signaling cascades reliant on HIF2α. Thus, the relationship between Cav-1 and MnSOD at this level of complexity suggests that their interaction may be critical not only in metabolic regulation, but also directly involved in other processes (e.g., EMT) that enhance tumor aggression. Investigation of the importance of the direct interaction of Cav-1 and MnSOD (e.g., through inhibition of this interaction by site mutagenesis of Cav-1 or possibly enhancement by CSD peptide) on the localization and migration of MnSOD and the resulting HIF2α-dependent EMT may indicate how MnSOD may influence invasion and metastasis.

It is also critical to understand how these changes in cellular redox status and metabolism could affect the tumor microenvironment. To analyze this more appropriately, using shCav-1 or MnSOD overexpressing cells in organotypic spheroid 3D culture in combination with a variety of other cell/tissue types (e.g., fibroblasts, adipocytes) may reveal how either loss of Cav-1 and/or gain of MnSOD may impact the cellular processes of cells outside of the ductal epithelium, and how this interplay may affect tumor development. While far outside the scope of the current project, a holistic approach using proteomic and other broad (yet in-depth) analyses using a wider range of cell and tissue types are required to fully understand the implication of these interactions on the course of the disease.

Lastly, given the potential tight regulation of Cav-1 on MnSOD expression and cellular redox status, it is still necessary to understand under what conditions Cav-1 is repressed. We had observed that mRNA expression of Cav-1 was inhibited strongly in patients with DCIS, consistent
with the rapid loss of Cav-1 expression early during transformation in vitro and the rapid loss of Cav-1 protein during tumor progression in clinical tissue samples. We had also observed that Cav-1 expression resulted in the downregulation of NADPH oxidases (NOX2 and NOX4) with simultaneous upregulation of catalase, glutathione peroxidase (GPx1) and thioredoxin reductase (TXNRD3); therefore, in addition to the observed enhancement of MnSOD/H$_2$O$_2$ by Cav-1 loss described in this work, it is likely that transcriptional inhibition of Cav-1 may be a critical event in the overall deregulation of redox homeostasis leading to a highly oxidative cellular environment. Thus, identification of how Cav-1 is lost in breast cancer may be critical in understanding the molecular predispositions that facilitate malignant transformation and tumor progression.
CITED LITERATURE


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APPENDIX

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