Myoglobin Attenuates Breast Cancer Cell Proliferation and Migration

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Bachelor of Science, Biochemistry, University of Wisconsin-Madison, 2018

Submitted to the Graduate Faculty of the

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2023

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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The monomeric heme protein myoglobin (Mb), traditionally thought to be expressed exclusively in cardiac and skeletal muscle, is now known to be expressed in approximately 40% of breast tumors. While Mb expression is associated with better patient prognosis, the molecular mechanisms by which Mb limits cancer cell proliferation and migration are unclear. In muscle, the predominant function of Mb is oxygen storage and delivery; however, prior studies demonstrate that the low levels of Mb expressed in cancer cells preclude this. Recent studies have determined that Mb binds to fatty acids, generates oxidants, and scavenges nitric oxide (NO•) in other tissues, but these functions have not been examined in breast cancer cells. We hypothesized that Mb decreases proliferation and migration through fatty acid binding, oxidant generation, and NO• scavenging in cancer cells. Because fatty acid oxidation (FAO) is often upregulated in cancer cells and drives cell migration, we tested whether Mb-mediated fatty acid binding modulates FAO to decrease breast cancer cell migration. We demonstrate that the stable expression of human Mb in MDA-MB-231 breast cancer cells decreases cell migration, mitochondrial respiration, and FAO. We performed site-directed mutagenesis to disrupt fatty acid binding (but not heme coordination) to Mb (Mb K46M) or heme incorporation alone was disrupted (Apo-Mb). Only Apo-Mb restored respiration and cell migration to that of the control cells, suggesting that Mb modulates respiration and migration via a heme-dependent mechanism rather than through fatty acid binding. Mb heme iron generates oxidants, which modulate the actin-binding proteins profilin and cofilin and

scavenging of oxidants restores migration. These data demonstrate that Mb decreases breast cancer FAO and migration, and this effect is due to Mb-dependent oxidant production rather than fatty acid binding. NO•, produced by nitric oxide synthases (NOS), promotes cancer cell proliferation. We report that the expression of Mb scavenges NO• and limits NOS activity to attenuate NO•-dependent proliferation in breast cancer cells. Collectively, the data presented herein demonstrate that Mb-dependent oxidant production and NO• scavenging contribute to Mb-mediated attenuation of cancer cell proliferation and migration. These results will be discussed in the context of breast cancer pathogenesis.

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Preface

I would like to acknowledge our collaborations with Dr. Jesus Tejero and Dr. Courtney Watkins to generate and characterize recombinant myoglobin constructs. Confocal microscopy experiments and the sharing of cell lines are credited to Dr. Partha Roy with assistance from Dave Gau. Collaborations with Dr. Eric Goetzman with help from Bob Zhang for ^{14}C-palmitic acid oxidation studies are greatly appreciated. The work presented in this dissertation would not have been possible without the expertise of these individuals.

I would like to thank Dr. Sruti Shiva for her mentorship and support as well as all members of her lab throughout my candidacy, specifically Drs. Krithika Rao and Andrea Braganza. Their assistance and mentorship have indispensably shaped my career development toward becoming an independent scientist.

Finally, I am emphatically grateful to Ms. Haley Ingersoll, close friends, colleagues, and family members who have provided their unwavering support. Each of these people has made my graduate career fulfilling and memorable.

This work was supported by the predoctoral NIH T32 Pharmacological Sciences Training Program Fellowship.

1.0 Introduction:

1.1 Myoglobin Structure and Function

Myoglobin (Mb) is a small, monomeric heme protein that was first identified in skeletal muscle and cardiomyocytes, where it is expressed at high micromolar concentrations and canonically functions as an oxygen store and transporter.[1, 2] In 1957, John Kendrew and Max Perutz solved the structure of Mb by X-ray diffraction,[3] making Mb the first protein to be described in atomic detail, and earning Kendrew and Perutz the 1962 Nobel Prize in Chemistry.[4] Mb's primary protein structure is 153 amino acids, which configure into eight α-helical secondary structures which encompass its heme prosthetic group (Fe-protoporphyrin IX; **Figure 1-1**). The heme group is stabilized by binding to the distal His64 and proximal His93 residues stemming from helices E and F, respectively. At the center of the heme is an iron ion (Fe), which is penta-coordinated: four bonds are formed with nitrogen atoms within the plane of the protoporphyrin ring, and one with the pyrrole side chain of the His93. This leaves a sixth ligand site to be occupied by small gaseous ligands, such as oxygen (O_2), nitric oxide (NO•), or carbon monoxide (CO). The distal His64 further stabilizes the binding of these ligands.[5-7] The majority of Mb functionality is dependent on its heme iron, which when it is in its reduced (ferrous) state (Fe^{II}) allows for stable coordination of oxygen and reaction with NO•. In contrast, oxidized (ferric) iron (Fe^{III}) is unable to stably bind oxygen and can catalyze the formation of reactive oxygen species (ROS).[8]

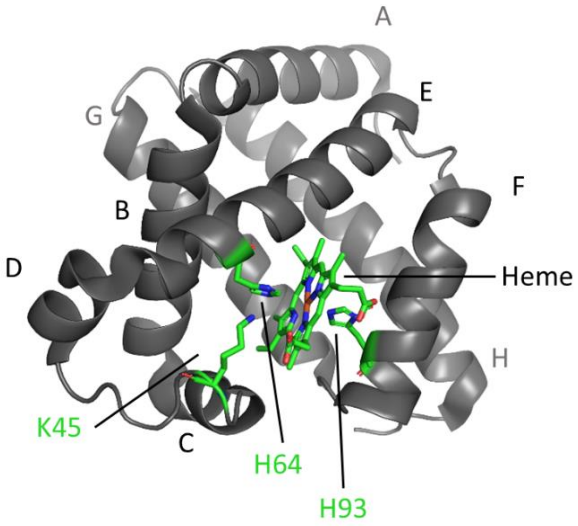

Figure 1-1: The ribbon structure of myoglobin.

Myoglobin contains eight α-helices (A-H) which encircle and coordinate the heme moiety by two histidine residues (H64, H93). The heme is required for the binding of small gaseous ligands. Lysine-45 (K45) has been implicated in fatty acid binding. Protein Data Bank (PDB) structure 1WLA was deposited by Maurus et al. and obtained under the Creative Commons License (CC0 1.0). 9

1.1.1 Canonical Functions: Myoglobin-Dependent Oxygen Storage and Delivery

Early work by Wittenberg and others showed that Mb facilitates oxygen storage and transport in myocytes. These studies characterized Mb's strong affinity for oxygen: Mb's association constant (k_A) with diatomic oxygen is 15-18x10^6 l•mol^{-1}•s^{-1}, while its dissociation constant (k_D) is 60 s^{-1},[10-12] and it possesses a low P_{50} O_2 of 3.1 µM (or 2.8mm Hg).[13] Modeling and animal experiments suggest that Mb is approximately half-saturated with oxygen in skeletal muscle working that is working near its maximum sustainable oxygen consumption.[14, 15] The ability of muscle tissues to maintain sustained energy production (through OXPHOS) and perform work is directly related to the concentration of Mb providing sufficient oxygen to mitochondria within the tissues. Indeed,

Mb concentrations in muscular tissues positively correlate with oxidative enzyme capacity and mitochondrial density.[16]

Along with storing oxygen, Mb promotes oxygen delivery throughout the cell.[1, 2] Towards the capillary, a cell's oxygen concentration will be significantly higher than the interior of the cell at the mitochondrion, where oxygen is consumed. Simple diffusion of oxygen is insufficient to sustain the mitochondria of working muscles. As such, Mb transports oxygen across through translational diffusion of oxy-Mb and deoxy-Mb, where Mb continually combines with and disassociates with oxygen to achieve equitable oxygen distribution throughout tissues.[12, 17] Collectively, early work established that it is the role of Mb is to support mitochondrial oxidative phosphorylation by storage and delivery of oxygen.[12, 17]

Despite its critical role in oxygen storage and delivery in cardiac and skeletal muscle function, genetic ablation of *MB* is non-lethal in mice. Garry *et al*. showed that whole-body Mb-knockout mice ($MB^{-/-}$) showed no phenotypic differences during rest, endurance exercises, or hypoxia.[18] An independent study that soon followed determined that the lack of phenotype was due to several compensatory mechanisms that are present due to Mb deletion, including narrower cell shape, increased capillary density, higher coronary flow, and elevated blood hematocrit.[19] Each of these contributed to a "plateauing" effect on the oxygen gradient in $MB^{-/-}$ cells and allowed normal physiological function of the $MB^{-/-}$ mice. While compensatory mechanisms seemingly explained the non-lethality of $MB^{-/-}$ mice, these studies sparked the investigation of alternative functions of Mb.[13, 18-25]

1.1.2 Canonical Functions: Myoglobin-Dependent Modulation of Nitric Oxide and Generation of ROS

A novel function of Mb is its NO• dioxygenase activity, which is due to the diffusion-limited reaction of NO• with the ferrous heme of oxy-Mb resulting in the oxidation of NO• to nitrate (**Equation 1; Figure 1-2A**).[26] Importantly, deoxygenated Mb (deoxy-Mb) also scavenges NO• through the formation of iron-nitrosyl heme (**Equation 2; Figure 1-2B***).*[27] Brunori and colleagues first proposed a physiological role for Mb-mediated NO• scavenging in which the high concentrations of Mb in the heart could scavenge NO• to decrease NO• bioavailability and downstream signaling.[28] Flogel *et al*. corroborated this role for Mb by showing that *MB*$^{-/-}$ mice were more susceptible to NO•-mediated depression of cardiac function than corresponding wildtype mice and this effect was associated with the formation of cardiac Mb iron-nitrosyl indicative of NO• scavenging in wildtype mice.[27]

$$Mb(Fe^{II})O_2 + NO\cdot \rightarrow Mb(Fe^{III}) + NO_3^- \qquad \textbf{Equation 1}$$

$$Mb(Fe^{II}) + NO\cdot \rightarrow Mb(Fe^{II})NO\cdot \qquad \textbf{Equation 2}$$

An additional function of Mb is its ability to regulate cellular reactive oxygen species (ROS) levels, such as superoxide (•O_2), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$ **Figure 1-2**). It is now well established that autoxidation (**Equation 3**) or low levels of ROS can lead to the formation of the oxidized met (Fe^{III}) and ferryl (Fe^{IV}) forms of Mb (**Equations 4** and **5**, respectively). Mb catalyzes the formation of superoxide and hydroxyl (•OH) radicals, the former of which is dismutated to H_2O_2 by superoxide dismutase (SOD) enzymes **(Figure 1-2C**).[29, 30] Ferryl Mb can oxidize proteins and lipids as well as catalyze the generation of much greater concentrations of H_2O_2 (**Equation 6**). Because Mb can produce superoxide and NO•, Mb may promote the spontaneous and diffusion-limited generation of $ONOO^-$ (**Equation 7**; **Figure 1-2D),**

a potent oxidant and driver of nitrosative stress.[31, 32] Peroxynitrite can also be scavenged by metMb to form nitrate, oxygen, and ferryl Mb.[33-35] Collectively, Mb plays a dual role in both the generation and detoxification of ROS. For subsequent catalysis, the oxidized iron atom of the heme group is reduced to its ferrous state primarily by cytochrome B5 reductase 3 (CYB5R3) and NADH.[36]

$$Mb(Fe^{II})O_2 \rightarrow \cdot O_2^- + Mb(Fe^{III}) \quad \textbf{Equation 3}$$

$$Mb(Fe^{II}) + H_2O_2 \rightarrow Mb(Fe^{III}) + \cdot OH + OH^- \quad \textbf{Equation 4}$$

$$Mb(Fe^{III}) + H_2O_2 \rightarrow \cdot Mb^+(Fe^{IV}) = O + H_2O \quad \textbf{Equation 5}$$

$$Mb(Fe^{III}) + H_2O_2 \rightarrow \cdot Mb^+(Fe^{IV}) = O + H_2O \quad \textbf{Equation 6}$$

$$\cdot O_2^- + NO \cdot \rightarrow ONOO^- \quad \textbf{Equation 7}$$

Studies in the *MB*$^{-/-}$ mouse have demonstrated that this pro-oxidant function of Mb plays a role in pathogenesis. For example, *MB*$^{-/-}$ mouse hearts show less oxidative stress and oxidative damage to the heart after ischemia/reperfusion injury.[20, 37] Further, Mb-catalyzed ROS generation has been implicated in the pathogenesis of rhabdomyolysis and crush injuries in which muscle Mb is released into the circulation.[38, 39]

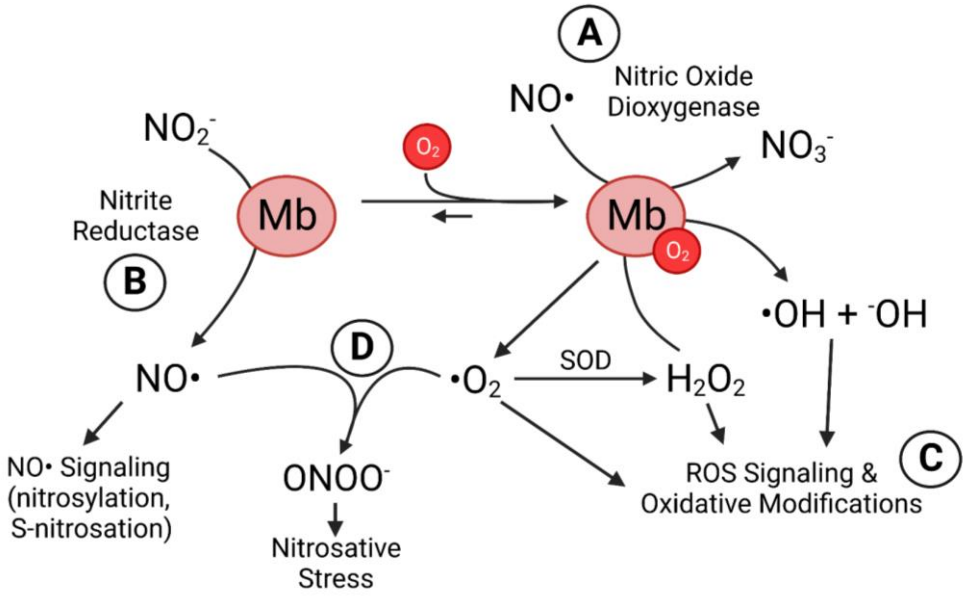

Figure 1-2: Mb-catalyzed modulations of reactive oxygen and nitrogen species.

A) OxyMb scavenges nitric oxide (NO•) to nitrate (NO_3^-) through its nitric oxide dioxygenase function. B) DeoxyMb reduces nitrite (NO_2^-) to NO•, leading to increased levels of bioactive NO•. C) Mb generates superoxide (•O_2) and hydroxyl (•OH) radicals through Fenton chemistry, which can induce signaling or oxidative modfications of proteins, lipids, or nucleic acids. D) Peroxynitrite ($ONOO^-$) is generated through the spontaneous reaction of NO• and •O_2, which leads to nitration of proteins and lipids.

1.1.3 Canonical Functions: Myoglobin-Dependent Fatty Acid Binding

Most recently, Mb has been shown to modulate cellular metabolism in the heart through the binding of fatty acids.[40-42] Mb-dependent fatty acid binding was postulated soon after its oxygen binding and delivery functions were characterized but were not demonstrated until recently.[41, 43-45] The ability for Mb to bind to fatty acids is dependent on its oxygenation state, where oxy-Mb can bind to fatty acids, but deoxy-Mb cannot.[12, 41, 46, 47] Oxy-Mb stably binds to the alkyl tail of long-chain fatty acids through hydrophobic interactions with the heme and residues in the B, C, and E α-helices. The dissociation constant between oxy-Mb and fatty acids is in the micromolar

range and inversely related to the alkyl tail length.[46, 47] Furthermore, oxy-Mb has been shown to bind fatty acids like palmitate and oleate through a highly conserved lysine residue, K45 (**Figure 1-1**).[44-48] The positively charged lysine residue forms electrostatic interactions with the electron-rich carbonyl group of the fatty acid or acylcarnitine.[47] In healthy breast tissue, oxy-Mb contributes to the production and secretion of lipids into breast milk by binding and transporting fatty acids.[44, 46] Mb has also been shown to promote fatty acid oxidation (FAO) in brown adipose tissue.[49] Moreover, in cardiomyocytes, which predominantly utilize fatty acids for energy, $MB^{-/-}$ has been shown to shift substrate utilization to glucose and increase triglyceride levels, indicating that Mb promotes cardiomyocyte FAO.[40, 42]

Despite the recognition of these non-oxygen storage functions of Mb, the exact role of these functions in different cell types, such as epithelial cancers, has not been elucidated.[49-51] The overarching goal of this thesis is to understand the role of these alternative functions of Mb in the context of breast cancer cell function.

1.2 Myoglobin Expression is Associated with Improved Outcomes in Breast Cancer Patients

In 2009 Flonta *et al*. first reported the detection of ectopic Mb expression in breast, prostate, colon, and non-small cell lung cancers, renal cell carcinoma, leukemic bone marrow, as well as mesenchymal stem cells.[50-52] Specifically in breast tumors, Mb is expressed in 40% of all tumors.[53] Importantly, accumulating data from independent studies have identified the tumor-specific transcriptional regulation of *MB* and revealed that Mb expression is associated with improved patient survival in breast cancer patients.[53, 54]

Clinically, the expression of Mb in breast tumors has been associated with increased overall survival in patients. From their 2010 study of 917 patients with invasive breast tumors, Kristiansen *et al*. found that Mb-positive tumors correlate with a significantly higher five-year survival rate of 83% compared to 75% for Mb-negative tumors.[53] They report that Mb-positivity is inversely correlated with tumor stage, where 88% of ductal carcinoma in situ (DCIS) cases were Mb-positive compared to 71% of invasive breast tumors. Moreover, Mb expression was weakly and inversely correlated with tumor grade, where Mb-expressing tumors were more differentiated than Mb-negative tumors.[53] In addition, the molecular markers associated with Mb expression were also detailed by Kristiansen *et al*.: Mb expression is positively correlated to HR expression, signifying that Mb expression is more common in the luminal subtype of breast tumors. Consistent with this, 71% of all hormone receptor-positive (HR+) tumors examined showed Mb protein expression and *MB* transcript levels were highest in luminal tumors. To account for differences in survival based on HR-positive tumors, Kristiansen *et al*. further showed that Mb expression is associated with an increased survival rate, where estrogen receptor negative (ER-) and Mb-positive tumors showed a higher cumulative survival than ER- and Mb-negative tumors; tumors that expressed either Mb or ER showed an intermediate survival rate (**Figure 1-2A**).[53] These survival data from 2010 are also supported by Aboouf *et al*.'s 2022 study, where Mb expression was also found to be correlated with p53 status.[55] p53 is frequently mutated in cancers and correlates with worse patient outcomes.[55] In 288 samples of invasive ductal carcinoma, Aboouf *et al*. found that Mb expression was correlated with wild-type (WT) p53 status: 67.1% of p53 mutant tumors showed mild, moderate, or high Mb expression compared to the 80.8% of p53 WT tumors. Moreover, cumulative survival rates were higher when tumors expressed Mb regardless of whether p53 was WT or mutated (**Figure 1-2B**).[55] In total, these

patient samples have revealed that Mb expression is associated with enhanced patient survival. Of note, in the following chapters, we focus on triple-negative breast cancer (TNBC) specifically to remove the confounding effects of hormone receptor expression.

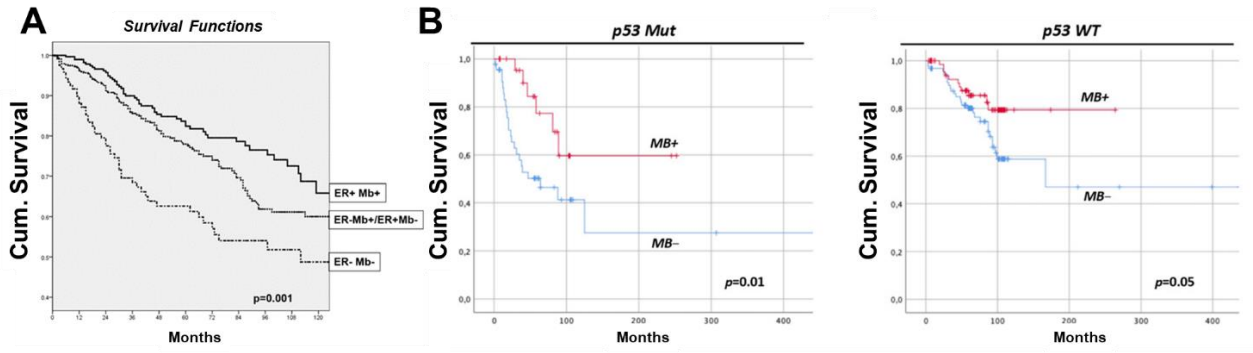

Figure 1-3: Myoglobin expression is associated with increased patient survival.

A) Cumulative survival rates are significantly increased in patients with tumors expressing both estrogen receptor (ER+) and myoglobin (MB+) compared to ER- and Mb- tumors. Patients with ER+/Mb- or ER-/Mb+ tumors show an intermediate survival rate. Data are obtained from 872 breast cancer patients, as published by Kristiansen et al. under a Creative Commons CC-BY-NC-SA license.[53] B) Cumulative survival rates are improved in patients with MB+ tumors whether P53 is mutated (left) or wild-type (WT; right). Data are obtained from 288 invasive ductal carcinomas, as published by Aboouf et al. under Creative Commons CC BY 4.0 license.[55]

While it is established that Mb improves patient survival in both HR+ and HR- tumors, the molecular mechanisms underlying Mb-mediated improvement in survival and outcomes are still unknown. The focus of this thesis is to elucidate the molecular mechanisms by which Mb expression modulates cancer cell function. Chapter 1.3 outlines the current understanding of the transcriptional regulation of Mb in breast cancer cells. Chapter 1.4 focuses on the roles of Mb-dependent ROS generation, NO• scavenging, and fatty acid binding functions in the regulation of key molecular processes in cancer pathogenesis.

1.3 Expression and Transcriptional Regulation of Myoglobin in Breast Cancer

Flonta *et al*. and Kristiansen *et al* reported that five breast carcinoma cell lines, but not healthy breast tissue, express Mb at both the mRNA and protein levels, and in patients, 30 of the 31 (97%) primary breast tumors express Mb to some degree.[50].[50] More recently in 2015, Bicker *et al*. identified six novel exons in the 5' untranslated region (5' UTR) of the *MB* gene within breast and colon cancer cell lines that aberrantly express *MB* (**Figure 1-3**). The updated *MB* gene spans 31,796 base pairs (bp) and includes 13 exons; 10 of which are untranslated ('u') and three encoding exons ('c').[56] The formerly identified exons encoding *MB* 1-3 have since been renamed to include the novel untranslated exons and now correspond to exons 9c, 11c, and 12c, respectively. The major difference between *MB* expression in these tissues is the 5'UTR, but the full-length *MB* transcripts in cancer cells encode the same gene product as in myocytes. MB transcription starts at sites 8u and 9u in myocytes and sites 5u and 4u in cancer cells. This is supported by 21 human breast cancer patient tumors showing that the combined total of *MB* transcripts (variants 9, 10, and 11) was 14-fold higher than variant 2. Taken together, variants 9, 10, and 11 are the predominant transcripts of *MB* within cancer cells.[56]

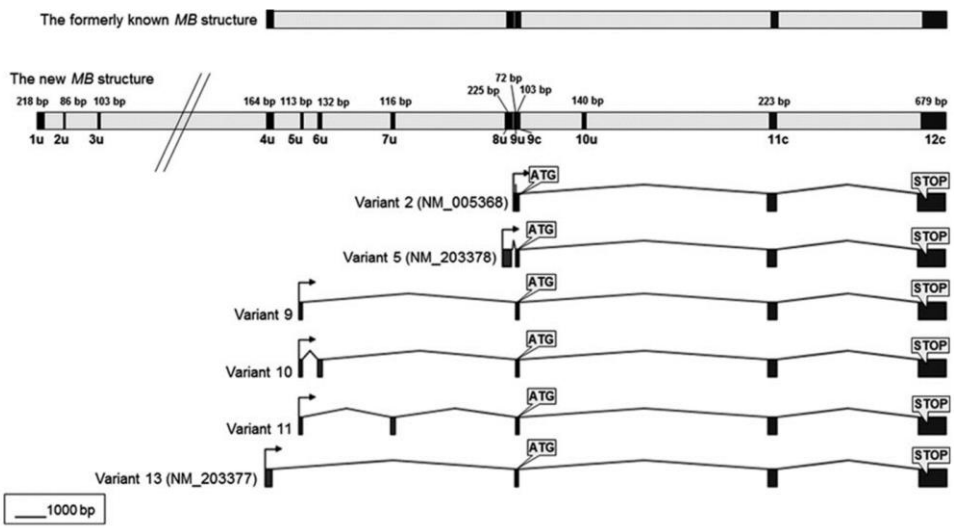

Figure 1-4: The revised exon and intron structure of the human MB gene.

Exons are represented as dark boxes with base pair sizes. Untranslated and coding exons are labeled with "u" or "c," respectively. Variants 2 and 5 are the predominant protein-encoding transcript variants in muscle cells, and variants 9-11, and 13 are within cancer-related samples. Figures are obtained from Bicker *et al.*'s 2014 publication through Oxford University Press (license agreement number 5512041423385).[56]

Given that the neoplastic and myocytic *MB* transcripts originate at different 5' UTR exons, the expression of *MB* is regulated by tissue-dependent mechanisms. In skeletal muscle, *MB* is induced upon hypoxia *and* exercise stimuli.[57] However, several studies have shown that hypoxia alone is sufficient to increase *MB* expression in cancer cells.[54, 56, 58] Bicker *et al.* identified a single enhancer region near the promoter of *MB* (termed A1) that contains both HIF-1α and HIF-1β binding peaks nearby, suggesting that the HIF-1α/β dimer binds near enhancer A1 under hypoxia.[54, 58] In alignment with the transcriptional regulation of *MB* within breast cancer cells being under the regulation of HIF-1α, several studies have reported that Mb expression also correlates with HIF-1α stabilization and protein expression in pre-clinical models and human tumors.[50, 53, 54, 59]

In contrast to hypoxia driving *MB* expression in breast cancers, *MB* expression is decreased upon treatment with estrogens. Early work by Kristiansen *et al*. showed that *MB*

expression is downregulated by 17β-estradiol (E2) treatment in MCF7 and MDA-MB-468 cells in a dose-dependent manner.[53]. Supporting these data, pre-menopausal breast cancer patients had significantly less Mb compared to post-menopausal patients.[53] Four estrogen-responsive elements were identified in region A1, further suggesting estrogen-downregulated *MB* expression in breast cancer cells. [58]

While the mechanisms of MB's transcriptional regulation have been well-characterized, the functional roles of the translated protein within breast cancer cells are not well known. Early studies highlighted that the levels of Mb protein (rather than *MB* mRNA) correlate with increased patient survival rates.[53] Elucidating the roles of Mb as a protein is therefore critical to understanding how Mb limits cancer progress and improves patient prognoses.

1.4 The Role of Myoglobin in Regulating Cellular Processes Underlying Breast Cancer Pathogenesis

Over the past three decades, Weinberg and Hanahan have identified several hallmarks of cancer cells. This section will focus specifically on three major hallmarks – metabolism, migration, and proliferation- and their potential regulation by Mb in breast cancer cells.

The level of Mb protein expression in breast tumors is far lower (3-10μM) than in myocytes (200-300μM). Given the low levels of endogenous Mb in breast cancer cells, it is accepted that Mb expression in tumors does not function to mitigate hypoxia. In agreement with this idea, Kristiansen *et al*.'s showed in 2011 that oxygen tension in MDA-MB-468 cells did not change when Mb was genetically silenced. In contrast, oxygen consumption increased when Mb expression was inhibited compared with Mb-expressing cells.[54] Given that the predominant

protective role of Mb does not appear to be the storage and delivery of oxygen in cancer cells, it has been proposed that the other functions of Mb predominate and modulate cancer cell function and phenotype. Thus, the following subchapters will focus on three non-canonical functions of Mb: fatty acid binding, ROS generation, and NO• scavenging.

1.4.1 Fatty Acid Oxidation is a Driver of Cancer Progression: A Potential Role for Mb-Mediated Fatty Acid Binding

Cancer cells initiate metabolic rewiring to increase bioenergetic and biosynthetic output, allowing them to sustain rapid tumor growth and migration.[60-64] Otto Warburg noted an increased reliance on glycolysis in the presence of normal oxygen levels (aerobic glycolysis, "the Warburg effect")[65, 66] and hypothesized that this shift in substrate utilization was due to dysfunctional mitochondria. However, this was later disproved. Instead of all cancers upregulating glycolysis uniformly, a consensus is that different tumors deregulate bioenergetics through different mechanisms; metabolic flexibility and efficient energy conversion drive malignant tumor growth.[65, 67] Cancer cells must be able to alter their carbon sources and interchangeably utilize glycolysis, FAO, and oxidative phosphorylation as means of energy production based on their surrounding environment.[65]

Lipid metabolism—both synthesis and oxidation—is a major pathway that has received renewed interest in understanding cancer progression. The processes of fatty acid synthesis and oxidation (FAS and FAO, respectively) are thought to be mutually exclusive to prevent a futile cycle of fatty acid generation and degradation through several key mechanisms. Malonyl-CoA, an early product of FAS, inhibits the rate-limiting enzyme of FAO, carnitine palmitoyl transferase-1 (CPT1). In contrast, when cellular energy levels are depleted (i.e.: the ratio of

AMP:ATP rises), AMP-activated protein kinase (AMPK) inhibits acetyl-CoA carboxylase (ACC) to inhibit FAS and instead promotes FAO for increased energy production.[68] Despite these feedback mechanisms and FAS and FAO, Corbet *et al*. have reported that some cancer cells can increase both lipid synthesis and oxidation.[69] Indeed, increased *de novo* lipid synthesis and lipid uptake are common features of cancers and are required for new cell membranes, and these processes in the context of breast cancer disease progression and treatment have been detailed elsewhere.[68, 70-75] On the other hand, cancer cells that upregulate FAO have been reported to have selective advantages because FAO is an efficient means of generating ATP and maintaining redox homeostasis.

The process of FAO requires several steps of fatty acid transport modification prior to repeated rounds of oxidation. First, fatty acids are either obtained through increased cellular uptake (via CD36) or through the cleavage of triglycerides (TGs) to generate acyl-CoAs or liberate free fatty acids, respectively.[71, 74] Fatty acids and acyl-CoAs that are destined for degradation through mitochondrial β-oxidation are shuttled to the mitochondrial via fatty acid binding proteins (FABP). At the outer mitochondrial membrane (OMM), long-chain acyl-CoA synthetase (ACSL1) activates free fatty acids by thiol-esterification to CoA.[76, 77] Acyl-CoAs are then chemically modified by CPT1, which substitutes the CoA with L-carnitine, producing acyl-carnitines that are then transported across the OMM and into the intermembrane space. The processes of conversion and transport catalyzed by CPT1 are the rate-limiting steps of FAO.[76-80] Next, carnitine-acylcarnitine translocase (CACT, i.e.: SLC25A20) mobilizes acylcarnitines across the inner mitochondrial membrane (IMM) and into the matrix. Once inside the matrix, CPT2 cleaves L-carnitine from acylcarnitines to liberate the free fatty acid. The conversion and import process of acylcarnitines by CPT1, CACT, and CPT2 and the recycling of L-carnitine is

collectively called the "carnitine cycle/shuttle" and has been reviewed previously.[77] Once inside the mitochondrial matrix, β-oxidation ensues, where four key enzymes work sequentially to remove two carbon units (in the form of acetyl-CoA) from the fatty acid repeatedly.[81] The enzymes are acyl-CoA dehydrogenase, hydroxyalkyl-CoA dehydrogenase, enoyl-CoA hydratase, and 2-ketoacyl-CoA thiolase. [82, 83] The cyclical oxidation of one 16-carbon molecule of the fatty acid palmitate (C16:0) generates eight acetyl-CoA molecules and 16 reducing equivalents (eight FADH2, eight NADH) compared to the breakdown of one glucose into two acetyl-CoA and two NADH through glycolysis.[80, 84, 85] This four-fold increase in acetyl-CoA generation from FAO allows for more efficient production of reducing equivalents through the TCA cycle per molecule of carbon substrate compared to glycolysis. Increased $FADH_2$ and NADH levels allow for heightened production of ATP through OXPHOS. Importantly, NADH can also be phosphorylated by mitochondrial NAD kinase (NADK2) to produce NADPH, which is a major reducing equivalent used for cancer cell anabolism.[68, 82, 86] These properties of FAO enable cancer cells to endure the challenges of different microenvironments and nutrient deprivation throughout the body during metastatic dissemination.

Previous studies have demonstrated that increased FAO in cancers enhances cell migration and metastasis, distinguishing FAO as a driver of cancer progression.[68, 82, 87-90] Primary tumors, which have not yet metastasized, are more likely to utilize glycolysis, yet cancer cells increase their reliance on FAO upon intravasation into the bloodstream or lymph and circulation throughout the body.[64] Loo *et al*. have reported that FAO is closely tied to TNBC cells' transition/dedifferentiation from the epithelial state to the mesenchymal state, and this is reversed by FAO inhibition with the CPT1 inhibitor etomoxir.[91] Shifting to FAO serves several purposes for metastatic cells, including utilization of internal lipid stores (e.g.: triglycerides) in times of

nutrient deprivation, attenuation of cytosolic and extracellular acidification from glycolytic lactate production, epigenetic regulation of gene transcription, and increased energy production efficiency to enhance cytoskeletal remodeling.[69, 90-94] Wright *et al*. have reported that increasing FAO in TNBC drives metastasis but enhancing lipid droplet accumulation is sufficient to decrease migration *in vitro*. Additionally, primary tumors showed higher levels of lipid droplets compared to metastatic tumors *in vivo*.[92] In agreement with this, Loo *et al*. observed that lipid droplets are associated with an epithelial cell state and decreased metastasis.[91] High rates of glycolysis and subsequent lactate production lead to an acidosis-induced inhibition of glycolysis and cellular proliferation; cancer cells circumvent this by shifting their metabolism towards FAO to avoid acidosis and cell cycle arrest.[69] Epigenetics are also influenced by FAO; the acetyl-CoA generated by FAO increases histone acetylation within the nucleus. FAO-induced acetylation changes have been shown to decrease the transcription of the FAS gene *ACC1* and further promote FAO; yet this is reversed by blocking FAO.[85, 95] Because FAO can generate more ATP per molecule of substrate, cancers upregulate FAO to fuel actin cytoskeleton remodeling during migration. In agreement with this, Park *et al*. have shown that the ATP generated from metastatic TNBCs increases Src autophosphorylation and downstream cell migration *in vitro* and metastasis *in vivo*. Inhibiting FAO pharmacologically or by genetic silencing of CPT1 and CPT2 decreases Src autophosphorylation and cell migration.[90] Together, these data suggest that increased mitochondrial FAO in breast cancer cells is a common feature of cancer progression and a driver of metastasis, and modulators of FAO may have a therapeutic benefit.

Few studies have explored the relationship between Mb-dependent fatty acid binding and its role in fatty acid metabolism in breast cancer cells. Kristiansen *et al*. noticed that Mb expression is correlated with FASN; they also observed that treating with the FASN inhibitor,

C75, *MB* mRNA and protein expression decreases.[53] From these data, they suggested Mb may play a role in fatty acid metabolism. RNA-Sequencing (RNA-Seq) and gene ontology (GO) term analysis by Bicker *et al*. revealed that *MB* knockdown led to a decrease in genes associated with fatty acid metabolism and biosynthesis in MDA-MB-468 cells.[96] A more recent study by Armbruster *et al*. shows that MDA-MB-468 cells have differing fatty acid profiles in the presence or absence of Mb expression; the expression of Mb also increases fatty acid solubility within the cytoplasm.[97] As a proxy for FAO, the levels of oleate (C18:1) were significantly less than hexadecenoate (C16:1) in Mb-expressing MDA-MB-468 cells. Because C18:1 is converted to C16:1 almost exclusively by oxidation, they reasoned that Mb promotes *limited* oxidation of oleate at normoxia. Together, Armbruster *et al*. hypothesize that Mb promotes fatty acid cytoplasmic solubility and limited oxidation within MDA-MB-468 and MCF7 cells under normoxic conditions, but severe hypoxia (0.2%) resulted in Mb deoxygenation and fatty acid deposition into lipid droplets.[97] These studies led us to investigate the functional role of Mb-dependent fatty acid binding in the regulation of cancer cell FAO and its link to cell migration, which will be detailed in Chapter 2.

1.4.2 Actin Cytoskeletal Remodeling in Cancer Metastasis and the Role of Myoglobin-Derived ROS

The process of metastasis involves cellular migration and invasion from the primary tumor site and dissemination through the blood or lymph vessels before establishing a micro-metastasis at a secondary site. Primary tumor cancer cells undergo an epithelial-mesenchymal transition (EMT) through dedifferentiation, allowing them more stem-like properties and enhanced migratory and invasive behavior.[98, 99] During directed migration towards a particular substance, called

chemotaxis, integrin activation leads to a signaling cascade of nascent focal adhesion formation, kinase activation, and downstream actin fiber remodeling. This process is highly dynamic, requiring cycles of focal adhesion turnover and actin reorganization. Migration is a locally coordinated process that requires the formation of the lamellipodium and myosin-directed movement towards the leading edge of the cell as well as simultaneous retraction at the rear of the cell.[100-105]

A multitude of actin-binding proteins (ABPs) is required for successful cytoskeletal reorganization and cell migration. Actin exists in a cycle of the monomeric globules (G-actin) or polymerized filaments (F-actin).[106] At the leading edge of the cell, actin polymerization drives the plasma membrane forward within the lamellipodia.[101] Actin is polymerized by the addition of ATP-bound G-actin at the barbed end of F-actin, then the ATP is hydrolyzed to ADP and the inorganic phosphate (Pi); both of ADP and Pi are coordinated by and required for stable F-actin.[107] The Arp2/3 complex promotes actin nucleation and branching at the lamellipodia along with the WASP and WAVE family proteins. Formin and Ena/VASP family proteins are the predominant ABPs for elongating actin filaments.[108, 109] Profilin-1 (PFN1) is a widely expressed ABP and primarily serves as a nucleotide exchange factor, switching ADP for ATP on G-actin monomers and promoting elongation of F-actin.[110] It has been reported that the levels of PFN1 directly influence cell motility by modulating the level of F-actin, where low levels of PFN1 enhance cell migration and high levels of PFN1 decrease migration.[110-118] Consistent with this, breast cancers commonly downregulate PFN1 levels to increase their migratory capacity.[111, 113, 114, 116] The PFN1 level-specific effects on migration are due to PFN1's stoichiometry to its binding partners. In addition to actin, PFN1 interacts with phosphatidylinositol-4,5-bisphosphate (PIP_2) in the plasma membrane and poly-L-proline (PLP) sequences of other ABP proteins (e.g., WASP, Ena/VASP).

Ding *et al*. have published a working model of how low levels of PFN1 promote actin polymerization at the barbed end and provide a scaffold for other ABP through its PLP region. At higher PFN1 levels, PFN1 binds to actin as well as PIP_2, and this blocks the recruitment of ABPs (e.g. Ena/VASP) to the lamellipodia and slows cell migration.[110] Together, these reports have detailed a pivotal role for PFN1 levels in facilitating breast cancer cell migration at low levels, while slowing cell migration at high levels.

As the cell begins to move forward, it must simultaneously disassemble focal adhesions and depolymerize F-actin towards the rear of the cell. ADP-Pi-actin releases the Pi and becomes destabilized, and F-actin is depolymerized into G-actin monomers.[101, 108] Cofilin (CFL1) is an actin-depolymerizing protein that facilitates the severing of F-actin.[119] CFL1 activity is regulated by several post-translational modifications, such as phosphorylation and oxidation of serine-3 and several cysteine residues, respectively. Phosphorylation at serine 3 (phospho-Ser3) at CFL1 by LIM-kinase (LIMK) renders CFL1 inactive and unable to sever actin. However, the phosphatases slightshot1 (SSH1) and chronophin can dephosphorylate CFL1 to activate it.[120, 121] Dephosphorylated CFL1 has been reported to promote actin reorganization by depolymerizing F-actin, promoting its turnover and reintegration into the expanding lamellipodium at the front of the cell and advancing migration.[122-124] Specifically in breast cancer, both the overexpression and dephosphorylation of CFL1 are associated with an increased risk of metastasis.[125, 126] CFL1 has four cysteine residues that are subject to oxidative modification by ROS and have been shown to inhibit CFL1 activity at the leading edge of the cell to prevent nascent F-actin from becoming severed during migration.[127, 128]

Actin cytoskeletal remodeling is energetically demanding and thus is tightly coordinated with mitochondrial trafficking to the lamella along microtubules.[100] To adapt to fluctuating

metabolic demands and the progression of the cell cycle, mitochondria undergo regular fusion and fission events, in which mitochondrial networks form and break apart.[80, 129] Mitochondrial fusion is regulated by several small GTPase proteins including mitofusins 1 and 2 (MFN1/2, collectively called MFNs). The overexpression of MFNs has been shown to suppress cell proliferation, lamellipodia formation, and migration, yet the loss of MFNs causes mitochondrial network fragmentation that leads to mitotic fission and cell growth.[129-131] In addition to mitochondrial dynamics and their role in coordinating cell migration, mitochondria also generate ROS, which has been shown to influence key signaling modules and downstream cellular activities, such as proliferation, invasion, energy sensing, and cell fate; each of which has been reviewed elsewhere.[109] Collectively, migration is dependent on the successful coordination of cytoskeletal remodeling by ABPs as well as mitochondrial bioenergetics and network dynamics, and each of these factors is a critical component of cell migration and the metastatic cascade**.**

The impact of Mb expression on cellular migration has not yet been determined. A handful of studies have reported conflicting findings, where Mb expression either slows or enhances cellular migration; authors have noted that this discrepancy is likely due to cell line-specific mutations and deletions.[54, 55, 132] In any case, it has been well-established that ROS affects cell migration by modulating the incorporation of actin and/or the activity of actin remodeling proteins, such as cofilin (CFL1) and Rho GTPases, as reviewed previously.[133] However, no studies have examined whether Mb-catalyzed ROS modulates cytoskeletal remodeling through the modulation of ABPs. Aside from ABPs, Braganza *et al*. demonstrated that Mb-dependent oxidant production led to the accumulation of mitofusins (MFNs), the predominant catalysts of mitochondrial fusion, and decreased cell proliferation *in vitro* and *in vivo*. MFNs also play a role in cell migration, but Mb-dependent alterations in MFNs and their effect on cell migration have

not yet been examined. The role of Mb-catalyzed ROS generation in the modulation of cancer cell migration will be investigated in Chapter 3.

1.4.3 Nitric Oxide (NO•) Promotes Enhanced Breast Cancer Cell Proliferation and the Potential Role of Myoglobin-Mediated NO• Scavenging

Aberrant cell cycle progression is a hallmark of cancer. Cancer cells achieve unchecked cell division through the mutation or deletion of tumor suppressors or the acquisition of mutations within oncogenes.[63, 134, 135] In addition, cell signaling factors can also drive cell division, leading to tumoral outgrowth. Nitric oxide (NO•) is a critical modulator of cancer cell proliferation that has been increasingly implicated in driving breast cancer breast disease progression.[136-138] Thus, identifying novel mechanisms that regulate NO• production and signaling to its downstream targets may provide therapeutic benefits by limiting cancer cell proliferation.

NO• is canonically generated through nitric oxide synthase (NOS) enzymes *NOS1-3*. Each NOS isoform catalyzes the formation of NO• and L-citrulline from molecular oxygen (O_2) and L-arginine, yet each isoform is expressed in different tissues and generates NO• in different amounts. *NOS1* and *NOS3* (i.e.: neuronal "n" NOS and endothelial "e" NOS, respectively) are expressed within the neurons and endothelial cells. nNOS and eNOS are constitutively expressed and generate nanomolar levels of NO• over short periods depending on intracellular calcium levels. *NOS2*, or inducible "i" NOS, becomes expressed upon exposure to bacterial pathogens or cytokines and is constitutively active, producing high levels of NO• to exert cytotoxic effects on pathogens. [139, 140] Although iNOS is mainly expressed within macrophages, other cells, such as breast cancer cells, can induce the expression of iNOS and upregulate NO• levels. iNOS expression is a negative prognostic indicator in TNBC patients and correlates with advanced

disease stage, disease recurrence, distant metastasis, and decreased survival.[137, 140-144] Additionally, NO• can be generated through the sequential reduction of nitrate and nitrite. Nitrate, obtained from one's diet, is reduced to nitrites by commensal bacteria. From there, proteins like hemoglobin, Mb, and xanthine oxidoreductase further reduce nitrite to bioactive NO• when deoxygenated.[140, 145-147] NO• is generated through several mechanisms and plays a key role in cell signaling.

In cancer cells, the flux of NO• determines whether it has pro-cancerous or cytostatic/cytotoxic effects. [138] Physiologically, NO• is classically known to mediate vasodilation by activating soluble guanylyl cyclase (sGC) in the neighboring smooth muscle cells. sGC converts GTP to cGMP, which then activates protein kinase G (PKG) to stimulate arterial relaxation and increased blood flow.[148] The level of NO• flux that activates sGC is approximately 10-100nM. This low level of NO• has also been shown to promote cancer cell proliferation through the activation of the PI3K-AKT pathway and mitogen-activated protein kinase (MAPK)/ERK cascade.[138, 140, 149, 150] Higher levels of NO• flux (100-500nM) are similar to the NO• levels generated by iNOS *in vivo* and are independent of the sGC-cGMP pathway.[138] At this level, the PI3K-AKT, and MAPK/ERK pathways are also activated through the NO•-dependent modification of thiol side chains of cysteine residues, called S-nitrosylation, to play enable unchecked proliferation.[137, 151, 152] NO•-dependent metabolic changes also occur through the inhibition of mitochondrial ETC complex IV as well as the stabilization of hypoxia-inducible-factor-1-alpha (HIF-1α).[138, 153] Nitrosative stress signaling occurs between 500-1000nM NO• flux and commonly activates p53 via phosphorylation. In cells with wildtype/unmutated p53, phosphorylation arrests the cell cycle and alters carbon utilization towards FAO; however, p53 is frequently mutated in cancers and nitrosation of p53 does not deter cell growth but may induce

apoptosis. NO• flux above one micromolar (μM) results in necrotic cell death through reactive nitrogen species (RNS).[138] The dichotomous role of NO• is perhaps best exemplified by the observation of low levels of iNOS being associated with breast tumor progression, while on the other hand, high levels of iNOS are associated with tumor regression.[154]

Because NO• is a short-lived and reactive free radical, methods aimed at attenuating NO• signaling are challenging. Current clinical efforts have utilized NOS inhibitors to directly block NO• production and downstream signaling. In a small clinical trial, a treatment regimen of pan-NOS inhibitors L-NNMA (in combination with a taxane) decreased tumor volumes in 15.4% of metastatic and 81.8% of chemo-refractory locally advanced TNBCs.[155] One area that has not been explored with TNBC is the interaction between Mb and NO• signaling. Because oxy-Mb is a NO• dioxygenase that scavenges NO• by oxidizing it to nitrate, the expression of Mb within tumors may therefore present a novel mechanism to modulate NO• signaling.[156]

Few studies have characterized the interaction of Mb and NO• within breast tumors. Bicker *et al*. conducted RNA-Seq and GO term analysis on MDA-MB-468 cells with and with Mb expression and noted the GO term 'regulation of NO• biosynthetic process' was decreased when *MB* was knocked down.[96] Quinting *et al*. recently showed that the addition of NO• (through the NO•-donor S-nitrosoglutathione, GSNO) limits MDA-MB-468 tumor spheroids necrosis; however, when *MB* is knocked down, NO• treatment led to increased necrosis.[157] They also showed that HIF-1α is stabilized by NO• treatment in cell monolayers, yet the knockdown of *MB* decreases HIF-1α proline hydroxylation and increases HIF-1α target gene transcription, suggesting that Mb attenuates HIF-1α stabilization and consequently its transcription factor activity.[157] These data suggest that Mb diminishes NO• signaling and HIF-1α stabilization via NO• scavenging. Quinting *et al*. explored the interplay between NO•, HIF-1α, and Mb further via

RNA-Seq. They identified significant enrichment in genes relating to cellular assembly and organization, cell death and survival, and cardiovascular system development and function with the treatment of NO• after *MB* knockdown. Within these categories, they identified 1029 differentially expressed genes, 45 of which are both target genes of HIF-1α and changed with *MB* knockdown with NO• treatment. Only three genes were significantly changed by ≥1.5-fold and validated by qPCR in MDA-MB-468 cells: *MKNK1*, *RAB8B*, and *SLC2A1* (i.e.: GLUT1). *MKNK1* was the only gene found to be upregulated in *MB* knockout cells treated with NO• and encodes MAP-kinase interacting kinases (MNKs). MNKs have been implicated in cancer progression and treatment resistance, yet these data suggest that Mb-mediated scavenging of NO• may attenuate MNK and MAPK signaling.[158] In contrast, *RAB8B* and GLUT1 are downregulated following MB silencing and NO• treatment; this may imply a decrease in intracellular membrane trafficking and glucose uptake.[159, 160] The authors suggest that deoxy-Mb generates NO• within the hypoxic tumor, yet these studies were conducted at normoxia and hypoxia (21% and 1% O_2, respectively), which corresponds to approximately 42% deoxy-Mb and 58% oxy-Mb, the latter being the predominant species acting as an nitrite dioxygenase (**Equation 1**).[161] Accordingly, the direct mechanism of Mb-dependent NO• scavenging through its NO• dioxygenase activity have not yet been examined within breast cancer cells. Therefore, we hypothesize that Mb affords improved patient survival rates by scavenging NO• and attenuating its signaling within TNBC. Our findings are detailed in Chapter 4.

1.5 Summary and Hypothesis

In summary, published data demonstrate that Mb is expressed at low levels in breast cancer cells and this expression is associated with improved patient prognosis. However, the molecular mechanisms that underlie this effect are unknown. While Mb expression levels are too low to regulate cancer cell oxygen gradients, the role of Mb-mediated fatty acid binding, ROS generation, and NO• scavenging has not been thoroughly tested in the context of cancer cell function and phenotype. The overarching hypothesis of this thesis is that Mb-dependent fatty acid binding, oxidant production, and NO• scavenging decrease cell migration and proliferation, which ultimately decrease cancer progression (**Figure 1-5**).

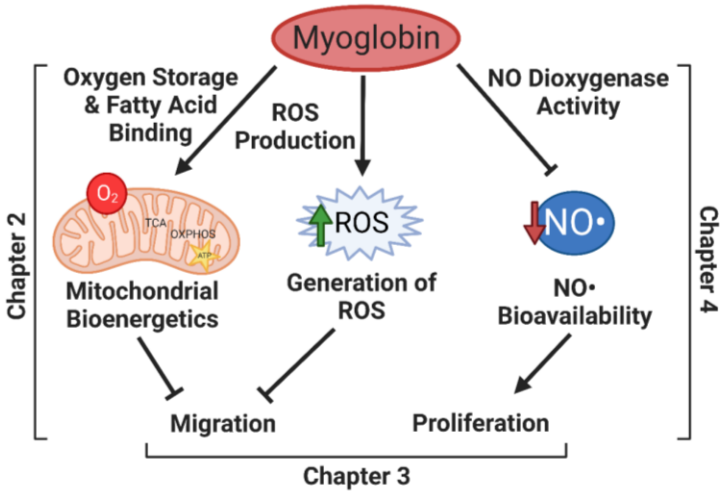

Figure 1-5: Graphical Hypothesis.

Mb attenuates breast cancer progression through the modulation of mitochondrial energetics, production of ROS, and regulation of NO• bioavailability.

Chapter 2 explores the role of Mb-dependent fatty acid binding in the modulation of FAO, and its relation to cell migration. Chapter 3 demonstrates that Mb-catalyzed ROS decreases the migratory capability of breast cancer cells through the modulation of the ABPs PFN1 and CFL1

phosphorylation. Chapter 4 reveals that Mb-dependent scavenging of NO• in breast cancer cells attenuates NO•-dependent downstream proliferative signaling. Finally, Chapter 5 will discuss the conclusions of these results, their overall contributions to our understanding of Mb-dependent regulation of breast cancer cell function, and how these results have sparked future directions for research in this area.

2.0 Myoglobin-Dependent Fatty Acid Binding Does Not Contribute to Myoglobin-Mediated Attenuation of Breast Cancer Cell Metabolism or Migration

2.1 Introduction

A hallmark of cancer cells is metabolic reprogramming, which allows for neoplastic cells to meet the energetic demands of aberrant cell migration in metastatic cancers.[63, 135] Specifically, fatty acid oxidation (FAO) is upregulated in breast cancers for its high yield of ATP per molecule of substrate, which can drive efficient actin cytoskeleton remodeling and promote metastatic spreading throughout the body.[71, 82, 162] Several studies have linked increased FAO with increased breast cancer cell migration and metastasis.[85, 90, 91] Specifically, Loo *et al*. showed that inhibition of FAO with the carnitine palmitoyl transferase (CPT1) inhibitor etomoxir suppresses epithelial-to-mesenchymal transition (EMT) and decreases metastases in animal models[91]. In agreement with this, Zeng *et al*. demonstrated that CPT1 levels correspond with increased breast cancer cell migration and metastasis.[163] However, the endogenous mechanisms that modulate FAO in cancer are not fully understood.

Kristiansen *et al*. (2010) identified that Mb is ectopically expressed in nearly 40% of all breast tumors.[53] Importantly, breast cancer patients with Mb expression show smaller, more differentiated tumors and fewer metastases, which correlate with a better survival outcome compared to patients without Mb, yet the mechanism(s) of how Mb improves patient outcome remains unclear. Only a handful of studies have shown that Mb modulates cancer cell migration. Galluzzo *et al*. showed that exogenous Mb expression in lung cancer cells decreases metastasis in animals.[132] Reports of *in vitro* cell migration have shown conflicting results as to whether Mb

increases or decreases migration.[54, 55] However, it remains unexplored whether Mb regulates cancer cell metabolism and whether it has any impact on breast cancer cell migration.

Mb is constitutively expressed at high concentrations in skeletal and cardiac muscle, where its canonical role is to store and deliver oxygen to mitochondria to maintain aerobic respiration in hypoxic conditions.[1, 2, 7, 13, 17, 97] In addition to oxygen storage, Mb regulates tissue nitric oxide levels as well as catalyzes cellular oxidant production.[13, 26, 145, 147] Notably, all of these functions of Mb are solely dependent on its heme prosthetic group. A growing body of literature has now demonstrated that Mb is able to bind to medium- and long-chain fatty acids and this binding has been shown to enhance FAO in cardiomyocytes. [40, 42, 44-48] The interaction between Mb and fatty acids is dependent on an oxygen-coordinated heme with a ferrous iron (Fe^{2+}) center of Mb (oxy-Mb), where deoxygenated Mb (deoxy-Mb) and metmyoglobin (Fe^{3+}; met-Mb), are unable to stably bind fatty acids.[44, 45, 48] More recently, computational models have identified that equine Mb's lysine 45 residue (human ortholog Mb K46) contributes to stabilizing fatty acids near the heme pocket.[46, 47] However, it remains to be clarified whether Mb-dependent fatty acid binding occurs in cancer cells and whether this binding modulates breast cancer cell metabolism and has any influence on migration.

Based on these prior studies, we hypothesized that the binding of Mb to fatty acids in breast cancer cells alters fatty acid metabolism, leading to decreased cell migration. In this chapter, we demonstrate that Mb expression decreases cell migration and FAO. To test whether fatty acid binding mediates these effects, we generated an Mb mutant (Mb K46M) and show that while it decreases the affinity of Mb for fatty acids, disrupting fatty acid binding has no significant effect on cellular respiration, FAO, or migration. Further, we show that Mb expression decreases

CPT1 levels, and re-expressing CPT1 restores FAO rates but not migration, demonstrating that increased FAO is not sufficient to increase migration in this context.

2.2 Materials and Methods

2.2.1 Cell Culture

MDA-MB-231 (CRM-HTB-26) and MDA-MB-468 (HTB-132) were purchased from The American Type Culture Collection (ATCC). MDA-MB-231 cell lines were cultured in DMEM with glutamine (4.5g/L glucose, Gibco) and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 1% penicillin-streptomycin antibiotic (50 Units/mL), and 15mM HEPES buffer (pH 7.4). MDA-MB-468 cell lines were cultured in a 1:1 mixture of DMEM/F-12 media (Gibco) and supplemented with 10% HI-FBS, 1% penicillin-streptomycin, and 15 mM HEPES as above. Cells were stored in a humidified incubator maintained at 37C, 21% O_2, and 5% CO_2.

2.2.2 Generation of Stable Myoglobin Expressing Cell Lines

MDA-MB-231 cells (90% confluence) were transduced with pLenti-C-mGFP-P2A-Purovectors (9.75x105 TU) expressing human myoglobin (MB; transcript variant 1; 231 Mb). Empty vector control cells (231 EV) were generated by transducing MDA-MB-231 cells with pLenti-C-mGFP-P2A-Puro particles (9.75x105 TU). Cells were incubated with viral particles (18 h) and then placed under puromycin selection (1µg/mL). Cells were expanded and maintained under puromycin selection media for at least 3 passages before experiments.

2.2.3 Transient Knockdown of Myoglobin by siRNA

MDA-MB-468 cells were treated with either 20 nmol of ON-TARGETplus non-targeting (siNT) or Mb-targeting SMARTpool siRNA (siMb; Dharmacon) and incubated overnight using Mirus Trans-ITx2 reagent as previously described.26 Transfection complexes were removed 24 hours after transfection and replaced with complete growth medium. Cells were subsequently assayed between 48 and 72 hours after transfection.

2.2.4 Protein Extraction and Western Blotting

Cells were lysed in RIPA buffer with protease (cOmplete; Roche) and phosphatase (PhosSTOP, Roche) inhibitors on ice for at least 20 minutes. Lysates were clarified by collecting the supernatant after centrifugation for 20 minutes at 20,000xg. Protein concentrations were estimated by bicinchoninic acid (BCA) assays. Western blot was performed as previously described.27 Primary antibodies used include Mb (catalog G-125-C, R&D Systems), CPT1a (catalog 97361, Cell Signaling), FASN (catalog 3180, Cell Signaling), FABP5 (catalog 39926, Cell Signaling), citrate synthase (catalog 14309, Cell Signaling). Blots were incubated in the appropriate fluorophore-conjugated secondary antibodies and then imaged using the LI-COR Biosciences Odyssey DLx imager and analyzed using the LI-COR Image Studio Lite (software version 5.2). All protein concentrations were normalized to the housekeeping protein alpha-tubulin (catalog: CP06, Calbiochem) unless otherwise noted.

2.2.5 Wound Closure and Transwell Migration Assays

Cells were seeded and achieved 95% confluence before being treated with MMC the following day. Cells were "scratched" using a P200 pipet tip, washed with PBS, and given complete growth media. Cells in the same field of view were immediately imaged under a brightfield microscope at t=0, 2, 4, and 6h using a reference line on the cell culture plate. The percentage of scratch/wound closure was calculated by measuring the area of the wound size via ImageJ and a ratio of the wound size at 0 hours. Data were fitted using a simple linear regression to calculate the rate of scratch/wound closure.

2.2.6 Seahorse Extracellular Flux (XF) Analysis

MDA-MB-231 and MDA-MB-468 cells (25,000/well) were seeded into XF96 cell culture microplates and oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured by Seahorse XFe96 Analyzer (Agilent). For mitochondrial stress tests, DMEM, oligomycin A (Oligo, 2.5 µM; Sigma), FCCP (1.0 µM for MDA-MB-231 cells; 0.75 µM for MDA-MB-468 cells; Cayman Chemical), and rotenone (2.5 µM; Sigma) were injected sequentially. For substrate-dependency assays, etomoxir (40µM or 100 µM; Cayman Chemical), UK5099 (10µM), and BPTES (10µM; Cayman Chemical) were added prior to injection of oligomycin, FCCP, and rotenone. Rates of respiration were calculated as previously described.[164]

2.2.7 ^{14}C-Palmitatic Acid Oxidation

Complete oxidation of ^{14}C-labeled palmitic acid (C_{16}) was measured as previously described.[165]

2.2.8 Metabolite Extraction and Liquid Chromatography-Mass Spectrometry

One million cells were scraped into 80% methanol and frozen at -80°C prior to analysis by HPLC-MS/MS as previously described. [166]

2.2.9 Site-Directed Mutagenesis of Myoglobin and Bacterial Transformation

Apo-Mb (Mb H94F) was generated as previously described.26 For Mb K46X mutants, site-directed mutagenesis (SDM) of Mb was achieved using the Phusion SDM Kit (Thermo Scientific) and the parent Mb (WT) plasmid (OriGene) according to manufacturer's instructions and custom primers with 5' phosphorylation modifications (**Table 1**). Plasmids were transformed into competent DH10B E. coli and selected for ampicillin resistance according to the manufacturer's instructions. Bacterial colonies were isolated and cultured in LB Miller Broth overnight with appropriate selection antibiotics. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer's instructions. Mutant Mb sequence alignments were validated using Sanger Sequencing and NCBI's Basic Local Alignment Search Tool (BLAST). MDA-MB-231 cells were then transiently transfected with Mb-expressing constructs as above and assessed 72 hours post-transfection. Mb protein levels were verified by western blot as above.

Table 1: Mb K46X Mutagenesis Primers

Primer	Forward Sequence	Reverse Sequence
Mb WT	GAGAAGTTTGACAAGTTCAAGCACC	CAGAGTCTCTGGGTGACCCTTAAAGAG
Mb K46M	GAGAAGTTTGACATGTTCAAGCACC	CAGAGTCTCTGGGTGACCCTTAAAGAG

2.2.10 Recombinant Protein Expression and Isolation

Human Mb constructs (WT Mb and Mb K46M) were cloned into pET28a(+) vectors with bacterially optimized codons and purchased from GenScript Biotech Corp. pET28 plasmids were transformed into Shuffle® T7 Express competent E. coli (catalog: C2029J, New England Biolabs) according to the manufacturer's instructions. Single bacterial colonies were selected, expanded, and stored as glycerol stocks. Expression and purification of WT and mutant Mb proteins were carried out by HPLC as described previously.31 WT Mb and Mb K46M proteins were assayed for lipid binding as isolated after purification from the HPLC column.

2.2.11 Oleate Binding via UV-VIS Spectroscopy

The binding of sodium oleate to WT and mutant Mb K46M constructs was assessed by UV-Vis spectroscopy as previously described (Kaliszuk *et al*., *Nitric Oxide*. 2022, 125-126, 12-22). We verified that WT Mb and Mb K46M recombinant proteins were 80-81% and 73-77% oxy-Mb, respectively, by spectra deconvolution analysis.[145] ORIGIN software was used to fit data using **Equation 8** to determine the dissociation constant (K_d) as previously described.[167]

$$\Delta \boldsymbol{Abs} = \Delta\boldsymbol{\varepsilon} \times \frac{(\boldsymbol{P} + \boldsymbol{L} + \boldsymbol{K_D}) - \sqrt{(\boldsymbol{P} + \boldsymbol{L} + \boldsymbol{K_D})^2 - 4\boldsymbol{PL}}}{\mathbf{2}} \qquad \textbf{Equation 8}$$

2.2.12 Transient Expression of Myoglobin Constructs and Carnitine Palmitoyl Transferase-1a Plasmids

MDA-MB-231 cells were transfected with either control GFP, Mb, or Apo-Mb DNA (2.5µg) constructs using TransIT-X2 transfection reagent following manufacturer's instructions (Mirus Bio) and assayed 72 hours after transfection. Apo-Mb was generated previously by mutating H94 to a phenylalanine (H94F).[168]

Plasmids encoding Myc-DDK-tagged full-length CPT1a transcript variant 1 (RC221740, OriGene) were transformed into competent DH10B *E. coli* and selected for kanamycin resistance according to the manufacturer's instructions. Bacterial colonies were isolated and cultured in LB Miller Broth overnight. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer's instructions. MDA-MB-231 cells were transiently transfected with DNA (2.5µg) constructs using MegaTran 2.0 transfection reagent according to the manufacturer's instructions and assessed 72 hours post-transfection. CPT1a protein levels were verified by western blot as above.

2.2.13 Cellular Thermal Shift Assay (CETSA)

MDA-MB-231 cells were plated and transiently transfected with either Mb (WT), H94F (Apo-Mb), or Mb K46M plasmids as above and treated with BSA-conjugated palmitate (BSA-PA; 25µM; 24 h). Cells were then washed, harvested, and lysed as previously described with some modifications.41 Briefly, cells were harvested and washed in PBS supplemented with protease inhibitors (PBS+PI) and then resuspended PBS+PI. Cell suspensions were freeze-thawed three times and centrifuged (20,000 x g; 20 minutes; 4°C). Cell lysates were heated across a range of

temperatures (40-95°C; 5 min) followed by cooling (4°C; 5 minutes) on a BIO-RAD C1000 Thermocycler. Heated samples were centrifuged (18,000 x g; 20 minutes; 4°C) to separate soluble from precipitated proteins. Supernatants were then analyzed by SDS-PAGE as above. For each thermal denaturation curve, protein concentrations were normalized to 47.5°C samples and expressed as a percent of the 47.5°C signal. Melting temperature (Tm) was determined by graphing protein signals and the corresponding temperatures as a Sigmoidal dose-response with a least-squares regression line fit using ORIGIN software, where T_m is equivalent to $logEC_{50}$.

2.2.14 Statistics

All statistical analyses were carried out using GraphPad Prism version 9.5 unless otherwise noted. Welch's t-test was used to determine the significance between two groups for Mb-knockdown in MDA-MB-468 cells, ECAR, ^{14}C-palmitic acid oxidation, and lipidomics. One-way ANOVA was used to determine the significance between CETSA T_m, Transwell with Mb K46M mutant. Two-way ANOVA with multiple comparisons tests was used for the following experiments: Transwell migration assays, wound closure, Seahorse XF experiments, western blot quantification,

2.3 Results

2.3.1 Myoglobin Expression Decreases Migration in MDA-MB-231 Breast Cancer Cells.

To determine whether myoglobin (Mb) expression modulates breast cancer cell migration, human Mb was stably expressed in MDA-MB-231 breast cancer cells that do not express endogenous

Mb (**Figure 2-1A**). In a transwell migration assay, stable expression of GFP-tagged Mb (231 Mb cells) significantly decreased the migration rate by approximately 30% compared to GFP empty-vector control cells (231 EV) (**Figure 2-1B-C**). To ensure that differential proliferation was not responsible for the difference in migration, we repeated the assay in the presence of the proliferation inhibitor mitomycin C (MMC; 10ug/mL). In the absence of proliferation, Mb expression still significantly decreased the migratory capacity of 231 cells by approximately 54% (231 EV+MMC: 100±19%; 231 Mb +MMC: 46±15%; **Figure 2-1B-C**). This effect was recapitulated in a scratch closure assay in which Mb expression significantly decreased the closure of the scratch compared to 231 EV cells (**Figure 2-1D-E**). These data demonstrate that Mb expression decreases breast cancer cell migration.

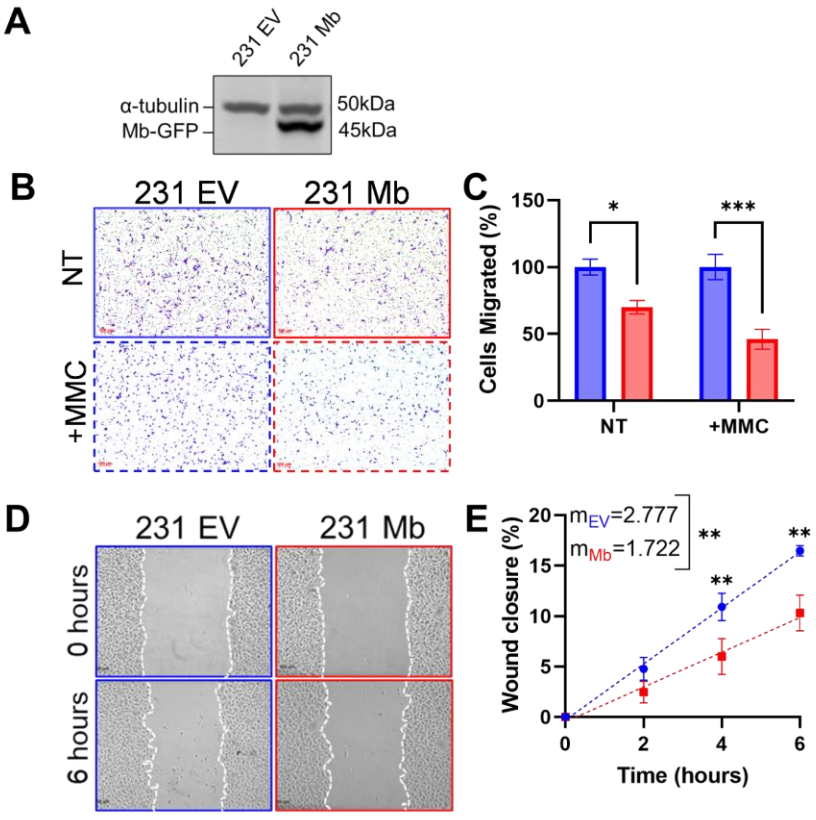

Figure 2-1: Mb expression decreases migration in MDA-MB-231 cells.

A) Representative western blot of stable MDA-MB-231 cell lines expressing either an empty vector GFP (231 EV) or GFP-tagged Mb (231 Mb). B) Representative brightfield images of migrated cells from 6-hour Transwell experiments stained with crystal violet and C) quantification of the percentage of cells migrated relative to the 231 EV (blue) cells with mitomycin C treatment (+MMC) or no treatment (NT) treatment after 6 hours. Two-way ANOVA with multiple comparisons tests; N=4. D) Representative images of 231 EV and 231 Mb cells in a scratch assay at 0 and 6 hours; dashed lines outline wound size. E) Quantification of results in assays such as in D showing the average percentage wound closure relative to the initial wound size (t=0) and fit using a simple linear regression (dashed lines) to determine the rate of migration/wound closure (m), simple linear regression and comparison of slopes; two-way ANOVA with multiple comparisons test; N=4. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001.

2.3.2 Myoglobin Expression Decreases Mitochondrial Oxygen Consumption Rate (OCR)

To determine whether the Mb-induced change in migration was associated with bioenergetic alterations, we measured cellular bioenergetics in each cell line. 231 Mb cells showed significant decreases in basal (66±13%), ATP-linked (53±10%), and maximal (121±19%) OCR compared to 231 EV cells (231 EV basal: 100±14% ATP-linked: 79±11; maximal: 191±25; **Figure 2-2A-B**). To test the effect of Mb in a second cell line, MDA-MB-468 cells, which express endogenous Mb, were treated with siRNA-targeting Mb (468 Mb-), which resulted in approximately a 54% decrease in endogenous Mb levels relative to 468 CTRL cells (**Figure 2C**). Measurement of bioenergetics in these cells showed that depletion of endogenous Mb significantly increased basal and maximal OCR compared to Mb-containing 468 CTRL cells (**Figure 2-2D-E**). Mb expression did not significantly change the basal ECAR of the MDA-MB-231 cells (**Figure 2-2F**). However, 468 Mb- cells showed a significant increase in ECAR relative to 468 CTRL cells (**Figure 2-2G**).

These data demonstrate that the expression of Mb significantly decreases the mitochondrial basal and maximal OCR in breast cancer cells.

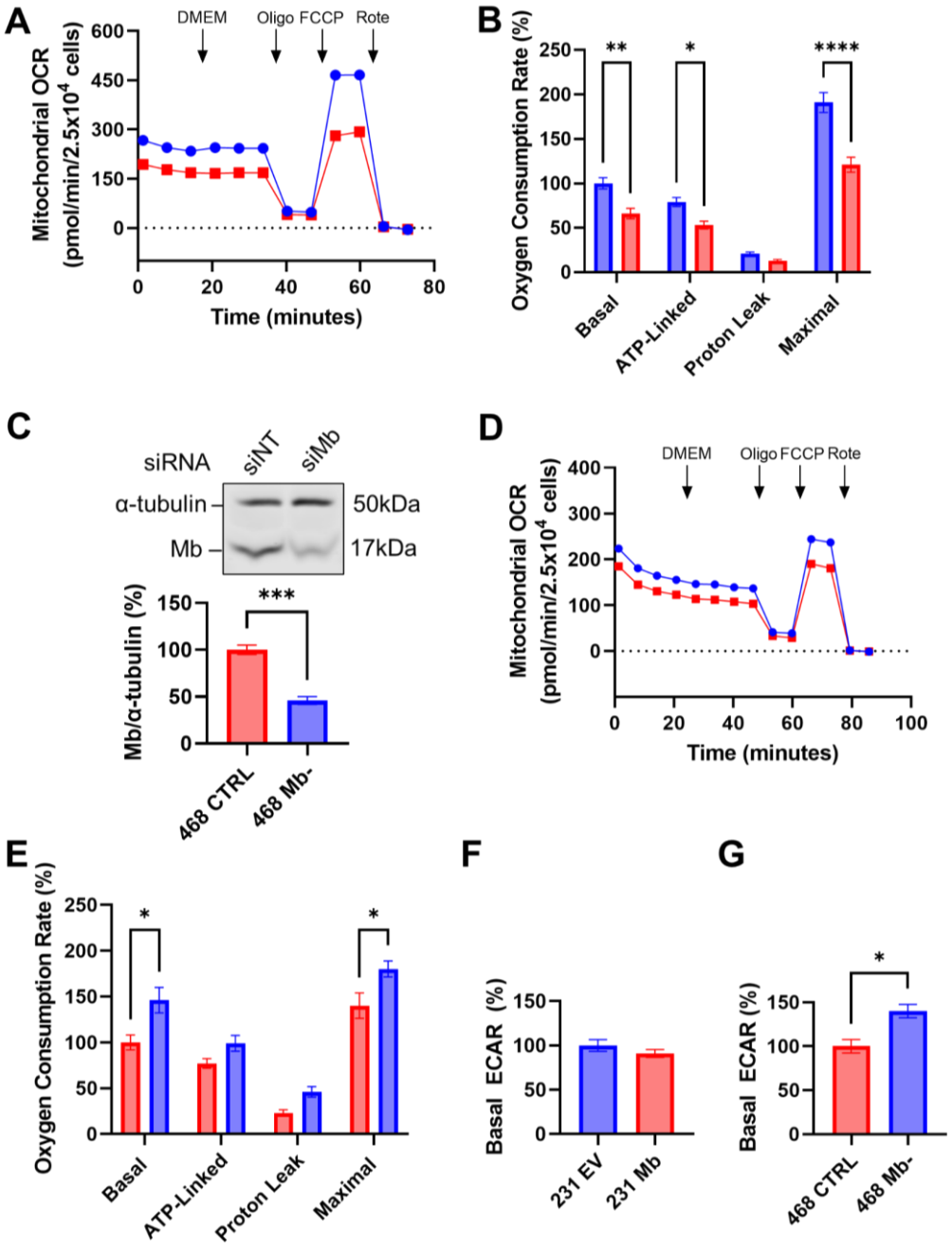

Figure 2-2: Mb expression decreases mitochondrial oxygen consumption rate (OCR).

A) Representative Seahorse extracellular flux (XF) analyzer traces of mitochondrial OCR in 231 EV (blue) and 231 Mb (red) cells. Dashed lines indicate etomoxir-treated cells. Arrows indicate the time of injection of

either DMEM, Oligomycin A (Oligo; 2.0μM), FCCP (1.0 μM), or Rotenone (Rote; 2.5μM). B) Quantitation of mitochondrial OCR in 231 EV (blue) and 231 Mb (red). Two-way ANOVA with multiple comparisons tests; N=5. C) Top: Representative western blot of 468 cells treated with non-targeting siRNA (siNT) or Mb-targeting siRNA (siMb) transfection. Bottom: Quantitation of siRNA-mediated knockdown of Mb in 468 Mb- cells. Welch's t-test; N=4. D) Representative traces of mitochondrial OCR in 468 CTRL (red) and 468 Mb- (blue) cells. E) Quantitation of mitochondrial OCR of 468 CTRL (red) and 468 Mb- (blue). Two-way ANOVA with multiple comparisons tests; N=3. F-G) Basal extracellular acidification rates (ECAR) of 231 (F) and 468 cells (G) with or without Mb expression. Welch's t-test; N=3 and N=5 for 231 and 468 cells, respectively. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001, ✱✱✱✱: p<0.0001.

2.3.3 Myoglobin Expression Decreases Fatty Acid Oxidation (FAO)

To determine whether alterations in substrate utilization contribute to the Mb-dependent decrease in basal and maximal OCR, we next assessed the contribution of pyruvate, fatty acid, and glutamine oxidation to OCR in each cell line by measuring OCR in the presence of inhibitors of the mitochondrial pyruvate carrier (UK5099; 10μM), CPT1 (etomoxir; 40μM or 100μM) and glutaminase (BPTES; 10μM). The contribution of pyruvate and glutamine utilization to OCR was not statistically different in 231 Mb cells compared to 231 EV (**Figure 2-3B**). However, inhibition of mitochondrial fatty acid import using etomoxir showed a significant decrease in fatty acid-dependent OCR in 231 Mb cells (28±6%) compared to 231 EV cells (41±6%; **Figure 2-3A-B**).

To confirm these results, we next directly assayed FAO by measuring the oxidation of ^{14}C-palmitate by the MDA-MB-231 cells. Mb expression significantly decreased palmitic acid oxidation in the 231 Mb cells (4.797±0.838 pmol/hour) compared to the 231 EV cells (5.789±0.729 pmol/hour**; Figure 2-3C**). Furthermore, using liquid chromatography coupled mass spectrometry, we observed that levels of long-chain myristoleic, stearic, and linoleic fatty acids

were significantly elevated (between ~11-29%) in 231 Mb cells relative to 231 EV cells (**Figure 2-3D**). Levels of free carnitine were not changed between cell lines, while acetyl-carnitine was significantly decreased by approximately 19% in 231 Mb cells, consistent with decreased FAO (**Figure 2-3E**). Consistent with Mb-dependent suppression of FAO, 468 Mb- cells also showed a higher contribution of FAO to overall OCR than 468 CTRL cells (**Figure 2-3F-G**). Collectively, these data show that Mb expression significantly decreases FAO and increases fatty acid storage in these breast cancer cells.

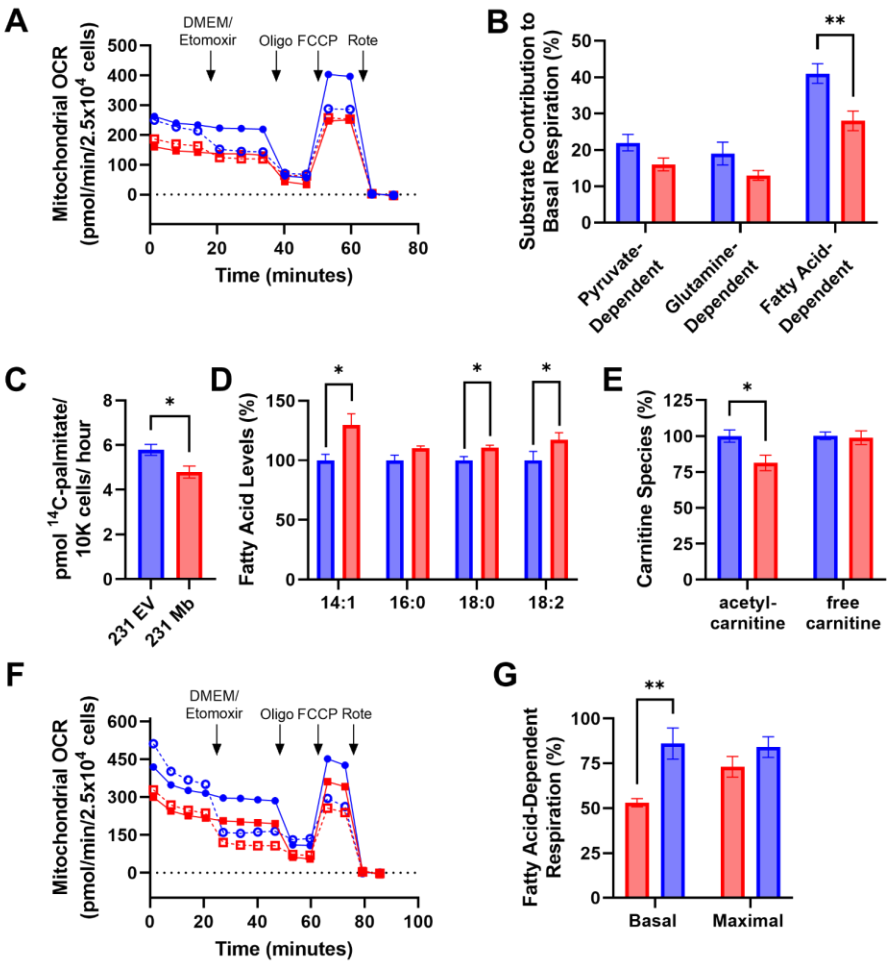

Figure 2-3: Mb expression decreases fatty acid oxidation

A) Representative traces of mitochondrial OCR of 231 EV (blue) and 231 Mb (red) cells. Dashed lines indicate etomoxir-treated cells (40uM). Arrows indicate the time of injection of DMEM or Etomoxir, Oligo, FCCP, or

Rote. B) The percent contribution of pyruvate-, glutamine-, and fatty acid-dependent respiration to total basal OCR in 231 EV (blue) and 231 Mb (red) cells. Two-way ANOVA with multiple comparisons tests; N=5. C) Rate of ^{14}C-palmitic acid oxidation in 231 EV (blue) and 231 Mb (red) cells. Welch's t-test; N=9-10. D) Levels of myristoleic (14:1), palmitic (16:0), stearic (18:0), and linoleic (18:2) fatty acids in 231 EV (blue) and 231 Mb (red) cells. Welch's t-test; N=6. E) Relative levels of carnitine species extracted from 231 EV (blue) and 231 Mb (red) cells as measured by LC-MS. Two-way ANOVA with multiple comparisons tests; N=6. F) Representative traces OCR in 468 CTRL (red) and 468 Mb- (blue) cells. Dashed lines indicate etomoxir-treated cells. Arrows indicate the time of injection of either Etomoxir, Oligo, FCCP, or Rote. G) The percent contribution of fatty acid-dependent respiration to total basal and maximal OCR in 468 CTRL (red) and 468 Mb- (blue) cells. Two-way ANOVA with multiple comparisons tests; N=3. Data are mean ±SEM. ✱: p<0.05, ✱✱: p<0.01.

2.3.4 Mutation of Myoglobin Lysine-46 to Methionine (K46M) Maintains Heme-Catalyzed Functions but Diminishes Fatty Acid Binding

Computational models of Mb-fatty acid binding suggest key electrostatic interactions between the positively charged lysine residue (K45) of equine oxy-Mb and the negatively charged carboxylate head of palmitic and oleic acids while the alkyl tail of the fatty acid tucks into a hydrophobic cleft near the heme prosthetic group of oxy-Mb.[46, 47, 169] To test whether Mb-dependent attenuation of FAO is due to its ability to bind fatty acids, we generated a mutant Mb protein in which the K46 (the human ortholog of equine Mb K45) was mutated from lysine to methionine (K46M). UV-Vis spectroscopy demonstrated no change in the Soret peak (~410nm) of the K46M mutant construct compared to WT, suggesting that heme incorporation is unaffected by mutagenesis (**Figure 2-4A**). The addition of increasing concentrations of oleate (0-200μM) decreased the absorbance of the Soret peak of both proteins (**Figure 2-4B**). Calculation of the

difference spectra and fitting of the kinetic binding curves showed distinctly different binding profiles for WT and K46M Mb (**Figure 2-4C-D**). Calculation of the equilibrium constant (K_d) showed a ~50-fold increase in the K_d of Mb K46M (13.13±6.75μM oleate) compared to that of WT Mb (0.24±0.10μM oleate), suggesting that the binding of oleate to Mb K46M is significantly weaker than its binding to WT Mb. These results were confirmed by a secondary assay of ligand binding, CETSA, in which binding of fatty acid to Mb was expected to stabilize the protein and increase its melting temperature (T_m) (**Figure 2-5**). Apo-Mb was used as a negative control, as the heme stabilizes the protein; therefore, preventing heme coordination is expected to decrease thermostability. Similarly, consistent with the disruption of Mb-fatty acid binding, the T_m for Mb was significantly decreased in cells expressing Mb K46M (59.8±0.71C) compared to WT Mb (63.0±0.6C; **Figure 2-5B**).

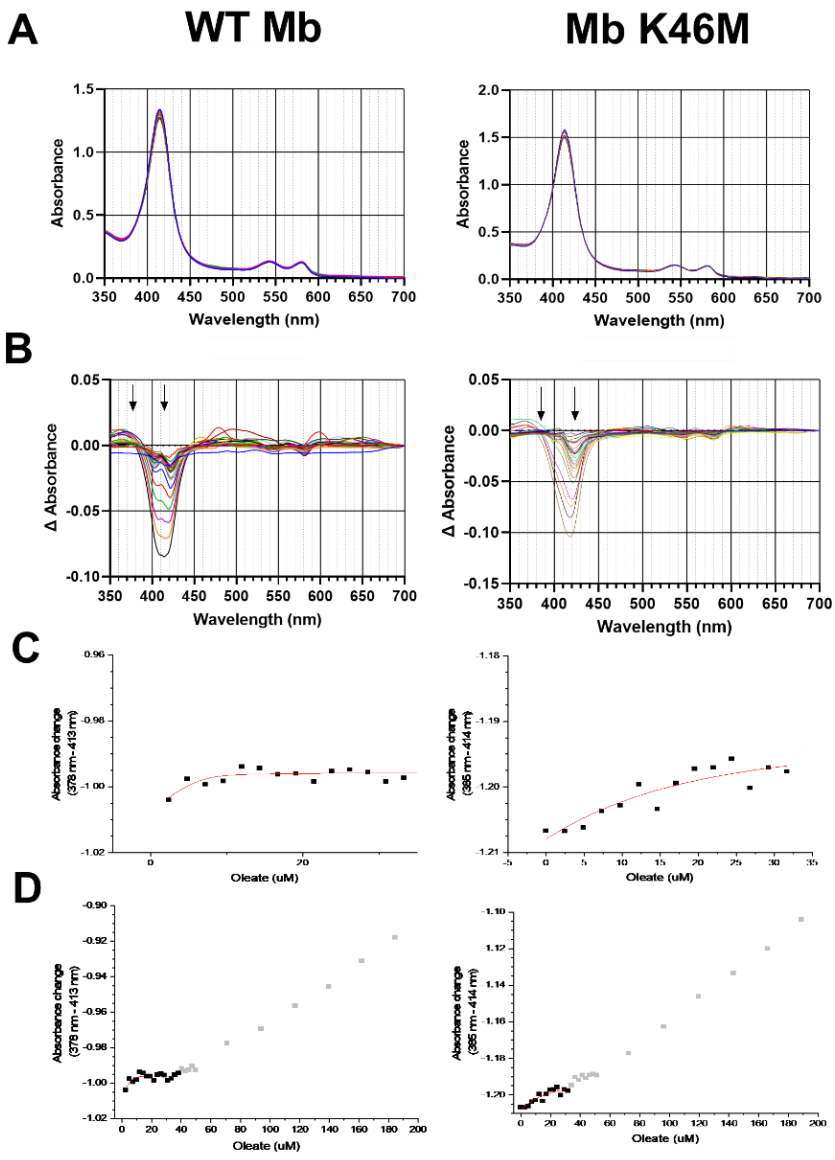

Figure 2-4: Mutation of Mb K46 to methionine (K46M) is sufficient to decrease Mb's affinity for oleate. **Representative absorbance spectra of WT Mb (left) and Mb K46M (right). B) Representative difference spectra of WT and K64M Mb with increasing concentrations of oleate (0-200μM). Arrows represent isosbestic and Soret peak wavelengths used to generate binding curves. C-D) Absorbance changes for each protein as a function of oleate concentration and fit. C is fit at low oleate concentrations using a binding model of 1:1 stoichiometry based on the equilibrium constant and Beer-Lambert equation. N=2. Data are Mean ±SEM.**

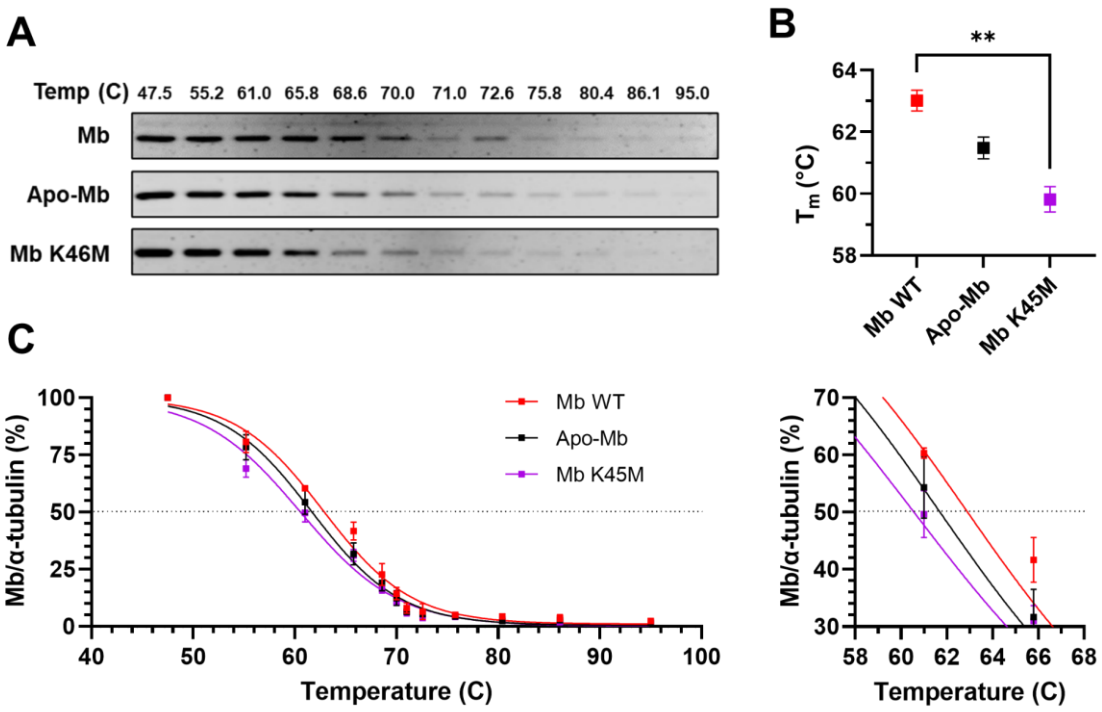

Figure 2-5: Mb K46M decreases protein stability by cellular thermal shift assay.

A) Representative western blots of MDA-MB-231 WT cells expressing either WT Mb, Apo-Mb, or Mb K46M in which protein concentration decreases as a function of increasing temperature. B) Calculated T_m of WT Mb (red), Apo-Mb (black), or Mb K46M (purple) protein from MDA-MB-231 cells. One-way ANOVA with Dunnett's multiple comparisons to $T_{m\ WT\ Mb}$. C) WT Mb (red), Apo-Mb (black), and Mb K46M (purple) protein levels transiently expressed in MDA-MB-231 cells decrease as a function of temperature. Protein signals are fit to a sigmoidal dose-response curve with a variable slope by least-squares at all temperatures (left) and near the melting temperature (T_m; right). For all panels, N=3. Data are Mean ±SEM. ✱✱: p<0.01.

2.3.5 Myoglobin-Dependent Fatty Acid Binding does not Alter OCR or Migration

To determine whether the Mb K46M mutant was able to restore the Mb-dependent decrease in mitochondrial basal and maximal OCR, we transfected 231 WT cells with either a GFP-control, WT Mb, or Mb K46M and measured bioenergetics (**Figure 2-6A-B**). As before, WT Mb plasmid

expression decreased basal, ATP-linked, and maximal mitochondrial OCR compared to 231 WT cells. However, the K46M Mb mutant cells showed no significant difference in OCR compared to WT Mb-expressing cells (**Figure 2-6B**). Further, K46M Mb expressing cells showed decreased migration (identical to WT Mb expressing cells) compared to 231WT cells (**Figure 2-6C-D**). Taken together, these data suggest that Mb decreases OCR and migration through a mechanism independent of Mb's ability to bind fatty acids.

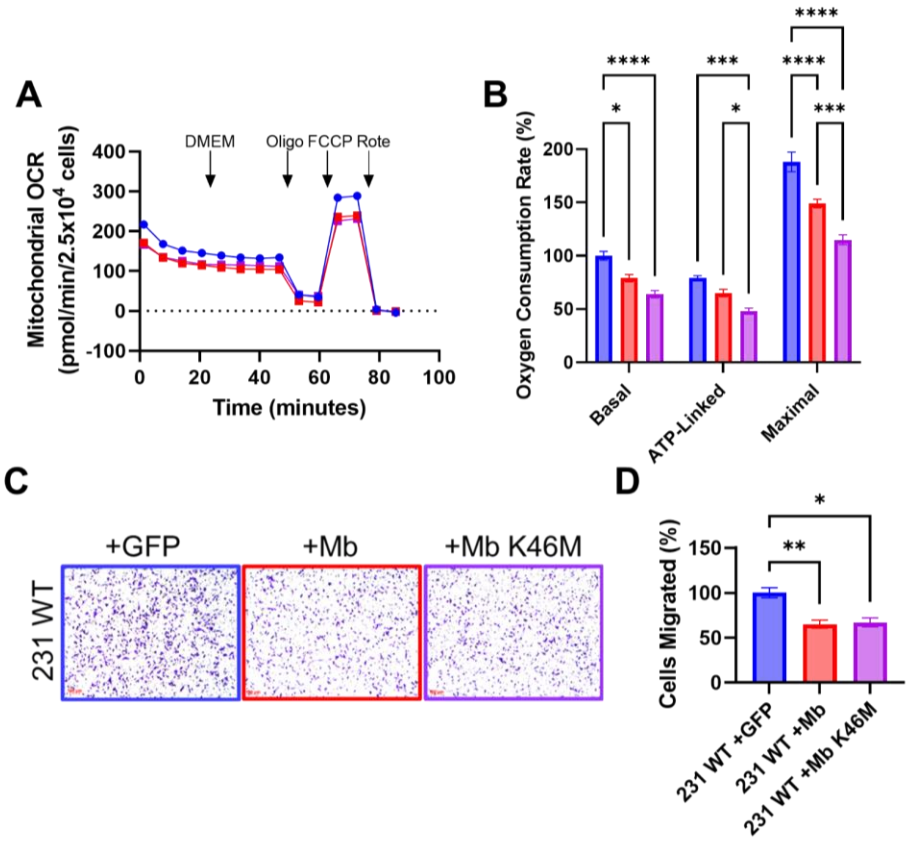

Figure 2-6: Expression of Mb K46M is not sufficient to rescue Mb-dependent decreases in mitochondrial OCR or migration.

A-B) Representative mitochondrial OCR traces (A) and corresponding quantitation (B) of MDA-MB-231 WT cells transiently expressing either GFP (blue), WT Mb (red), or Mb K46M (purple). Arrows in (A) indicate the time of injection of either DMEM, Oligo, FCCP, or Rote. Two-way ANOVA with Dunnett's multiple comparisons tests; N=3. C) Representative brightfield images of migrated cells from 6-hour Transwell

experiments stained with crystal violet. D) Percent of GFP- (blue), WT Mb- (red), or Mb K46M- (purple) expressing cell migration at 6 hours. One-way ANOVA with Dunnett's multiple comparisons tests; N=4. Data are Mean ±SEM; ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001, ✱✱✱✱: p<0.0001.

2.3.6 Myoglobin Expression Decreases Carnitine Palmitoyl Transferase-1 Levels

To determine the mechanism of Mb-mediated attenuation of FAO, we investigated key enzymes involved in fatty acid synthesis, transport, and oxidation. We did not observe differences in FASN, FABP5, or citrate synthase (CS), suggesting that fatty acid synthesis, cytosolic transport, and mitochondrial content remain unchanged with Mb expression (**Figure 2-7A-B**). However, we observed a significant decrease in the level of CPT1a in 231 Mb cells, suggesting decreased fatty acid transport into the mitochondrion (**Figure 2-7A-B**). Overexpression of CPT1a in 231 Mb cells increased CPT1 levels by 18-20-fold (**Figure 2-7C-D**) and restored the Mb-dependent decrease in FAO (**Figure 2-7E**) to that of the GFP-transfected 231 EV cells (231 EV+GFP: 31±4%; 231 Mb +CPT1a: 30±7%). Taken together, these data demonstrate that decreased CPT1a expression is responsible for the Mb-mediated decrease in FAO; however, restoration of FAO does not restore migration in 231 Mb-expressing cells (**Figure 2-7F**).

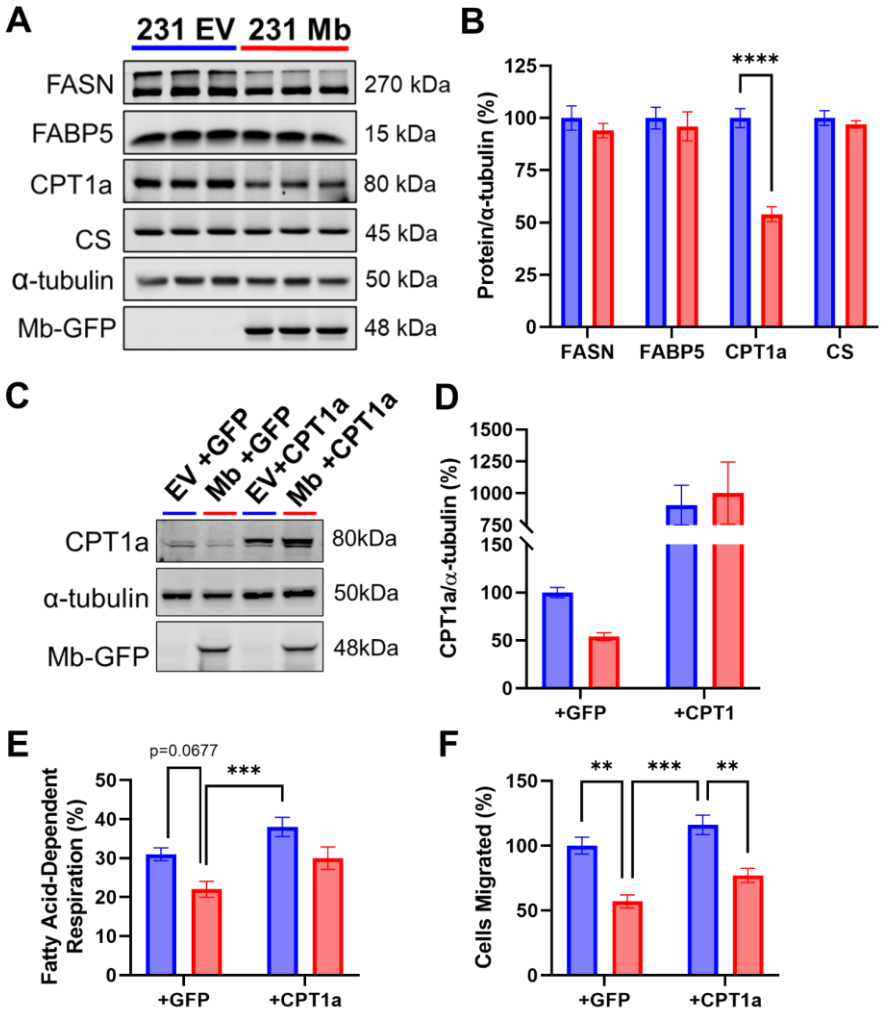

Figure 2-7: Mb expression decreases CPT1a levels and re-expressing CPT1a is sufficient to increase fatty acid-dependent OCR but not migration in 231 Mb cells.

A-B) Representative western blots (A) and quantitation of fatty acid synthase (FASN), fatty acid binding protein 5 (FABP5), carnitine palmitoyl transferase 1a (CPT1a), and citrate synthase (CS) in 231 EV (blue) or 231 Mb (red) cells (B). Two-way ANOVA with multiple comparisons tests; N=3-4. C-D) Representative western blots (C) and quantitation of CPT1a levels 72 hours after transfection (D) in 231 EV (blue) or 231 Mb (red) cells. N=3. E) Percent contribution of fatty acid-dependent respiration to basal OCR in 231 EV (blue) or 231 Mb (red) cells. Two-way ANOVA with multiple comparisons tests; N=6. F) Quantitation of migrated 231 EV (blue) and 231 Mb (red) cells transiently expressing GFP or CPT1a. Two-way ANOVA with multiple comparisons tests; N=4. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001.

2.3.7 Myoglobin Decreases Mitochondrial Oxygen Consumption through a Heme-Dependent Mechanism

Because altering the interaction between Mb and fatty acids did not restore basal respiration or migration rates, we next tested whether the heme group of Mb underlies this effect. We expressed either GFP, WT Mb or Apo-Mb (which cannot coordinate heme) in WT 231 cells and assayed cell respiration (**Figure 2-8A-B**). Importantly, we did not observe any differences in the levels of WT Mb and Apo-Mb in the cell lysates by western blot (data not shown), indicating that both constructs were stably expressed. The expression of Mb trends toward a decrease in basal OCR and significantly decreases maximal OCR by approximately 21% and 39%, respectively (231 EV basal: 100±7%, maximal: 188±16%; 231 Mb basal: 79±6%, maximal: 149±7%). The expression of Apo-Mb, however, abolishes the differences between 231 +GFP cells (Apo-Mb basal: 97±10%, maximal: 175±22%). From these data, we conclude that Mb decreases cellular respiration through a heme-dependent mechanism.

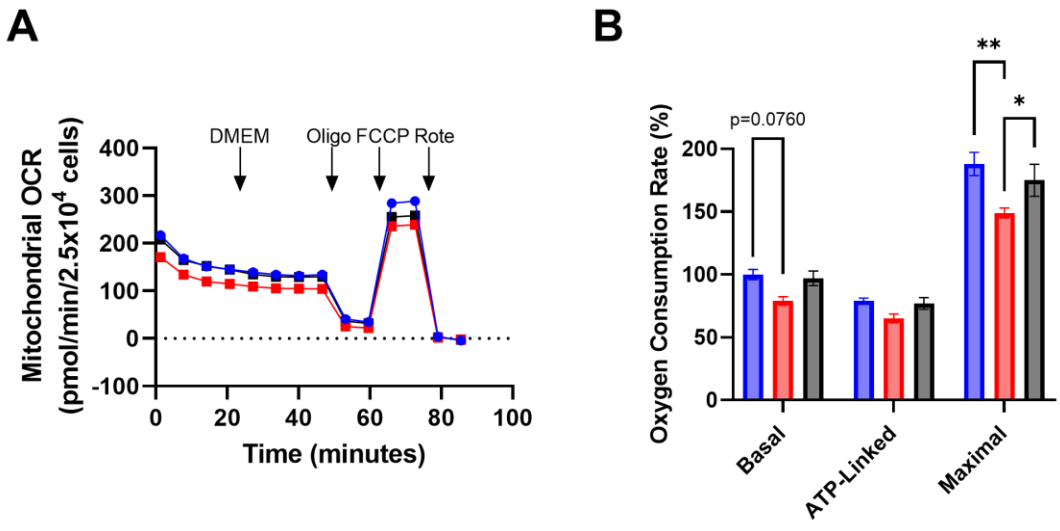

Figure 2-8: Expression of Apo-Mb rescues Mb-dependent decreases in mitochondrial OCR.

A-B) Representative mitochondrial OCR traces (A) and corresponding quantitation (B) of MDA-MB-231 WT cells transiently expressing either GFP (blue), WT Mb (red), or Apo-Mb (black). Arrows in (A) indicate the time of injection of either DMEM, Oligo, FCCP, or Rote. Two-way ANOVA with Dunnett's multiple comparisons tests; N=3. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01.

2.4 Discussion

The data presented in this chapter demonstrate that Mb expression decreases cell migration and mitochondrial respiration. We tested whether these differences were due to Mb-dependent regulation of FAO achieved through the binding of fatty acids. We report that Mb decreased FAO through the downregulation of CPT1 rather than through fatty acid binding but restoring FAO rates in 231 Mb cells was not sufficient to significantly increase migration rates.

Our data align with previous reports of Mb decreasing cellular oxygen consumption and migration rates. Kristiansen *et al*. showed that the knockdown of Mb in MDA-MB-468 cells increased respiration. [54] The expression of Mb decreases cell migration in MDA-MB-231s and also aligns with previous studies.[55, 132] Others have speculated that Mb-dependent fatty acid binding may underlie the Mb-dependent decreases in respiration, but to our knowledge, this has never been tested experimentally.[54, 96] Our *in vitro* experiments with Mb K46M protein isolates and cells lysates corroborate *in silico* modeling by Chintapalli *et al*. and underscore the critical role of the human Mb K46 in stabilizing fatty acids within the heme pocket of oxy-Mb.[46] However, we demonstrate that diminished Mb-dependent fatty acid binding (through the use of Mb K46M) does not affect cellular respiration or migration. This may be due to low concentrations of Mb in cancer cells and/or specific cancer cells having a low metabolic reliance

on fatty acids compared to cardiac muscle or Myc-overexpressing tumors, for example.[40, 42, 52, 53, 170]

Prior studies have associated increases in FAO levels with metastasis through the upregulation of several mechanisms, such as EMT, suppression of anoikis, and ERK activation.[163, 171, 172] Metabolically, supplementing cells with acetyl-CoA to circumvent FAO-inhibited decrease in acetyl-CoA has been shown to increase mammosphere formation and histone acetylation, which are markers of EMT.[91] However, the increase in acetyl-CoA and energy production as a result of increasing FAO has not been reported to sufficiently increase cell migration alone. It is likely that increased FAO promotes EMT, which then allows for increased cell migration. To this end, Mb expression has been reported to decrease the expression of EMT genes, but no metabolic alterations were reported.[55] Future studies may shed light on whether Mb affects EMT through the modulation of FAO as well as other metabolic pathways that were not considered here.

Aside from our studies, only Armbruster *et al*. have experimentally interrogated fatty acid metabolism in MDA-MB-468 and MCF7 breast cancer cell lines.[97] They report that Mb expression promotes cytoplasmic solubility and limited oxidation through fatty acid binding, yet this was only based on lipidomic measurements. In contrast, we utilized direct approaches of oxygen consumption- and radioactivity-based measurements to validate our findings in addition to metabolomic approaches. Armbruster *et al*. also showed that Mb promotes fatty acid deposition into lipid droplets upon desaturation of oxygen under hypoxia. Importantly, lipid droplet accumulation has been associated with decreased metastasis.[92] Data presented in our studies help support previous findings of Mb binding to fatty acids and limiting their oxidation in breast cancer cells when oxygenated. In instances of hypoxia, deoxy-Mb promotes lipid droplet formation,

which may also decrease the metastatic potential of hypoxic cancer cells. Altogether, our data suggest that Mb is an endogenous inhibitor of FAO within breast tumors through the redox regulation of CPT1 and not through fatty acid binding.

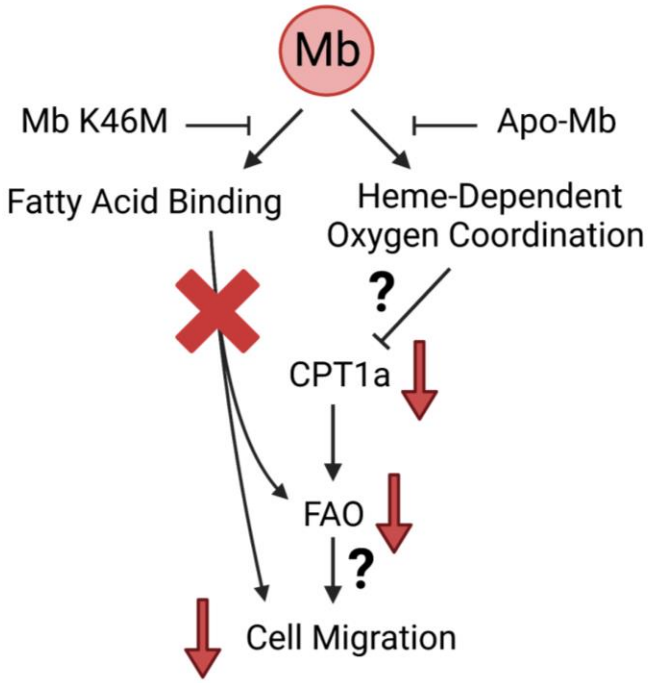

Figure 2-9: Graphical Model—Mb decreases FAO of breast cancer cells independent of fatty acid binding. Mb-dependent fatty acid binding is disrupted by K46M mutation but changes in binding do not rescue FAO or migration rates of 231 Mb cells. This suggests that Mb modulates FAO through a heme-dependent mechanism; however, FAO does not modulate cell migration in our model. Red arrows indicate Mb-dependent decreases.

3.0 Myoglobin-Dependent ROS Production Decreases Cell Migration.

3.1 Introduction

Cell migration, which is critical for *in vivo* metastasis, is an integrated process that requires the spatiotemporal coordination and reorganization of multiple cytoskeletal proteins. During chemotaxis, integrin activation leads to a signaling cascade of nascent focal adhesion formation, kinase activation, and downstream actin fiber remodeling. This process is highly dynamic, requiring constant steps of focal adhesion turnover, cell contraction, and actin reorganization.[100-105] Importantly, migration also requires the recruitment of mitochondria to the cell's leading edge to generate ATP and fuel the actin cytoskeleton remodeling. Post-translational modifications of actin-binding proteins (ABPs) initiate the actin cytoskeletal remodeling namely through phosphorylation; however, studies have only recently begun to understand the redox-sensitive mechanisms of actin remodeling and their effects on cell motility.[133]

Two ABPs that are implicated in cancer progression are profilin-1 (PFN1) and cofilin-1 (CFL1). PFN1 has a pro-migratory role in cells and catalyzes the GTP nucleotide exchange on G-actin monomers, supplying polymerization-competent G-actin monomers for incorporation into F-actin. Aside from actin, PFN1 interacts with phosphoinositide lipids at the plasma membrane and poly-L-proline regions of other actin-polymerizing proteins. Specifically in breast cancer cells, the levels of PFN1 are inversely related to cell migration. Breast cancer cells have been shown to downregulate PFN1 levels to become more motile;[173] in contrast, the overexpression of PFN1 slow cell migration by saturating phosphoinositide sites at the plasma membrane and blocking actin polymerizing protein recruitment at the plasma membrane.[110] CFL1

is an actin-severing protein that is often upregulated in metastatic cancers. CFL1 activity is locally controlled within cells through the phosphorylation of Ser 3, which inhibits F-actin severing. LIM kinase (LIMK) inactivates CFL1, while slingshot-1 (SSH1) phosphatase activates CFL1.[121, 174, 175] The levels of phospho-Ser3 CFL1 are inversely correlated with increased metastasis in breast cancer patients. The endogenous mechanisms that regulate PFN1 and phosphorylation of CFL1, particularly in different subtypes of cancer, are not completely clear.

Mb is expressed in approximately 40% of breast tumors and is associated with increased patient survival.[53, 55] Aboouf *et al*. showed that the expression of Mb decreases *in vitro* breast cancer cell migration by mitigating the epithelial-to-mesenchymal transition, which is associated with increased expression of migratory proteins such as matrix metalloproteases.[55] Moreover, the expression of Mb in A549 lung cancer cells decreased the formation of metastases in animal models.[132] However, the molecular mechanisms by which Mb modulates migratory pathways and whether ABP expression and activity are changed by Mb expression, remain unclear.

It is established that the heme center of Mb catalyzes the production of reactive oxygen species (ROS). Superoxide is produced through the autooxidation of ferrous iron (Fe^{II}) to ferric iron (Fe^{III}; **Equation 3**), and ferrous iron can also catalyze Fenton-type chemistry to generate hydroxyl radicals (**Equation 4**).[176] Further, metmyoglobin can be oxidized to ferryl (Fe^{IV}) by reacting with hydrogen peroxide from other sources in the cells, such as the mitochondrion or other oxidases (**Equation 5**). Ferryl iron can catalyze peroxidase chemistry to oxidize lipids and proteins (**Equation 6**) and further potentiate hydrogen peroxide production.[29, 176]

Braganza *et al*. showed that Mb-dependent ROS (i.e.: $\bullet O_2$ and H_2O_2) production increases the levels of mitofusin-1 and mitofusin-2 (MFN1 and MFN2, collectively called MFNs) in breast cancer cells. This increase in MFNs, small GTPases that potentiate mitochondrial fusion, was

responsible for Mb-dependent inhibition of cell proliferation.[168] Notably, mitochondrial fission and fusion are implicated in cell migration, and genetic silencing of MFNs has been shown to increase cancer cell migration by promoting fragmented mitochondria, which are more readily shuttled to the leading edge of mitochondria during cell migration compared to elongated mitochondria.[129, 131, 177]

However, whether Mb-dependent generation of ROS alters cell migration through the modulation of ABPs or MFNs has not been explored. Based on these studies, we hypothesize that Mb-dependent ROS generation modifies ABP and MFN expression to decrease cancer cell migration.

3.2 Materials and Methods

3.2.1 Cell Culture

MDA-MB-231 (CRM-HTB-26) and MDA-MB-468 (HTB-132) were purchased from The American Type Culture Collection (ATCC). MDA-MB-231 cell lines were cultured in DMEM with glutamine (4.5g/L glucose, Gibco) and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 1% penicillin-streptomycin antibiotic (50 Units/mL), and 15mM HEPES buffer (pH 7.4). MDA-MB-468 cell lines were cultured in a 1:1 mixture of DMEM/F-12 media (Gibco) and supplemented with 10% HI-FBS, 1% penicillin-streptomycin, and 15 mM HEPES as above. Cells were stored in a humidified incubator maintained at 37C, 21% O_2, and 5% CO_2.

3.2.2 Generation of Stable Myoglobin Expressing Cell Lines

MDA-MB-231 cells (90% confluence) were transduced with pLenti-C-mGFP-P2A-Purovectors (9.75x105 TU) expressing human myoglobin (MB; transcript variant 1; 231 Mb). Empty vector control cells (231 EV) were generated by transducing MDA-MB-231 cells with pLenti-C-mGFP-P2A-Puro particles ($9.75x10^5$ TU). Cells were incubated with viral particles (18 h) and then placed under puromycin selection (1µg/mL). Cells were expanded and maintained under puromycin selection media for at least three passages before experiments.

3.2.3 Transient Expression of GFP, Myoglobin, and Apo-Myoglobin

MDA-MB-231 cells were transfected with either control GFP, Mb, or Apo-Mb DNA (2.5µg) constructs using TransIT-X2 transfection reagent following manufacturer's instructions (Mirus Bio) and assayed 72 hours after transfection. Apo-Mb was generated previously by mutating H94 to a phenylalanine (H94F).[168]

3.2.4 Protein Extraction and Western Blotting

Cells were lysed in RIPA buffer with protease (cOmplete; Roche) and phosphatase (PhosSTOP, Roche) inhibitors on ice for at least 20 minutes. Lysates were clarified by collecting the supernatant after centrifugation for 20 minutes at 20,000xg. Protein concentrations were estimated by bicinchoninic acid (BCA) assays. Western blot was performed as previously described.[178] Primary antibodies used include Mb (catalog G-125-C, R&D Systems), PFN1 (catalog 3237, Cell Signaling), CFL1 (catalog 5175, Cell Signaling), phospho-Ser3 CFL1 (catalog

3313, Cell Signaling), LIMK1 (catalog 3842, Cell Signaling). Blots were incubated in the appropriate fluorophore-conjugated secondary antibodies and then imaged using the LI-COR Biosciences Odyssey DLx imager and analyzed using the LI-COR Image Studio Lite (software version 5.2). All protein concentrations were normalized to the housekeeping protein alpha-tubulin (catalog: CP06, Calbiochem) unless otherwise noted.

3.2.5 Wound Closure and Transwell Migration Assays

Cells were seeded and achieved 95% confluence before being treated with MMC the following day. Cells were "scratched" using a P200 pipet tip, washed with PBS, and given complete growth media. Cells in the same field of view were immediately imaged under a brightfield microscope at t=0, 2, 4, and 6h using a reference line on the cell culture plate. The percentage of scratch/wound closure was calculated by measuring the area of the wound size via ImageJ and a ratio of the wound size at 0 hours. Data were fitted using a simple linear regression to calculate the rate of scratch/wound closure.

For some Transwell assays, cells were treated with the proliferation inhibitor mitomycin C (10µg/mL MMC) for 2 hours one day prior to serum starvation with (0.5% FBS) overnight. On the day of the assay, cells were disassociated and added to the upper chamber of Corning Transwell polycarbonate membrane cell culture inserts (24-well format with 6.5mm inserts, 8.0-micron pore sizes). Full FBS (10%) or low serum (0.5% FBS) was added to the lower chamber of the plate as a chemoattractant or an undirected migration control, respectively, and incubated for 6 hours. For certain experiments, N-acetyl cysteine (NAC; 1mM) was added to the upper chamber. Cells were collected by fixing them in methanol and stained with crystal violet (0.5%). Cells that did not migrate to the lower plane of the membrane were removed using a cotton-tipped

applicator and allowed to dry overnight. Migrated cells were imaged under a Zeiss brightfield microscope with a 5x objective in 5 unique fields of view per transwell insert. Migrated cells were quantified using a custom ImageJ software plugin.

3.2.6 Reactive Oxygen Species Generation Assays

The hydrogen peroxide production rate in MDA-MB-231 cells was measured by Amplex™ Red dye at a final concentration of 7.5µM, as previously described.[168]

3.2.7 N-Acetylcysteine and PEG-Catalase Antioxidant Treatments

For NAC antioxidant treatments, cells were treated with 1mM NAC (+NAC) or untreated (NT). NAC was added to cells immediately before starting the six-hour migration assays or Amplex Red ROS Assays. Cell lysates were collected 24 hours after starting NAC treatment. For pegylated-catalase (PEG-Catalase, 200 units (U) were added to cells for 24 hours before collecting cell lysates.

3.2.8 Quantitative Polymerase Chain Reaction

Migration genes were measured by qPCR using the QIAGEN RT2 Profiler PCR Human Cell Motility Array (PAHS-128ZF-6) according to the manufacturer's instructions.

3.2.9 Genetic Depletion of Profilin-1 by shRNA

Cells stably expressing either non-targeting shRNA (shNT) or PFN1-targeting shRNA (shPFN1) were created by transducing MDA-MB-231 cells with lentiviral particles as previously described.[173]

3.2.10 Statistics

All statistical analyses were carried out using GraphPad Prism version 9.5. Welch's t-test was used to determine significant differences in PFN1 levels. One-way ANOVA was used for the analysis of GFP, Mb, and Apo-Mb ROS production and wound closure. Two-way ANOVA was used to determine statistical significance for NAC-treated ROS production and protein levels as well as Transwell migration assays.

3.3 Results

3.3.1 Myoglobin Expression Decreases Cell Migration

As previously shown in Chapter 2, we utilized both Transwell and wound closure assays to determine whether myoglobin (Mb) expression modulates breast cancer cell migration. Human Mb was stably expressed in MDA-MB-231 breast cancer cells that do not express endogenous Mb (**Figure 3-1A**). Control cells were transduced with an empty vector (231 EV). In a transwell migration assay, stable expression of GFP-tagged Mb (231 Mb cells) significantly decreased the

migration rate by approximately 30% compared to GFP empty-vector control cells (231 EV). We inhibited proliferation to ensure that it would not interfere with migration rates by treating cells with mitomycin C (MMC; 10µg/mL) and observed a ~54% decrease in 231 Mb cells that migrated compared to 231 EV cells (**Figure 3-1B-C**). We also measured migration using a wound closure assay; as in the Transwell assay, Mb-expressing cells migrate at a significantly decreased rate compared to control 231 EV cells (**Figure 3-1D-E**). As previously shown in **Figure 2-1**, these data show that Mb expression in breast cancer cells decreases migration.

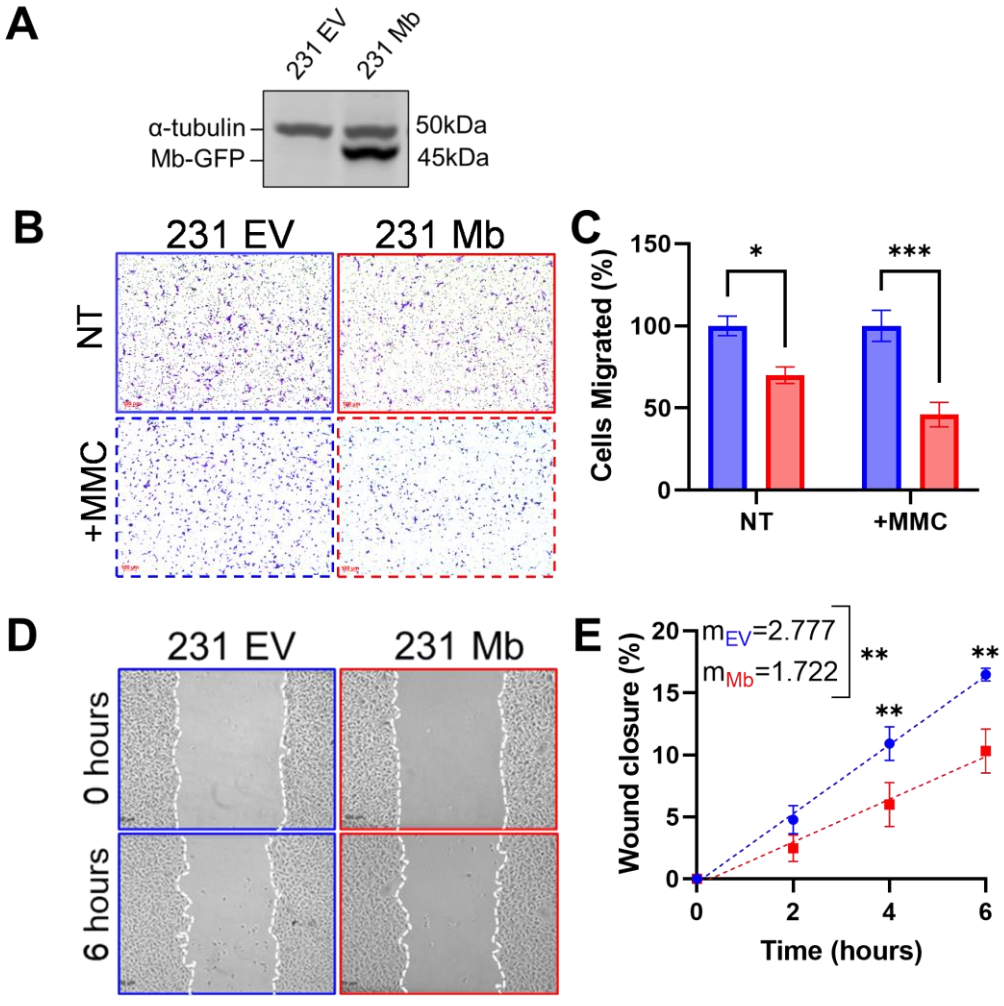

Figure 3-1: Mb expression decreases migration in MDA-MB-231 cells.

A) Representative western blot of stable MDA-MB-231 cell lines expressing either an empty vector GFP (231 EV) or GFP-tagged Mb (231 Mb). B) Representative brightfield images of migrated cells from 6-hour Transwell experiments stained with crystal violet and C) quantification of the percentage of cells migrated relative to the 231 EV (blue) cells with mitomycin C treatment (+MMC) or no treatment (NT) treatment after 6 hours. Two-way ANOVA with multiple comparisons tests; N=4. D) Representative images of 231 EV and 231 Mb cells in a scratch assay at 0 and 6 hours; dashed lines outline wound size. E) Quantification of results in assays such as in D showing the average percentage wound closure relative to the initial wound size (t=0) and fit using a simple linear regression (dashed lines) to determine the rate of migration/wound closure (m), simple linear regression and comparison of slopes; two-way ANOVA with multiple comparisons test; N=4. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001.

3.3.2 Myoglobin Expression Increases ROS Production and Scavenging ROS Restores Migration of 231 Mb Cells

To determine whether the heme group was required for the Mb-dependent attenuation of migration, we expressed either GFP, WT Mb, or Apo-Mb, in which heme is not incorporated due to a mutation in His94 to phenylalanine (H94F), and assayed migration by the wound closure assay (**Figure 3-3A-B**). Mb expression significantly decreased migration by approximately 24% compared to control cells (231 WT +GFP: 74±6%; 231 WT+GFP: 50±7% relative to initial wound size at 0 hours). Importantly, the expression of Apo-Mb restored the Mb-dependent decrease in migration, making the wound closure rate indistinguishable from the 231 WT +GFP cells (73±6%). Collectively, these data demonstrate that Mb-dependent attenuation of migration is dependent on Mb heme moiety.

We next tested whether heme-catalyzed ROS production was responsible for the attenuation of migration. Consistent with heme-dependent oxidant production, 231 Mb cells

generated significantly more H_2O_2 than 231 WT cells, and this effect was abolished in 231 Apo-Mb expressing cells (**Figure 3-2C**). To determine whether Mb-dependent oxidant production led to decreased cell migration, 231 EV and 231 Mb cells were treated with the antioxidant and cysteine donor N-acetylcysteine (+NAC) to scavenge the Mb-derived H_2O_2 and measured migration (**Figure 3-2E-F**). Treatment with NAC mitigated 231 Mb oxidant production to a level indistinguishable from 231 EV cells (**Figure 3-3D**), and NAC treatment restored the rate of cell migration in 231 Mb cells in a Transwell assay (**Figure 3-3E-F**).

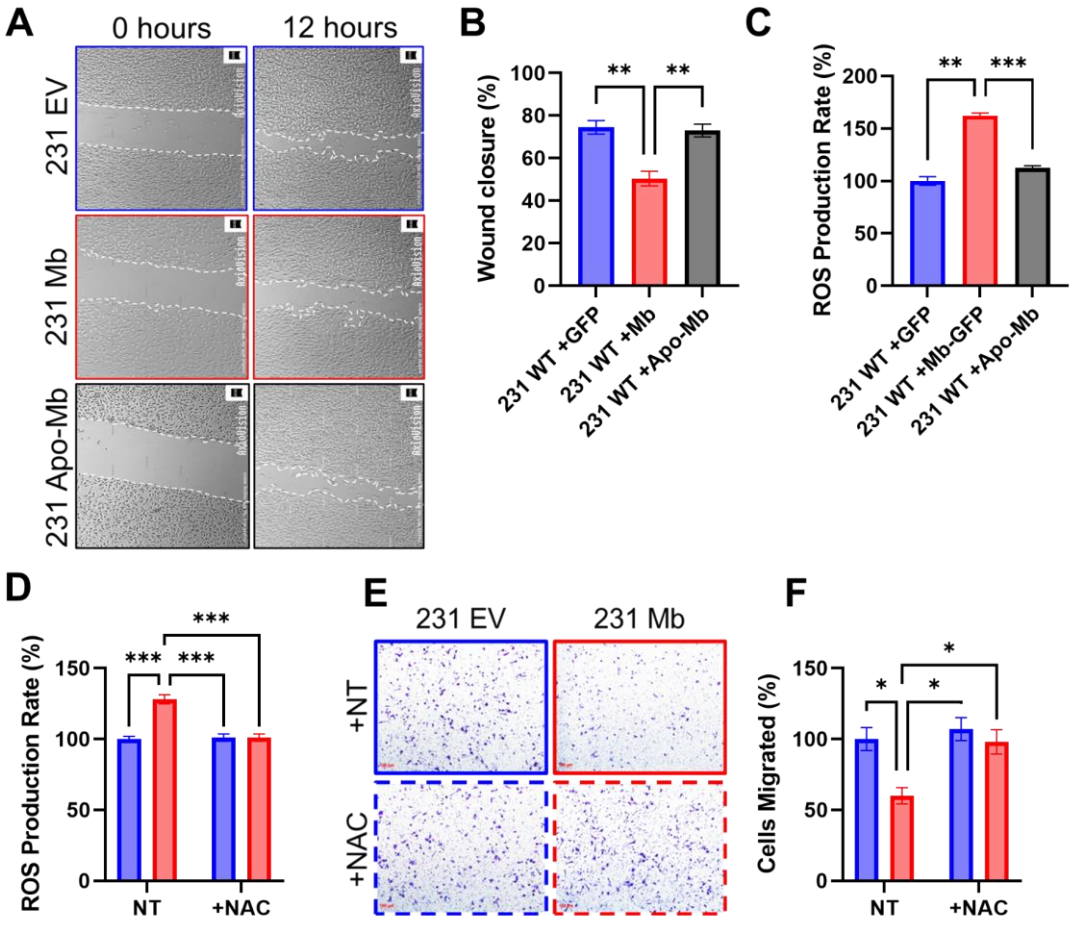

Figure 3-2: Mb-dependent ROS production downregulates cell migration

A-B) Representative brightfield images of scratch assays of 231 WT with either GFP, Mb, or Apo-Mb results were quantified as a percentage relative to the initial wound size after 12 hours (B). One-way ANOVA with

multiple comparisons tests; N=4 C) Cellular H_2O_2 production rates of 231 WT cells transiently expressing either GFP, Mb-GFP, or Apo-Mb as measured by Amplex Red dye. One-way ANOVA with multiple comparisons tests; N=1. D) Cellular ROS production rates of 231 EV (blue) and 231 Mb (red) cells with no treatment (NT) or treated with N-acetyl cysteine (1mM NAC). Two-way ANOVA with multiple comparisons tests; N=3. E) Representative brightfield images at 6h of migrated 231 EV and 231 Mb. F) Transwell migration assay results of 231 EV (blue) and 231 Mb (red) cells with or without NAC treatment at 6 hours. Two-way ANOVA with multiple comparisons tests; N=3. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001.

3.3.3 Myoglobin Decreases Migration Independent of MFNs

In a previous study by Braganza *et al*., Mb expression increased the levels of MFNs and resulted in decreased proliferation rates.[168] We tested whether Mb-dependent MFN accumulation also affects cell migration by genetically silencing MFNs with siRNA and then measuring cell migration. As expected, the deletion of either MFN1 or MFN2 significantly increased cell migration rates in 231 EV cells (**Figure 3-3A-B**). However, MFN deletion had no significant effect on the migration rates of 231 Mb cells. These results suggest that the Mb-dependent decrease in cell migration is not dependent on the levels of MFN.

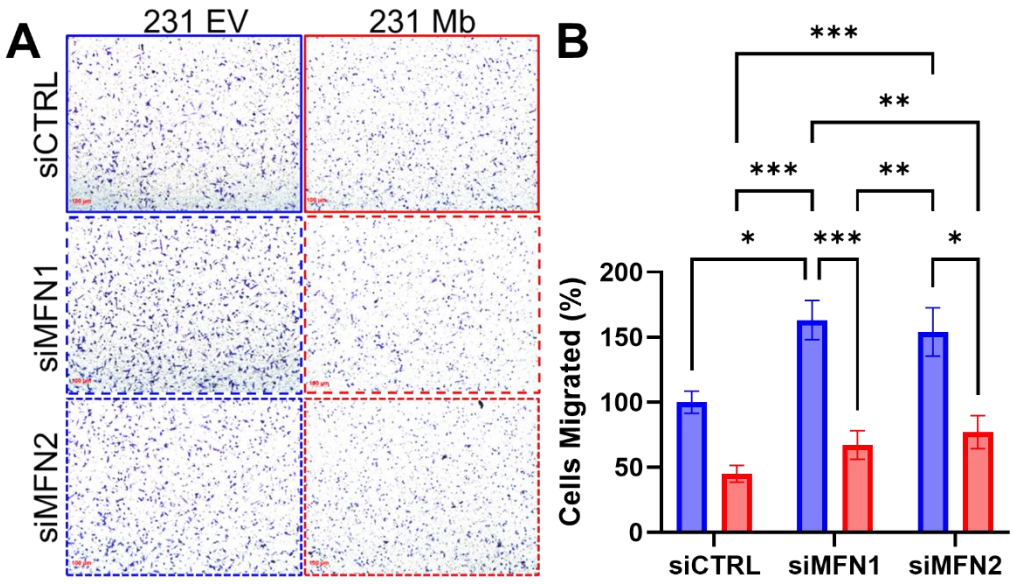

Figure 3-3: Depletion of MFNs does not restore migration rates of 231 Mb cells.

A) Representative brightfield images of migrated 231 EV and 231 Mb cells after control (siCTRL), MFN1 (siMFN1), or MFN2 (siMFN2) knockdown Transwell experiments stained with crystal violet. B) Transwell migration assay results of 231 EV (blue) and 231 Mb (red) cells with siRNA-mediated knockdown of MFNs were quantified as a percentage relative to the 231 EV siCTRL cells after 6 hours. Two-way ANOVA with multiple comparisons tests; N=3-4.

3.3.4 PFN1 and CFL1 are Targets of Mb-Catalyzed Oxidant Generation that Modulate Cancer Cell Migration

To determine whether Mb enhances cancer cell migration through proteins that regulate actin skeleton rearrangement, we first examine the RNA levels of *PFN1* and *CFL1* in 231 EV and 231 Mb cells using a QIAGEN RT^2 qPCR array. We observed that 231 Mb cells showed greater levels of *PFN1* and *CFL1* RNA (**Figure 3-4A**) and thus focused on these two ABPs. Notably, measurement of protein levels showed that PFN1 protein was increased (**Figure 3-4B**) but CFL1

was not (data not shown) in 231 Mb cells. PFN1 levels were also decreased by NAC treatment, consistent with the hypothesis that Mb-dependent ROS increases PFN1 levels in 231 Mb cells (**Figure 3-4C**). To determine whether the Mb-mediated decrease in migration was due to increased levels of PFN1 in 231 Mb cells, we utilized cells expressing either a *PFN1*-targeting shRNA (shPFN1) or control shRNA (shNT) and transiently transfected them with either GFP or Mb and assayed migration (**Figure 3-4F)**. The knockdown of PFN1 lead to an approximate 47% and 44% increase in Transwell migration of GFP- and Mb-expressing cells, respectively (**Figure 3-4C-D**). These data demonstrated that inhibition of PFN1 on its own is not sufficient to restore migration in the 231 Mb cells.

While Mb expression did not change CFL1 expression, we did see an increase in the phosphorylation of serine 3 (phospho-Ser3) on CFL1, consistent with decreased migration. Measurement of the levels of LIMK, the kinase responsible for CFL1 phosphorylation also showed that Mb expression increased LIMK levels (**Figure 3-4E-F**). Importantly, levels of both phospho-Ser3 CFL1 and LIMK appeared to be decreased in the presence of the ROS scavenger pegylated-Catalase, but this effect did not reach statistical significance (**Figure 3-4E-F**). These data suggest that Mb's heme-catalyzed production of ROS may regulate the levels of PFN1 and activity of CFL1 through LIMK1, which may decrease cell migration.

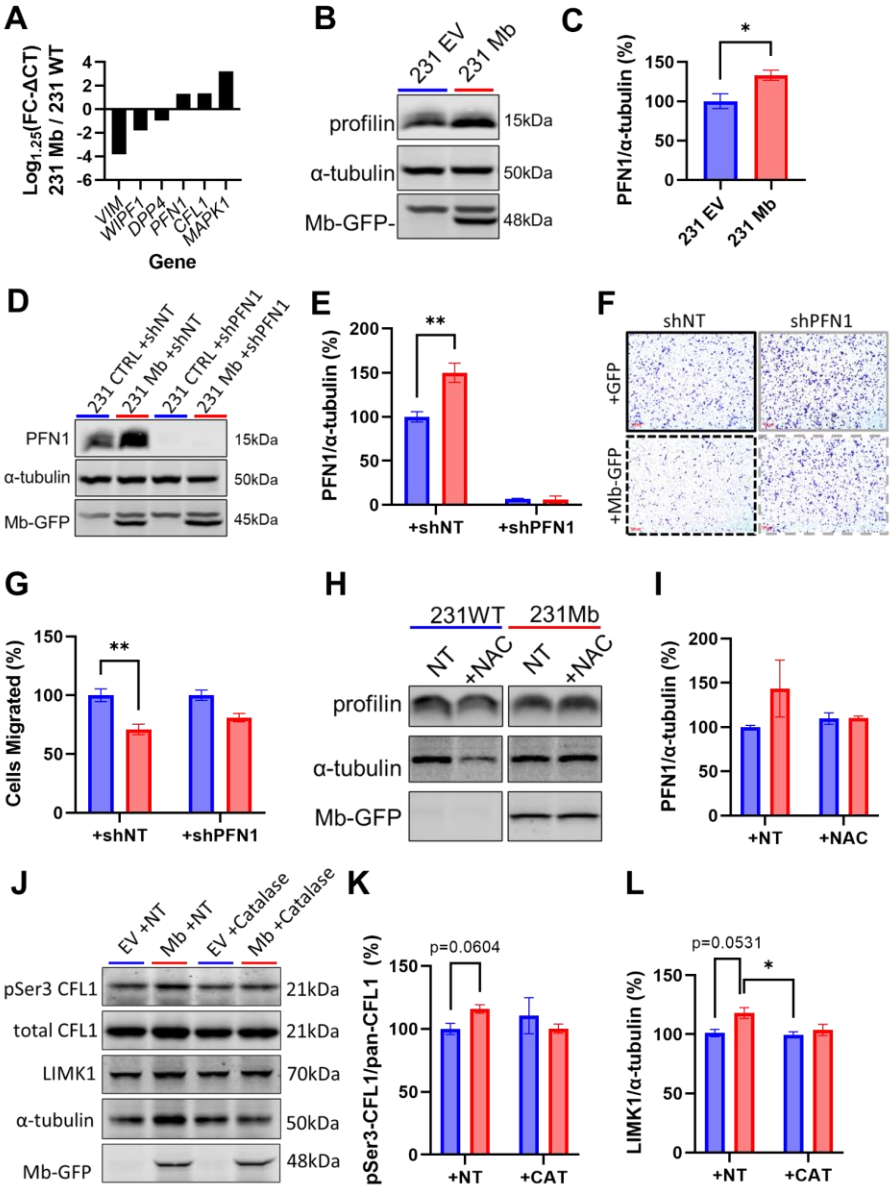

Figure 3-4: Mb-dependent ROS production modulates ABPs PFN1 and CFL1.

A) Quantitative PCR revealed expression changes of at least 25% for vimentin (*VIM*), WAS/WASL-interacting protein family member 1 (*WIPF1*), dipeptidyl peptidase-4 (*DPP4*), profilin-1 (*PFN1*), cofilin-1 (*CFL1*), and (mitogen-activated-protein kinase-1 (*MAPK1*) relative to the 231 EV cells. N=1. B) Representative western blots of profilin-1 (PFN1) in 231 EV and 231 Mb cells. C) Quantitation of western blots is expressed as a percent relative to 231 EV cells (blue). Welch's t-test; N=4. D) Representative western blot of constitutive profilin knockdown (shPFN1) and transient Mb-GFP expression in 231 WT cells 72 hours after transfection.

E) Quantitation of western blots is expressed as a percent relative to 231 +GFP +shNT cells (blue). Two-way ANOVA with multiple comparisons test; N=3. F) Representative western blot of profilin levels expression of either GFP or Mb. F) Representative brightfield images of migrated 231 +GFP (blue) and 231 +Mb-GFP (red) cells with non-targeting and PFN1-targeting shRNAs (shNT and shPFN1, respectively) from 6-hour Transwell experiments stained with crystal violet. G) Transwell migration assay results of F were quantified as a percentage relative to the 231 +GFP cells after 6 hours. Two-way ANOVA with multiple comparisons tests; N=4. H) Representative western blot of profilin levels 6 hours after NAC treatment. I) Quantification of western blots in H is expressed as a percent relative to 231 WT +NT cells; N=1. J) Representative western blots of phospho-Ser3 CFL, total CFL1, and LIMK1 levels in 231 EV and 231 Mb cells 24 hours after 200U PEG-catalase treatment. K) Quantification of phospho-Ser3 CFL1 in J of nontreated (NT) or PEG-catalase-treated (+CAT) in 231 EV (blue) and 231 Mb (red) cells. Two-way ANOVA with multiple comparisons tests; N=4. L) Quantification of LIMK1 in nontreated (NT) or PEG-catalase-treated in 231 EV (blue) and 231 Mb (red) cells. Two-way ANOVA with multiple comparisons tests; N=3. Two-way ANOVA with multiple comparisons tests; N=3. Data are Mean ±SEM. ✱: $p<0.05$.

3.4 Discussion

The data presented in this chapter are the first to demonstrate that Mb heme-dependent oxidant production attenuates cell migration. Mb increases the levels of *PFN1* and the activity of CFL1, as indicated by its phosphorylation at Ser3. Importantly, these increases were attenuated by ROS scavenging in Mb-expressing cells. Notably, silencing of PFN1 alone was not sufficient to restore migration in Mb-expressing cells.

Though we found that PFN1 levels were modulated by Mb-dependent ROS generation (i.e.: H_2O_2), PFN1 deletion did not completely restore migration in the Mb-expressing cells. This is likely due to the fact that multiple ABPs are regulated by ROS. This is evidenced by the changes

we observed in phospho-Ser3 CFL1 levels. Future studies will entail larger proteomic experiments to determine the spectrum of ABPs and regulators that are modulated by Mb. Once identified, migration assays in which multiple ABPs are silenced will give deeper insight into the exact pathways that are modulated by Mb to attenuate migration.

Low levels of ROS are implicated in carcinogenesis by modulating cell signaling through proteins and nucleic acids oxidative modifications that result in altered protein function and mutations, yet cancer cells may tolerate ROS through the upregulation of antioxidant systems.[179] However, higher levels of unregulated ROS production lead to stalled cell cycle progression and apoptosis. Our findings implicate Mb-derived ROS as mediators of cellular migration by interfering with PFN1 levels and CFL1 activity. Studies in melanoma have shown that NAC leads to an increase in metastasis in murine xenografts, and the authors of this study suggested this was due to the cysteine donating properties of NAC that lead to increased glutathione levels. While we did not measure antioxidant capacity or glutathione levels in our study, the short time course (six hours) of our study suggests that NAC is more likely acting as an antioxidant through cysteine donation. Further, a second antioxidant (PEG-catalase) confirmed the ROS-mediated effects on CFL1 phosphorylation. It is interesting to speculate that Mb expression is potentially generating enough ROS to overwhelm the cell's antioxidant capacity. This is demonstrated by the ability to directly measure hydrogen peroxide from the Mb-expressing cells.

We tested whether the Mb-dependent upregulation of MFN1 and MFN2 attenuates cell migration. While MFN depletion increased cell migration in 231 EV cells, migration was not significantly increased in 231 Mb cells suggesting that Mb-dependent regulation of migration is not dependent on MFNs. To this end, actin polymerization-dependent protrusion of the plasma membrane is the initiating step in cell migration. Because the polymerization of actin precedes

the migration-induced trafficking of the mitochondria, we may speculate the Mb-dependent modulation of ABPs has a superseding effect on cell migration than mitochondrial dynamics. Interestingly, prior studies demonstrate that Mb-mediated attenuation of proliferation is dependent on MFN. Thus, though Mb-dependent ROS attenuates both proliferation and migration, the cellular targets modulated by Mb-catalyzed ROS are distinct.

Our data show that PFN1 levels are increased with Mb expression in a ROS-dependent manner. This may be explained by an oxidant-driven activation of PFN1's transcription factor, serum-response factor (SRF), or the inhibition of its degradation pathway. The transcription factor activity of SRF is stimulated by myocardin-related transcription factors (MRTFs). [180] Within smooth muscle cells, SRF-MRTF transcriptional activity is dependent on the oxidants generated by NAPDH Oxidase-4.[181, 182] In regard to the latter, PFN1 is marked for degradation by the E3 ubiquitin ligase CHIP. While we did not specifically examine CHIP in these studies, others have shown that CHIP decreases proliferation and metastasis in TNBC.[183]. Similar to the Mb-dependent oxidation of the E3 ubiquitin ligase Parkin described by Braganza *et al*., it is interesting to speculate that CHIP activity may also be affected by Mb-derived oxidants.[168]. To this end, decreased CHIP protein levels have been implicated in the progression of neurological disorders.[184] However, redox-dependent modulation of either of these proteins has not been demonstrated. However, to our knowledge, evidence of direct modulation of either SRF-MRTF or CHIP activities by oxidants has not been described within cancer cells.

CFL1 activity correlates with increased metastasis in breast cancer patients. Our findings demonstrate that Mb-dependent ROS production decreases the activity of CFL1. We also observe that the levels of LIMK1 are elevated in Mb-expressing cells but decreased in catalase-treated samples. Corcoran and Cotter have previously reviewed the redox-dependent mechanisms of

kinase activation by ROS. To this end, at least in smooth muscle cells, Src family kinases are activated by ROS, which stimulates actin remodeling and RhoA-ROCK1-LIMK1, leading to inhibited increased CFL1 levels.[185] It stands to reason that ROS from Mb increases LIMK1 activity but Mb-dependent PFN1 increases mitigate cell migration. However, studies are required to determine whether Mb-derived oxidants regulate the catalytic LIMK activity. Alternatively, Mb-dependent accumulation of LIMK1 may be explained by oxidative inactivation of its E3 ubiquitin ligase Rnf6. LIMK is marked for proteasomal degradation by Rnf6 in neurons, but this has not yet been examined in cancer cells.[186]

The utilization of antioxidants, such as vitamin C, carotenoids, and NAC, has provided mixed results within clinical trials.[187] Antioxidants are often administered in addition to standard chemotherapeutics to limit their side effects. In breast cancer specifically, independent trials have demonstrated that antioxidants limit carcinogenic ROS signaling.[187] In contrast, others have noted that antioxidants increase disease progression, aligning with our findings above.[188, 189] Specifically, Jung and colleagues report that disease progression was increased in post-menopausal breast patients prescribed antioxidants.[190]. Since Mb expression tends to be higher in post-menopausal breast cancer patients, it is interesting to speculate that differing results in these studies may stem from the expression of Mb within breast tumors as a major source of ROS.[53] Future studies that stratify patients based on Mb-expression may reveal whether Mb underlies the differing effects of antioxidants in breast cancer disease progression.

The data presented in this chapter show for the first time that Mb expression modulates ABPs PFN1 and CFL1 through the production of ROS (**Figure 3-6**). Our data contribute to the overall understanding of how Mb expression mitigates the pathogenesis of breast cancer migration.

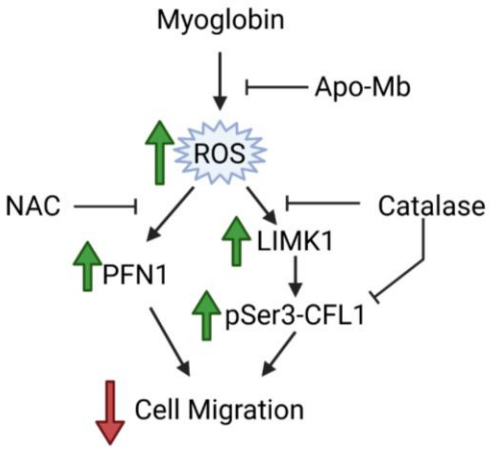

Figure 3-5: Graphical Model—Mb-Dependent ROS Production Decrease Cell Migration Through the Modulation of PFN1 Levels and CFL1 Activity.

Myoglobin's heme-dependent production of ROS leads to increased levels of PFN1 and phospho-Ser3 CFL1, which decrease migration. Treatment with the antioxidants NAC and catalase are able to lower levels of PFN1 and phospho-Ser3 CFL1, which increase cell migration. The green and red arrows indicate Mb-dependent increases and decreases, respectively.

4.0 Myoglobin Attenuates Nitric Oxide-Dependent Proliferation in Triple-Negative Breast Cancer through its Nitric Oxide Dioxygenase Activity.

4.1 Introduction

Breast cancer is the most common disease in women, and patients' treatment and outcome vary depending on tumor biology.[191] Triple-negative breast cancer (TNBC) accounts for up to 15% of all breast tumors and is a particularly aggressive subtype that presents at advanced stages. In addition, the lack of targeted therapies for TNBC tumors leaves patients with significantly worse outcomes compared to patients with other breast tumor subtypes.[191-194] Approximately 87% of TNBC tumors express inducible nitric oxide synthase (iNOS, i.e., *NOS2*) and the expression of this protein is correlated with decreased patient survival. [143, 144] Within iNOS-positive tumors, survival can also vary: Garrido *et al*. previously demonstrated that low and moderate levels of iNOS expression led to significantly worse survival rates than high iNOS levels, exemplifying a dual role of NO• signaling in tumors.

NOS enzymes catalyze the conversion of L-arginine and oxygen to L-citrulline, generating a molecule of nitric oxide (NO•) in the process.[139] At the molecular level, NO• has a biphasic concentration-dependent effect in tumors, with low levels (<1μM flux) promoting cell proliferation and higher levels of NO• (≥1μM flux) inducing cell death.[138, 141, 143, 195] The canonical physiological signaling pathway mediated by NO• is the propagation of vasodilation in the vasculature and occurs through the activation of soluble guanylyl cyclase (sGC) to produce cGMP, leading to the subsequent activation PKG.[148] However in tumor cells, the NO•-sGC-cGMP pathway has also been shown to promote cancer cell proliferation.[150] Independent of the

cGMP pathway, NO• has been shown to activate a number of other pro-proliferative cascades in cancer cells. For example, Switzer *et al*. have shown that NO• exposure increases the activation of EGFR by S-nitrosylation and its downstream effectors including Src, AKT, and ERK1; they also found that NO• promotes c-Myc stabilization and inactivates protein phosphatase 2A (PP2A).[196] Importantly, inhibition of EGFR prevents TNBC growth even in the presence of NO• production.[138, 143, 150, 196, 197] Despite the recognition that NOS-dependent NO• production propagates tumor cell proliferation, there is little known about the endogenous mechanisms that modulate NO•-mediated signaling and NOS expression in TNBC tumor cells.

Myoglobin (Mb) is a critical regulator of NO• bioavailability in skeletal and cardiac muscle, but its role in modulating NO• levels in cancer cells remains unclear. Mb limits NO• bioavailability through its dioxygenase reaction in which oxygen-bound Mb (oxy-Mb) oxidizes NO• to nitrate (NO_3^-; **Equation 1**).[156]

Bicker *et al*. utilized RNA-Seq to identify how Mb in MDA-MB-468 cells alters gene expression and reported that genes related to the "regulation of NO• biosynthetic process[es]" are decreased in the Mb-silenced cells at hypoxia and normoxia; this suggests that Mb may be involved with NO• production. However, the direct effect of Mb-dependent NO• scavenging on proliferative pathways has not yet been tested. Further, while it is well established that Mb directly reacts with NO•, the role of Mb in regulating the expression of NOS has not been examined. In this chapter, we hypothesize that endogenous Mb expression in breast tumor cells decreases NO• bioavailability, leading to the attenuation of pro-proliferative signaling. We demonstrate that the expression of Mb in cultured TNBC breast cancer cells inhibits NO•-mediated cGMP production and MAPK signaling, which attenuates cell proliferation. Further, we show data demonstrating that Mb expression decreases NOS expression in TNBC cells and tumors, suggesting a second

mechanism by which Mb limits NO• bioavailability. These results will be discussed in the context of Mb-mediated mechanisms of TNBC attenuation as well as the development of targeted therapies for TNBC.

4.2 Materials and Methods

4.2.1 Cell culture

MDA-MB-231 (CRM-HTB-26) and MDA-MB-468 (HTB-132) were purchased from The American Type Culture Collection (ATCC). MDA-MB-231 cell lines were cultured in DMEM with glutamine (4.5g/L glucose, Gibco) and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 1% penicillin-streptomycin antibiotic (50 Units/mL), and 15mM HEPES buffer (pH 7.4). MDA-MB-468 cell lines were cultured in a 1:1 mixture of DMEM/F-12 media (Gibco) and supplemented with 10% HI-FBS, 1% penicillin-streptomycin, and 15 mM HEPES as above. Cells were stored in a humidified incubator maintained at 37C, 21% O_2, and 5% CO_2. Mb was transfected into MDA-MB-231 cells as previously described.[168] *MB* was genetically silenced in MDA-MB-468 cells using siRNA as previously described. [168]

4.2.2 Cell proliferation assays:

Cell proliferation was determined by crystal violet staining of DNA as previously described. [168] DETA NONOate (DETA-NO; catalog 82120, Cayman Chemical Co.) was resuspended in 0.1mM sodium hydroxide (NaOH) and diluted to a final concentration of 25μM. Vehicle-treated controls

were treated with the equivalent volume of NaOH. The pan-AKT inhibitor GSK690693 (catalog 16891, Cayman Chemical Co.) was added to cells at a final concentration of 100μM.

4.2.3 Measurement of cyclic GMP (cGMP):

Generation of cGMP was quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (catalog: 581021; Cayman Chemical Co.). Cells were treated with the pan phosphodiesterase inhibitor IBMX (catalog 13347; Cayman Chemical Co.) and DETA-NO for six hours.

4.2.4 Measurement of iron-nitrosyl (Fe-NO):

Fe-NO was measured by reductive chemiluminescence as previously described. [145]

4.2.5 Protein extraction and western blot analysis:

Cells were lysed in RIPA buffer with protease (cOmplete; Roche) and phosphatase (PhosSTOP, Roche) inhibitors on ice for at least 20 minutes. Lysates were clarified by collecting the supernatant after centrifugation for 20 minutes at 20,000xg. Protein concentrations were estimated by bicinchoninic acid (BCA) assays. Western blotting was performed as previously described in cell lysates.[178] Primary antibodies used include Mb (catalog G-125-C, R&D Systems), phospho-Thr202/phospho-Tyr204 ERK1 (catalog 4370, Cell Signaling Technology), ERK1 (catalog 4695, Cell Signaling Technology), phospho-Ser473 AKT (catalog 4060, Cell Signaling Technology), AKT (catalog 4691, Cell Signaling Technology), eNOS (catalog

ab76198, abcam); each was incubated overnight according to manufacturer's instructions. Blots were incubated in the appropriate fluorophore-conjugated secondary antibodies and then imaged using the LI-COR Biosciences Odyssey DLx imager and analyzed using the LI-COR Image Studio Lite (software version 5.2).

4.2.6 Nitric oxide synthase (NOS) activity assay:

MDA-MB-231 cells were treated with interferon-γ (INFγ) and tumor necrosis factor-α (TNFα) to promote iNOS expression as previously described.[198] NOS activity was determined by measuring the conversion of radiolabeled arginine to citrulline as described previously.[199]

4.2.7 Murine xenograft models:

Animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (protocol number 12030315B-7). Xenografts were carried out as previously described.[168] Briefly, ten 4–5-week-old NOD scid female mice were purchased from The Jackson Laboratory (NOD.CB17-Prkdc^{scid}/J; strain 001303). Both inguinal mammary fat pads of mice were injected with 3 x 10^6 of either 231 EV or 231 Mb cells. Studies were terminated when the average tumor volume of the 231 EV control group approached 1.5cm^3 (25 days). Primary tumors were harvested from each animal, and their mass and dimensions were recorded.

4.2.8 Human Protein Atlas mRNA correlation:

Data were obtained from the Human Protein Atlas (proteinatlas.org).[200] Fragments per kilobase million (FPKM) for genes of interest were organized by patient identifiers and analyzed using Spearman's nonparametric correlation matrix.

4.2.9 Statistical analyses:

All statistical analyses were carried out using GraphPad Prism software (version 9.5). Proliferation rates were determined using simple linear regression. Percent increases in proliferation were compared using two-way ANOVA with multiple comparisons test. Two-way ANOVA with multiple comparisons tests were used to compare levels of cGMP, Fe-NO, phospho-ERK, phospho-AKT, NOS activity, and metabolomics. Spearman's nonparametric correlation matrix was used for all Human Protein Atlas analyses. All data are expressed as mean ±SEM.

4.3 Results

4.3.1 Myoglobin expression attenuates NO•-dependent increases in cellular proliferation

To determine whether Mb modulates NO•-induced TNBC cell proliferation, we treated MDA-MB-231 (231 EV) cells and identical cells in which we expressed human Mb (231 Mb) with the NO• donor compound DETA NONOate (DETA-NO; 25μM; 48 hours) and measured cellular

proliferation. NO• treatment increased the proliferation rate of 231 EV cells by approximately 13.5% relative to vehicle-treated cells over 48 hours (**Figure 4-1A-B**). Consistent with prior studies by Braganza *et al*., 231 Mb cells showed significantly decreased proliferation relative to 231 EV cells.16 The proliferative response of 231 Mb cells to DETA-NO was significantly blunted compared to DETA-NO-treated 231 EV cells. These data suggest that Mb attenuates NO•-dependent proliferative signaling.

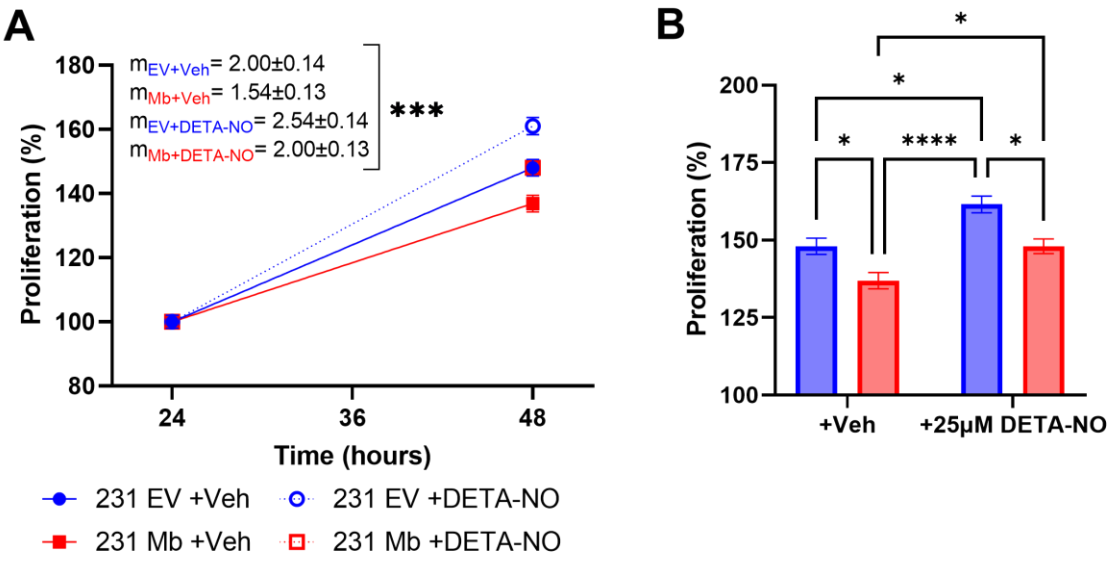

Figure 4-1: Mb decrease NO-dependent increases in cell proliferation.

A) Cell proliferation rates after 48 hours of treatment with vehicle (+Veh) or 25μM DETA NONOate (+DETA-NO) relative to proliferation at 24 hours. Data are fit using a simple linear regression to determine the rate of proliferation (m), simple linear regression and comparison of slopes; N=5. B) Proliferation rates of 231 EV (blue) and 231 Mb (red) cells after 48 hours are quantified as a percentage relative to 24 hours; two-way ANOVA with multiple comparisons test; N=5. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001, ✱✱✱✱: p<0.0001.

4.3.2 Myoglobin scavenges NO• through its NO• dioxygenase activity

To determine whether Mb scavenges NO• *in vitro*, we treated 231 EV and 231 Mb cells with DETA-NO and measured the production of cGMP, a downstream product of NO-mediated activation of sGC, in the presence of phosphodiesterase inhibitor IMBX. Basal cGMP levels were not different between 231 EV and 231 Mb cells, and treatment with DETA-NO led to a dose-dependent increase in cGMP formation in both cell types (**Figure 4-2A-B**). Importantly, the presence of Mb decreased the DETA-NO-dependent production of cGMP. In MDA-MB-231 cells, 50μM DETA-NO increased cGMP production by 33% (182 ±12 pmol/min/mg) in 231 EV cells relative to 231 Mb cells (122±16 pmol/min/mg). DETA-NO (100μM) led to an increase in cGMP production in 231 EV cells (262 ±19 pmol/min/mg) compared to 231 Mb cells (188±21 pmol/min/mg). 200μM led to a 34% difference in cGMP production between 231 EV (345±27 pmol/min/mg) and 231 Mb (227±18 pmol/min/min; **Figure 4-2A**). As an indicator of direct heme-dependent NO• scavenging, Fe-NO was measured after DETA-NO treatment. In 231 Mb cells, Fe-NO levels were increased compared to 231 EV cells, though these levels did not reach statistical significance (231 EV: 9±3 pmol/mg; 231 Mb: 15±5pmol/mg; **Figure 4-2B**).

To confirm the role of Mb in NO• scavenging, we utilized a second TNBC cell line, MDA-MB-468 in which Mb is endogenously expressed. We treated wild-type cells (468 CTRL) and those with endogenous Mb deleted (468 Mb-) with DETA-NO and measured cGMP as well as Fe-NO (**Figure 4-2C-D**). Similar to the MDA-MB-231 cell line, we found that Mb expression mitigates cGMP production. 50μM DETA-NO increased cGMP levels to 221±19 pmol/min/mg in 468 CTRL cells, but 468 Mb- cells produced 288±21pmol/min/mg. 468 Mb- cells generated 347±19 pmol/min/mg after 100μM DETA-NO treatment, but 468 CTRL cells generated significantly less (319±21 pmol/min/mg). 200μM led to a 10% difference in cGMP production

between 468 CTRL (421±11 pmol/min/mg) and 468 Mb- cells (468±13 pmol/min/min; **Figure 4-2C**). Fe-NO was also decreased in Mb-expressing 468 CTRL cells (468 CTRL: 17±2 pmol/mg; 468 Mb-: 11±4pmol/mg). Together, these data suggest that the expression of Mb diminishes NO• signaling by NO• scavenging.

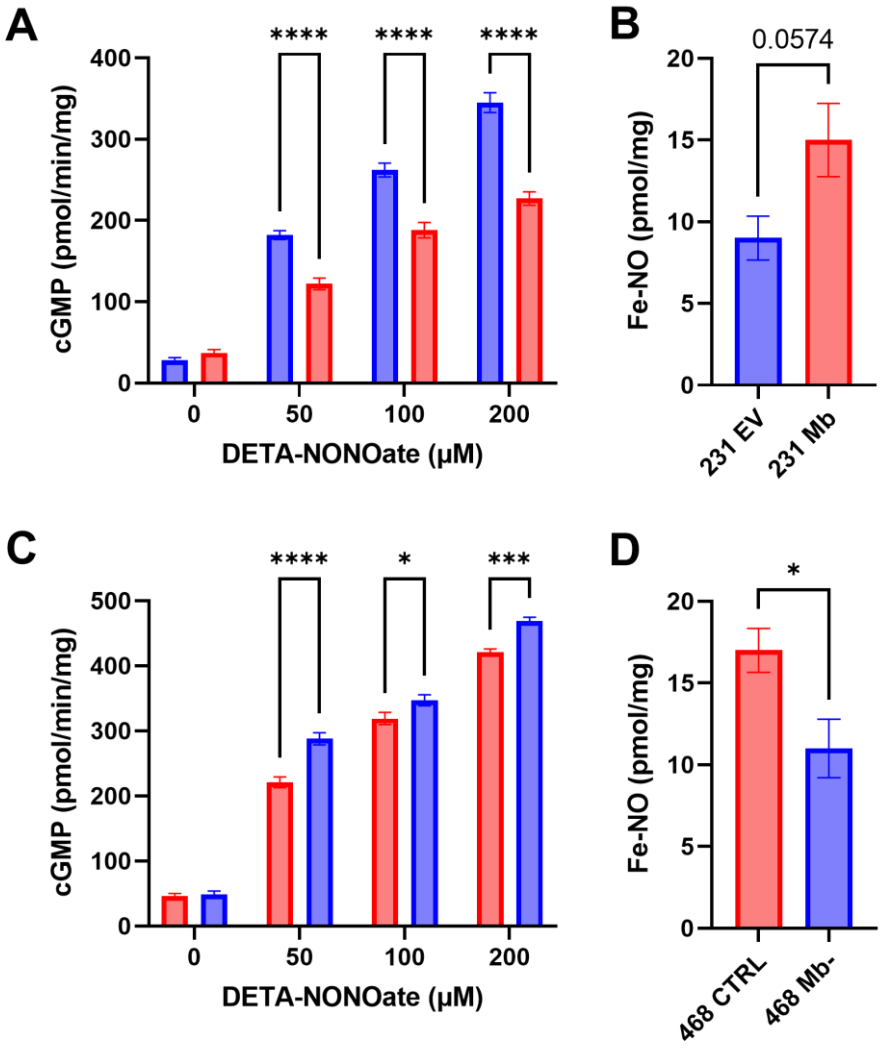

Figure 4-2: Mb-dependent NO• scavenging decreases cGMP formation and produces iron nitrosyl.

A, C) NO-sGC-cGMP levels are decreased in presence of Mb (red) within MDA-MB-231 (A) and MDA-MB-468 (C) cells compared to control cells without Mb (blue). Two-way ANOVA with multiple comparisons test; N=5. B, D) Iron nitrosyl levels are increased upon the addition of DETA-NO within MDA-MB-231 (B) and MDA-MB-468 (D) cells that express Mb (red) compared to cells without Mb (blue). Two-way ANOVA with

multiple comparisons test; N=5. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001, ✱✱✱✱: p<0.0001.

4.3.3 Myoglobin attenuates NO-dependent ERK1 and AKT activation

To determine whether Mb expression attenuates other NO-induced mitotic pathways that are implicated in TNBC progression, we treated 231 EV and 231 Mb cells with DETA-NO (100μM; 6h) and measured the Raf-MEK-ERK MAPK cascade and EGFR-PI3K-AKT pathway.10 Treatment of the cells with DETA-NO led to an approximately 68% increase in phospho-Thr202/phospho-Tyr204 ERK1 in 231 EV cells (172±20%) relative to vehicle-treated 231 EV cells (104±7%); however, the expression of Mb attenuated the NO-dependent activation of ERK1 by approximately 49% (231 Mb +Veh: 111±9%; 231 Mb +DETA-NO: 123±18%; **Figure 4-3A**) compared to 231 EV cells treated with DETA-NO. Additionally, NO• treatment increased phospho-Ser473 AKT exclusively in 231 EV (231 EV +Veh: 98±9%; 231 EV +DETA-NO 161±18%) but not 231 Mb cells (231 Mb +Veh: 106±12%; 231 Mb +DETA-NO: 130±14%) compared to their respective vehicle-treated controls.

Like ERK1 in NO-treated 231 Mb cells, Mb significantly attenuated NO-dependent AKT activation relative to 231 EV DETA-NO-treated cells (231 EV +DETA-NO: 161±18%; 231 Mb +DETA-NO: 130±14%; **Figure 4-3B**). To determine the role of AKT in NO-dependent proliferation, we assayed cell proliferation after the addition of DETA-NO in the presence of the pan AKT inhibitor GSK690693. NO• significantly increased cell proliferation in the 231 EV cells (231 EV +Veh: 100±5%; 231 EV +DETA-NO: 156±9%) but not in the 231 Mb cells (231 Mb +Veh: 80±9%; 231 Mb +DETA-NO: 96±6%; **Figure 4-3C**). GSK690693 decreased NO-mediated cell proliferation (118±11%) relative to the DETA-NO-treated 231 EV cells (156±9%).

There were no statistically significant changes in 231 Mb cell proliferation between the three treatment groups (231 Mb +Veh: 80±9%; 231 Mb +DETA-NO: 96±6%; 231 Mb +DETA-NO +GSK690693: 90±7%). These data support the hypothesis that Mb impedes NO-dependent cell proliferation through multiple pathways.

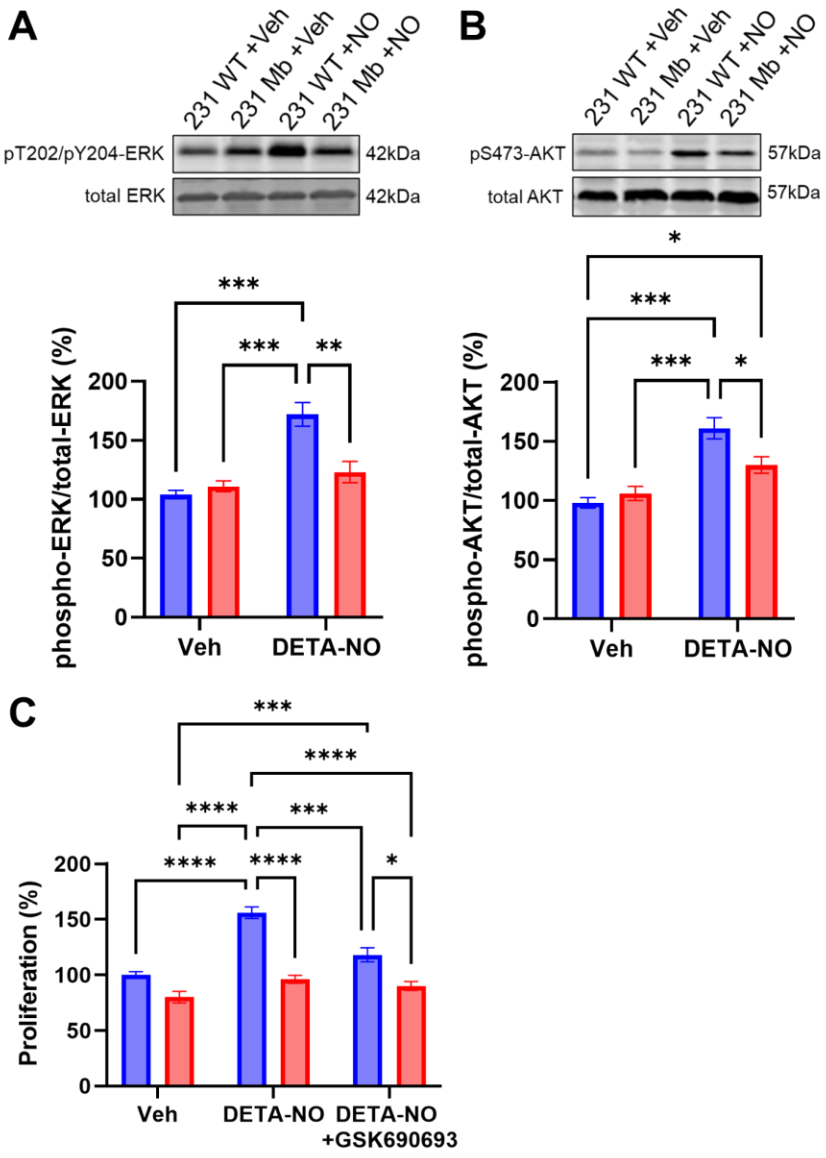

Figure 4-3: NO-dependent activation of ERK1 and AKT are inhibited by Mb expression, preventing increases in cell proliferation.

A-B) Top: Representative western blots showing ERK1 (A) and AKT (B) phosphorylation increases upon the addition of DETA NONOate (+DETA-NO) and is prevented by Mb expression in MDA-MB-231 cells. Bottom: Quantitation of phosphorylated ERK1 (A) and AKT (B) relative to total ERK1 and AKT, respectively. Two-way ANOVA with multiple comparisons tests; N=4. C) Cell proliferation increases with the addition of NO• but is attenuated by the expression of Mb and the AKT inhibitor GSK690693 in MDA-MB-231 cells. Two-way ANOVA with multiple comparisons tests; N=3. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001, ✱✱✱✱: p<0.0001.

4.3.4 Myoglobin decreases NOS activity and NOS levels *in vivo*

We next tested whether Mb regulates endogenous NO• levels through modulation of the NOS enzyme. Since both MDA-MB-231 and MDA-MB-468 cells do not express NOS enzymes endogenously, we induced the expression of iNOS by treating cells with interferon-γ (INFγ) and tumor necrosis factor-α (TNFα; **Figure 4-4A-B**) as previously described.[198] There were no differences in baseline iNOS activity between cells that do or do not express Mb. INFγ/TNFα treatment significantly increased iNOS activity in both MDA-MB-231 and MDA-MB-468 cells (**Figure 4-4A-B**, respectively). However, Mb expression significantly attenuated the INFγ/TNFα-induced NOS activity compared to control cells. 231 Mb (231 Mb +INFγ/TNFα: 1.21±0.27 nmol/min/mg) cells had approximately 57% less iNOS activity than 231 EV cells (231 EV + INFγ/TNFα: 2.78±0.18 nmol/min/mg; **Figure 4-4A**), and 468 CTRL cells (468 CTRL +INFγ/TNFα: 3.45±0.31 nmol/min/mg) had an average of 29% less iNOS activity than 468 Mb-cells (468 Mb-: +INFγ/TNFα: 4.88±0.46 nmol/min/mg; **Figure 4-4B**). To test whether Mb alters NOS levels *in vivo*, we used a murine xenograft tumor model. Twenty-five days after tumor implantation, primary tumors were extracted and analyzed for NOS expression. We identified that tumors formed from 231 Mb cells have significantly lower NOS levels than those formed

from 231 EV cells (**Figure 4-4C**). Metabolomic analysis in 231 EV and 231 Mb cells showed that levels of L-arginine and L-citrulline were not significantly changed with Mb expression. However, levels of asymmetric dimethylarginine (ADMA) an endogenous inhibitor of NOS enzymes, were increased nearly two-fold in 231 Mb cells (231 EV: 100±42%; 231 Mb: 198±51%). These data suggest that Mb modulates NOS through the inhibition of its protein expression as well as its activity through increased levels of ADMA.

To investigate whether the Mb-dependent changes in NO• signaling we observed *in vitro* are reflected *in vivo*, we used the Human Protein Atlas. Using data from 1075 samples, we determined whether the expression of *MB* correlates with the expression of NO-related proteins. We saw a small inverse correlation between *MB* and all *NOS1-3* isoforms (*NOS1*: r=-0.12, $p=5.11e^{-5}$; *NOS2*: r=-0.09, p=0.003; *NOS3*: r= -0.10, p=0.001; **Figure 4-4E**). Both sGC subunits (*GUCY1A1*, *GUCY1B1*) were also negatively correlated with *MB* expression (*GUCY1A1*: r=-0.264, $p=1.52e^{-18}$; *GUCY1B1*: r=-0.278, $p=1.39e^{-20}$) but positively correlated with *NOS2* (*GUCY1A1*: r=0.148, $p=1.10e^{-6}$; *GUCY1B1*: r=0.313, $p=8.31e^{-26}$). These data indicate that Mb may have a role in downregulating NO• production and signaling at the level of transcription within breast tumors. In contrast, the expression of *ERK1* and *AKT* are weakly correlated with MB expression (*ERK1*: r=0.275, $p=4.81e^{-20}$; *AKT*: r=0.166, $p=4.57e^{-8}$). Collectively, these data underscore Mb as a key modulator of NO• production and signaling that translates from *in vitro* cell models to human patients.

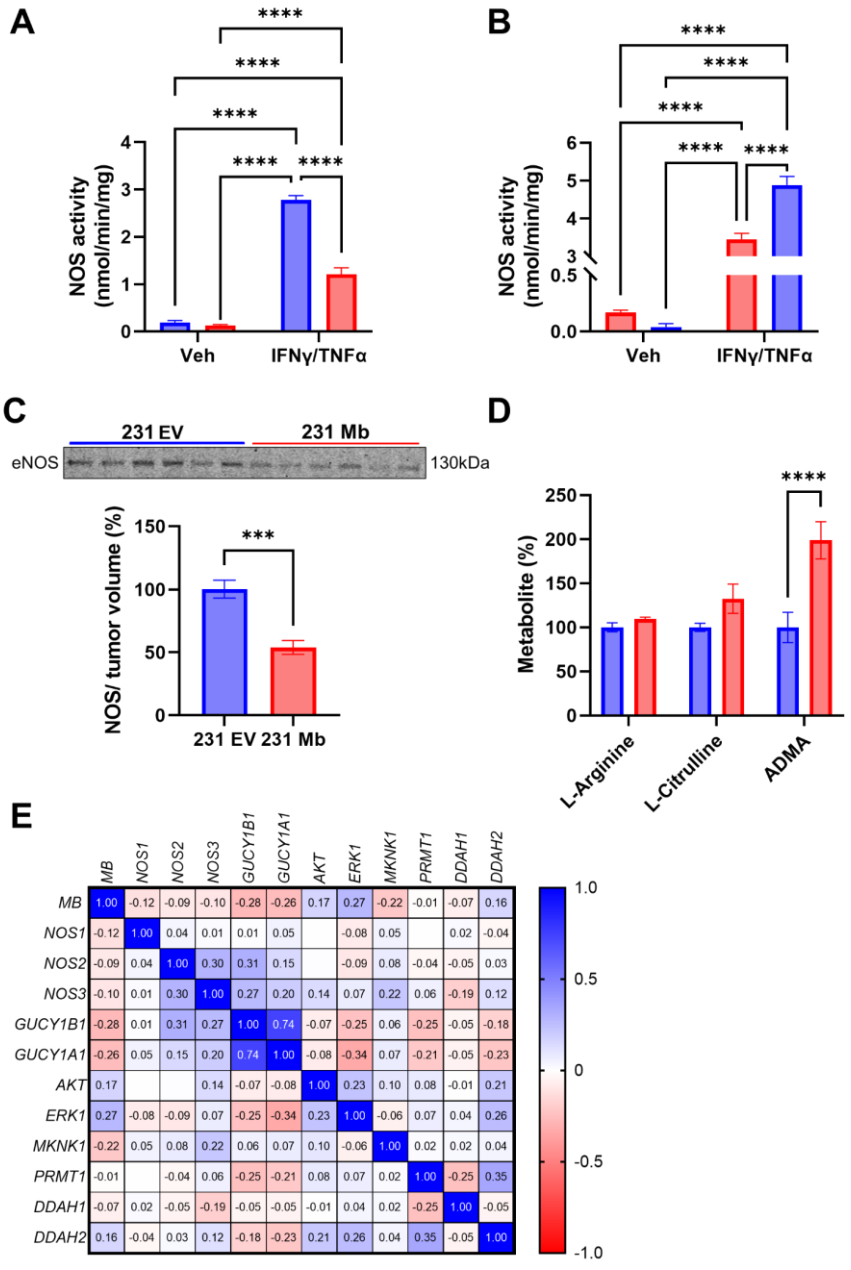

Figure 4-4: Mb decreases NOS induction *in vitro* and *in vivo*.

A-B) Mb expression decreases interferon-γ (INFγ) and tumor necrosis factor-α (TNFα) induced iNOS activity within MDA-MB-231 (A) and MDA-MB-468 (B) cells. Two-way ANOVA with multiple comparisons tests; N=4. C) Top: representative western blot of endothelial NOS (eNOS) expression from primary tumor xenografts of MDA-MB-231s 25 days after implantation. Bottom: Western blot quantitation of eNOS expression from primary xenograft tumors relative to tumor volume; Welch's t-test; N=6. D) Levels of L-

arginine, L-citrulline, and asymmetric dimethylarginine (ADMA) differ with Mb expression. Two-way ANOVA with multiple comparisons test; N=6. E) Spearman correlation matrix of mRNA expression of Mb and NO-related genes from 1075 breast cancer patient samples. Data are Mean ±SEM. ✱: p<0.05, ✱✱: p<0.01, ✱✱✱: p<0.001, ✱✱✱✱: p<0.0001.

4.4 Discussion

The data presented in this chapter collectively show that Mb expression attenuates NO• signaling at multiple levels. We have shown that the endogenous expression (within MDA-MB-468 cells) and transient overexpression (within MDA-MB-231 cells) of Mb are able to limit cell proliferation as well as NO-stimulated proliferation. Oxy-Mb achieves this through scavenging NO, resulting in decreased cGMP production and decreased ERK1 and AKT activation. We show that Mb expression also limits NOS induction and activity *in vitro* and *in vivo*, which may be explained by increased levels of ADMA in 231 Mb cells. Finally, we examined gene expression data from 1075 human patient samples. *MB* inversely correlates with all isoforms of *NOS* expression (*NOS1-3*) and both sGC subunits (*GUCY1A1*, *GUCY1B1*), suggesting that Mb may also play a role in modulating *NOS* expression at the level of transcription.

NOS induction and activity are sharply limited by Mb expression. ADMA is a potent inhibitor of NOS enzymes that is generated by post-translational modification of arginine residues (rather than free L-arginine) by protein arginine methyltransferase (PRMT) type II enzymes. ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH) and DDAH inhibition leads to accumulated ADMA. Importantly, the activity of DDAH has been shown to be impaired by oxidative stress. [201] Mb is known to generate ROS. Therefore, it is plausible that ADMA may

accumulate in 231 Mb cells by Mb-derived ROS-dependent DDAH inhibition, leading to decreased ADMA and subsequent NOS activity inhibition. Using mRNA data from the Human Protein Atlas, *MB* expression does not correlate with *PRMT1* expression. Mb expression negatively correlates with *DDAH1 (r= -0.07, p=*0.030) and positively correlates and *DDAH2* ((*r= 0.16, p=1.66e*$^{-7}$; **Figure 4-4E**). The differential correlations between MB and these genes may be explained by their tissue-specific expression patterns that have been previously reported: *DDAH1* is predominantly expressed with nNOS, and *DDAH2* is expressed with eNOS.[201] These analyses are limited, however, as mRNA expression levels may not necessarily correlate with protein levels or enzymatic activity. Altogether, the Mb-specific increase in ADMA (possibly through the downregulation of DDAH activity) offers a potential mechanism of decreased NOS activity that warrants further investigation.

To our knowledge, only two other studies have examined the interaction between Mb and NO• within breast cancer cells. Bicker *et al*. performed RNA-Seq within MDA-MB-468 cells and identified several gene ontology (GO) terms that are modulated with and without Mb expression. Specifically, they report an increased expression of genes relating to the "regulation of NO• biosynthetic processes" within *MB* knockdown cells.[96] These data are consistent with our results reported above, where Mb downregulates NOS induction and activity (**Figure 4-4A-C**). However, a major confounding factor of Bicker *et al*.'s study is that hypoxic experiments were conducted at 1% oxygen. Mb's high affinity for oxygen still allows it to remain 58% oxygenated at 1% oxygen, making the predominant species oxy-Mb that functions as a NO• dioxygenase (**Equation 1**).[161] Therefore, we would expect Mb to predominantly function as a NO• scavenger at 1% oxygen, but we cannot exclude the possibility that deoxy-Mb may also produce NO• (through its nitrite reductase activity, **Equation 2**) within areas of the cell that are more severely

hypoxic.[37, 145] In another study, Quinting *et al*. utilized RNA-Seq to identify differentially regulated genes Mb-silenced MDA-MB-468 cells treated with the NO• donor S-nitrosoglutathione (GSNO). Out of 1029 differentially expressed genes, they identified *MKNK1* as the only upregulated gene, and *RAB8B* and *SLC2A1* (i.e.: GLUT1) as two downregulated genes with fold-changes ≥1.5 and validated their findings by quantitative polymerase chain reaction (qPCR).[157] *MKNK1* encodes MAP-kinase interacting kinases (MNKs), which are implicated in cancer progression and resistance.[158] In agreement with their study, our genetic analysis shows that Mb downregulates *MKNK1* expression in patient samples (**Figure 4-4E**). These data suggest that MNK expression as well as downstream MAPKs (i.e.: ERK1) activity (as shown in **Figure 4-3A**) is modulated by Mb expression within normoxic tumor cells and patients. Together, the data presented here align with Quinting *et al*.'s findings and support the notion that Mb-mediated scavenging of NO• attenuates pro-mitotic MNK and MAPK signaling and cancer cell proliferation.

The data reported herein are the first to show mechanistic evidence of Mb functioning as a NO• dioxygenase that attenuates NO• signaling within breast cancer cells (**Figure 4-5**). It is important to note that the oxygenation of Mb will determine whether it functions as either a NO• scavenger or a generator within patient tumors. Therefore, Mb may function as a double-edged sword: within severely hypoxic tumors, Mb is expected to produce NO• which may improve tumor oxygenation but potentially drive disease progression; in contrast, in mildly hypoxic or oxygenated tumors, Mb is expected to scavenge NO• and limit its signaling, as supported in this study. However, these two seemingly juxtaposing roles of Mb may work together by limiting the severity of tumor hypoxia (through NO• production) in some regions of the tumor while also blocking excessive pro-mitotic NO• signaling in more oxygenated areas. Several pre-clinical

studies as well as a small clinical trial by Chung *et al*. have reported the efficacy of NOS inhibitors in limiting breast cancer progression.[143, 144, 155, 195] We demonstrate here that Mb acts as an endogenous inhibitor within tumors by limiting NOS expression and activity and downstream NO• signaling; therefore, we suggest that Mb expression should be examined within tumors as a possible prognostic indicator and biomarker to determine patient treatment regimens.

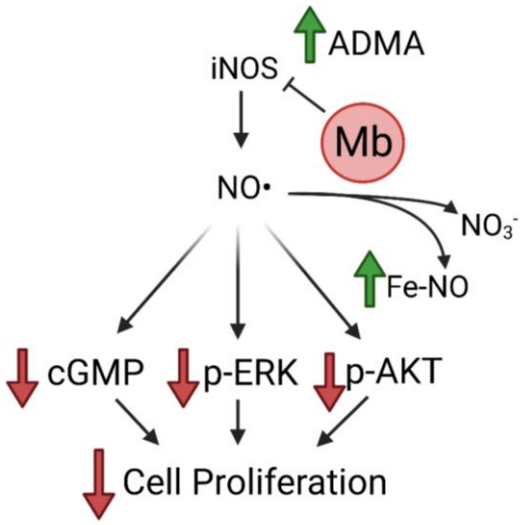

Figure 4-5: Graphic Model—Mb-dependent downregulation of NO• attenuates cancer cell proliferation. Oxy-Mb scavenges NO•, producing iron nitrosyl (Fe-NO) and nitrate while decreasing NO•-dependent activation of ERK, AKT, and cGMP and limiting cell proliferation. The green and red arrows indicate Mb-dependent increases and decreases, respectively.

5.0 Discussion

The data presented in the previous chapters demonstrate how Mb expression modulates three unique properties of cancer cells—cell metabolism, migration, and proliferation—through the heme-dependent production of ROS (Chapters 2 and 3) or scavenging of NO• (Chapter 4). The novelty of these results will be discussed in the following subchapters. Moreover, the shortcomings of each of these results will be expanded upon in the following subchapters as well as future research questions that still remain to be addressed.

5.1 Summary

The overarching goal of this thesis was to investigate the mechanisms by which Mb regulates breast cancer cell function. Prior studies established that Mb is expressed in approximately 40% of breast cancer tumors, and this expression is associated with a better prognosis. However, the molecular mechanisms by which Mb confers this protection remain unclear. While it is well established that in skeletal and cardiac muscle (where Mb is constitutively expressed), the protein serves a number of functions beyond the storage of oxygen including catalysis of ROS production, scavenging of NO•, and modulation of fatty acid metabolism, the role of these Mb functions in cancer cells has not thoroughly been examined. Thus, we hypothesized that Mb contributes to improving patient outcomes by attenuating cancer cell migration and proliferation through heme-dependent mechanisms. We found that Mb expression modulates three unique properties of

cancer cells—cell metabolism, migration, and proliferation—through the heme-dependent production of ROS (Chapters 2 and 3) or scavenging of NO• (Chapter 4).

In Chapter 2, we investigated the effect of Mb expression on cellular bioenergetics and migration. We found that Mb downregulates cellular oxygen consumption rate through decreased mitochondrial fatty acid oxidation (FAO) and decreases cell migration. By creating a novel mutant of Mb (K46M) to disrupt the electrostatic interactions between Lys46 and the fatty acid carboxyl, we tested whether Mb-fatty acid binding regulates breast cancer cell FAO and migration. While the mutation of Lys46 significantly decreased the affinity of Mb for oleate, it did not affect FAO or cellular migration when expressed in cancer cells. Further mechanistic investigation demonstrated that Mb expression decreased the cellular levels of CPT1, and overexpression of CPT1 in these cells restored fatty acid-dependent cellular oxygen consumption rates but not cellular migration. In contrast, we found that the expression of Apo-Mb, which lacks heme coordination, restored cellular oxygen consumption rates. These data demonstrated that the Mb-mediated decrease in cellular respiration and FAO are dependent on Mb's heme group, rather than fatty acid binding.

Chapter 3 explored how Mb expression leads to decreased cell migration *in vitro*. We hypothesized that ROS production catalyzed by Mb's heme iron impairs actin cytoskeletal remodeling. We reported that the treatment of cells with the antioxidant N-acetylcysteine (NAC) reverses the Mb-dependent downregulation of cell migration. We examined actin-binding proteins (ABPs) and found that both profilin (PFN1) levels and cofilin (CFL1) activity are changed in 231 Mb cells. Both PFN1 protein levels and phosphorylation of CFL1 at Ser3 are increased in Mb-expressing cells, which aligns with decreased cell migration. Notably, while genetically silencing *PFN1* alone was not sufficient to restore cell migration to Mb-expressing

cells, ROS scavenging both decreased PFN1 levels and phospho-Ser3 CFL1 levels to that of the 231 EV cells as well as restored cell migration in 231 Mb cells. Together, these data demonstrate two novel targets of Mb-dependent ROS generation that lead to decreased cell migration.

Chapter 4 hypothesized that oxy-Mb serves as a NO• dioxygenase that oxidizes NO• to nitrate, blocking downstream NO• signaling and subsequent cell proliferation. We confirmed that when expressed in cancer cells Mb acts as a NO• scavenger using two unique cell lines (MDA-MB-231 and MDA-MB-468) in which Mb expression decreased cGMP production and enhanced Fe-NO formation. We show that in Mb-expressing cells, NO• scavenging also decreases cell proliferation by blocking NO•-dependent activation of ERK1 and AKT. Furthermore, we investigated whether Mb regulates the expression of *NOS* and found that Mb attenuates NOS induction and activity, possibly through the accumulation of ADMA. Together, these data confirm the role of Mb as a NO• scavenger and describe a novel role for the protein as a regulator of *NOS* expression.

In the following subchapters, we discuss these results and their limitations in the context of understanding the role of Mb in breast cancer pathogenesis and outlining future directions.

5.2 Questions Remaining about Myoglobin-Dependent Regulation of Cell Metabolism

5.2.1 How Does Myoglobin Regulate CPT1 Levels?

Our data demonstrate that Mb expression decreases CPT1 protein levels, and that overexpression of CPT1 or deletion of the Mb heme iron restores FAO or respiration, respectively. However, it remains unclear how Mb (and particularly the heme iron) decreases CPT1 expression and whether

Mb-derived ROS regulates CPT1 activity. For example, it is possible that Mb-derived ROS decreases CPT1 activity. Setoyama *et al*. has shown that the activity of CPT1a is decreased by hydrogen peroxide in a dose-dependent manner and reversed by catalase treatment.[202] In another study, Liu *et al*. performed scanning mutagenesis of CPT1b (muscle isoform) cysteines to either serine or alanine residues and identified that Cys305 is critical for CPT1b enzymatic activity and inhibition by malonyl-CoA.[203] Cys305 is believed to be a part of a catalytic triad (Cys305-Asp454-His473) in the C-terminal domain of CPT1. Liu *et al*. hypothesized that Cys305 is a reactive thiolate, serving as a nucleophile towards the carbonyl carbon of the acyl-CoA.[203] This triad is conserved within CPT1a (liver and epithelial isoform); the equivalent cysteine residue within CPT1a is Cys304. Importantly, the redox state of cysteine residues (i.e.: thiols) is critical for protein structure and function. To this end, thiols react with either ROS or NO•, creating sulfenic acid or S-nitrosocysteine via oxidation or S-nitrosylation, respectively.[204] It is therefore plausible that Mb expression may lead to the oxidation of Cys305 of CPT1 and inactivate or lessen its activity through Mb-dependent generation of ROS. This mechanism of decreased CPT1a may be similar to Braganza *et al*.'s previous finding of Mb-dependent parkin oxidation leading to decreased enzymatic activity and levels.[168] However, further studies are required to examine this relationship between Mb-dependent ROS production and CPT1 activity and oxidative modifications of cysteine residues of all proteins more broadly.

At the level of gene expression, Mb may modulate CPT1 expression through the regulation of peroxisome proliferator-activated receptor-gamma (PPARγ), which is a transcriptional regulator of lipid metabolism gene expression that is activated by the binding of fatty acids.[205] PPARγ activation increases the expression of enzymes involved in fatty acid metabolism, including CPT1, and hydrogen peroxide has been shown to downregulate PPARγ.[206]

Importantly, we observed that PPARγ levels are decreased in 231 Mb cells compared to 231 EV cells (**Figure 5-1**), and this may provide a link between Mb heme-catalyzed ROS generation and CPT1 downregulation in our model. Cheng *et al*. previously reviewed PPAR transcription factors and noted conflicting results for PPARγ as an activator and inhibitor of cancer progression.[207] Although we observe higher levels of free fatty acids in 231 Mb cells, which may act as agonists for PPARγ activation, the decrease in total PPARγ levels likely has a greater effect on decreasing FAO. The decrease in PPARγ levels in 231 Mb cells also aligns with decreased mitochondrial bioenergetics we report in Chapter 2. Other studies have reported that PPARγ is more active within mesenchymal state breast cancer cells than the epithelial state, hinting towards a possible mechanism of Mb-dependent inhibition of dedifferentiation.[50, 53] Aside from this, NO•-sGC-cGMP signaling has been shown to drive PPARγ activation in skeletal muscle cells.[208] Mb-dependent scavenging of NO• may also provide an alternative mechanism to mitigate PPARγ signaling. Collectively, we may speculate that Mb downregulates PPARγ levels, transcriptional activity, and CPT1 as well as FAO altogether through several mechanisms in breast cancer cells.

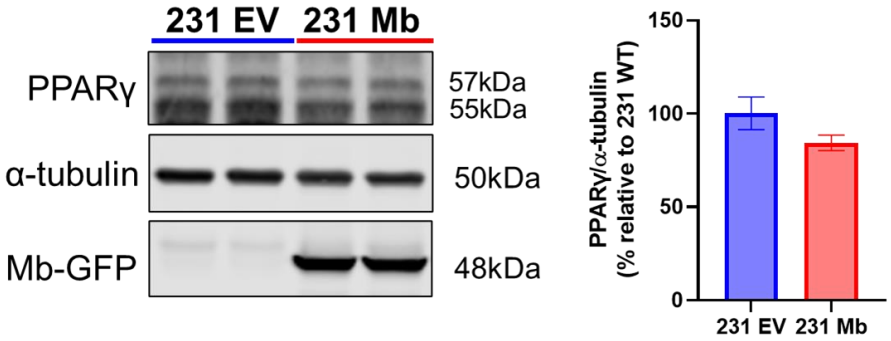

Figure 5-1: Mb decreases PPARγ levels.

Left: Representative western blots of PPARγ in 231 EV and 231 Mb cells. Right: Quantitation of PPARγ relative to α-tubulin levels. N=2. Data are Mean ±SEM.

Of relevance to the observation of the Mb-dependent downregulation of PPARγ, it is important to note that we did not fully characterize the expression of all the major fatty acid metabolism genes—both FAO and FAS. Future experiments should examine CD36 as the major fatty acid importer, all isoforms of FABPs, fatty acid-activating proteins (e.g.: ACOT), other enzymes within the carnitine shuttle (i.e.: CACT and CPT2), as well as fatty acid synthesis proteins outside of FASN. Concerning the latter, Kristiansen *et al*. report that *MB* expression correlates with *FASN*, but the mechanism of their co-expression and its implications for cancer progression remain to be defined and could involve Mb-catalyzed ROS and PPARγ as discussed above.[53]

5.2.2 Considerations of Cancer Cell Type and Genetic Background

It is important to note that Mb differentially affects FAO in muscle versus cancer cells, with Mb promoting FAO in cardiomyocytes while inhibiting FAO in cancer cells. We and others have shown that Mb promotes FAO in the heart, with Mb knockdown decreasing respiration (unpublished data from K. Rao). [40, 42] In contrast, Mb inhibits FAO in cancer cells, as shown in Chapter 2 and by others.[97] The differences between these two observations are likely explained by differing gene expression patterns, energetic requirements, and nutrient availability in cardiac muscle compared to neoplastic cells. Future studies may distinguish these genetic differences through RNA-Seq in both cardiac and cancer cells in the presence and absence of Mb. Comparing mRNA expression profiles between these cell types for FAO genes, for example, may reveal additional factors of genetic regulation that are cell-specific and influence the Mb-dependent regulation of FAO. RNA-Seq analyses may also elucidate the genetic differences as to why *MB* is only expressed in some cancer cells.

Breast tumor subtypes and genetic background may play a role in the up- versus down-regulation of FAO genes and their effects on tumor biology. Independent studies by Monaco *et al*. and Aiderus *et al*. both report that TNBCs tend to decrease FAO genes compared to HR+ and HER2+ tumors.[75, 209] However, Camarda *et al*. report that Myc-overexpressing TNBCs have a high reliance on FAO for energy production, and inhibiting FAO *in vivo* decreases tumor burden.[170] Similarly, Casciano *et al*. showed that Myc-overexpressing TNBCs had decreased migration rates in the presence of the CPT1 inhibitor etomoxir.[210] Together, these suggest that Myc-overexpressing TNBC tumors may be susceptible to FAO inhibition and should be further investigated for patient future therapies.[82] Our *in vitro* models of TNBC (MDA-MB-231 and MDA-MB-468 cell lines) used do not express high amounts of Myc. Based on data presented by Janghorban *et al*., both of these cell lines show relatively low levels of phospho-Ser62 Myc, an indicator of stabilized and transcriptionally active Myc. In contrast, other TNBC cell lines, including BT549, HCC1937, and MDA-MB-436, express two- to three-fold higher levels of phospho-Ser62 compared to MDA-MB-231 and MDA-MB-468 cell lines.[211] Future studies should investigate whether other TNBC cell lines with Myc overexpression may be more impacted by Mb-dependent modulation of FAO and its effect on cell migration.

5.2.3 Consequences of Myoglobin-Dependent Downregulation of Metabolism Beyond Migration

Though we observed that Mb-mediated downregulation of FAO did not alter cell migration, this change in fatty acid metabolism may have other consequences in cancer cells. For example, a change in FAO may affect gene expression through epigenetic modulation if the liberation of acetyl-CoA from β-oxidation increases global histone acetylation.[69, 93, 94, 212] **Figure 5-2** shows a

decrease in histone lysine acetylation within 231 Mb cells, suggesting that Mb expression indirectly regulates gene expression through epigenetics. This is important because prior studies have shown that histone acetylation disrupts the electrostatic interactions between DNA and histones, leading to more "open" chromatin and increased gene transcription of oncogenes.[213] Mb may therefore be able to mitigate hyperacetylation-induced gene transcription; however, these studies require further investigation. Future studies with the Mb K46M mutant that does not bind fatty acids may shed light on the metabolic role of fatty acid binding and delivery within more Mb-rich tissues, such as the heart and skeletal muscle. Specifically, future studies utilizing chromatin-immunoprecipitation may distinguish wild-type Mb- versus mutant Mb K46M-dependent alterations in FAO that link to histone acetylation changes.

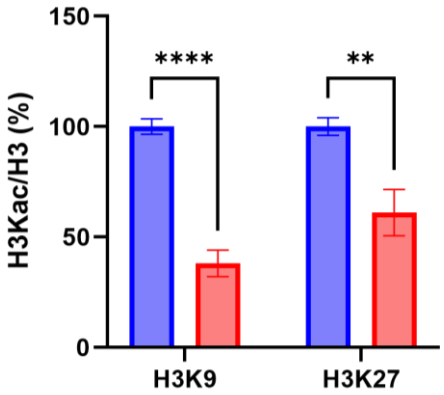

Figure 5-2: Mb expression decreases histone acetylation.

Quantitation of histone-3 lysine-9 acetylation (H3K9ac) and lysine-27 acetylation (K27ac) by mass spectrometry in 231 EV (blue) and 231 Mb (red). Two-way ANOVA with multiple comparisons test; N=4. Data are Mean ±SEM.

5.3 Future Directions: Myoglobin-Dependent Downregulation of Cell Migration

Our data demonstrating increased PFN1 protein levels in 231 Mb cells is consistent with decreased migration.[111] Consistent with this idea, we have observed a small increase in actin stress fibers in 231 Mb cells (**Figure 5-3**). Stress fibers are bundles of F-actin that are used for myosin contraction and cell motility; however, elevated PFN1 levels increase and stabilize F-actin, which is consistent with decreased cell migration.[214] Future studies will test whether PFN1 protein levels are increased in human tumor samples and perform more in-depth analysis on mRNA and protein stability both in cell culture as well as xenograft models. Interestingly, the deletion of PFN1 by shRNA in Mb-expressing cells did not attenuate the Mb-induced decrease in migration (**Figure 3-4).** Though this is surprising based on prior studies in different cell types showing that deletion of *PFN1* increases cell migration, it is possible that several ABPs are regulated by Mb and deletion of *PFN1* alone is not sufficient to reverse the decreased migration induced by Mb. Moreover, the small effect size we observe with PFN1 levels and phosphor-Ser3 CFL1 (~15-30% difference) may require greater biological replicate sampling. Future studies will not only investigate the mechanism by which Mb increases PFN1 levels but also whether simultaneous modulation of PFN1 with other ABPs (such as CLF1, discussed below) reverses the Mb-dependent decrease in migration. It should also be noted that the small effect size we observed with PFN1 levels and phosphor-Ser3 CFL1 (~15-30% difference) may require greater biological replicate sampling.

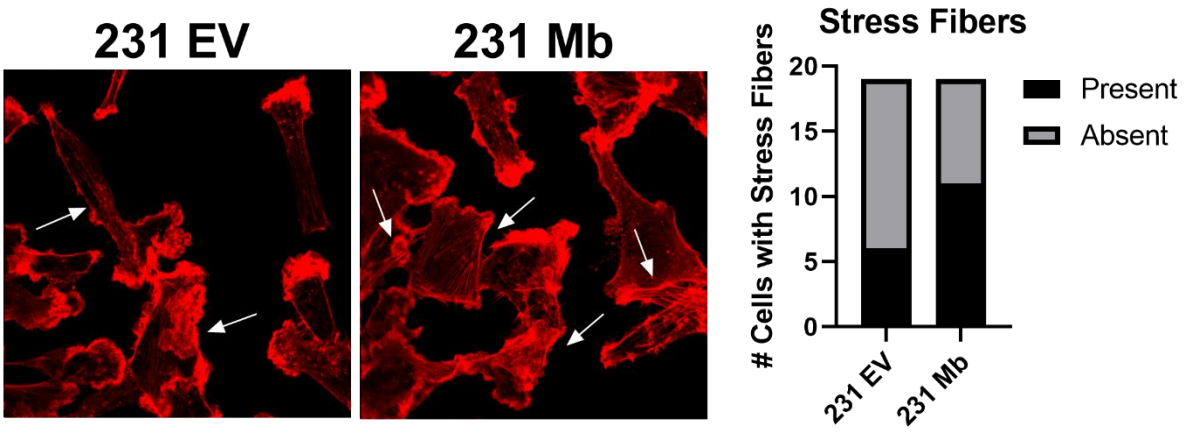

Figure 5-3: Mb expression tends to increase stress fibers.

Left: Representative images of F-actin in 231 EV and 231 Mb cells with stress fibers (white arrows). Right: Quantification of cells that display stress fibers. N=19 cells.

5.3.1 The Role of Cofilin Phosphorylation in Myoglobin-Dependent Attenuation of Migration

In contrast to PFN1, we did not observe any changes in CFL1 levels. Instead, phospho-Ser3 CFL1 was increased in 231 Mb cells, corresponding to decreased CFL1 activity.[124, 215] Increased CFL1 levels have been reported in cancer cells, yet the activity (i.e., phosphorylation) is more indicative of metastasis.[124, 126, 215] LIM kinase-1 (LIMK1) and slingshot-1 (SSH1) phosphatase antagonize and activate CFL1 activity, respectively. We observe that Mb increases levels of phospho-Ser3 CFL1 and LIMK1, and this is reversed with catalase treatment. However, we have not described the redox-dependent mechanism underlying these changes in LIMK1 levels.

To this end, LIMK1 levels are regulated by E3 ubiquitin ligase Rnf6 in the brain. Whether Mb-derived ROS inhibits Rnf6-mediated degradation of LIMK1, however, has not been examined within breast cancer cells.[186] The activity of LIMK1 has been reported to change with

levels of oxidative stress. Early work by Nagata *et al*. showed that cysteine residues in the LIM domains of LIMK1 (Cys49 and Cys108) regulate the kinase domain, and substituting these cysteine residues with glycine results in a seven-fold increase in kinase activity.[216] It is interesting to speculate that LIMK activity is redox-sensitive based on the negative regulation by Cys49 and Cys 108 residues and that Mb-dependent generation of ROS decreases LIMK activity and downstream actin remodeling by CFL1. In addition to this, LIMK1 is a downstream target of the Rho-ROCK-LIMK-CFL1 cascade, and others have reported that these kinases are regulated by redox signaling.[217] Therefore, it is plausible that Mb downregulates this signaling cascade at multiple levels to decrease cell migration, but additional studies, particularly those measuring LIMK expression and activity in 231 EV and 231 Mb cells in the presence and absence of antioxidants, are required to establish this. Alternatively, unbiased proteomic approaches may also be used to identify an multitude of proteins with thiol oxidative modifications.[218]

Unabated cell migration and proliferation of cancer cells require vast tolerance towards oxidative stress through increased redox signaling regulation. Increased oxidative stress is a common trait that drives tumorigenesis and cancer progression, but unregulated oxidative stress leads to cell death. To evade cell death, cancers upregulate antioxidant signaling pathways, such as Nrf2, to increase the expression of superoxide dismutase (SOD) and catalase and mitigate oxidative stress from tipping toward cell death.[219, 220] In this regard, it is possible that the expression of Mb results in ROS levels that overwhelm the antioxidant mechanisms within cancer cells, decreasing cancer cells' threshold of oxidative stress tolerance to interfere with pro-migratory. Future studies are required to determine whether Mb expression alters antioxidant response gene expression.

5.3.2 The Role of Myoglobin-Dependent Regulation of Mitochondrial Dynamics in Cell Migration

Aside from ABPs, we examined the role of mitochondrial dynamics in Mb-dependent attenuation of migration. We previously reported that Mb leads to the upregulation of MFNs, the predominant catalyst of mitochondrial fusion, causing cell cycle arrest and decreased tumor volumes *in vivo*.[168] MFNs regulate mitochondrial dynamics and consequently, cell proliferation and migration.[130, 131, 221, 222] Mitochondria undergo fission and fusion at different phases of the cell cycle. To adapt to increased energetic demands for DNA replication, mitochondrial form fused, elongated networks.[129] Once mitosis initiates, mitochondria fragment so that each daughter cell obtains sufficient mitochondria. During migration, mitochondria undergo fission before they are shuttled toward the leading edge of the cell to fulfill the increased energy demands of cytoskeletal reorganization.[109, 223] Zhao *et al*. have shown that metastatic cells maintain a fragmented mitochondrial network either through the overexpression of the mitochondrial fission protein Drp1 or the depletion of MFNs, and this promotes cell migration. In contrast, migration is significantly decreased by overexpressing MFNs.[130] To test whether Mb decreases migration through the upregulation of MFNs, we depleted MFNs and assayed migration. *MFN1* and *MFN2* depletion increased the migration rates of 231 EV cells, as expected, but did not increase the migration rates of 231 Mb cells (**Figure 3-3**). This suggested that Mb alters migration through a mechanism independent of MFNs, but MFN accumulation may be an additional "fail-safe" to decrease cell migration. Future studies will test whether Mb expression affects mitochondrial trafficking and localization to the leading edge of migrating cells.

5.3.3 Considerations of Cancer Cell Types and Models of Metastasis

An important limitation of our migratory studies is the utilization of one cell line. In contrast to MDA-MB-231 cells, the knockdown of Mb in MDA-MB-468 cells results in a non-significant decrease in cell migration (**Figure 5-4**), and this has been corroborated by other studies.[54, 96] The expression of Mb and its role in cell migration has also been examined in the HR+ MCF7 as well as the HER2+ SKBR3 cell lines by Aboouf *et al*.[55] Consistent with our studies in MDA-MB-231 cells, *MB* knockdown in MCF7 cells increases cell migration but only under hypoxia (0.2% O_2). However, *MB*-depleted SKBR3 cells showed significantly slower migration rates in normoxia exclusively, lining up with the results from MDA-MB-468 studies. Researchers have speculated that these differing results stem from genetic differences within each cell line, such as HR+/HER2+ expression and p53 mutation status.[55] Further analyses examining the expression of Mb and genetic mutations and/or gene amplifications within a variety of breast cancer cell lines may elucidate a clear relationship between Mb expression and cell migration. Establishing this relationship would benefit patients by further stratifying them for selective cancer therapies aimed at treating metastasis.

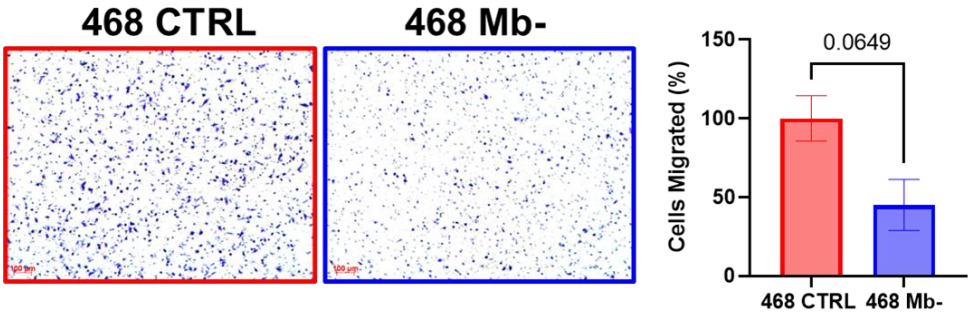

Figure 5-4: Mb expression increases migration in MDA-MB-468 cells.

Left: representative images of migrated cells with endogenous Mb expression (468 CTRL) or Mb knocked down (468 Mb-). Right: Quantification of cells migrated. Welch's t-test; N=3. Data are Mean ± SEM.

Although we tested chemotactic migration using two different *in vitro* methods, we did not examine cell invasion or metastasis. An important barrier for cancer metastasis is the surrounding tissues, and cancer cells secrete matrix metalloproteases to facilitate tissue infiltration. Aboouf *et al*. (2022) showed that invasion of MCF7 cells is increased when Mb is knocked down through increased *MMP3* expression, suggesting that Mb also decreases invasion in some cancer cells.[55] Another shortcoming of our study is that we did not examine metastasis using an *in vivo* model. Intravasation into vessels, extravasation out of vessels, and establishing secondary tumors are all limiting steps in the metastatic cascade.[224-226] Galluzzo *et al*. showed that A549 lung cancer cell expressing Mb decreases tumor metastasis *in vivo*.[132] However, Mb's influence on metastasis has not yet been examined within breast cancers. Future studies to determine whether Mb modulates metastasis *in vivo* through xenograft and/or syngeneic models would strengthen our understanding of Mb-mediated protection of breast cancers in patients.

5.4 Future Directions: Elucidating Myoglobin-Dependent Regulation of Nitric Oxide Proliferative Signaling

It has been shown previously that Mb scavenges NO• in the skeletal and cardiac muscles.[26] However, only two other studies have experimentally examined the impact of Mb on NO• signaling in breast cancer cells.[97] First, the RNA-Seq and gene ontology (GO) analysis by Bicker *et al*. (2020) showed that Mb expression decreases genes associated with "the regulation of biosynthetic processes" in MDA-MB-468 cells. A second study by Quinting *et al*. showed that NO• leads to HIF-1α stabilization when Mb is knocked down in both normoxic and hypoxic (1%

O_2) MDA-MB-468 cells, triggering transcription of hypoxic-response genes. Each of these studies analyzed the NO• modulation functions of Mb in both normoxia and hypoxia (1% O_2). Even at 1% oxygen, Mb (P_{50} O_2= 3.1μM) is 58% oxygen-saturated, functioning partially as a scavenger of NO• rather than a producer. Their studies fail to recognize this flaw, as they do not demonstrate mechanistic evidence of NO• scavenging.[96] Our study presents novel data that support oxy-Mb's NO• scavenging properties, as evidenced by decreased cGMP and increased Fe-NO. Mb also attenuates the activation of NO• targets ERK1 and AKT. Overall, our data agree with Bicker *et al*. (2020) and Quinting *et al.*'s prior studies and highlight additional pro-cancerous pathways that are attenuated by Mb expression.

NO• signaling is associated with increased cancer metastasis and inflammation; however, we did not examine whether Mb attenuates NO•-dependent migration, invasion, inflammation, or metastasis in these studies.[137] Previous work by Switzer *et al*. showed that NO• donors increase EMT markers, cyclooxygenase-2 levels, and prostaglandin E2 levels.[196] Additionally, Garrido *et al*. showed that *MMP1* and *MMP9* expression was increased after NO• treatment in an EGFR-dependent manner.[143] We may expect that Mb would attenuate these signaling modes, as shown in **Figure 4-3,** but similar studies have not yet been conducted in presence of Mb expression. Adding to this, syngeneic models will be required to examine the interactions between NO•, Mb, and the immune system within the surrounding tissues to understand whether Mb alters NO•-dependent breast cancer metastasis.

Another limitation of these studies is that we did not examine the Mb-dependent generation or scavenging of peroxynitrite ($ONOO^-$). Because Mb catalyzes the formation or scavenging of NO• as well as the formation of superoxide ($•O_2$; **Figure 1-2**), it is interesting to speculate whether Mb expression also promotes the formation of $ONOO^-$ (**Equation 7)** and if this

impacts the functions of proliferative and migratory proteins and/or the oxidative and nitrosative stress responses of breast cancer cells.[31, 32, 35] On the other hand, metMb has been shown to scavenge $ONOO^-$ and promote its isomerization to nitrate.[33, 34] Whether Mb expression in breast cancer cells leads to either the production or the scavenging of $ONOO^-$ remains to be investigated, but we may speculate that the role of Mb will depend on the context of the tumor microenvironment (e.g. NO•, O_2 and CO_2 levels) and the expression of other ROS scavenging and producing enzymes (e.g. SOD, NADPH Oxidases).[31, 32]

Aside from kinase activation, epigenetic modifications are regulated by NO•. DNA and histone epigenetic "writers" and "erasers" include DNA methyltransferases (DNMT), histone methyltransferases (KMT), histone demethylases (KDM), histone acetyltransferases (HAT), and histone deacetylases (HDAC); each of these is affected by NO• signaling.[227-229] Recently, a major area of focus is the NO•-dependent methylation of DNA and histones that regulates cancer gene expression. Switzer *et al*. showed that *NOS2* expression decreases DNA methyltransferase-1 levels and correlates with DNA hypomethylation, which is a common marker within cancer cells.[229] Though we did not specifically examine the effect of Mb on NO•-mediated methylation, we anticipate that Mb would scavenge NO• and prevent methylation changes. In terms of acetylation, a well-described mechanism of NO•-mediated epigenetic changes is through HDAC enzymes: S-nitrosylation of HDAC decreases its activity, causing hyperacetylation and increased gene transcription.[227, 228] We briefly examined the Mb- and NO•-dependent epigenetic changes in MDA-MB-231 cells in **Figure 5-2.** Mb-expressing cells tended to show a lower basal level of H3K9ac and H3K27ac levels, but we did not examine whether NO• treatment affects histone acetylation. More in-depth studies are required to elucidate whether Mb modulates NO•-mediated epigenetic changes.

For the first time, we present data demonstrating that Mb directly decreases NOS transcription and activity (**Figure 4-4**). The expression of *NOS2* (as well as other *NOS* isoforms) is inversely correlated with *MB* expression within 1075 patient samples from the Human Protein Atlas, and these are corroborated by our *in vitro* NOS activity assays and *in vivo* xenografts. We speculate that elevated levels of ADMA within 231 Mb cells may downregulate NOS activity through ROS-dependent inhibition of DDAH activity, but this warrants further investigation. Furthermore, activation of the JNK MAPK and NF-κB pathways have been shown to activate iNOS independent of inflammatory cytokines (TNFα, INFγ); this suggests that Mb may restrict these pathways of iNOS induction but is not currently defined.[230]

Tumor oxygenation will vary within patients; however, Mb's high affinity for oxygen allows it to function as a dioxygenase in a majority of all oxygen tensions reported in tumors (0.3-4.2% O_2).[231] Mb's activity as a NO• dioxygenase is dependent on coordinated oxygen at the heme. In contrast, regions of severe hypoxia (≤0.2% O_2) will result in deoxy-Mb making up 67% of the protein species, and therefore may generate NO• from nitrite and increase vasodilation.[161] This allows for Mb to play a dual role in tumors depending on oxygenation gradients that may limit tumor hypoxia and proliferation simultaneously. Based on this, a critical limitation of NO•-modulation breast cancer therapies in Mb-expressing tumors may be their ability to shift the cytotoxic threshold of NO• through scavenging. High levels of NO• induce apoptosis, yet the expression of Mb within these tumors would be expected to mitigate NO• flux and therefore attenuate cell death, having a negative effect on patients that may accelerate tumor progression. Both Mb and NOS levels should be examined within patient tumors prior to initiating high-NO• flux therapies.

5.5 Conclusions

Collectively, data presented throughout these chapters build a basis for novel functions of Mb in cancer cell proliferation and migration with potential implications for determining breast cancer tumor biology. Mb limits breast cancer cell growth and migration through oxidant production and scavenging mechanisms that disrupt fatty acid metabolism, cell migration, and NO•-induced proliferation. Each of these mechanisms is dependent on the heme moiety of Mb. The *in vitro* and *in vivo* studies we conducted align with previously published pre-clinical and clinical findings that report Mb expression providing some degree of protection against cancer disease progression in patients by modulating three key hallmarks of cancer cells: dysregulated cell metabolism, persistent migration, and sustained proliferation.[134] Future, more expansive studies that reveal the mechanisms of Mb on cancer cell properties in a variety of genetic backgrounds and breast tumor subtypes within the complex tumor microenvironment will have a substantial benefit in developing personalized medicine for breast cancer patients in the clinic.

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