# METABOLIC STRESS IN NON-METASTATIC AND METASTATIC MURINE MAMMARY CANCER CELLS

by

**Violet Abigail Kiesel**

**A Dissertation**

*Submitted to the Faculty of Purdue University*

*In Partial Fulfillment of the Requirements for the degree of*

**Doctor of Philosophy**


Department of Nutrition Science

West Lafayette, Indiana

December 2020

# THE PURDUE UNIVERSITY GRADUATE SCHOOL
# STATEMENT OF COMMITTEE APPROVAL

**Dr. Dorothy Teegarden, Chair**

Department of Nutrition Science

**Dr. Kimberly Buhman**

Department of Nutrition Science

**Dr. Qing Jiang**

Department of Nutrition Science

**Dr. Michael Wendt**

Department of Medicinal Chemistry and Molecular Pharmacology

**Approved by:**

Dr. Amanda Seidl

## ACKNOWLEDGMENTS

This work was partly supported by the Purdue University Cancer Prevention Internship Program fellowship and the Purdue University Bilsland Fellowship.

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| | |
|---|---|
| $1,25(OH)_2D$ | 1,25 dihydroxyvitamin $D_3$ |
| αKG | α-ketoglutarate |
| αKGDH | α-ketoglutarate dehydrogenase |
| ADAM | A disintegrin and metalloproteinase |
| ATF4 | Activating transcription factor 4 |
| ATP | Adenosine triphosphate |
| BMP | Bone morphogenic protein |
| BNIP3 | BCL2 Interacting Protein 3 |
| BNIP3L | BNIP3-like |
| CMF-PBS | Calcium and magnesium-free phosphate buffered saline |
| CSC | Cancer stem cell |
| CXCL12 | Bone-derived stromal cell-derived factor 1 |
| DCFH-DA | 2',7'-dichlorofluorescin diacetate |
| DEC2 | Differentially expressed in chondrocytes 2 |
| DMαKG | Dimethyl α-ketoglutarate |
| DTC | Disseminated tumor cell |
| ECM | Extracellular matrix |
| EGFR | Epidermal growth factor receptor |
| ER | Endoplasmic reticulum |
| FABP | Fatty acid binding protein |
| eIF2α | Eukaryotic translation initiation factor 2α |
| EMT | Epithelial to mesenchymal transition |
| ETC | Electron transport chain |

| | |
|---|---|
| $FADH_2$ | Flavin adenine dinucleotide |
| GCLC | Glutamate cysteine ligase catalytic subunit |
| GCLM | Glutamate cysteine ligase modifier subunit |
| GLS | Glutaminase |
| GLUD | Glutamate dehydrogenase |
| GLUL | Glutamine synthetase |
| GLUT | Glucose transporter |
| GOT | Aspartate aminotransferase |
| GPT | Glutamic pyruvate transaminase |
| GSH | Reduced glutathione |
| GSS | Glutathione synthetase |
| GSSG | Oxidized glutathione |
| HIF | Hypoxia inducible factor |
| HRE | Hypoxia response element |
| IDH | Isocitrate dehydrogenase |
| ISR | Integrated stress response |
| L1CAM | L1 cell adhesion molecule |
| LDH | Lactate dehydrogenase |
| LIFR | Leukemia inhibitory factor receptor |
| LOX | Lysyl oxidase |
| LOXL | LOX-like |
| MCT | monocarboxylate transporter |
| ME1 | Malic enzyme 1 |
| mTORC1 | mammalian target of rapamycin complex 1 |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |

| | |
|---|---|
| NAC | N-acetylcysteine |
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa B |
| $NH_4Cl$ | Ammonium chloride |
| NR2F1 | Nuclear receptor subfamily 2 group F member 1 |
| NRF2 | Nuclear factor erythroid 2-related factor 2 |
| PCK1 | Phosphoenolpyruvate carboxykinase-1 |
| PEI | Polyethylenimine |
| PERK | Double-stranded RNA-dependent protein kinase-like endoplasmic reticulum kinase |
| PC | Pyruvate carboxylase |
| PDH | Pyruvate dehydrogenase |
| PDK | Pyruvate dehydrogenase kinase |
| PHD | Prolyl hydroxylase |
| PI3K | Phosphoinositide 3-kinase |
| PIK3CA | Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha |
| PK | Pyruvate kinase |
| PKR | Double-stranded RNA-dependent protein kinase |
| poly-HEMA | Polyhydroxyethylmethacrylate |
| PSAT | phosphoserine aminotransferase |
| PTEN | Phosphatase and tensin homolog |
| ROCK1 | Rho-associated protein kinase |
| ROS | Reactive oxygen species |
| SIAH2 | Seven in absentia homolog 2 |

| | |
|---|---|
| SLC1A5 | Solute carrier family 1 member 5 |
| SOCS3 | Suppressor of cytokine signaling 3 |
| SOX | Sex determining region y-box 2 |
| STAT3 | Signal transducer and activator of transcription 3 |
| TCA | Tricarboxylic acid |
| TGFβ | Transforming growth factor β |
| UPR | Unfolded protein response |
| VEGF | Vascular endothelial growth factor |

# ABSTRACT

Breast cancer is a major public health concern, with one in eight women in the United States expected to be diagnosed throughout the course of her lifetime. Metastasis of cancer to secondary sites in the body is the primary cause of death among breast cancer patients, highlighting the critical need to understand mechanisms that contribute to metastatic progression. Throughout metastatic progression, cancer cells are exposed to cell stresses, including metabolic, oxidative, and hypoxic cell stress, which cells must overcome in order to survive and progress. In the present studies, we determined the effects of metabolic cell stresses in non-metastatic M-Wnt and metastatic metM-Wnt$^{lung}$ murine mammary cancer cell lines. Culturing both cell lines in high (4 mM) compared to low (2 mM) glutamine conditions suppressed viability of metM-Wnt$^{lung}$ cells. M-Wnt cells had no change in viability in response to glutamine concentration, and high glutamine concentrations decreased mRNA levels of genes involved in glutamine catabolism in M-Wnt cells only. In accordance with the differences in glutamine metabolism, metM-Wnt$^{lung}$ cell demonstrated an increase in glutamine flux into the TCA cycle in high glutamine, whereas M-Wnt cells had no change in glutamine flux in response to glutamine concentration. metM-Wnt$^{lung}$ cells were significantly more sensitive to treatment with ammonium, a byproduct of glutamine catabolism, suggesting that a high rate of metabolism and ammonium production may decrease cell viability in high glutamine conditions. These data suggest that glutamine utilization and metabolism change in cancer cells at different stages of metastatic progression. In addition to metabolic stress from variable nutrient availability, changes in oxygen availability are a source of metabolic stress for cancer cells. Hypoxia, or low oxygen tension, is associated with metastasis and reduced survival, making it an important biological process to study in the context of cancer. Culturing non-metastatic and metastatic cells in hypoxia increased mRNA levels of genes related to antioxidant defense only in metM-Wnt$^{lung}$ cells. Hypoxia also induced expression of the integrated stress response effector protein activating transcription factor 4 (ATF4) and its target gene glutamic pyruvic transaminase (Gpt2) in metM-Wnt$^{lung}$ cells. Furthermore, genetic depletion of ATF4 reduced survival of hypoxic metM-Wnt$^{lung}$ cells in detached conditions. These results suggest that cancer cells accumulate cell stress throughout the course of progression and must adapt their gene expression for continued survival throughout metastatic processes. The results of these two studies highlight metabolic adaptations and vulnerabilities of cancer cells at different

stages of progression. These data will contribute to improving our understanding of therapeutic targets to prevent or delay metastasis in cancer patients, thereby reducing cancer mortality.

# CHAPTER 1. INTRODUCTION

## 1.1 Cancer and Metastasis

### 1.1.1 Introduction

It is estimated that 606,520 individuals will die of cancer in the United States in 2020 (1). Furthermore, 90% of all cancer-related deaths are attributed to metastasis (2). The high level of cancer mortality and contribution of metastasis to patient mortality indicate a critical need in the field for strategies to prevent metastasis. Cancer metastasis occurs through initiation of a multi-step process, the metastatic cascade, which cancer cells must undergo to successfully form clinically relevant secondary lesions. Key steps of the metastatic cascade include invasion through the extracellular matrix (ECM), intravasation into the bloodstream, extravasation at the metastatic site, dormancy, and colonization (2). Elucidating the mechanisms that underlie these major steps is of utmost importance to identifying processes that can be halted or reversed with targeted therapies to prevent metastatic progression.

### 1.1.2 Migration, Invasion, and the Epithelial to Mesenchymal Transition

Migration and invasion are two processes that underlie cancer cell motility and are necessary for metastasis. In order for cancer cells to ultimately travel to distant sites in the body, they must first invade through a layer of epithelial basement membrane, traverse through layers of ECM, and finally invade through another layer of vascular basement membrane to enter the bloodstream (3). Cancer cells alter expression of adhesion and junction proteins to facilitate this process. For example, expression of the adhesion protein, E-cadherin, as well as the junction proteins occludin and desmoplakin, are typically lost in motile cells (4). Loss of adhesion between cancer cells permits single cells to escape the primary tumor mass and invade into their surroundings, thereby promoting metastasis. In addition, cancer cells form protrusions to facilitate locomotion. Cancer cells utilize invadopodia, specialized protrusions which secrete matrix metalloproteases-2 and -9, to degrade ECM (5–9). Other types of cellular protrusions, such as lamellipodia and pseudopodia, do not possess proteolytic activity but contribute to cell motility cycles (3). In these motility cycles, the cell protrusion extends forward and the opposite end of the cell contracts, resulting in

movement (3,10,11). Formation of cellular protrusions is mediated by cytoskeletal rearrangement, including polymerization of F-actin, which is enriched in the protrusions (3,10). Thus, cancer cells must alter expression of key adhesion proteins, remodel their cytoskeletons to form motility protrusions, and upregulate expression of matrix-degrading enzymes to migrate and invade.

Reversible induction of the epithelial to mesenchymal transition (EMT) is one mechanism by which cancer cells increase their migration and invasion. The EMT program is induced by several cellular signals, including Transforming Growth Factor β (TGFβ) signaling, activation of stem cell-associated pathways including Wnt, Notch, and Hedgehog signaling, and by microenvironmental factors (12–14). The EMT is coordinated by a series of EMT transcription factors, including Snail, Slug, Twist, and Zeb, that repress epithelial genes and transactivate mesenchymal genes to increase cell motility (4,15,16).

Modulation of gene expression by EMT transcription factors can directly modify cell motility. For example, suppression of E-cadherin by Snail or Slug is a signature marker of an EMT and increases invasion of cancer cells (17–19). Conversely, expression of E-cadherin decreases invasion and metastasis (20,21). The induction of EMT is also associated with decreased expression of other junction and adhesion proteins, including occludin, desmoplakin, and cytokeratin (4). In addition, mesenchymal cells also have reorganized cellular architecture which mediates their increased motility. Mesenchymal cells, for example, are characterized by actin-rich lamellipodia which facilitate cell movement through expression of N-cadherin (12,22). Finally, mesenchymal cells also increase expression of matrix metalloproteinases-2 and -9, promoting ECM degradation to permit cell invasion (12,23). Thus, induction of EMT decreases cell-cell adhesion, increases pro-migratory cytoskeletal rearrangements, and enhances secretion of pro-invasive enzymes, which poises cells to migrate out from the primary tumor. In accordance with this, expression of Twist, Snail, and the mesenchymal protein Vimentin are associated with increased invasion and metastasis (24–26).

It is important to note that the extent of EMT activation plays a critical role in determining the overall success of cancer metastasis. An intermediate EMT phenotype, that is, cells that express a partial mesenchymal shift while retaining some epithelial characteristics, are more successful at various steps involved in metastatic progression compared to fully mesenchymal cells (27–31).

For example, groups of cells possessing mixed epithelial and mesenchymal phenotypes participate in collective migration, which is associated with more successful migration compared to single cell migration (32–34). An intermediate EMT phenotype is also particularly important for colonization of tumor cells at a secondary organ. For instance, forced induction of a fully mesenchymal phenotype in breast cancer cells reduced metastatic colonization in an animal model (27). Collectively, these data indicate that while acquisition of mesenchymal traits is necessary for tumor progression, a complete shift to a mesenchymal phenotype hinders metastasis. In agreement with this observation, tumor cells that undergo EMT reversal, a mesenchymal to epithelial transition, may have increased metastatic success (35–38). Thus, the balance between epithelial and mesenchymal traits in cancer cells plays a determining role in advancement of tumor cells throughout the metastatic cascade.

### 1.1.3 Intravasation, Survival in Circulation, and Extravasation

Upon exiting the primary tumor, cancer cells enter the circulation in a process termed intravasation. While tumor cells only subside in circulation for seconds or minutes, they face several obstacles to maintaining survival. These obstacles include the shear stresses of blood flow, detachment from ECM, loss of interaction with pro-survival elements of the primary tumor microenvironment, and interaction with circulating immune cells (2,39). Cancer cells that survive in circulation utilize a number of mechanisms to circumvent these inhibitory factors. For example, while the majority of circulating tumor cells travel as single cells, tumor cells that travel in small clusters are more likely to successfully metastasize (40). These cellular clusters are composed of primary tumor cells at various stages along the epithelial-mesenchymal spectrum, loosely adhered to one another in a cluster or chain-like structure (2,40). In addition to primary tumor cells, cell clusters may also contain stromal cells and components from the primary tumor microenvironment. Presence of these components in the circulating tumor cell clusters provides scaffolding to reduce cell stress from matrix detachment and maintains a partial EMT phenotype, thereby improving survival of the cancer cells (40–43). Clusters of circulating tumor cells also interact with platelets in the bloodstream, which protects the cluster from immune attack and secrete TGFβ to further sustain EMT (44–46). Thus, increased cell-cell interactions promote survival of cancer cells in circulation.

Tumor cells that survive in circulation must ultimately extravasate in order to colonize a secondary site. Cancer cells may undergo trans-endothelial migration into the parenchyma at the secondary site, or may arrest and divide within the blood vessel leading to rupture of the blood vessel followed by subsequent invasion into the secondary site and colonization (47–50). Specific sites at which primary tumor cells extravasate in the body have emerged and vary by primary cancer type with the lung, liver, and bone representing the most common metastatic sites across cancers (51). In certain cancers, the major metastatic site for primary cancers is determined by anatomy. Colon cancer cells, for example, readily enter into portal veins that surround the colon for transport to the liver, thereby increasing the burden of cells at this site (52). However, not all types of cancer follow this anatomy-driven model. The seed and soil hypothesis posits that primary tumor cells preferentially grow at secondary microenvironments that are conducive to growth, thereby increasing the proportion of cells which metastasize to those sites (53,54). The seed and soil hypothesis is supported by evidence that some secondary sites undergo remodeling to support arrival of primary tumor cells, thereby creating a pre-metastatic niche (55).

### 1.1.4 The Pre-Metastatic Niche

The likelihood of successful extravasation can be improved by pro-metastatic actions of primary tumor cells leading to formation of a pre-metastatic niche at secondary sites. Specifically, primary tumor cells secrete factors which localize to the secondary site to modify its structure by direct or indirect mechanisms. Primary tumor cells, for example, secrete angiopoietin-like 4 that disrupts endothelial cell-endothelial cell junctions and increases vascular permeability at the parenchyma of the secondary site, thereby improving the odds that cancer cells will lodge and colonize the secondary site (56). Tumor cells also secrete vascular endothelial growth factor (VEGF) (57,58) and A disintegrin and metalloproteinase (ADAM) proteins (59) that directly remodel the pre-metastatic niche to increase colonization. Cancer cells also release vesicles, including exosomes, that carry tumor-derived RNA to secondary sites, thereby indirectly modifying the structure of the secondary site by reprogramming the transcriptome of host cells (60,61). Exosomes may also contain ligands to stimulate pathway activation at the secondary site, including activation of epidermal growth factor receptor (EGFR) signaling, to increase metastasis (62), as well as factors that stimulate EMT, migration, and invasion (63–68). In sum, the signals secreted by cells within the primary tumor play a key role in shaping the pre-metastatic niche to enhance cell colonization.

### 1.1.5 Dormancy and Colonization

Tumor cells that successfully extravasate the blood vessel and embed into the parenchyma at the metastatic site often enter a dormant state in which cells divide and undergo apoptosis at equal rates (69,70). These disseminated tumor cells (DTCs) can reside in a dormant state for years before expanding into clinically significant macroscopic lesions (71). The defining characteristics of DTCs contribute to their prolonged survival and the latent arrival of macroscopic tumor colonies. DTCs, for example, possess characteristics of cancer stem cells (CSCs) including slow proliferation and expression of Sex determining region y-box 2 (SOX2) and SOX9 transcription factors (72). In accordance with this, DTCs often occupy local stem cell niches in the secondary tissue that are rich in factors which maintain a quiescent, stem-like state, including thrombospondin-1 (73,74). These observations lend support to the notion that CSCs are responsible for colonization of secondary tumors, while also shedding light on the mechanisms by which these cells survive.

DTCs may enter dormancy through passive or active processes. Cells can passively enter dormancy from the absence of pro-proliferative signals that previously sustained their growth in the primary tumor, including growth factors, nutrients, oxygen, or ECM (75,76), or as a result of immunosuppression at the secondary site (75). Conversely, DTCs may actively enter a state of dormancy upon arrival at the secondary site in the presence of pro-dormancy signaling factors. Examples of these pro-dormancy factors include bone-derived stromal cell-derived factor 1 (CXCL12), TGFβ2, and bone morphogenic protein (BMP) ligands (77). The combined presence of these factors and low mitogenic signaling at the secondary site results in $ERK^{low}/p38^{high}$ phenotype, entry into $G_0/G_1$ arrest, and quiescence (77). Regardless of whether entry into dormancy is passive or active, tumor cells in a dormant state exist in equilibrium in which tumor cell proliferation is equally matched with elimination.

Awakening from cellular dormancy is necessary for formation of a clinically significant secondary tumor and can occur through a number of mechanisms. Acquisition of gene expression that combats pro-dormancy signals (78–80) or induce a mesenchymal to epithelial transition (81) may be sufficient to induce proliferation. The mechanism by which cells gain expression of these pro-proliferative genes is poorly characterized and is speculated to be the result of random chance with each proliferative cycle of the DTC (2). The notion that cells gain necessary gene activation to

support proliferation in a stochastic manner is supported by the observation that DTCs may take years to emerge from dormancy and form metastatic lesions, as such an inefficient process may take several rounds of proliferation to gain activation of genes that stimulate proliferation (2). Similarly, tumor cells can awaken from dormancy as a result of pro-angiogenic signaling from endothelial cells or surrounding fibroblasts (74,82–84), or by loss of immune repression at the secondary site when the host is under stress, such as chronic inflammation (85,86). Awakening from dormancy is a poorly understood, critical step for completion of the metastatic cascade.

### 1.1.6 Conclusions

Cancer metastasis is a complex biological process that contributes significantly to poor patient outcomes. While the early steps of the metastatic cascade have been outlined and researched in great detail, further research is still needed to fully elucidate the processes required for metastasis once cells have left the primary tumor. For example, further efforts are needed to determine factors that contribute to metastatic tropism of cancer cells. Understanding the genetic, proteomic, and metabolomic profiles of cells that track to specific organs will be imperative in evaluating primary tumors of cancer patients to determine which organs may be susceptible to metastasis, and designing targeted therapies to prevent metastatic progression. It will also be important to identify signals from the primary tumor that promote cell metastasis, and how these signals are retained once cancer cells leave the primary tumor. Improving our understanding of the major steps of the metastatic cascade will ultimately pave the way to more advanced therapies to stop or delay metastatic progression and reduce cancer mortality.

## 1.2 Metabolism in Cancer Progression

### 1.2.1 Introduction

Dysregulated metabolism is a hallmark of cancer and plays a critical role in cancer cell survival. Furthermore, recent evidence shows that changes in cell metabolism are necessary for metastatic progression (87,88). Given that metastatic disease is the primary cause of patient mortality, this evidence highlights metabolic adaptations as a potential therapeutic target to improve overall survival in cancer patients. Here we describe changes in glucose and glutamine metabolism that

occur in transformed compared to untransformed cells and detail how these adaptations change throughout metastatic progression.

### 1.2.2 Reprogramming Glycolysis and the TCA Cycle in Cancer Cells

Cancer cells display well-characterized upregulation of glucose metabolism. Tumor cells consume and metabolize up to ten-fold higher amounts of glucose relative to untransformed, non-proliferative cells while simultaneously suppressing entry of pyruvate in the tricarboxylic acid (TCA) cycle, a phenomenon known as the Warburg effect (89,90). Upregulation of glucose metabolism via glycolysis occurs in both cancer cells and highly proliferative untransformed cells (90). Unique to cancer cells, however, is the mechanism driving glucose utilization. While untransformed cells rely on growth factors or ECM detachment to stimulate glucose utilization (91–93), cancer cells accumulate mutations in pro-proliferative genes that enhance glucose use in the absence of exogenous signals (94). Cancer cells, for example, carry activating mutations in phosphoinositide 3-kinase (PI3K) and Ras pathways which induce transcription of genes necessary for glucose use, including the glucose transporter GLUT1 and enzymes in the glycolytic pathway, such as pyruvate kinase M2 (PKM2) (94). Thus, somatic mutations harbored by cancer cells alter the metabolic landscape of the cell to increase metabolism of glucose through glycolysis.

The high glycolytic and low oxidative phenotype of cancer cells is driven by oncogenes and partially maintained by allosteric regulation by NADH and ATP. Conversion of pyruvate to lactate via lactate dehydrogenase consumes NADH and produces $NAD^+$ (94). Because NADH is an allosteric inhibitor of glycolysis, reduction of NADH to $NAD^+$ in tandem with lactate synthesis relieves inhibition of glycolysis. High levels of ATP, such as those produced at the electron transport chain during respiration, also allosterically inhibit glycolysis (95). Thus, restricting entry of carbon into the TCA cycle by converting pyruvate to lactate limits excess ATP production, enabling proliferating cells to maintain high glycolytic activity to support cellular biosynthetic demands. In sum, maintenance of high glycolytic activity through allosteric regulation maintains oncogene-driven metabolic reprogramming; this metabolic reprogramming is critical for cell survival.

Upregulation of glycolysis and suppressed flow of carbon into the TCA cycle creates a survival advantage for cancer cells. Metabolites of glucose that are produced in glycolysis are used for

several biosynthetic reactions essential for cell growth, including the production of reducing equivalents to maintain reductive-oxidative (redox) balance in the cell, synthesis of nucleotides and pentose sugars to promote production of genetic material, synthesis of nonessential amino acids to support protein synthesis and one-carbon metabolism, and synthesis of lipids to support membrane production (94,96). Upregulation of glycolysis therefore provides the cancer cell with a host of carbon-based intermediates required for proliferation. While glycolysis produces a relatively small amount of ATP (2 molecules of ATP for each molecule of glucose) compared to oxidative phosphorylation, this increase is in line with the relatively small increase in cellular demand for ATP as cells transition from a quiescent to a proliferative state (97). Increasing glycolysis and reducing oxidative metabolism also reduces the production of reactive oxygen species (ROS) as byproducts of respiration, protecting cancer cells from ROS-induced cell death or senescence (98,99). These data collectively highlight that upregulation of glycolysis provides cells with substrates and ATP necessary for proliferation, and protects cells from cell death.

Although entry of pyruvate into the TCA cycle is decreased in cancer cells as a result of increased lactate dehydrogenase activity, metabolism of nutrients through the TCA cycle also plays an important role in cancer cell survival. Entry of moderate amounts of carbon into the TCA cycle of proliferating cancer cells is advantageous for cell growth, as this pathway promotes ATP production and provides carbon-based intermediates necessary for anabolic reactions. The TCA cycle intermediate citrate, for example, may be used as a precursor for fatty acid synthesis (100–102). In addition, the TCA cycle intermediate oxaloacetate may be used as a precursor for synthesis of the nonessential amino acids aspartate and asparagine (103,104). Maintenance of TCA cycle activity is therefore often essential for proliferation, despite the metabolic shift to increase glycolysis in cancer cells.

Utilization of TCA cycle intermediates for anabolic reactions increases cellular demand for replenishment, or anaplerosis, of the TCA cycle. Several metabolites are used for anaplerosis of the TCA cycle, including acetate, pyruvate, and glutamine. Acetate is converted to acetyl CoA and used for *de novo* fatty acid synthesis in glioblastoma cells and in cells which have metastasized to the brain, suggesting that acetate may be a particularly important substrate in the brain where glucose and other nutrients are primarily consumed by resident glial cells (105–107). Pyruvate contributes to TCA cycle replenishment through the enzyme pyruvate carboxylase, which converts

pyruvate to oxaloacetate. Pyruvate carboxylase activity is associated with *de novo* fatty acid synthesis and oxidative stress protection in breast cancer cells, highlighting the role of pyruvate carboxylase and TCA cycle anaplerosis in the regulation of lipid metabolism and cancer progression (108,109). In addition, pyruvate carboxylase activity is critical for TCA cycle anaplerosis and cell survival when cancer cells are grown with low or no glutamine (110–113). Glutamine has been identified as a major anaplerotic substrate of the TCA cycle (94). In order to replenish the TCA cycle, intracellular glutamine is sequentially converted first to glutamate, then to α-ketoglutarate (αKG). The cellular preference for glutamine as an anaplerotic substrate may be related to the diverse array of reactions to which glutamine contributes in proliferating cells.

### 1.2.3 Reprogramming Glutamine Metabolism

Glutamine is a unique nutrient that provides carbon and reduced nitrogen to the cell to support proliferation through both catabolic and anabolic reactions. Glutamine enters the cell through solute carrier family members including solute carrier family 1 member 5 (SLC1A5) and is the second-most highly consumed nutrient among several types of cancer *in vitro* (114,115). Glutamine has several potential context-dependent cell fates following uptake, including use in exchange systems for other amino acids, participation in anabolic reactions, and participation in catabolic reactions. First, intracellular glutamine fluxes through antiporter channels on the cell membrane in reactions which simultaneously transport other amino acids, including leucine, isoleucine, methionine, phenylalanine, tryptophan, tyrosine, and valine into the cell (116,117). Exchange of glutamine for leucine is of particular relevance to cell proliferation, as intracellular leucine positively regulates mammalian target of rapamycin complex 1 (mTORC1) activity, thereby contributing to pro-growth signaling in the cell (118). Both glutamine and its metabolite glutamate can be exchanged for extracellular cysteine, an amino acid necessary for glutathione synthesis (119). Maintaining high levels of glutamine uptake are therefore critical for maintaining pools of other essential amino acids for cancer cell growth.

Glutamine participates in both anabolic and catabolic reactions in the cell. Following uptake, glutamine can be used directly for synthesis of nascent proteins (120). Alternatively, glutamine can be catabolized to glutamate through glutaminase-1 or -2 in a reaction which produces ammonium (Figure 1.1, 120). Glutamate may be used for synthesis of proteins or the antioxidant

glutathione, or may be further metabolized to αKG for entry into the TCA cycle (120). Conversion of glutamate to αKG is mediated by several enzymes in the cell, including glutamate dehydrogenase, which deaminates glutamate and produces ammonium, or by transaminases (121). Transaminases reversibly transfer the amino group from glutamate to α-keto acids including pyruvate and oxaloacetate, while simultaneously generating nonessential amino acids such as alanine and aspartate (121). In this way, glutamine and its metabolite glutamate are important molecules for synthesis of nonessential amino acids, including synthesis of asparagine downstream of aspartate. Glutamine-derived αKG can either be oxidized through the forward TCA cycle leading to energy production or may undergo reductive carboxylation to citrate, which is exported from the mitochondria and used for fatty acid synthesis (120). The several potential cell fates of glutamine implicate the importance of its metabolism in proliferation and redox balance.

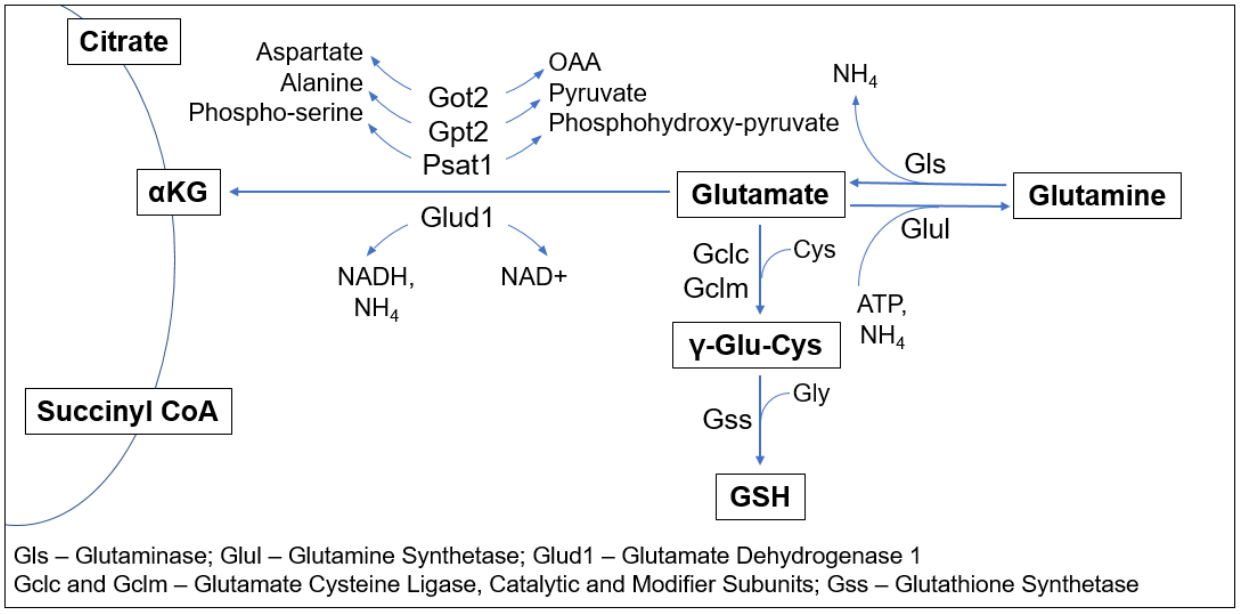


Figure 1.1 Overview of glutamine metabolism.

Glutaminolysis is mediated by glutaminase enzymes to produce glutamate. Glutamate has several potential cell fates, including conversion to glutathione or to αKG by one of several reversible enzymes.

Finally, glutamine and other amino acids contribute to cell signaling via the integrated stress response. The integrated stress response is a mechanism to detect extrinsic cell stresses, including amino acid starvation, and transduce these signals into a response which restores cell homeostasis,

or induces apoptosis if homeostasis cannot be achieved (122). In the integrated stress response pathway, cell stresses induce phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) by one of four kinases. Phosphorylated eIF2α globally suppresses mRNA translation in the cell, while simultaneously upregulating translation of select transcripts, including activating transcription factor 4 (ATF4). ATF4 transactivates genes related to induction of autophagy and antioxidant defense to promote cell survival (123,124). In response to amino acid starvation, ATF4 binds to amino acid response elements in the promoters of genes involved in synthesis and uptake of amino acids, including asparagine synthetase (125). Thus, the integrated stress response pathway serves as a system to respond to amino acid starvation and other cell stresses, making it a critical pathway for cancer cells which grow in microenvironments with limited nutrients.

Despite the several mechanisms by which glutamine metabolism contributes to cancer cell growth, dependence on glutamine metabolism varies widely among different types of cancer and experimental models. Cancers of the kidney, liver, and pancreas, as well as basal breast cancers, require glutamine for survival, as demonstrated by glutaminase inhibition (126–131). Conversely, lung and brain cancer cells are more likely to synthesize glutamine and display resistance to glutaminase inhibition (111,132–134). Luminal breast cancers, in contrast to basal breast cancers, also display glutamine independence (129), further emphasizing the heterogeneity of glutamine dependence.

Heterogeneity in the dependence of tumor cells on glutamine for survival is driven by factors such as the genetic makeup of the tumor cells and signals from the tumor microenvironment. For example, the proto-oncogene c-Myc is frequently mutated in several types of cancer, and positively regulates glutaminase expression in liver tumors (128,133). However, c-Myc also induces transcription of glutamine synthetase and can promote glutamine synthesis, as observed in non-small cell lung cancer tumors (134). Signals from the tumor microenvironment can also dictate glutamine metabolism in cancer cells. The pyruvate/glutamine ratio was three-fold higher in the lung microenvironment compared to the blood in an animal model (135). In accordance with this, lung cancer cells *in vivo* display higher dependence on pyruvate and its metabolism through pyruvate carboxylase for replenishment of the TCA cycle, and less utilization of glutamine (135). Cell stress also regulates glutamine fate, as non-small cell lung cancer cells cultured in attached conditions oxidize glutamine for energy production, whereas cells grown in matrix-detached

conditions shunt glutamine-derived citrate through isocitrate dehydrogenase 2 (IDH2) to produce mitochondrial NADPH (136). Finally, proliferative cells utilize transaminases for the conversion of glutamate to αKG while quiescent cells use glutamate dehydrogenase, although the mechanism controlling this shift in enzyme use is not clear (137,138). A more complete understanding of the factors that affect glutamine dependence in different types of cancer will help identify which tumors may be most responsive to glutamine-focused therapies.

### 1.2.4 Metabolism throughout Progression

The metabolic profile of tumor cells changes dramatically throughout progression. While a common set of metabolic changes has not been identified across all types of cancer, several studies have elucidated key gene-level changes that alter metabolism and increase metastatic potential in cell and animal models. Changes in cell metabolism may also be mediated by signals from the tumor microenvironment as cells adapt to rapidly changing nutrient availability. Understanding the changes in metabolism that occur in progression may help identify strategies to prevent metastasis and decrease cancer mortality.

Both glycolytic and oxidative metabolic metabolism in cancer cells have been associated with metastasis and poor patient survival. In early tumor development, cancer cells rapidly increase glucose utilization, and this enrichment of glycolytic activity is associated with poor prognosis in cancer patients (139). In agreement with this, suppressing glycolysis in cancer cells was associated with decreased metastasis in animal models (140,141). In contrast, in an examination of the interaction between cancer cells and the surrounding stroma, there is some evidence that cancer cells with an oxidative metabolic phenotype are more likely to metastasize. This mechanism is via the Reverse Warburg effect. In the Reverse Warburg effect, cancer-associated fibroblasts rely on glycolysis for their energy needs and secrete lactate and pyruvate, which may subsequently be oxidized by cancer cells for energy (142,143). The cellular preference for glycolytic or oxidative metabolic programs may correspond to survival at specific secondary sites. For example, lung- and bone-tropic murine mammary cancer cell lines were more oxidative compared to their pantropic counterparts (144,145), whereas liver-tropic cell lines were more glycolytic (144). The diversity of available evidence suggests that no single metabolic phenotype is required for

successful metastasis and that both glycolytic and oxidative phenotypes can contribute to metastatic progression in a context-dependent manner.

Metabolic adaptations drive specific processes throughout the metastatic cascade, including migration and invasion, at the molecular level. One well-characterized mechanism by which changes in metabolism drive migration is through extracellular acidification. Upregulation of the glycolytic enzymes hexokinase 2 and PKM2 in cancer cells, as observed in the Warburg effect, promotes high levels of glycolysis and lactate secretion (146,147). Lactate secretion acidifies the tumor microenvironment, leading to activation of matrix metalloprotease-9 through stimulation of nuclear factor kappa B (NF-κB) (148). Activation of NF-κB also induces EMT, suggesting that extracellular acidification can promote migration and invasion through multiple mechanisms (148). Similarly, oxidative metabolism can promote migration in some models. High levels of oxidative metabolism can induce formation of moderate levels of ROS which activate Src and increase migration and metastasis (149,150). As such, evidence from different models of cancer support roles of both glycolytic and oxidative phenotypes in promotion of migration and invasion.

Cancer cell metabolism continues to change as cells advance through the metastatic cascade, including in ECM detachment. ECM detachment increases oxidative stress, and cancer cells must enrich their antioxidant defense mechanisms to survive this step of metastasis (151). ErbB2 expression in MCF10A breast epithelial cells is sufficient to activate the pentose phosphate pathway and induce production of NADPH in detached conditions, thereby improving cell survival (93). Non-small cell lung cancer cells also increase their antioxidant response to matrix detachment by shunting glutamine and citrate metabolism through isocitrate dehydrogenase 2 (IDH2) to enrich production of mitochondrial NADPH (136). In fibrosarcoma cells, matrix detachment and metabolic stress increase ATF4 and nuclear factor erythroid 2-related factor 2 (NRF2) to counter ROS production and confer resistance to anoikis (152). Changes in metabolism to favor production of antioxidants in low attachment conditions are necessary for cancer cells to progress through the metastatic cascade to the secondary site.

Cancer cells adapt their metabolism to increase colonization at distant organs through two major mechanisms. First, cancer cells can prime the pre-metastatic niche to alter metabolism of resident cells at the secondary site. For example, primary breast tumor cells secrete exosomes that track to

secondary organs and suppress glucose metabolism of resident stromal cells via miR-122-mediated inhibition of pyruvate kinase and GLUT1 (153). This metabolic reprogramming increases the availability of glucose for use by the cancer cells upon arrival at the secondary organ (153). Cancer cells also reprogram their own metabolism to compete with host cells for nutrients at the secondary site. Colon cancer cells that metastasize to the liver increase expression and activity of creatine kinase brain-type to utilize phosphocreatine as an energy source (154). Similarly, cancer cells that metastasize to the brain increase use of glutamine and branched-chain amino acids thereby reducing their dependence on glucose, which is rapidly consumed by resident glial cells (106,110,155). In addition, breast cancer cells that metastasize to the lung have increased expression of pyruvate carboxylase (112,156). These data are in line with studies showing increased flux of glucose through pyruvate carboxylase in lung cancer cells at the primary site and suggest that cancer cells residing in the lung microenvironment metabolize pyruvate to improve their survival (112,135). In sum, metabolic adaptations in cancer cells plays a key role in enabling survival at secondary sites.

A final consideration in understanding changes in metabolism which occur throughout progression is the tumor microenvironment. Interactions between multiple cell types that make up the tumor microenvironment contribute to metabolic adaptation of cancer cells. For example, cancer cells secrete factors that stimulate the release of fatty acids from adipocytes, which may be used as a fuel source for cancer cells (157). In addition, secretion of lactate by cancer cells or by cancer-associated fibroblasts as observed in the Reverse Warburg effect (142,143) increases extracellular acidification (158), leading to induction of angiogenesis (159), increased cancer cell invasion through enhanced matrix metalloproteinase activity (148), and an overall immune permissive environment (159–162). Cross talk between cancer cells and stromal cells may play a role in promoting metabolic plasticity in cancer cells, thereby increasing their metastatic potential. These factors are important considerations when designing therapies that may need to target multiple cell types within the tumor microenvironment to prevent cancer progression.

### 1.2.5 Conclusions

In conclusion, changes in cell metabolism are instrumental in permitting cancer cell proliferation and progression. These changes in metabolism are driven by oncogenic mutations and sustained

through allosteric regulations, and result in a metabolic program that efficiently produces carbon-based intermediates for proliferation. Cumulating evidence has shown a great deal of heterogeneity in metabolic adaptations between different types of cancer in accordance with the underlying genetic profile of the cancer cells and signals from the tumor microenvironment. The metabolic profile of cancer cells is also transient in nature, with changes in preferred methods of nutrient utilization occurring at different stages of the metastatic cascade and at different sites in the body. Identifying the mechanisms underlying these diverse metabolic adaptations throughout progression will help elucidate treatment strategies to prevent metastasis and improve patient outcomes.

## 1.3 Hypoxia and Cancer

### 1.3.1 Introduction

The presence of hypoxia, or low oxygen tension, in solid tumors is positively associated with therapy resistance and metastasis and is inversely associated with patient survival (163,164). Hypoxia may develop when epithelial cell proliferation outpaces neovascularization or when cells rapidly utilize oxygen for metabolism, depleting oxygen faster than it is supplied. Because rapid proliferation and increased metabolism are both common characteristics of cancer cells, many solid tumors have regions of hypoxia (163,165). Hypoxic conditions mediate cell signaling to promote activation of several cell survival genes and pathways. Thus, understanding the biology in response to hypoxia is critical for the prevention of cancer progression.

### 1.3.2 Regulation of Gene Expression by Hypoxia

A major mechanism by which hypoxia drives changes in gene expression is through hypoxia-inducible factors (HIFs) (166). HIFs are multi-subunit proteins composed of a stably expressed beta subunit (HIFβ) and an oxygen-regulated alpha subunit possessing three isoforms (HIF1-3α), with HIF1α being the most well-characterized (167). Each of the three HIFα isoforms undergo similar processing in the cell and participate in similar interactions with their binding partner HIFβ. The HIFα subunits are continuously transcribed and translated in both normal and cancer cells and regulation of their expression by oxygen levels occurs at the post-translational level. In normal oxygen conditions (normoxia), prolyl hydroxylases (PHDs) use molecular oxygen and αKG as

substrates to hydroxylate HIFα on two proline residues (166). In this reaction, one oxygen atom from $O_2$ is transferred to HIFα, and the second atom is transferred to αKG to yield succinate and carbon dioxide. Hydroxylated HIFα is bound and ubiquitinated by von Hippel-Lindau tumor suppressor protein, a component of the E3 ubiquitin protein ligase complex. Ubiquitination of HIFα leads to its proteasomal degradation. In sum, in the canonical pathway, the presence of molecular oxygen in normoxia leads to HIFα degradation.

In hypoxia, the low oxygen tension limits hydroxylation of HIFα, relieving HIFα from ubiquitin-mediated degradation. This leaves HIFα free to translocate to the nucleus where it dimerizes with HIFβ, recruits p300/CBP at hypoxia response elements (HREs), and modulates transcription. HIFα subunits can also be stabilized in normoxia, particularly in the context of cancer (168). For example, mutations in succinate dehydrogenase and fumarate hydratase within cancer cells can lead to accumulation of succinate, which allosterically inhibits the activity of PHDs and therefore inhibits HIFα hydroxylation (169). In addition, overexpression of isocitrate dehydrogenase 3α increases conversion of αKG to isocitrate, reducing the availability of αKG to serve as a substrate for the PHD reaction and increasing HIFα protein retention (170). Similarly, c-Myc protein can physically interfere with the HIFα-von Hippel-Lindau complex, protecting HIFα from ubiquitination and subsequent degradation (171). Finally, HIFα transcription can be induced by ERK/MAPK, JAK/STAT, and PI3K pathways (172), by mutations in phosphatase and tensin homolog (PTEN) or p53 (168), or by moderate levels of ROS (173), all of which are common features of tumors. Collectively, HIFα protein can be stabilized by hypoxia or by mutations that block its degradation in normoxia.

Whether HIFα is stabilized by hypoxia or by mutations that lead to its retention, HIF complexes binding to HREs leads to alterations in gene expression. It has been estimated that there are over 2500 HIF target genes in the cell (167,174–176). The primary purpose of HIF-mediated gene transactivation is to restore oxygen levels in tissues to alleviate hypoxia. There are multiple mechanisms by which HIF signaling mediates restoration of cellular oxygenation, including induction of cell cycle arrest to reduce the proliferation of oxygen-consuming cells, secretion of pro-angiogenic factors to increase the formation of new blood vessels, and reprogramming cell metabolism to limit oxygen consumption and ROS generation from oxidative phosphorylation (167,177).

Reprogramming cell metabolism in hypoxia to reduce oxidative phosphorylation is imperative for cell survival. Oxidative phosphorylation is a metabolic process in which electrons from the cofactors NADH and $FADH_2$, which are generated at the TCA cycle, are used to drive production of ATP at the electron transport chain in an oxygen-dependent reaction. While previous literature suggested that oxidative phosphorylation was reduced in hypoxic cells because of limited oxygen availability, more recent evidence indicates that low oxygen levels are not rate-limiting for ATP production at the ETC in hypoxia (178). Mouse embryo fibroblasts with genetic depletion of HIF1α consume higher levels of oxygen and produce higher levels of ATP compared to their wildtype counterparts when grown in hypoxia, suggesting that oxidative phosphorylation remains active, despite low oxygen availability (178). In addition, there was no difference in oxygen consumption or ATP production in HIF1α-depleted cells grown in 21% or 1% oxygen, providing further evidence that oxidative phosphorylation remains active in HIF1α-depleted cells (178). Interestingly, HIF1α-depleted cells undergo cell death from excess ROS production within 48 h of hypoxic incubation (179). Hypoxia has been shown to increase the production of ROS at complex III in the electron transport chain (173). Taken together, the data show that hypoxia-driven generation of ROS at the electron transport chain leads to cell death in the absence of HIF1α. Importantly, viability of hypoxic HIF1α-depleted cells can be rescued through overexpression of genes that prevent entry of metabolites into the TCA cycle and therefore limit production of NADH and $FADH_2$, two potent drivers of ROS production, highlighting the importance of metabolic reprogramming for survival in hypoxia (178,179).

Another adaptation for survival and successful progression in hypoxia, is changes in gene and protein expression through activation of the integrated stress response (122,180). In hypoxia, double-stranded RNA-dependent protein kinase (PKR)-like endoplasmic reticulum (ER) kinase (PERK) autophosphorylates to become active (181). Active PERK phosphorylates eIF2α which globally suppresses protein translation while simultaneously inducing translation of the transcription factor ATF4. ATF4 activation in hypoxia regulates genes required for autophagy (123), and has been shown to play a role in enabling migration (182,183) and resistance to anoikis (152) in cancer cells. Thus, activation of ATF4 through the integrated stress response pathway represents a HIF-independent mechanism of modulation of gene expression in hypoxic cells.

### 1.3.3 Effect of Hypoxia on Cell Metabolism

***Effect of Hypoxia on Glucose and Pyruvate Metabolism***

ypoxia dramatically shifts cell metabolism to increase the flux of glucose through glycolysis and suppress oxidative metabolism. Upregulation of glycolysis in hypoxic cells is induced by HIF1α-mediated changes in gene expression. For example, HIF1α induces expression of the glucose transporters GLUT1 and GLUT3 to enable increased flux of glucose into the cell (167). Upregulation of GLUT1 protein was observed as early as four hours after hypoxic incubation in βTC6 pancreatic beta cells (184). In addition, HIF1α transactivates several genes encoding glycolytic enzymes, including hexokinase 1 and 2, phosphofructokinase, aldolase, phosphoglycerate kinase 1, enolase 1 and 2, and PKM2 (167,185–189). This transcriptional induction strongly promotes a glycolytic phenotype in hypoxic cells. For example, MDA-MB-231 breast cancer cells produce 75-80% of their total ATP from glycolysis when cultured in hypoxia, compared to 38% of ATP coming from glycolysis in normoxia (190). Increase reliance on glycolysis through metabolic reprogramming is critical to protect cells from metabolism-induced cell death or senescence.

In order to further reduce oxidative metabolism in hypoxic cells, HIF1α also activates expression of genes that prevent entry of pyruvate, the end-product of glycolysis, into the TCA cycle. Three major mechanisms by which HIF1α prohibits entry of pyruvate into the TCA cycle include activation of mitophagy, inhibition of pyruvate dehydrogenase (PDH), and activation of lactate dehydrogenase A (LDHA). First, HIF1α suppresses entry of pyruvate into the TCA cycle through its activation of mitochondrial autophagy, or mitophagy. Mitophagy is upregulated in hypoxic conditions in a process that requires co-expression of the HIF1α target genes BCL2 Interacting Protein 3 (BNIP3) and BNIP3-like (BNIP3L) (178,191). Genetic depletion of BNIP3 and BNIP3L in PC3 prostate cancer cells suppressed hypoxia-induced mitophagy, and suppression of mitophagy in hypoxic conditions increased ROS generation and cell death (178,191). These data support that activation of mitophagy, and by extension inhibition of mitochondrial metabolism, is critical to block oxidative phosphorylation and accumulation of cytotoxic ROS.

A second mechanism is that hypoxia and HIF1α indirectly suppress the PDH enzyme complex, which converts pyruvate to acetyl CoA for entry into the TCA cycle. Pyruvate dehydrogenase

kinase (PDK) enzymes phosphorylate PDH, inhibiting its activity. PDK1 is directly transactivated by HIF1α, leading to decreased PDH activity and reduced conversion of pyruvate to acetyl CoA when HIF1α is stabilized (179,192). Overexpression of PDK1 in HIF1α-depleted cells is sufficient to rescue cells from ROS-induced cell death in hypoxia (179), suggesting that HIF1α-mediated PDK1 activation is necessary for cell survival in hypoxia.

Finally, HIF1α induces expression of LDHA to reprogram pyruvate and lactate metabolism. LDHA is a subunit of the bidirectional enzyme lactate dehydrogenase, which reversibly converts pyruvate to lactate. Transactivation of the LDHA gene increases incorporation of LDHA protein subunits into the lactate dehydrogenase enzyme complex, increasing the LDHA:LDHB subunit ratio and causing the reaction to favor the reduction of pyruvate to lactate (185,193,194). Lactate is then exported from the cell by monocarboxylate transporters (MCTs), including the HIF1α target MCT4 (195,196). Secretion of lactate from the cell is critical, as accumulation of intracellular lactate causes acidification, which slows the rate of glycolysis, thereby limiting the major route of ATP production in hypoxic cells (197). Collectively, metabolic reprogramming of pyruvate and lactate metabolism provides a shunt for glycolysis-derived pyruvate which limits entry of carbon into the TCA cycle.

Emerging evidence shows that hypoxia alters utilization of glucose stored as glycogen in cancer cells. Hypoxia increases expression of genes required for both glycogen synthesis and glycogenolysis (198–200). Importantly, hypoxia induces expression of glycogen phosphorylase, a rate-limiting enzyme required for glycogenolysis (200). Suppression of glycogen phosphorylase in hypoxic U87 glioblastoma cells increased the pool of intracellular glycogen and led to ROS accumulation and cellular senescence (200). These data suggest that hypoxic cells have increased reliance on stored glucose as a substrate for energy production and that in its absence, cell death may occur from oxidation of alternate substrates.

### *Effect of Hypoxia on Glutamine and Lipid Metabolism*

Glutamine is a key source of nitrogen and carbon for proliferating cells, but its oxidation is often restricted in hypoxic cells (201). Glutamine enters cells primarily through the transporter SLC1A5 and may be subsequently metabolized to glutamate and the TCA cycle intermediate αKG (201). Glutamine-derived αKG has two potential fates: reductive carboxylation to isocitrate and citrate

(202–206), or oxidation to succinyl CoA and subsequent TCA cycle intermediates (207–209). Here we discuss the metabolism of glutamine through reductive carboxylation, as this is the major pathway of glutamine metabolism in hypoxic cells.

HIF1α drives the reductive carboxylation of glutamine-derived αKG to citrate. Reprogramming metabolism of glutamine-derived αKG to favor reductive carboxylation is accomplished by suppression of the α-ketoglutarate dehydrogenase enzyme complex (αKGDH), an enzyme complex required for forward oxidation of αKG to succinyl CoA. HIF1α induces expression of the E3 ubiquitin-protein ligase Seven in Absentia Homolog 2 (SIAH2), which ubiquitinates αKGDH, leading to its degradation. High levels of HIF1α therefore block activity of αKGDH and prevent oxidation of αKG to succinyl CoA, shunting αKG to isocitrate and citrate (206). Citrate is then used for fatty acid synthesis (206), a process required for production of cell membranes and proliferation. Hypoxic glioblastoma cells that were dosed with [U-$^{13}$C]-labeled glutamine for four hours showed enrichment of the citrate M+5 pool, indicating that glutamine was being converted to αKG and reduced to citrate (205). No M+4 labeled citrate was detected in hypoxic cells, indicating that glutamine-derived citrate was not being produced from oxidative metabolism of glutamine through the forward-TCA cycle (205). Interruption of this reductive pathway by glutamine starvation or depletion of isocitrate dehydrogenase 2 causes growth arrest in glioblastoma cells cultured in hypoxia (205). Thus, glutamine is an essential nutrient for fatty acid synthesis and proliferation of hypoxic cancer cells.

In addition to synthesis of fatty acids from glutamine, cancer cells cultured in hypoxia can also synthesize fatty acids from exogenous acetate. Addition of $^{13}C_2$-acetate into the media of cells cultured in hypoxia resulted in incorporation of labeled carbon into over 50% of the acetyl CoA pool, whereas normoxic cells given the same $^{13}C_2$-acetate had incorporation of labeled carbon into <10% of the acetyl CoA pool (107). Acetate is converted to acetyl CoA via the enzyme acetyl-CoA synthetase, which is upregulated in hypoxia, and acetyl CoA is subsequently incorporated into fatty acids (107). In addition, acetate-derived carbon was used to a significantly greater extent for phosphatidylcholine synthesis in hypoxic cells compared to normoxic cells, supporting a role of acetate in fatty acid synthesis in hypoxic cells (107). HIF1α also increased fatty acid uptake through upregulation of fatty acid binding proteins-3 and -7 (FABP3/FABP7) in glioblastoma cells, which enhanced the formation of cytoplasmic lipid droplets (210). Lipid droplets were then

used as an energy source when cells were re-exposed to oxygen (210). These data further demonstrate increased fatty acid uptake and synthesis in hypoxic cells, a process which may have implications for cell survival following re-oxygenation.

Whereas fatty acid synthesis is induced by hypoxia, fatty acid oxidation is repressed in hypoxic conditions (211). HIF1α indirectly suppresses the enzymes medium- and long-chain acyl CoA dehydrogenase, which are required for the first step of β-oxidation of fatty acids in the mitochondria (212). Suppression of fatty acid oxidation reduces oxidative metabolism and spares cells from cytotoxic ROS production while promoting utilization of carbon for biosynthesis rather than energy production.

In sum, hypoxia-mediated changes in cell metabolism include (1) increased reliance on glucose and glycolysis for ATP production, (2) remodeling of pyruvate metabolism to prevent acetyl-CoA production and oxidation, and (3) reprogramming of glutamine and lipid metabolism to promote fatty acid synthesis and prevent fatty acid oxidation. The net effect of these changes is reduced flow of carbon into the TCA cycle, limited production of ROS, and avoidance of cell death.

### 1.3.4 Hypoxia and Metastasis

#### *Introduction*

Strong evidence supports that hypoxia and HIF1α are required for metastasis. Several models have shown that overexpression of HIF1α drives metastasis, and that HIF1α depletion conversely blocks metastasis, suggesting that HIF1α signaling is sufficient and necessary for metastasis (165,213). Interestingly, depletion of HIF1α or HIF2α in a murine model of melanoma did not affect primary tumor growth but did suppress metastasis (214). The notion that HIF1α is necessary specifically for metastatic progression is further supported by a study showing that cancer cells that were incubated in hypoxia *ex vivo* prior to IV injection into animals had increased lung colonization (215). Understanding the mechanisms by which hypoxia increases metastasis will help elucidate targeted therapies for prevention of metastatic progression.

The mechanisms underlying hypoxia-driven metastasis have been extensively researched both *in vitro* and *in vivo*. It is important to note that while *in vitro* work is critical to elucidating molecular

mechanisms related to metastasis, the complexity of the tumor microenvironment – including presence of immune, endothelial, and red blood cells, cell stresses, and oxygen gradients – is generally lost in *in vitro* work. In addition, *in vitro* studies typically compare 21% (environmental) oxygen to ~1% oxygen, but normal tissue levels of oxygen are 3-7.4%, a range of oxygen levels sometimes referred to as "physoxia" (216). In accordance with this, comparison of gene activation in response to hypoxia *in vivo* versus *in vitro* showed that approximately 800 genes induced by hypoxia *in vitro* are not induced by hypoxia *in vivo* (217), suggesting that factors in the tumor microenvironment may contribute to differences between cell- and animal-based work. Nonetheless, hypoxia signaling has been shown to affect several, if not all, of the steps of the metastatic cascade in several models of cancer. Here we review literature of the effect of hypoxia on key processes of metastasis.

***Hypoxia, Migration, and Invasion***

A vast body of research has examined the effect of hypoxia on migration and invasion on cancer cells and collectively supports a pro-invasive role of hypoxia. Data from *in vivo* analyses have revealed that HIF1α promotes invasion of cancer cells (218–220). Injection of HIF1α-depleted GL261 glioblastoma cells into brain tissue of mice showed impaired invasion compared to HIF1α-expressing control cells (220). These data demonstrate that HIF1α, likely through hypoxia signaling, is required for invasion in this model, and are in line with previous reports that HIF1α is required for metastasis *in vivo* (221–223). There are several mechanisms by which hypoxia or hypoxia signaling may drive successful cancer cell invasion, including modifying crosstalk between diverse cell types in the primary tumor, modifying motility speed, and oxygen-mediated chemotaxis.

One mechanism by which hypoxia and HIF1α may drive invasion is through altering interactions between different cell types within the tumor microenvironment. Among *in vitro* models that include multiple types of cells, hypoxia shows a consistent effect of increasing cell motility (224,225). For example, hypoxia increased migration of estrogen receptor-negative EII and estrogen receptor-positive pII breast cancer cells towards one another in a wound-healing assay using an Ibidi chamber (224). Similarly, co-culturing gastric cancer cell lines with macrophages in hypoxia increased migration as measured by a 3D dynamic migration imaging system (225). In

an animal model, hypoxia in the primary tumor stimulated release of factors that led to recruitment of mesenchymal stem cells to the primary tumor (226). Cross talk between mesenchymal stem cells and tumor cells increased invasion and, in turn, increased metastasis *in vivo* (226–229). Importantly, co-culturing mesenchymal stem cells with breast cancer cells with genetic HIF1α or HIF2α depletion limited metastatic potential when the cells were injected into mice (226,227). Similar results were obtained when the HIF1α target genes chemokine receptor CXCR3 or chemokine ligand CXCL16 were depleted in breast cancer cells, suggesting that HIF-mediated expression of either of these factors is critical for successful crosstalk between tumor cells and mesenchymal stem cells (226,227). These data collectively suggest that hypoxia signaling modifies interactions between cell types within the primary tumor to promote invasion.

Another mechanism by which hypoxia signaling may drive invasion is by increasing the speed at which cancer cells migrate or invade. MDA-MB-231 breast cancer cells cultured in a microfluidic device coated with collagen I moved at 15 μm/h in hypoxia compared to 10 μm/h in normoxia (228). Another *in vitro* report, however, showed that hypoxia did not affect the speed at which 4T1 breast cancer cells moved through a 3D matrix containing fibrillar collagen, with both normoxic and hypoxic conditions showing speeds of 6 μm/h (230). This model did, however, show that hypoxia induced a switch from collective to single-cell amoeboid locomotion, a phenotype associated with increased invasion (230). Additional *in vitro* studies have shown mixed effects of hypoxia on motility speeds (216,225,231,232), making it unclear if changes in cell kinetics are responsible for increased invasion in hypoxia.

*In vivo* assessment of motility speed has shown that hypoxia slows motility of cancer cells but increases invasion and metastasis. Cancer cells in primary murine mammary cancer are capable of two distinct locomotion speeds: fast (~69 μm/h) and slow (~ 8 μm/h) (233). Interestingly, only slow-migrating cells expressed invadopodia which possessed MMP activity. These slow-moving, invasive cells are critical for successful metastasis, as genetic depletion of Tks5, an invadopodia-associated protein involved in matrix degradation, impaired breast to lung metastasis without affecting fast-moving cells of the primary tumor (233). An independent study showed that hypoxic cancer cells in the primary tumor migrated more slowly than physoxic cancer cells (24 μm/h in hypoxia vs 60 μm/h in normoxia); however, hypoxic cancer cells were also shown to have increased matrix degradation, more invadopodia, and to be more chemotactic towards blood

vessels compared to physoxic cells (234). These data suggest that while the speed of migration may decrease in hypoxia compared to physoxia, the cells gain invasive characteristics which may translate to more successful intravasation (234).

Finally, various analyses have shown that oxygen may function as a chemoattractant to drive migration and invasion of cancer cells. MDA-MB-231 or 4T1 breast cancer cells that were transfected with hypoxia-responsive fluorescent probes and injected into animals showed that hypoxic cancer cells migrate out of the hypoxic core and move towards the more oxygenated invasive front of the tumor towards blood vessels (217). In agreement with this, cancer cells that had undergone radiation therapy *in vivo* exhibit HIF1α stabilization which induced translocation towards blood vessels (235). This movement towards blood vessels could be blocked with HIF1 inhibitors (235), suggesting that HIF1α or hypoxia signaling are required to respond to molecular oxygen. Data from these *in vivo* studies are supported by an independent *in vitro* analysis which showed that sarcoma tumor biopsies grafted into hydrogels with robust oxygen gradients (0.5-4% to 21% oxygen) had increased invasion compared to biopsies grafted into hydrogels with narrower oxygen gradients (10-15% to 21% oxygen) (232). Conversely, the MDA-MB-231 breast CSC subpopulation preferentially moved towards lower oxygen tensions when cultured on a microfluidic device coated with fibronectin possessing an oxygen gradient of 1.3-6.8% oxygen (236). No significant effect of oxygen on directional migration was observed in the total MDA-MB-231 cell population (236), suggesting that this phenomenon may be unique to the cancer stem cell population. Collectively, these data suggest that oxygen may serve as a chemoattractant to induce directional motility of hypoxic cancer cells, although further research is required to study this phenomenon in the cancer stem cell population.

A number of molecular mechanisms by which hypoxia may drive migration and invasion of cancer cells have been identified *in vitro*, including direct and indirect mechanisms. Both hypoxia and HIF1α drive EMT and invasion in cancer cells (237–239). HIF1α drives EMT directly via transactivation of ZEB1 (240), Snail (241), and Twist (242), all of which contribute to a mesenchymal, motile phenotype. HIF1α also increased cell motility through upregulation of RhoA and Rho-associated protein kinase (ROCK1) mRNA and protein levels in MDA-MB-231 breast cancer cells (231). Hypoxia and HIF1α promote activation of signaling pathways such as TGFβ, Notch, Wnt, Hedgehog, and through stimulation of integrin-linked kinases and tyrosine kinase

receptors (165). Activation of these pathways in hypoxia contributes not only to a shift towards a mesenchymal phenotype but also towards de-differentiation to a CSC phenotype (243,244). The concept that hypoxia induces a CSC phenotype is supported by a study showing that freshly isolated hypoxic mammary tumor cells showed enrichment of the CSC population compared to physoxic tumor cells, and reinjecting the hypoxic cells into animals resulted in enhanced tumor formation compared to injecting the physoxic cells (245). These data collectively indicate that hypoxia enforces a CSC phenotype *in vivo* and that this phenotype is associated with increased tumorigenicity (245). Modification of these cellular mechanisms may contribute to enhanced hypoxia-mediated invasion in cancer cells.

In sum, several potential mechanisms may drive hypoxia-mediated cancer cell invasion. Interactions between mixed cell populations in hypoxia are likely to play a critical role in permitting successful invasion, whereas hypoxia may affect cell motility speeds differently *in vivo* compared to *in vitro*. Further research on the chemoattractive role of oxygen and oxygen gradients is needed, as different models have shown mixed results on the effect of oxygen on directionality of cell movement.

### *Hypoxia, Intravasation, Survival in Circulation, Extravasation, and the Pre-Metastatic Niche*

Hypoxic cells may have increased intravasation and extravasation efficiencies compared to their normoxic counterparts. When MDA-MB-231 breast cancer cells were implanted into mice, 25% of the cells in the primary tumor were hypoxic (217). However, approximately 50% of the circulating tumor cells isolated from the same animal model were previously hypoxic, suggesting that hypoxic primary tumor cells reach the bloodstream with greater efficiency than physoxic primary tumor cells (217). This was shown to be related to lower levels of ROS in previously-hypoxic primary tumor cells compared to previously-physoxic cells, as high levels of oxidative stress in circulation are often rate-limiting in determining survival of circulating tumor cells (217,246). These data are in agreement with literature demonstrating that cells from the interior of the tumor, rather than the leading edge, are responsible for intravasation and subsequent metastasis (216). Collectively, these data support that hypoxia enhances intravasation of tumor cells into the circulation.

Once in circulation, tumor cells must bind to endothelial cells lining the blood vessel walls at the secondary site, a process called margination, and subsequently extravasate into the tissue. Hypoxia activates expression of L1 cell adhesion molecule (L1CAM), a protein involved in binding to endothelial cells, on the surface of breast cancer cells *in vitro* (247). Genetic depletion of L1CAM in breast cancer cells reduced lung colonization when cells were intravenously injected into animals and inhibited breast to lung metastasis when cells were injected into the mammary fat pad of animals (247). However, when previously-hypoxic or previously-physoxic mammary tumor-derived cells were tail vein injected in equal numbers into mice, both cell types seeded into the lungs with the same efficiency 48 h after injection (217). These conflicting data may suggest that physoxia, rather than hypoxia, is sufficient to permit margination of primary tumor cells. Thus, the role of hypoxia in modulating cell margination is unclear, and evaluation of L1CAM expression hypoxic compared to physoxic conditions *in vivo* is needed to clarify this point.

Hypoxia plays a critical role in establishing the pre-metastatic niche, in part through activating expression of secreted factors. For example, the pro-metastatic factors lysyl oxidase (LOX) and LOX-like (LOXL) are induced by hypoxia and secreted from primary tumor cells (57,248–253). LOX and LOXL proteins are secreted from primary mammary tumors and disseminate to the lungs, where they reshape the local collagen matrix. Modification of the collagen matrix attracts $CD11b^+$ bone marrow-derived cells to the lungs, where they secrete chemokines that circulate back to the primary tumor, leading to increased extravasation of tumor cells at the lung (248). Importantly, progression was partially blocked when LOX or LOXL4 were depleted in cancer cells before injection, and metastasis was decreased when HIF1 was chemically inhibited (254). In addition to its role in enhancing breast to lung metastasis, LOX may mediate breast to bone metastasis. This notion is supported by the observation that LOX secretion is significantly upregulated in a bone-tropic derivative of MDA-MB-231 breast cancer cells compared to the parental cell line, which metastasizes to multiple sites *in vivo* (255). Another secreted factor induced by hypoxia is angiopoietin-like 4, which suppresses endothelial cell-endothelial cell interactions, creating vascular leakiness at secondary organs such as the lung, thereby permitting extravasation of cancer cells (56,247). Depletion of angiopoietin-like 4 in MDA-MB-231 cells did not affect primary tumor formation when cells were implanted into mice but significantly suppressed lung metastases (56). Other secreted factors, including placental growth factor, VEGF, and TGFβ are stimulated by HIF1α and have been shown to play a role in shaping the pre-

metastatic niche (256). Collectively, modulation of the cellular secretome in hypoxia alters the secondary site to increase metastatic progression.

Finally, recent work has evaluated the effect of hypoxia on priming the pre-metastatic niche through tumor-derived exosomes. Exosomes derived from PC3 prostate cancer cells cultured in hypoxia increased matrix metalloproteinase expression and activity at metastatic sites when injected *in vivo* compared to mice injected with normoxia-derived exosomes (257). Injecting hypoxia-derived exosomes also increased fibronectin, collagen, and infiltration of $CD11b^+$ bone marrow-derived cells into metastatic organs compared to normoxia-derived exosomes (257). In agreement with these data, injection of hypoxia-derived exosomes in xenograft models of esophageal squamous cell carcinoma increased primary tumor size and lung metastases compared to normoxia-derived exosomes (258). In sum, hypoxia alters expression of genes in primary tumor cells that directly increase their ability to intravasate and extravasate, while also modifying the profile of exosomes to prime the pre-metastatic niche.

***Hypoxia, Dormancy, and Colonization***

Recent evidence suggests that hypoxia in the primary tumor plays a role in programming a dormancy phenotype in cancer cells that have arrived at the secondary site. Dormant cells have a stem-like phenotype characterized by slow proliferation and low metabolism (259). Hypoxia enriches the $CD44^+/CD24^-/ESA^+$ CSC population in breast cancer cells, which may contribute to dormant survival of cancer cells at the secondary site (260). In addition, recent evidence has shown that hypoxia in the primary tumor programs a dormancy gene signature in breast cancer cells, including increased expression of the dormancy-associated genes nuclear receptor subfamily 2 group F member 1 (NR2F1), differentially expressed in chondrocytes 2 (DEC2), and p27 (261). Importantly, expression of these genes was retained in cells that had metastasized to the lung despite loss of HIF1α expression, suggesting that expression of dormancy genes can be sustained in physoxia (261). Thus, hypoxia signaling at the primary tumor can contribute to a lasting gene signature that confers cellular protection from elimination at the secondary site.

Increasing evidence suggests that hypoxia also plays a role in escaping dormancy at the secondary site. Tail vein-injecting equal numbers of previously hypoxic or previously physoxic primary tumor cells resulted in twice as many hypoxic tumor-derived cells in the lung 25 days after

injection compared to physoxic-derived cells (217). Both cell types were shown to colonize and proliferate at the same rate in the lungs, suggesting that exposure to hypoxia in the primary tumor increases the likelihood of escaping dormancy (217). Mechanistically, hypoxia has been shown to disrupt LIFR-STAT3-SOCS3 (leukemia inhibitory factor receptor-signal transducer and activator of transcription 3-suppressor of cytokine signaling 3) signaling in breast cancer cells which have metastasized to bone, a signaling axis which maintains cells in a dormant state (262,263). Disruption of this signaling axis by hypoxia may be sufficient to release disseminated tumor cells from dormancy. Collectively, these data suggest that early exposure to hypoxia plays an important role in entry into and exit from dormancy at the secondary site.

In conclusion, hypoxia plays a critical pro-metastatic role in cancer progression. Several potential mechanisms driving this pro-metastatic role of hypoxia have been identified, including more successful invasion, improved survival in low attachment, and increased survival at the secondary site. Given its importance for several steps along the metastatic cascade, understanding the effects of hypoxia in physiologically relevant systems may help elucidate mechanisms to prevent cancer metastasis and improve patient outcomes.

### 1.3.5 The Intersection: Metastasis via Hypoxia-Mediated Metabolic Reprogramming

While it has been well established that hypoxia drives changes in cell metabolism and metastatic potential, there is currently little research on the effect of hypoxia-driven metabolic adaptation on metastasis. The limited data suggest that changes in cancer cell metabolism contribute to the increased metastatic potential seen in hypoxic tumors, either by remodeling the primary tumor microenvironment or by adapting gene expression in primary tumor cells.

Hypoxia-mediated changes in pyruvate metabolism in cancer cells can increase metastatic characteristics of cancer cells through several mechanisms. Hypoxia-mediated metabolic reprogramming promotes lactate dehydrogenase-mediated reduction of pyruvate to lactate, which is exported from cells through MCT proteins (197,264,265). Exporting pyruvate from the cell promotes extracellular acidification, which increases cell survival, migration, invasion, and resistance to radiotherapy in cancer cells (266,267). In addition, the lactate exported from hypoxic cells may be used as an energy source for normoxic cells within the tumor (162). A clinical trial in non-small cell lung cancer patients using $^{13}$C-lactate showed incorporation of labeled carbon

into TCA cycle intermediates in tumor cells (268), suggesting that human tumor cells can utilize lactate for energy production, and similar results were found in an analogous animal study (268). Furthermore, genetic depletion of MCT1 in non-small cell lung cancer cells blocked uptake and utilization of lactate by tumor cells in an animal model (268). These data collectively highlight the pro-metastatic effect of modified pyruvate metabolism in hypoxia at the primary tumor and the potential clinical value of targeting MCTs in the treatment of cancer.

In addition to its role in promoting progression of cells residing in the primary tumor, hypoxia may enhance metastasis through metabolic reprogramming in ECM-detached cells. ECM detachment is a common feature of cancer progression and occurs at multiple steps throughout the metastatic cascade. Tumor cell lines grown in detached conditions were more dependent on glycolysis and demonstrated increased reductive carboxylation of αKG to citrate compared to their attached counterparts (269), similar to the metabolic phenotype observed in hypoxia. Indeed, detached cells growing in clusters had hypoxic cores with increased expression of HIF1α and BNIP3 (269). Forcing detached cells to grow as single cells blocked reductive carboxylation, increased mitochondrial ROS production, and promoted oxidative stress (269). As previous literature has shown that cells grown in hypoxic 3D culture conditions shift to single-cell migration (230), further research is required to determine how hypoxia affects cell clustering *in vivo*. Nonetheless, these data may suggest that hypoxia and HIF1α signaling promote oxidative stress protection through metabolic reprogramming in cells detached from ECM, which may lead to improved cell survival and metastatic progression.

Hypoxia-driven expression of metabolic enzymes is required for hepatic colonization and metastasis in animal models. Genetic depletion of PDK1 in liver-tropic mammary cancer cells significantly reduced hepatic metastasis in an animal model (144). Hepatic tissue often contains regions of hypoxia (150), suggesting that metabolic adaptation to hypoxia within the primary tumor may be required for successful colonization of cancer cells at hypoxic secondary sites. The gluconeogenic enzyme phosphoenolpyruvate carboxykinase-1 (PCK1) is also required for metastatic colonization at the liver. PCK1 depletion in SW480 colon cancer cells suppressed liver colonization when cells were injected into portal circulation (271). Interestingly, mice were injected with doxycycline-inducible shPCK1 SW480 cells and doxycycline treatment of animals began after hepatic colonization had initiated. PCK1 depletion in this model decreased tumor

growth at the liver, suggesting PCK1 is specifically required for cell proliferation at the liver (271). *In vitro*, PCK1 expression was not required for SW480 cell growth in normoxia but became conditionally required for nucleotide synthesis and subsequent proliferation when cells were cultured in hypoxia (271). Thus, hypoxia-mediated metabolic reprogramming is required for colonization at the liver, likely to promote survival in the hypoxic liver microenvironment. Targeting PDK1 or PCK1 expression or activity may therefore be a useful strategy to prevent metastatic growth in liver tissue.

Hypoxia-driven alterations may also impact metastasis to the lung. The glutaminolysis enzyme glutaminase is directly transactivated by HIF1α in hypoxia, and depletion of glutaminase in HT29 colon cancer cells blocked hypoxia-mediated invasion *in vitro* and lung colonization *in vivo* (272). Glutaminase depletion also suppressed vimentin expression *in vivo*, suggesting that glutamine metabolism may contribute to an EMT in colon cancer cells (272). Further research is required to determine the mechanism by which modulation of glutamine metabolism mediates colon-to-lung metastasis.

Collectively, the limited evidence in this field supports the presence of a critical overlap between metabolic reprogramming and metastasis in hypoxia. Nonetheless, some gaps remain unanswered, including the effects of hypoxia-mediated metabolic reprogramming on processes upstream of colonization, including migration and invasion, in 3D or *in vivo* models. Further research is also needed to understand how hypoxia-mediated metabolic reprogramming affects colonization at different metastatic sites, including bone. Accumulating evidence in this field may lay the foundation for therapies targeting metabolic adaptation to hypoxia for prevention of metastatic progression.

### 1.3.6 Conclusions

Hypoxia dramatically modifies gene expression in normal and cancer cells alike. The cellular response to hypoxia collectively functions to restore normal oxygen homeostasis, while maintaining cell survival (166). Because hypoxia can dramatically increase ROS production secondary to nutrient oxidation, reprogramming cell metabolism to increase substrate-level phosphorylation at glycolysis and suppressing oxidative metabolism is imperative to avert ROS-mediated cell death.

Another critical cell response to hypoxia is activation of genes that directly restore oxygenation of cells. Hypoxic cells can achieve this effect through induction of angiogenesis and modifying cell motility, potentially in a manner that is physically directed towards oxygen-rich microenvironments. In the context of cancer cells, these changes in gene expression manifest as increased metastatic potential and increased patient mortality.

While previous literature has parsed out these two effects of hypoxia, metabolic reprogramming and changes in gene expression which promote metastasis, it may be more appropriate to categorize metabolic reprogramming as an example of a mechanism by which hypoxia promotes metastasis. Given the classification of metabolic reprogramming as a hallmark of cancer (273) and several emerging roles of metabolic adaption specifically in development of metastases, it is crucial to understand how hypoxia-driven changes in metabolism contribute to metastasis. Further elucidation of this relationship may uncover druggable targets to help prevent metastatic progression in patients with hypoxic tumors.

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# CHAPTER 2. GLUTAMINE CONCENTRATION REPROGRAMS GLUTAMINE METABOLISM IN NON-METASTATIC MURINE MAMMARY CANCER CELLS

## 2.1 Abstract

Glutamine is often an essential nutrient for cancer cell survival. Several models of cancer, including breast cancer, have shown dependence on glutamine metabolism for proliferation and progression. In the present study, we aimed to identify the differences in glutamine metabolism in non-metastatic compared to metastatic murine mammary cancer cell lines in response to glutamine concentration. Our results show that high glutamine reduced viability only in metastatic cells, and that this decrease in viability was accompanied by an increase flux of glutamine into the TCA cycle, assessed using stably labeled glutamine. Non-metastatic cells, in contrast, demonstrated no change in glutamine flux in high glutamine media concentration and showed reduced mRNA expression of genes related to glutamine metabolism. While increased glutamine metabolism occurred in tandem with a decrease in the reduced/oxidized glutathione ratio in metastatic cells, the ratio of NADPH/$NADP^+$ was unchanged and treatment with the antioxidant molecule N-acetylcysteine did not rescue viability of metastatic cells in high glutamine concentrations. Finally, we found that metastatic cells cultured in low glutamine were more sensitive to ammonium chloride treatment leading to reduced viability compared to their non-metastatic counterparts, suggesting that reprogramming glutamine metabolism in non-metastatic cells is important to avert nitrogen cytotoxicity. These results overall demonstrate the presence of a system to detect and respond to increasing glutamine concentrations in cancer cells that may be lost during the course of progression to metastasis.

## 2.2 Introduction

Glutamine is the second-most commonly consumed nutrient in cancer cells following glucose, and its metabolism is required for cell proliferation in several models of cancer (1–3). Glutamine has several potential cell fates, including incorporation into nascent peptides, contribution to nucleotide synthesis (4), participation in antiport exchange for other amino acids including leucine (5–8), a nitrogen source for synthesis of other amino acids, or it may be catabolized for energy

production. In the catabolic pathway, glutamine is converted first to glutamate, and subsequently catabolized to α-ketoglutarate (αKG) for entry into the tricarboxylic acid (TCA) cycle for oxidation and energy production (9,10). The many potential cell fates of glutamine highlight its importance for growth of cancer cells.

A battery of enzymes is required to mediate the conversion of glutamine to its metabolites. First, glutaminase 1 and 2 convert glutamine to glutamate in a reaction which produces ammonium as a byproduct (11). Conversion of glutamate to αKG is then mediated by one of three transaminases (aspartate aminotransferase, Got; glutamic pyruvic transaminase, Gpt; or phosphoserine aminotransferase, Psat), or by glutamate dehydrogenase (Figure 2.1F) (3,10). Transaminase enzymes transfer the amine group from glutamine to an α-keto acid, producing an amino acid, while glutamate dehydrogenase deaminates glutamate to produce ammonium.

Ammonium has previously been associated with decreased cell viability in models of cancer and untransformed cells (11), suggesting that glutamine catabolism through glutaminase or glutamate dehydrogenase may have cytotoxic effects. Of note, recent evidence has shown that rapidly proliferating cells preferentially utilize transaminases while quiescent cells use glutamate dehydrogenase (12), suggesting that glutaminase is likely the primary producer of ammonium in cancer cells. While the precise mechanism by which ammonium suppresses cell viability has not been fully elucidated, evidence suggests that intracellular acidification secondary to ammonium production may induce apoptosis (13), or that excess ammonium may contribute to N- and O-glycosylation of proteins (14,15), potentially reducing cell viability.

While it is generally recognized that cancer cells demonstrate increasing metabolic reprogramming through progression (16,17), adaptation to glutamine availability in non-metastatic compared to metastatic breast cancer cells has not yet been evaluated. Here we determined the effect of variable glutamine concentration in cell culture media in non-metastatic M-Wnt and metastatic metM-Wnt$^{lung}$ murine mammary cancer cell lines (18). Previous work in these cell lines has shown that metM-Wnt$^{lung}$ cells have higher levels of oxidative metabolism compared to their non-metastatic counterparts (18), suggesting that these cells may be prone to higher levels of glutamine oxidation, and therefore ammonium toxicity. In the present studies, we determined the effect of variable glutamine concentration on viability, glutamine flux, oxidative stress, and expression of genes

involved in glutamine metabolism. We hypothesized that increased glutamine metabolism in high glutamine culture conditions suppresses viability of metastatic cells through production of ammonium. This work suggests a metabolic vulnerability of metastatic compared to non-metastatic cells, and highlights targeting glutamine metabolism as a strategy to prevent metastatic progression.

## 2.3 Methods

### 2.3.1 Chemicals and reagents

N-acetylcysteine, ammonium chloride, dimethyl α-ketoglutarate, and glutaminase inhibitor 968 were purchased from Sigma.

### 2.3.2 Cell Culture

Non-metastatic M-Wnt and metastatic metM-Wnt$^{lung}$ murine mammary cancer cells (18) were constitutively cultured in DMEM (Sigma, St. Louis, MO) with 5 mM glucose and either 2 mM or 4 mM glutamine, as indicated. Complete cell culture media contained a final concentration of 1% penicillin/streptomycin antibiotic solution (Gibco, Waltham, MA) and 10% fetal bovine serum (Gibco).

### 2.3.3 MTT Assay

Cells were seeded at equal densities into 96 well plates, attached overnight, and treated with reagents indicated for 48 hours. Cell viability was determined through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's recommendations (Sigma St. Louis, MO). Briefly, cell culture media was removed and 1X MTT reagent added in serum-free media, and incubated for two hours at 37°C. Following incubation, media was removed, and crystals were dissolved in DMSO. Absorbance was measured at 570 nm.

### 2.3.4 RNA Isolation and qRT-PCR

RNA was isolated from cell samples using TRI-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. RNA was reverse transcribed to cDNA with MMLV

reverse transcriptase (Promega, Madison, WI). PCR was conducted with a LightCycler 480 instrument with LightCyler 480 SYBR Green I Master Mix (Roche, Indianapolis, IN). The comparative Ct method ($2^{-\Delta Ct}$) was used for data normalization, with 2 mM glutamine culture conditions as the reference group.

### 2.3.5 Glutamine Flux

Cells constitutively grown in 2 mM or 4 mM glutamine were seeded into 60 mm dishes and grown to 80% confluence. Media was removed and replaced with fresh media containing 100% of either 2 mM or 4 mM universally labeled $^{13}C_5$-glutamine for two hours at 37°C prior to harvesting samples in 70% ethanol heated to 70°C. As an internal standard, 1 µg norvaline was added to each sample, vortexed, and incubated at 95° for 5 minutes. Samples were cooled on ice for 5 minutes and centrifuged at 14,000 RPM for 5 minutes at room temperature. Cell pellets were analyzed for protein content with a bicinchoninic acid assay (BCA) assay (ThermoFisher, Waltham, MA). Supernatants were dried and derivatized with methoxylamine hydrochloride (MOX) in pyridine and prepared with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% (wt/wt) *tert*-butyldimethylchlorosilane (MTBSTFA + 1% (wt/wt) TBDMSClS) for analysis with gas chromatography-mass spectrometry (Thermo TSQ 8000 triple quadrupole mass spectrometer coupled with a Thermo Trace 1310 gas chromatrophagy) (19).

### 2.3.6 $NAD^+$/NADH Assay

Cells constitutively grown in 2 mM or 4 mM glutamine were seeded into white-walled clear bottom 96 well plates (Corning, Corning, NY) overnight. Cells were washed once with calcium and magnesium-free phosphate buffered saline (PBS), and $NAD^+$ and NADH were detected using $NAD^+$/NADH-Glo Assay kit (Promega) according to the manufacturer's instructions. Luminescence was measured using a Synergy H1 Multi-Mode reader.

### 2.3.7 Oxidative Stress Assays

Cells in 2 mM or 4 mM glutamine were seeded into white-walled clear bottom 96 well plates (Corning). On day 2, ratios of NADPH/NADP+ and GSH/GSSG ratios were measured with

NADP/NADPH-Glo and GSH/GSSG-Glo Assays (Promega) according to the manufacturer's instructions. Luminescence was measured using a Synergy H1 Multi-Mode reader.

### 2.3.8 ROS Assay

Cellular reactive oxygen species (ROS) levels were measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA) (20,21). Cells were plated into black-walled clear bottom 96-well plates (Corning). For time-course ROS assays, cells grown in 2 mM glutamine were seeded and the next day, media was changed to 4 mM glutamine for two, six, twelve, or twenty-four hours. Following treatment, media was removed and cells were washed once with PBS. Cells were then incubated at 37°C in 10 μM DCFH-DA in PBS for 20 minutes. Fluorescence was measured using a Synergy H1 Multi-Mode reader (excitation/emission 485/530). Fluorescence measures were normalized to cell viability, as measured by MTT.

### 2.3.9 Statistical Analysis

Values presented as means + SEM. Statistics were analyzed using SAS software version 9.4. P values $< 0.05$ were considered significant.

Table 1. Primers used for qRT-PCR.

| | |
|---|---|
| Gls | Forward: 5'-CTACAGGATTGCGAACATCTGAT-3'<br>Reverse: 5'-ACACCATCTGACGTTGTCTGA-3' |
| Gls2 | Forward: 5'-CAGAGGGACAGGAGCGTATC-3'<br>Reverse: 5'-TTCTTTCGGAATGCCTGAGTC-3' |
| Glul1 | Forward: 5'-TGAACAAAGGCATCAAGCAAATG-3'<br>Reverse: 5'-TGAACAAAGGCATCAAGCAAATG-3' |
| Got2 | Forward: 5'-GGACCTCCAGATCCCATCCT-3'<br>Reverse: 5'-GGTTTTCCGTTATCATCCCGGTA-3' |
| Gpt2 | Forward: 5'-AACCATTCACTGAGGTAATCCGA -3'<br>Reverse: 5'-GGGCTGTTTAGTAGGTTTGGGTA -3' |
| Psat1 | Forward: 5'-CAGTGGAGCGCCAGAATAGAA-3'<br>Reverse: 5'-CCTGTGCCCCTTCAAGGAG-3' |
| Glud1 | Forward: 5'-CCCAACTTCTTCAAGATGGTGG-3'<br>Reverse: 5'-AGAGGCTCAACACATGGTTGC-3' |

## 2.4 Results

We maintained M-Wnt and metM-Wnt$^{lung}$ cells in 2 mM or 4 mM glutamine in order to determine how variable glutamine concentrations affected viability of non-metastatic compared to metastatic cells. We show that 4 mM glutamine conditions decreased viability of metM-Wnt$^{lung}$ cells by 48% (Figure 2.1), but, in contrast, viability of M-Wnt cells was not affected by glutamine concentration. These results suggest that glutamine concentrations differentially affect viability of non-metastatic and metastatic cancer cells.

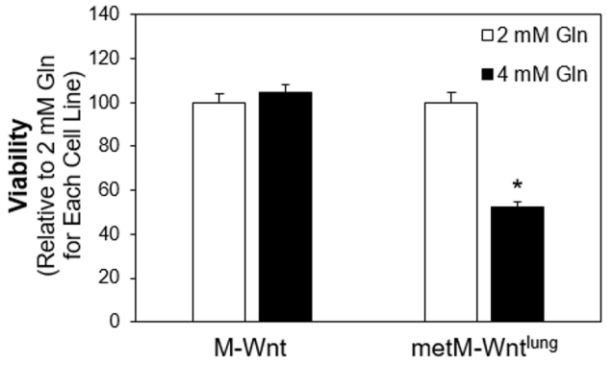


Figure 2.1. Effect of glutamine concentration on viability.

Viability of M-Wnt and metM-Wnt$^{lung}$ cells maintained in 2 mM or 4 mM glutamine was assessed by MTT (A). Results are expressed as means + SEM. Asterisk (*) indicates $P < 0.05$ relative to 2 mM glutamine.

We also determined if the changes in cell viability in variable glutamine concentrations corresponded to changes in mRNA levels of genes related to glutamine metabolism. Catabolism of glutamine occurs through a two-step process in which glutamine is first converted to glutamate by glutaminase (Gls), and glutamate is subsequently converted to αKG by either glutamate dehydrogenase 1 (Glud1) or by transaminases (3). Compared to cells grown in 2 mM glutamine, culturing M-Wnt cells in 4 mM glutamine significantly decreased relative mRNA level of Glud1 by 47% and the transaminases Got2 and Gpt2 by 95% and 34%, respectively (Figure 2.2A). In contrast, 4 mM glutamine suppressed the level of only Got2 by 79% in metM-Wnt$^{lung}$ cells (Figure 2.2B). Synthesis of glutamine from glutamate is mediated by glutamine synthetase (Glul) (3). There was a 48% decrease in Glul mRNA level in 4 mM compared to 2 mM glutamine culture conditions in M-Wnt cells, whereas Glul was not affected by glutamine concentration in metM-Wnt$^{lung}$ cells (Figure 2.2A). These results suggest that glutamine concentration reduces expression of genes related to glutamine catabolism in M-Wnt cells, but not in metM-Wnt$^{lung}$ cells.

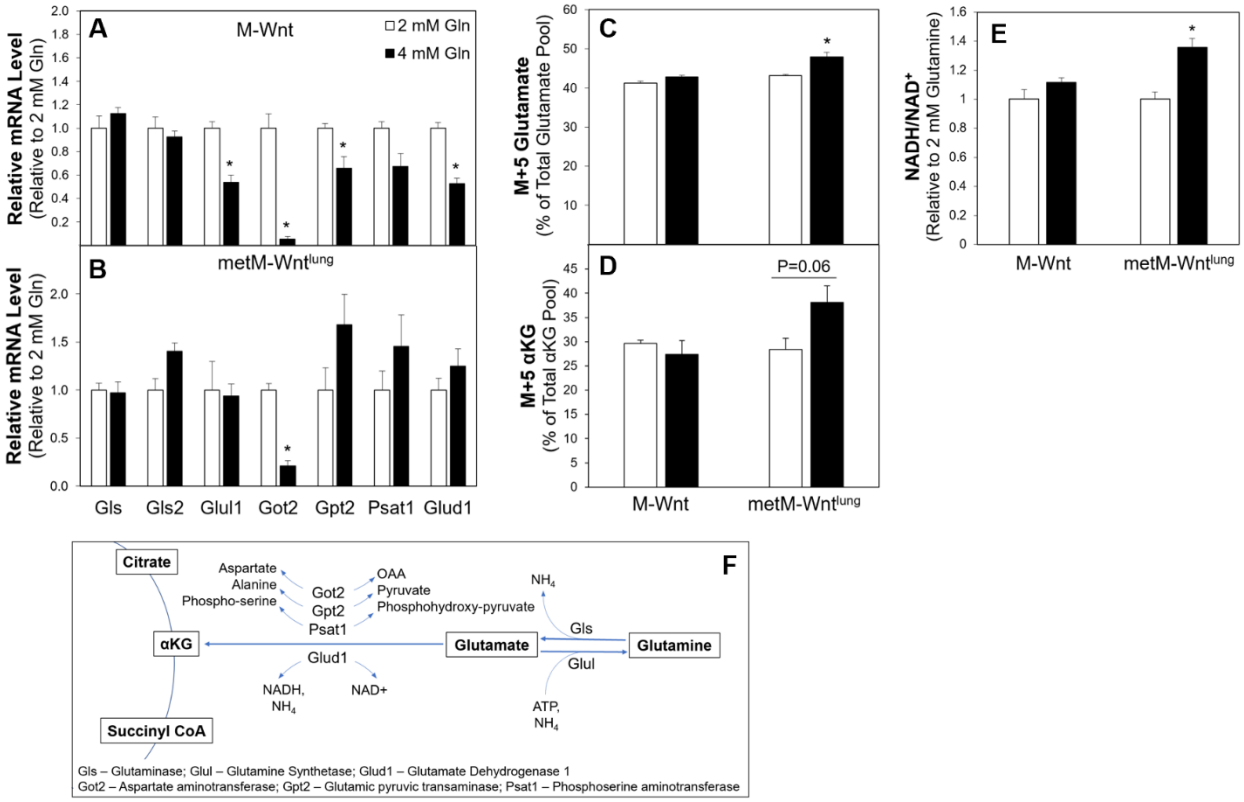


Figure 2.2. Glutamine metabolism in variable glutamine concentrations.

Cells were maintained in 2 or 4 mM glutamine and mRNA level of genes involved in glutamine metabolism in M-Wnt (A) and metM-Wnt$^{lung}$ cells (B) determined; labeled $^{13}C_5$-labeled glutamine was used to determine labeling of the downstream metabolites glutamate (C) and α-ketoglutarate (D); and NADH/NAD$^+$ ratios were measured (E). Overview of glutaminase catabolism (F). Results are expressed as means + SEM. Asterisk (*) indicates $P < 0.05$ relative to 2 mM glutamine.

We further tested if the changes in gene expression corresponded to differences in flux of glutamine to glutamate or αKG in either culture condition utilizing uniformly stably labeled glutamine. We show that metM-Wnt$^{lung}$ cells had a significant enrichment (11%) of M+5 glutamate (Figure 2.2C), and a trend towards enrichment (34%) of M+5 αKG (Figure 2.2D) in 4 mM glutamine compared to 2 mM glutamine (22) (Figure 2.2D). In contrast, there was no enrichment of M+5 labeling of glutamate or αKG in M-Wnt cells in 4 mM compared to 2 mM glutamine. These results suggest that metM-Wnt$^{lung}$ cells have increased glutamine flux in 4 mM glutamine concentrations, whereas glutamine concentration in the media does not affect flux in M-Wnt cells.

Because the NADH/$NAD^+$ ratio is an indicator of the reductive-oxidative (redox) balance and bioenergetic status of the cell and is reported to be increased in breast cancer cells (23,24), we assessed the effect of glutamine concentration on the NADH/$NAD^+$ ratio in both cell lines. Culturing cells in 4 mM glutamine significantly increased the NADH/$NAD^+$ ratio by 36% in metM-$Wnt^{lung}$ cells, but had no effect on the NADH/$NAD^+$ ratio in M-Wnt cells (Figure 2.2E). Collectively, these data suggest that M-Wnt cells suppress glutamine metabolism in response to 4 mM glutamine, while metM-$Wnt^{lung}$ cells do not regulate glutamine metabolism in variable glutamine concentrations.

Nutrient oxidation for energy production is a multistep process involving generation of NADH and/or $FADH_2$, which drives ATP production at the electron transport chain (ETC). Production of ATP at the ETC is linked with production of reactive oxygen species (ROS), which are capable of modifying transcription and may be cytotoxic in excess. We investigated whether the changes in cell viability and gene expression observed in 4 mM glutamine corresponded to changes in intracellular ROS or overall redox balance. We determined the ratios of reduced/oxidized glutathione (GSH/GSSG) and NADPH/$NADP^+$, two important redox systems used to neutralize intracellular ROS (25). We found that there was a 14% reduction in the GSH/GSSG ratio in metM-$Wnt^{lung}$ cells grown in 4 mM glutamine compared to 2 mM glutamine, and that the GSH/GSSG ratio was not affected by glutamine concentration in M-Wnt cells (Figure 2.3A). However, higher glutamine culture conditions did not affect the NADPH/$NADP^+$ ratio in either cell line (Figure 2.3B). In order to determine if the changes in GSH/GSSG in metM-$Wnt^{lung}$ cells were related to elevated oxidative stress, we measured intracellular ROS in cells grown in variable glutamine. Neither M-Wnt nor metM-$Wnt^{lung}$ cells grown in 4 mM glutamine culture conditions had higher ROS levels compared than their counterparts in 2 mM glutamine (Figure 2.3C, 2.3D). In order to determine if ROS levels transiently changed in response to changes in glutamine concentration, we changed the media to 4 mM glutamine in cells previously growing in 2 mM glutamine and measured ROS two, six, twelve, and twenty-four hours later. Short-treatment with 4 mM glutamine did not increase ROS formation at any time point in either cell line (Figure 2.3C, 2.3D), suggesting that increasing glutamine concentration does not increase intracellular ROS production. Further, in order to determine if reducing oxidative stress would increase viability of metM-$Wnt^{lung}$ cells in 4 mM glutamine, we cultured cells with the antioxidant molecule N-acetylcysteine. While a 1 mM dose of N-acetylcysteine reduced ROS by 39% and 24% in M-Wnt and metM-$Wnt^{lung}$

cells (Figure 2.3E), respectively, this dose did not significantly affect viability of either cell line grown in 4 mM glutamine (Figure 2.3F). These data suggest that changes in redox balance in 4 mM glutamine do not affect cell viability.

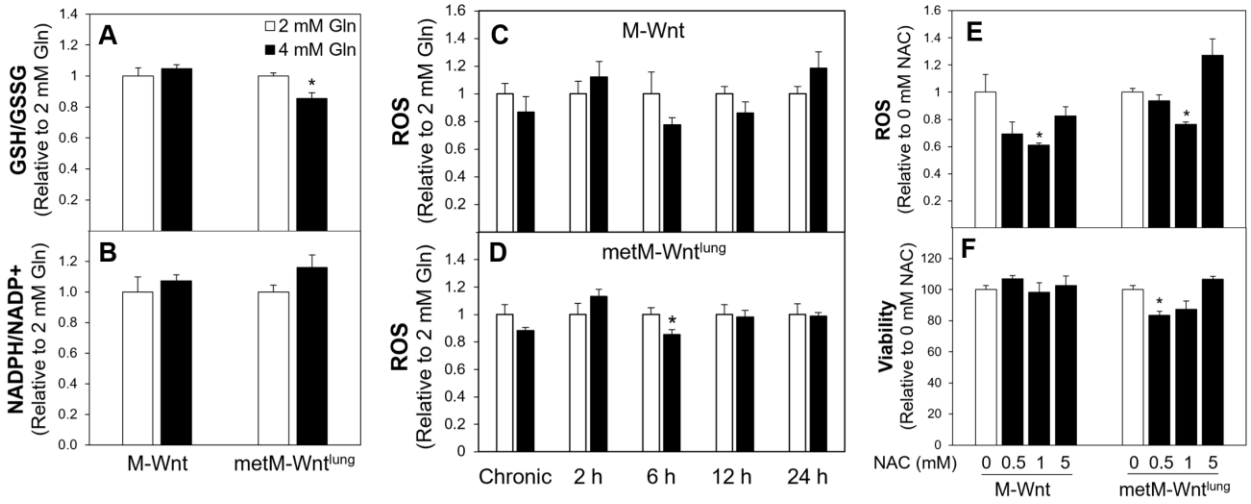


Figure 2.3. Effect of glutamine concentration on oxidative stress markers.

GSH/GSSG (A) and NADPH/NADP$^+$ (B) were measured in M-Wnt and metM-Wnt$^{lung}$ cells. ROS levels were assessed in M-Wnt (C) and metM-Wnt$^{lung}$ cells (D) chronically grown in 2 mM or 4 mM glutamine, and in cells grown in 4 mM glutamine for indicated times. The effects N-acetylcysteine (NAC) on ROS (E) and viability (F) were assessed in cells grown in 4 mM glutamine. Results are expressed as means + SEM. Asterisk (*) indicates $P < 0.05$ relative to 2 mM glutamine (in A and D) or relative to 0 mM NAC (E and F).

A potential mechanism for the effect of 4 mM glutamine on viability of metM-Wnt$^{lung}$ cells may be a result of increasing concentrations of carbon or nitrogen in the cell. In order to test the supply of carbon mechanism, we treated cells grown in 2 mM glutamine with 1 or 2 mM dimethyl α-ketoglutarate (DMαKG), a membrane-permeable form of αKG. Neither dose of DMαKG affected viability in either cell line (Figure 2.4A), suggesting that the carbon supplied in 4 mM glutamine culture conditions is not the source of decreased viability in metM-Wnt$^{lung}$ cells. During glutaminolysis, the amide nitrogen of glutamine is lost as ammonium in the glutaminase reaction, and the amine nitrogen is either lost as ammonium in the glutamate dehydrogenase reaction or transferred to an α-keto acid by transaminases (3,10). We therefore treated cells growing in 2 mM glutamine with ammonium chloride to determine if increasing ammonium concentrations reduces viability of metM-Wnt$^{lung}$ cells. While a low dose (1 mM) of ammonium chloride significantly

decreased viability of metM-Wnt$^{lung}$ cells by 31%, the same dose had no effect on viability of M-Wnt cells (Figure 2.4B). Similarly, a high dose (5 mM) of ammonium chloride suppressed viability by 57% in metM-Wnt$^{lung}$ cells and 27% in M-Wnt cells (Figure 2.4B, 2.4C), indicating that metM-Wnt$^{lung}$ cells are significantly more sensitive to ammonium chloride treatment compared to M-Wnt cells.

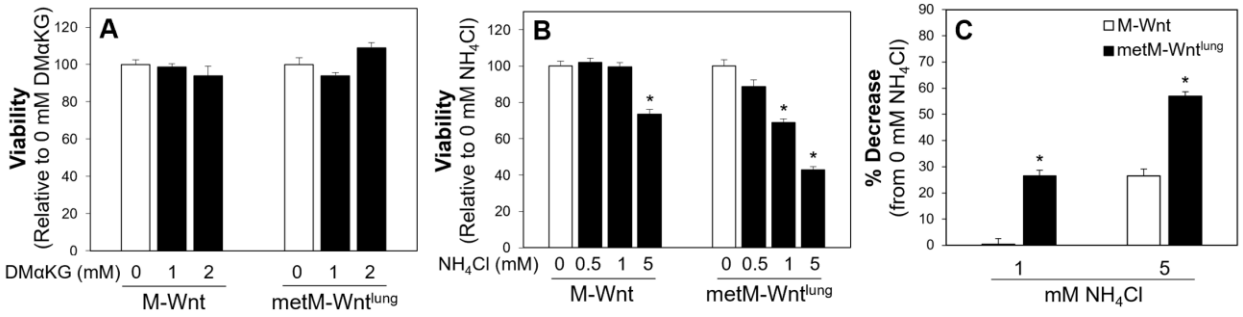


Figure 2.4. Effect of dimethyl α-ketoglutarate and ammonium chloride on cell viability.

The effect of ectopic addition of a membrane-permeable form of α-ketoglutarate (dimethyl α-ketoglutarate, DMαKG) (A) or ammonium chloride ($NH_4Cl$) (B) on viability was assessed in cells constitutively grown in 2 mM glutamine (A). Percent decrease in viability of cells treated with ammonium chloride is shown relative to 0 mM ammonium chloride (C). Results are expressed as means + SEM. Asterisk (*) indicates $P < 0.05$ relative to 0 mM ammonium chloride (B) or relative to M-Wnt cells (C).

Given that recent literature has shown preferential utilization of transaminases compared to glutamate dehydrogenase in proliferating cancer cells (12), we hypothesized that ammonium is primarily produced by the glutaminase reaction, and that ammonium production at this reaction suppresses viability of metM-Wnt$^{lung}$ cells. In order to test if the glutaminase reaction was responsible for the decrease in viability of metM-Wnt$^{lung}$ cells in 4 mM glutamine, we treated cells with the glutaminase inhibitor 968. Contrary to our hypothesis, treatment with the glutaminase inhibitor 968 suppressed viability of both cell lines to an equal extent (Figure 2.5A). Culturing cells with glutaminase inhibitor 968 and 2 mM DMαKG did not rescue viability compared to treatment with inhibitor alone (Figure 2.5B). These results suggest that inhibiting glutaminase activity does not rescue viability of metM-Wnt$^{lung}$ cells in 4 mM glutamine.

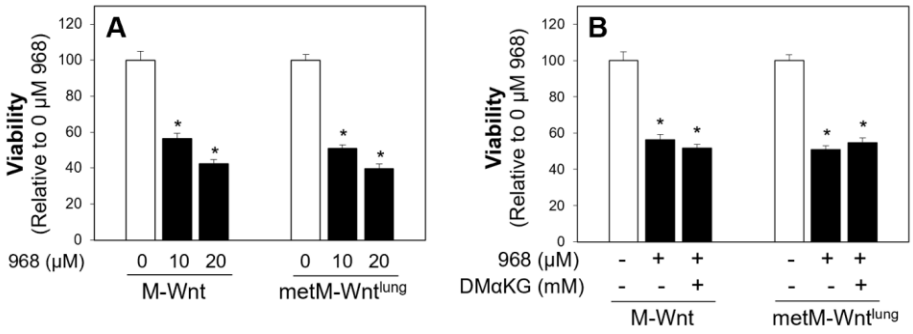


Figure 2.5. Effect of glutaminase inhibitor on cell viability.

The effect of the glutaminase inhibitor 968 alone (A) or in combination (10 μM) with DMαKG (2 mM) (B) on viability was assessed in cells constitutively grown in 4 mM glutamine. Results are expressed as means + SEM. Asterisk (*) $P < 0.05$ relative to 0 μM glutaminase inhibitor.

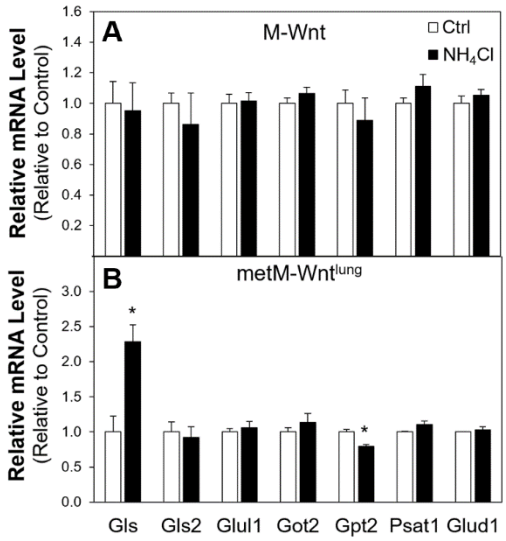


Figure 2.6. Effect of ammonium chloride treatment on gene expression.

Relative mRNA level was determined by qRT-PCR in M-Wnt (A) and metM-Wnt$^{lung}$ (B) cells grown in 2 mM glutamine with 1 mM ammonium chloride ($NH_4Cl$) or sodium chloride (Ctrl) for 48 h. Results are expressed as means + SEM. Asterisk (*) $P < 0.05$ relative to Ctrl.

Finally, we explored whether ammonium chloride treatment recapitulates the changes in mRNA levels we observed in M-Wnt cells cultured in 4 mM glutamine compared to 2 mM glutamine. We show that ammonium chloride treatment had no effect on expression of genes related to glutamine catabolism in M-Wnt cells (Figure 2.6A). Ammonium chloride treatment increased glutaminase (Gls) expression, and decreased Gpt2 expression in metM-Wnt$^{lung}$ cells (Figure 2.6B). These results suggest that increased ammonium concentrations are not responsible for the changes in gene expression induced by 4 mM glutamine in M-Wnt cells.

## 2.5 Discussion

In this work we investigated the effects of variable glutamine concentrations on metabolic reprogramming and viability in non-metastatic compared to metastatic murine mammary cancer cells. We show that metastatic metM-Wnt$^{lung}$ cells did not alter glutamine metabolism when cultured in 4 mM glutamine while non-metastatic M-Wnt cells suppress glutamine metabolism.

Previous work demonstrates that glutamine utilization varies considerably between different types of cancer, and limited research has shown that glutamine utilization varies with degree of cancer progression. One factor that may alter the use of glutamine is the tumor microenvironment. Evidence suggests that while breast cancer cells which use glutamine at the primary tumor, the same cells switch to pyruvate carboxylase-mediated anaplerosis of the TCA cycle following metastasis to the lung (26). These data are in agreement with research showing resistance to glutaminase inhibition by primary lung cancer cells and preferential use of pyruvate carboxylase for anaplerosis at the lung (27–29). While these data support a key role of glutamine metabolism in primary breast tumors, work in different models of breast cancer have shown that glutamine utilization varies by molecular subtype of breast cancer. For example, luminal breast cancer cells, which express hormone receptors, do not require glutamine for growth (30), while basal breast cancers are sensitive to glutaminase inhibition (30,31). These data collectively suggest that while the tumor microenvironment plays an important role in dictating glutamine utilization, the genetic profile of the tumor cells also contributes to glutamine utilization. Indeed, regulation of glutaminase is partly under control of oncogene expression, including expression of c-Myc and N-Myc (32–34), K-ras (35), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) (36), suggesting that more advanced cancer cells may have increased upregulation

of glutaminase and glutamine metabolism. In accordance with this, recent work has shown that more aggressive prostate cancer cell lines increase glutamine flux into the TCA cycle compared to their less metastatic counterparts (37). Our current model of non-metastatic and metastatic mammary cancer supports that more advanced cancers have increased utilization of glutamine, and that utilization of glutamine increases in response to glutamine dose in metastatic cells.

In the current study, maintaining mammary cancer cells in variable glutamine concentrations showed that metM-Wnt$^{lung}$ cells were sensitive to 4 mM glutamine concentrations, whereas glutamine concentration did not affect viability of M-Wnt cells (Figure 2.1). An interesting finding of this work was that only M-Wnt cells reprogrammed their glutamine metabolism in response to 4 mM glutamine, whereas metM-Wnt$^{lung}$ cells did not modify glutamine metabolism in response to increasing glutamine concentration. M-Wnt cells transcriptionally downregulated expression of transaminases and glutamate dehydrogenase in 4 mM glutamine, and there was no increase in flux to αKG or glutamate from labeled 4 mM glutamine in these cells (Figure 2.2). In contrast, there was significant enrichment of M+5 glutamate from labeled glutamine in metM-Wnt$^{lung}$ cells in 4 mM glutamine (Figure 2.2), suggesting that only M-Wnt cells are able to reprogram metabolism to reduce glutamine catabolism and flux into the TCA cycle. Given that glutaminase activity is inhibited when cells are in a high energy state, and that the metM-Wnt$^{lung}$ cells have increased NADH/NAD$^{+}$ in 4 mM glutamine, it is surprising that metM-Wnt$^{lung}$ cells have decreased glutaminase activity in 4 mM glutamine. These data may suggest that other modes of regulation contribute to sustained high glutaminase activity in metM-Wnt$^{lung}$ cells. Collectively, these data show that only non-metastatic M-Wnt cells are able to reprogram glutamine metabolism in response to high glutamine concentration.

One mode of signal transduction that translates metabolic stress information into changes in transcriptional activity is oxidative stress. Intermediate levels of ROS can stimulate transcription of genes associated with antioxidant defense and cell survival (38). We investigated whether an increase in oxidative stress in 4 mM glutamine explained the changes in transcriptional activity observed in the M-Wnt cells or viability in metM-Wnt$^{lung}$ cells. We showed no effect of increasing glutamine concentration on measures of ROS or redox balance in M-Wnt cells (Figure 2.3), suggesting an alternate mechanism reprograms metabolism in these cells. In addition, while 4 mM

glutamine culture conditions decreased the GSH/GSSG ratio in metM-Wnt$^{lung}$ cells, suggesting oxidative stress, there was no change in the NADPH/NADP$^{+}$ ratio, nor did treating cells with the antioxidant molecule N-acetylcysteine rescue viability, suggesting that oxidative stress in metM-Wnt$^{lung}$ cells is not responsible for reduced viability in 4 mM glutamine.

Interestingly, we found that ectopic ammonium chloride treatment more dramatically suppressed viability of metM-Wnt$^{lung}$ cells compared to M-Wnt cells, potentially explaining their reduced viability in 4 mM glutamine (Figure 2.4). However, treatment with the glutaminase inhibitor 968 did not rescue viability of cells cultured in 4 mM glutamine (Figure 2.5). In addition, ammonium treatment did not regulate transcriptional activity in M-Wnt cells (Figure 2.6). As such, it is currently unclear how M-Wnt cells detect high levels of glutamine, and translate this signal into a transcriptional response to reprogram glutamine metabolism.

The data presented in this study support that cancer cells at different stages of progression differentially reprogram metabolism in response to glutamine availability. While non-metastatic cells appear to utilize a system to detect and respond to high levels of glutamine, this mechanism is lost in metastatic cells. Further research is needed to elucidate the mechanism by which higher levels of glutamine are translated into changes in transcriptional activity in M-Wnt cells, and why this regulatory system is lost or impaired in metM-Wnt$^{lung}$ cells. Future work is also required to determine the mechanism by which M-Wnt cells mediate their resistance to ammonium chloride treatment, and how this mechanism is altered or lost in metM-Wnt$^{lung}$ cells. Overall, our work provides new evidence of differences in glutamine metabolism and utilization that occur over the course of cancer progression.

## 2.6 Acknowledgements

The authors acknowledge the use of the facilities of the Bindley Bioscience Center, a core facility of the NIH-funded Indiana Clinical and Translational Sciences Institute.

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# CHAPTER 3. HYPOXIA-MEDIATED ATF4 INDUCTION PROMOTES SURVIVAL IN DETACHED CONDITIONS IN METASTATIC MURINE MAMMARY CANCER CELLS

## 3.1 Abstract

Regions of hypoxia are common in solid tumors and drive changes in gene expression that increase risk of cancer metastasis. Tumor cells must respond to the stress of hypoxia by activating genes to modify cell metabolism and antioxidant response to improve survival. The goal of the current studies was to determine the effect of hypoxia on cell metabolism and markers of oxidative stress in non-metastatic M-Wnt compared to metastatic metM-Wnt$^{lung}$ murine mammary cancer cell lines. We show that hypoxia increased expression of genes involved in antioxidant response and induces a greater suppression of glutamine to glutamate conversion in metastatic cells. Interestingly, hypoxia increased the mRNA level of the transaminase glutamic pyruvic transaminase 2 (Gpt2, 7.7-fold) only in metM-Wnt$^{lung}$ cells. The change in Gpt2 expression was accompanied by transcriptional and translational induction of the integrated stress response effector protein activating transcription factor 4 (ATF4), which mediated survival of hypoxic metastatic cells in detached conditions. These data indicate that more aggressive, metastatic cancer cells rely on activation of metabolic reprogramming and induction of antioxidant defense for survival following in hypoxic conditions.

## 3.2 Introduction

Regions of hypoxia, or low oxygen tension, are prevalent in solid tumors and are inversely associated with survival in cancer patients (1,2). Hypoxia drives the production of mitochondrial reactive oxygen species (ROS) at complex III of the electron transport chain (3,4) which, at high levels, impairs cell survival. Cancer cells respond to hypoxia and oxidative stress through multiple overlapping mechanisms, including stabilization of the transcription factor hypoxia inducible factor 1α (HIF1α) and activation of the integrated stress response (ISR) (5–8). Activation of these pathways reduces oxidative stress by limiting production or increasing neutralization of ROS, thereby increasing cancer cell survival.

In hypoxia, the transcription factor HIF1α transactivates genes to reduce oxidation of nutrients in the tricarboxylic acid (TCA) cycle, consequently limiting production of ROS at the electron transport chain (9–13). Hypoxia, for example, reduces flux of pyruvate into the TCA cycle by upregulating pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA) (9,10). Similarly, hypoxia reduces flux of glutamine into the TCA cycle as α-ketoglutarate (αKG), and promotes reductive carboxylation of αKG to citrate (12). Finally, HIF1α activates genes required for production of the antioxidant molecules NADPH and glutathione, thereby increasing clearance of ROS in hypoxia (6). Thus, metabolic reprogramming by HIF1α represents an important axis of reducing oxidative stress in hypoxia.

Hypoxia also activates the ISR pathway. In this pathway, kinases are activated by specific cell stresses, including endoplasmic reticulum (ER) stress, oxidative stress, and hypoxic stress (8). The activated kinases phosphorylate elongation initiation factor 2α (eIF2α), which globally reduces mRNA translation while simultaneously increasing translation of select transcripts, including activating transcription factor 4 (ATF4) (8,14). ATF4 is the main effector protein of the ISR, and it modifies gene expression to reduce cell stress and restore cell homeostasis, or induce apoptosis if the degree of cell stress is too high to be resolved (8). Mechanisms by which ATF4 reduces cell stress include altering cell metabolism and upregulating oxidative stress response (15,16). Given its integral role in reducing cell stress, previous work has identified ATF4 as key protein involved in metastatic processes such as migration (17,18) and resistance to anoikis (19), suggesting that its expression is not only critical to cell survival, but also to cancer progression.

Although previous work has examined the role of ATF4 in cancer cell metabolism (20) and pro-metastatic outcomes (17–19), currently no research is available examining ATF4 induction in response to hypoxia throughout the course of cancer progression. Here we used non-metastatic M-Wnt and metastatic metM-Wnt$^{lung}$ murine mammary cancer cell lines (21) to model the cellular stress response to hypoxia. In the present work, we assessed the effect of hypoxia on metabolic adaptation and processes involved in metastatic progression in M-Wnt and metM-Wnt$^{lung}$ cells. We hypothesized that ATF4 mediates survival of hypoxic metM-Wnt$^{lung}$ cells in detached conditions. Identification of ATF4 as a protein important for metastatic progression will further highlight ATF4 as a potential therapeutic target to prevent metastasis in cancer patients.

## 3.3 Methods

### 3.3.1 Cell Culture

Murine mammary cancer M-Wnt and metM-Wnt$^{lung}$ cells (21) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO) with 5 mM glucose, 2 mM glutamine, no sodium pyruvate, with 10% final concentration fetal bovine serum (Gibco, Waltham, MA) and 1% final concentration penicillin-streptomycin (Gibco). For experiments in hypoxia, cells were seeded and attached in normoxia. The following day, media was changed and cells were transferred to a Billups-Rothenberg modular incubator chamber (Billups Rothenberg, Del Mar, CA). The modular incubator chamber was flushed with at least 100 L of 1% oxygen gas mixture, and cells were incubated at 37°C. Cells were routinely cultured at 37°C with 5% carbon dioxide.

### 3.3.2 RNA Isolation and qRT-PCR

RNA was isolated using TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNA was reverse transcribed using MMLV reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR was performed using LightCyler 480 SYBR Green I Master Mix (Roche, Indianapolis, IN). The comparative Ct method ($2^{-\Delta Ct}$) was used to normalize mRNA data, with normoxia or siCtr as the reference group.

### 3.3.3 Western Blotting

Cell samples were washed twice with ice-cold 1X phosphate-buffered saline (PBS) prior to harvesting in radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling, Danvers, MA) with 1% phenylmethanesulfonyl fluoride protease inhibitor (PMSF, Cell Signaling) and phosphatase inhibitor cocktail 2 (P5726, Sigma). Cells were lysed with sonication and vortexing, and cell debris was pelleted with centrifugation at 14,000 RPM for 15 minutes. Protein concentration was determined with Pierce bicinchoninic acid assay (BCA) protein assay kit (ThermoFisher, Waltham, MA). Equal amounts of protein (25 μg) were separated on polyacrylamide gels and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad, Hercules CA). Membranes were probed with antibodies for actin and ATF4 (Cell Signaling) overnight in 5% non-fat dry milk. Protein was detected using an Odyssey CLx imaging system (Li-Cor, Lincoln, NE).

### 3.3.4 Low Attachment Survival Assay

Cells were incubated for 48 h in normoxia or hypoxia. Cells were then removed from the modular incubator chamber, trypsinized, and 50 μL of cell suspension was rapidly re-seeded at equal cell densities into polyhydroxyethylmethacrylate (poly-HEMA) coated plates. To assess viability, 5 μL of 10X MTT solution (Sigma) was added to each well and the plate was incubated at 37°C for two hours. MTT crystals were dissolved in 150 μL DMSO and absorbance was read at 570 nm utilizing a Biotech spectrophotometer.

### 3.3.5 Transwell Migration Assay

Cells were incubated in normoxia or hypoxia for 48 h. Following incubation, cells were removed from the modulator incubator chamber, trypsinized, pelleted, and re-suspended in serum-free media. Cells were seeded into transparent transwells with 8 μm pore size (Corning, Corning, NY) mounted in 24 well plates. Serum-containing media was added below the transwells, and cells were incubated in normoxia for 15 h at 37°C. Migration was assessed by fixing cells in methanol and staining with crystal violet. Photos of five random fields in the transwells were captured and cells were counted for analysis. Data are presented as proportion of migrated cells from total cell count.

### 3.3.6 GSH and NADPH Assay

Cells were seeded into white walled, clear bottom 96 well plates (Corning) and grown overnight in normoxia. The next day, plates were transferred to hypoxia or maintained in normoxia for 48 h. Following incubation, cells were washed once with PBS, and analyzed for GSH/GSSG or NADPH/$NADP^+$ ratios using GSH/GSSG-Glo Assay or NADPH/$NADP^+$ Glo Assay kits (Promega) according to the manufacturer's instructions.

### 3.3.7 ROS Assay

Intracellular ROS was measured with 2',7'-dichlorofluorescin diacetate (DCFH-DA) as described previously (22,23). Cells were seeded into black walled 96 well plates and grown overnight in normoxia. Plates were then incubated for 48 h in normoxia or hypoxia. After incubation, media was removed and cells were washed once with PBS. Cells were incubated in 10 μM DCFH-DA

in PBS for 20 minutes prior to measuring fluorescence in a Synergy H1 Multi-Mode reader (excitation/emission 485/530). Cell viability was determined using the MTT assay immediately following fluorescence measurement and used to normalize fluorescence.

### 3.3.8 Metabolic Flux Analysis

Cells were seeded into 60 mm dishes and grown in normoxia overnight. The following day, media was changed and dishes were either maintained in normoxia or transferred to a two-port zipper-lock AtmosBag glove bag (gas volume 50 L, Sigma). The glove bag was flushed with at least 750 L of 1% oxygen gas, sealed, and incubated at 37°C. All dishes were incubated for 46 h. After incubation, media on all dishes was replaced with fresh media containing 100% 5 mM universally labeled $^{13}C_6$ glucose (with 2 mM unlabeled glutamine) or 100% 2 mM universally labeled $^{13}C_5$ glutamine (with 5 mM unlabeled glucose). For hypoxia samples, media was changed inside the glove bag to maintain hypoxia for the duration of the experiment. Dishes were incubated with labeled media for two hours at 37°C. Dishes were then removed from normoxia or the glove bag and rapidly scraped into a 70% ethanol solution heated to 70°C (24). Cells were vortexed, heated at 95°C for 5 minutes, and chilled on ice for 5 minutes. Cell debris was pelleted by centrifugation at 14,000 x g for 5 minutes. Supernatants were dried under a stream of nitrogen and the dried samples were derivatized with methoxylamine hydrochloride (MOX) in pyridine and prepared with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% (wt/wt) *tert*-butyldimethylchlorosilane (MTBSTFA + 1% (wt/wt) TBDMSClS). Samples were analyzed with gas chromatography mass spectrometry (Thermo TSQ 8000 triple quadrupole mass spectrometer coupled with a Thermo Trace 1310 gas chromatrophagy).

### 3.3.9 siRNA Transfection

Cells were transfected with ON-TARGETplus SMARTpool siRNA against murine ATF4 or non-targeting control (Dharmacon, Lafayette, CO) using polyethylenimine (PEI, Polysciences, Warrington, PA) in serum-free media. After 12 h, transfection media was replaced with fresh serum-containing media, and cells were grown for an additional 48 h in hypoxia or normoxia prior to analysis.

### 3.3.10 Statistical Analysis

All values are presented as means + SEM. All statistics were analyzed using SAS software version 9.4. P values < 0.05 were considered significant.

Table 2. Primers used for qRT-PCR.

| | |
|---|---|
| Gclc | Forward: 5'-GGGGTGACGAGGTGGAGTA-3'<br>Reverse: 5'-GTTGGGGTTTGTCCTCTCCC-3' |
| Gclm | Forward: 5'-AGGAGCTTCGGGACTGTATCC-3'<br>Reverse: 5'-GGGACATGGTGCATTCCAAAA-3' |
| Gss | Forward: 5'-CAAAGCAGGCCATAGACAGGG-3'<br>Reverse: 5'-AAAAGCGTGAATGGGGCATAC-3' |
| Me1 | Forward: 5'-TCAACAAGGACTTGGCTTTTACT-3'<br>Reverse: 5'-TGCAGGTCCATTAACAGGAGAT-3' |
| Ldha | Forward: 5'-AAACCGAGTAATTGGAAGTGGTTG-3'<br>Reverse: 5'-TCTGGGTTAAGAGACTTCAGGGAG-3' |
| Pdk1 | Forward: 5'-ACAAGGAGAGCTTCGGGGTGGATC-3'<br>Reverse: 5'-CCACGTCGCAGTTTGGATTTATGC-3' |
| Got2 | Forward: 5'-GGACCTCCAGATCCCATCCT-3'<br>Reverse: 5'-GGTTTTCCGTTATCATCCCGGTA-3' |
| Gpt2 | Forward: 5'-AACCATTCACTGAGGTAATCCGA -3'<br>Reverse: 5'-GGGCTGTTTAGTAGGTTTGGGTA -3' |
| Psat1 | Forward: 5'-CAGTGGAGCGCCAGAATAGAA-3'<br>Reverse: 5'-CCTGTGCCCCTTCAAGGAG-3' |
| Glud1 | Forward: 5'-CCCAACTTCTTCAAGATGGTGG-3'<br>Reverse: 5'-AGAGGCTCAACACATGGTTGC-3' |
| Atf4 | Forward: 5'-CCTGAACAGCGAAGTGTTGG-3'<br>Reverse: 5'-TGGAGAACCCATGAGGTTTCAA-3' |

## 3.4 Results

Previous research has shown that hypoxia affects viability of cancer cells, as well as processes related to progression through the metastatic cascade (25), thus we sought to determine if hypoxia would differentially regulate viability of non-metastatic M-Wnt compared to metastatic metM-Wnt$^{lung}$ cells. Hypoxia reduced survival of both M-Wnt cells (31%) in M-Wnt cells and metM-Wnt$^{lung}$ cells (29%) (Figure 3.1A). Pre-incubating cells in hypoxia similarly suppressed migration of M-Wnt and metM-Wnt$^{lung}$ cells by 33% and 42%, respectively (Figure 3.1B). Hypoxia had no significant effect on survival in detached conditions for either cell line (Figure 3.1C). These data collectively suggest that hypoxia does not differentially affect viability or processes involved in metastasis in cells from different stages of metastasis.

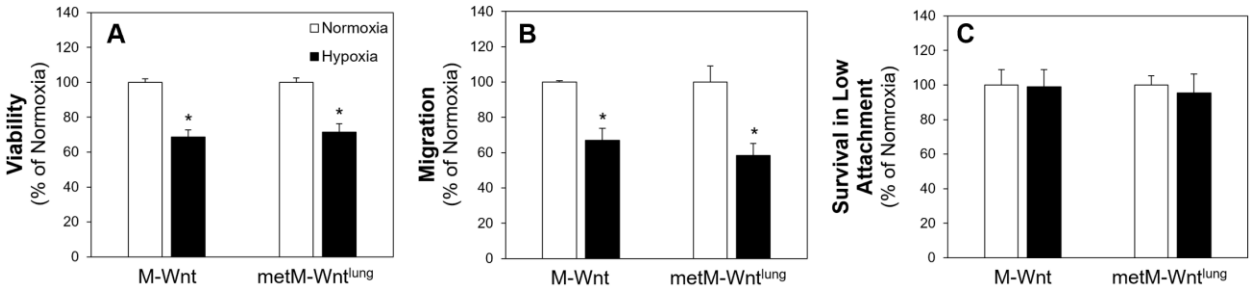


Figure 3.1. Effect of hypoxia on survival and migration.

Viability after 48 h in hypoxia or normoxia was assessed by MTT (A). Cells were pre-incubated in normoxia or hypoxia for 48 h and re-plated into transwells to assess migration (B) or low attachment plates to assess survival in detached conditions (C). Results are expressed as mean + SEM. Asterisk (*) indicates $P < 0.05$ relative to normoxia.

Hypoxia increases oxidative stress, which is detected and cleared by multiple systems in the cell (3,4,6). Because metastatic cells are reported to have higher levels of oxidative stress, we assessed the effect of hypoxia on antioxidant defense and reductive-oxidative (redox) balance in non-metastatic and metastatic cells. Culturing M-Wnt cells in hypoxia suppressed expression of genes required for *do novo* glutathione synthesis including glutamate cysteine ligase-catalytic subunit (Gclc) and –modifer subunit (Gclm), and glutathione synthetase (Gss) (Figure 3.2A). Conversely, hypoxia increased mRNA level of Gclc and Gclm by 1.9-fold in metM-Wnt$^{lung}$ cells (Figure 3.2B). Hypoxia also significantly increased mRNA level of malic enzyme 1 (Me1), an enzyme which

produces the antioxidant NADPH from $NADP^+$, in metM-$Wnt^{lung}$ cells (Figure 3.2B). We next evaluated the effect of hypoxia on overall redox balance by assessing the ratio of reduced-to-oxidized glutathione (GSH/GSSG) and NADPH/$NADP^+$. We found that hypoxia did not affect GSH/GSSG in either cell line (Figure 3.2C), but did significantly reduce the NADPH/$NADP^+$ ratio by 38% in metM-$Wnt^{lung}$ cells (Figure 3.2D). Finally, we found that hypoxia increased intracellular ROS by 21% in M-Wnt cells (Figure 3.2E). These data suggest that hypoxia may increase oxidative stress in metastatic metM-$Wnt^{lung}$ cells, but not M-Wnt cells, and that the increase in oxidative stress is countered by increased antioxidant response.

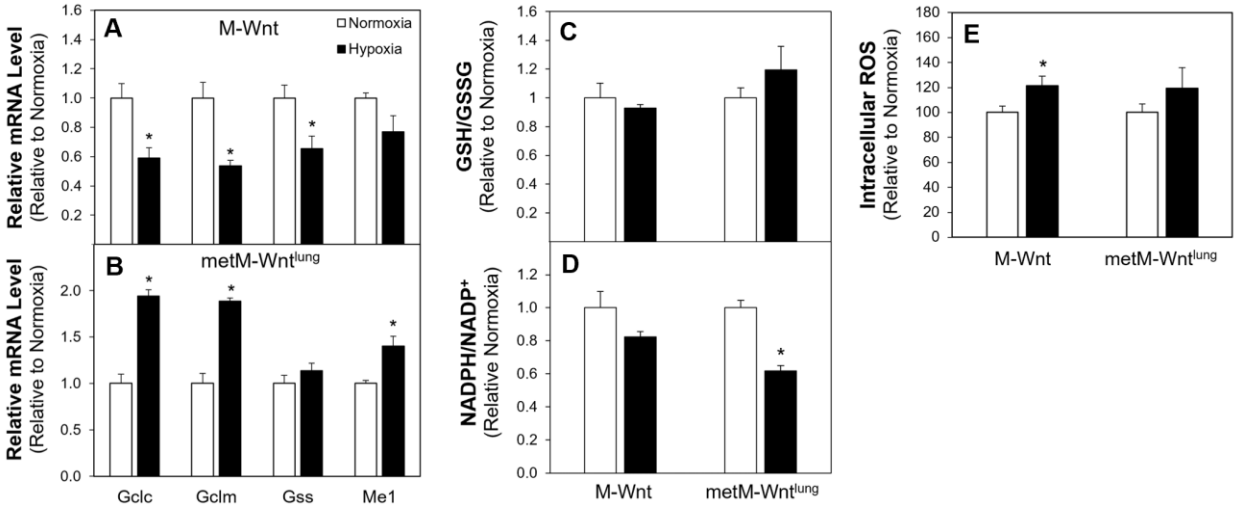


Figure 3.2. Effect of hypoxia on oxidative stress markers.

Levels of genes involved in antioxidant defense were assessed by qRT-PCR in normoxic and hypoxic M-Wnt (A) and metM-$Wnt^{lung}$ (B) cells. Ratios of GSH/GSSG (C), NADPH/$NADP^+$ (D), and levels of intracellular ROS (E) were determined. Results are expressed as mean + SEM. Asterisk (*) indicates $P < 0.05$ relative to normoxia.

Reprogramming glucose and glutamine metabolism are major mechanisms by which hypoxic cells reduce mitochondrial ROS production and consequently reduce oxidative stress (6). We assessed expression of genes related to glucose and glutamine metabolism and metabolic flux to determine if metabolic reprogramming contributes to antioxidant defense in hypoxic metM-$Wnt^{lung}$ cells. Hypoxia increased mRNA level of Pdk1, an enzyme involved in blocking flow of pyruvate into the TCA cycle, in both M-Wnt (Figure 3.3A) and metM-$Wnt^{lung}$ cells (Figure 3.3B). Hypoxia did

not affect expression of genes related to glutamine catabolism in M-Wnt cells (Figure 3.3A), but decreased expression of the transaminase aspartate aminotransferase (Got2) by 33% and induced expression of the transaminase glutamic pyruvic transaminase (Gpt2) 7.7-fold in metM-Wnt$^{lung}$ cells (Figure 3.3B). Metabolic flux using stably labeled $^{13}C_5$ glutamine or $^{13}C_6$ glucose revealed that hypoxia reduced flux of labeled glutamine to M+5 glutamate in both cell lines (Figure 3.3C), but to a greater extent in metM-Wnt$^{lung}$ cells (15%) compared to M-Wnt cells (9%) (Figure 3.3E). In addition, hypoxia reduced flux of labeled glutamine to M+5 αKG to similar extents in M-Wnt cells (40%) and metM-Wnt$^{lung}$ cells (38%) (Figure 3.3D, 3.3F). Finally, hypoxia reduced flux of labeled glucose to M+3 alanine, a product of the Gpt2 reaction, in both cell lines (Figure 3.3G), but this inhibition occurred to a greater extent in M-Wnt cells (39%) compared to metM-Wnt$^{lung}$ cells (25%) (Figure 3.3H). Thus, in metM-Wnt$^{lung}$ cells, hypoxia increases mRNA levels of Gpt2 and leads to weaker suppression of glucose flux to M+3 alanine compared to hypoxic M-Wnt cells. Collectively, while hypoxia suppresses conversion of glutamine to αKG to an similar extent in both cell lines, these results may suggest that non-metastatic and metastatic cell lines utilize different mechanisms to suppress glutamine catabolism in hypoxia.

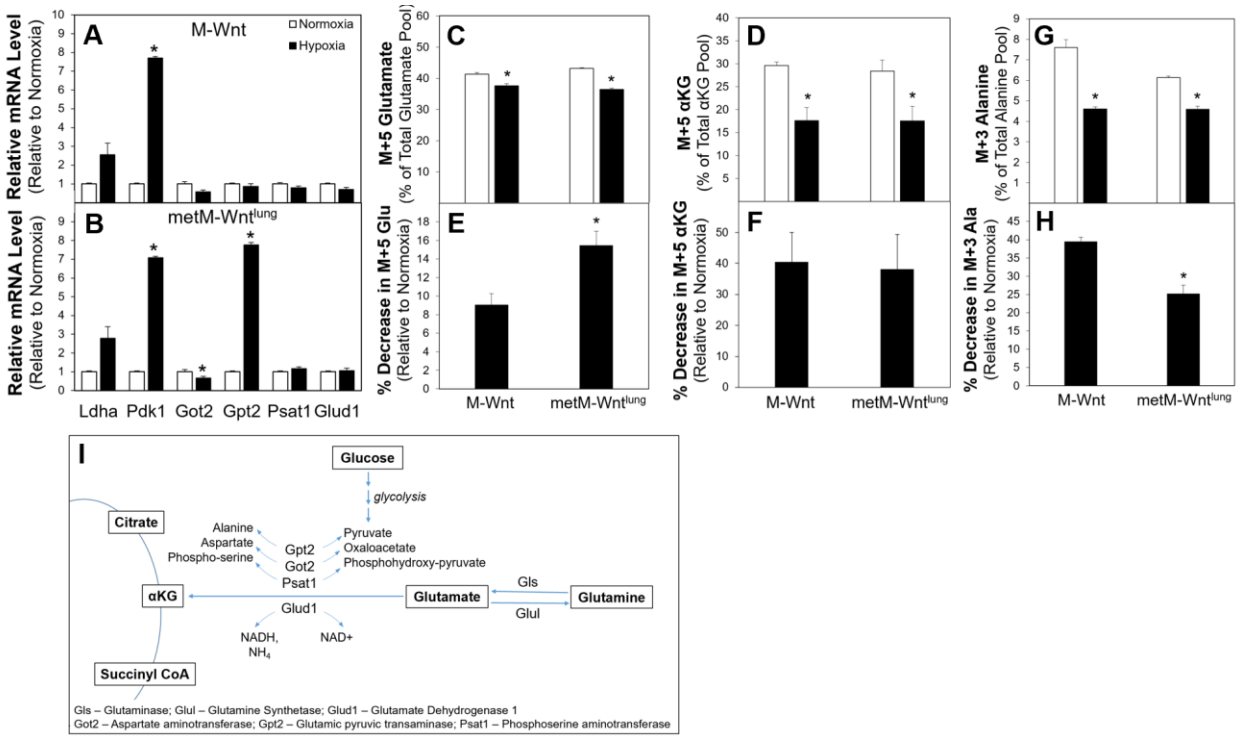


Figure 3.3. Metabolic adaptation in hypoxia.

Levels of genes involved in glucose and glutamine metabolism were analyzed by qRT-PCR in M-Wnt (A) and metM-Wnt$^{lung}$ (B) cells. Stably labeled $^{13}C_5$ glutamine was used to determine metabolism to glutamate (C) and α-ketoglutarate (D). Percent decrease in labeling of glutamate (E) and α-ketoglutarate (F) were determined relative to normoxia for each cell line. Stably labeled $^{13}C_6$ glucose was used to determine metabolism of glucose to alanine (G). Percent decrease in alanine labeling (H) was determined relative to normoxia for each cell line. Overview of glutamine and glucose metabolism (I). Results are expressed as mean + SEM. Asterisk (*) indicates $P < 0.05$ relative to normoxia (A-D,G) or relative to M-Wnt (E,F,H).

Because Gpt2 is transcriptionally regulated by the ISR effector protein ATF4, we evaluated its expression in normoxia and hypoxia in both cell lines. We show hypoxia increased ATF4 mRNA levels and protein expression in metM-Wnt$^{lung}$ cells (Figure 3.4). In contrast, hypoxia did not affect ATF4 expression in M-Wnt cells (Figure 3.4). In order to determine if ATF4 contributes to metastatic processes in hypoxic cells, we used siRNA to genetically deplete ATF4 and measured cell viability, migration, and survival in low attachment. First, genetic depletion of ATF4 prior to hypoxic pre-incubation significantly reduced viability of both cell lines, but had no effect on viability of either cell line in normoxia (Figure 3.5B). ATF4 depletion had no effect on migration in either cell line cultured in normoxia or hypoxia (Figure 3.5C). In contrast, ATF4 depletion

reduced survival of hypoxic metM-Wnt$^{lung}$ cells seeded into detached conditions by 10% (Figure 3.5D). ATF4 depletion had no effect on survival in detached conditions in normoxic metM-Wnt$^{lung}$ cells, or in M-Wnt cells in normoxia or hypoxia. These results suggest hypoxic metM-Wnt$^{lung}$ cells utilize ATF4 for survival in low attachment, whereas hypoxic M-Wnt cells rely on an ATF4-independent mechanism to facilitate their survival in detached conditions.

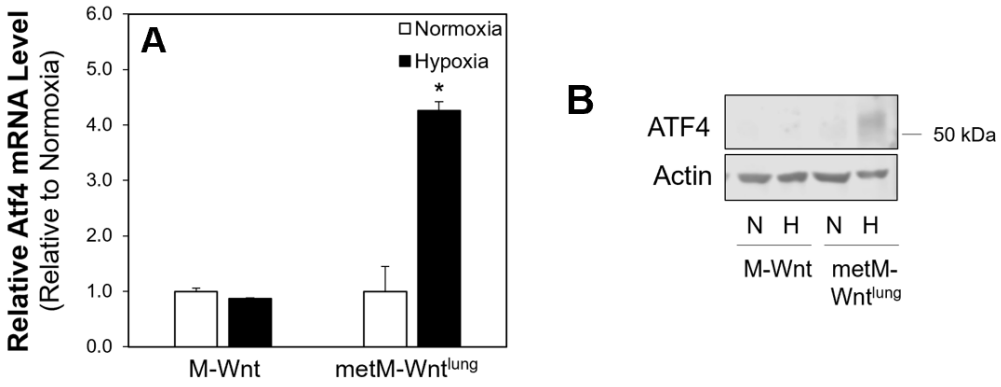


Figure 3.4. Effect of hypoxia on ATF4 expression.

mRNA (A) and protein (B) expression of ATF4 was determined using qRT-PCR and Western blotting in M-Wnt and metM-Wnt$^{lung}$ cells incubated in hypoxia (H) or normoxia (N) for 48 h. Results are expressed as mean + SEM. Asterisk (*) indicates $P < 0.05$ relative to normoxia.

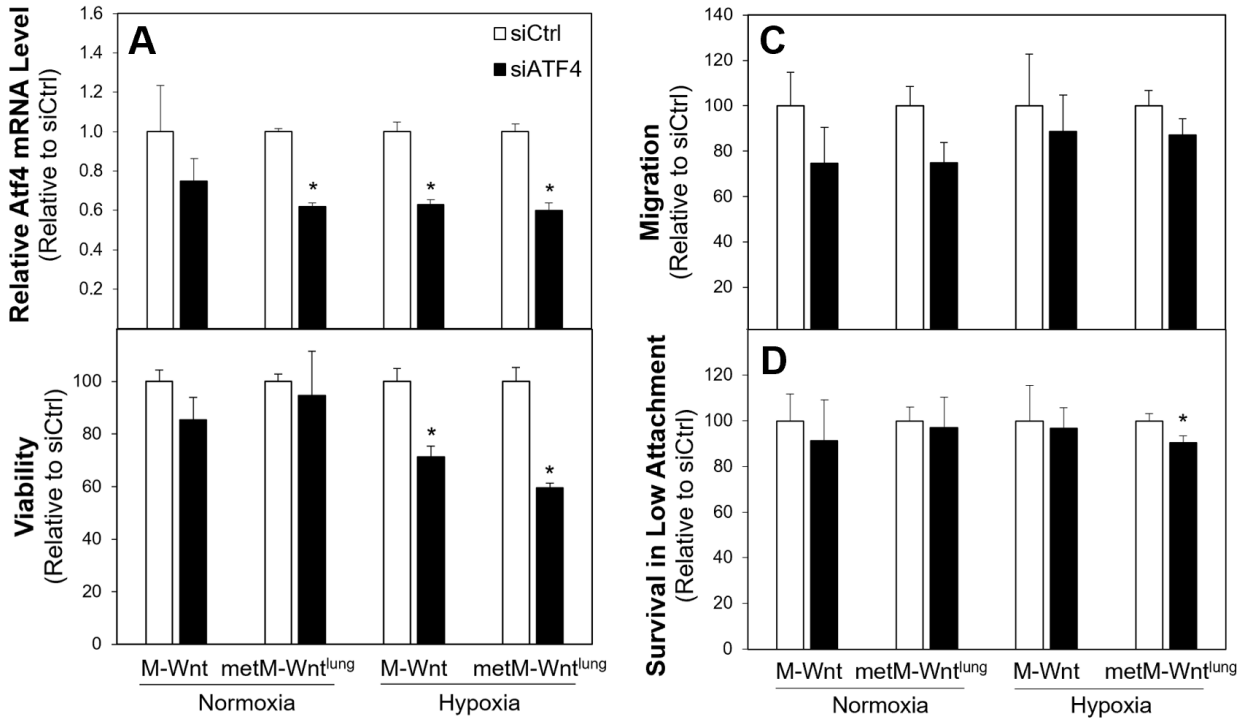


Figure 3.5. Effect of ATF4 depletion in normoxia and hypoxia.

Cell were transfected with siCtrl or siATF4 and incubated in hypoxia or normoxia for 48 h. ATF4 depletion was confirmed by qRT-PCR (A). Cell viability (B) was assessed at the end of incubation. Cells pre-incubated in hypoxia or normoxia were seeded into transwells to assess migration (C) or low attachment plates to assess survival in detached conditions (D). Results are expressed as mean + SEM. Asterisk (*) indicates $P < 0.05$ relative to siCtrl.

## 3.5 Discussion

In the current studies, we show that hypoxia drives differential responses to cell stress in non-metastatic compared to metastatic murine mammary cancer cells. Hypoxia induced transcription of genes associated with antioxidant defense, and had a greater effect in suppressing glutamine catabolism in metastatic metM-Wnt$^{lung}$ cells (Figure 3.2 and 3.3). Furthermore, hypoxia stimulated expression of the ISR effector protein ATF4, which mediated survival of hypoxic cells in low attachment (Figure 3.4 and 3.5) in metM-Wnt$^{lung}$ cells, but not non-metastatic M-Wnt cells.

Hypoxia is a state of high oxidative stress, and continued cell survival in hypoxic conditions requires activation of antioxidant response systems to reduce ROS (6). An interesting finding of this study is that metastatic and non-metastatic cells have different antioxidant defenses in response to hypoxia. We showed that in hypoxia, only metastatic metM-Wnt$^{lung}$ cells increased mRNA

levels of genes related to *de novo* synthesis of the antioxidant glutathione and cellular regeneration of NADPH (Figure 3.2B). Despite the increased mRNA levels of genes related to antioxidant defense, the NADPH/$NADP^+$ ratio was significantly lower in hypoxic metM-$Wnt^{lung}$ cells compared to normoxic cells (Figure 3.2). This observation may suggest a higher load of oxidative stress in metastatic cells compared to non-metastatic cells in hypoxia, and is consistent with observations from previous literature that showing increasing levels of ROS and ROS-related cell damage throughout the course of metastatic progression (26). Interestingly, hypoxia significantly increased intracellular ROS accumulation only in M-Wnt cells, which may suggest that antioxidant defense mechanisms activated in metM-$Wnt^{lung}$ cells inhibited hypoxia-mediated ROS production in metM-$Wnt^{lung}$ cells.

We also showed that metabolic reprogramming in hypoxia, particularly of glutamine metabolism, varied between non-metastatic and metastatic cells. Previous literature has shown that hypoxia suppresses glutamine catabolism in cancer cells (12). We showed that while hypoxia suppressed conversion of glutamine to glutamate in both cell lines, the percent decrease in M+5 labeled glutamate in hypoxia was significantly higher in metM-$Wnt^{lung}$ cells compared to M-Wnt cells (Figure 3), suggesting that hypoxia suppresses glutaminolysis to a greater extent in metastatic cells. Interestingly, we also found that hypoxia significantly induced the mRNA level of the transaminase Gpt2 only in metM-$Wnt^{lung}$ cells (Figure 3.3). Gpt2 transfers the amino group from glutamate to pyruvate, producing αKG and alanine (27). We show that hypoxia significantly reduced the metabolism of glucose to alanine in both cell lines, but that this reduction occurred to a greater extent in M-Wnt cells. Stated another way, these data suggest that hypoxia only modestly prohibits alanine synthesis in metM-$Wnt^{lung}$ cells compared to the robust suppression observed in hypoxic M-Wnt cells. Increased Gpt2 mRNA levels in hypoxic metM-$Wnt^{lung}$ cells may contribute to the poorer inhibition of alanine synthesis in hypoxia compared to M-Wnt cells.

Previous literature shows that Gpt2 mRNA levels can be induced by AFT4, the main effector protein of the ISR pathway (20). Our results show that ATF4 is activated in hypoxic metM-$Wnt^{lung}$ cells, and contributed to survival of hypoxic metM-$Wnt^{lung}$ cells in detached conditions (Figures 3.4 and 3.5). Our results are in agreement with previous work which showed that ATF4 induction in cancer cells contributes significantly to anoikis resistance (19).

Interestingly, the same study showed that nuclear factor erythroid 2-related factor 2 (NRF2) was also a key player mediating anoikis resistance (19). NRF2 is an antioxidant response pathway that is stimulated by oxidative stress, including hypoxia (28,29). The results of our study may suggest that hypoxia activates the NRF2 pathway in metM-Wnt$^{lung}$ cells, but not M-Wnt cells. First, NRF2 drives transcriptional activation of Atf4, while hypoxia only induces translational activation of ATF4 (7,14). Given that we observed transcriptional induction of Atf4 in hypoxic metM-Wnt$^{lung}$ cells (Figure 3.4), our results may suggest that hypoxia induces Atf4 through Nrf2 activation. Furthermore, we showed increased mRNA levels of genes related to antioxidant defense in hypoxic metM-Wnt$^{lung}$ cells (Figure 3.2). While the mechanism driving upregulation of these genes in metM-Wnt$^{lung}$ cells is currently not known, it is interesting to note that the genes profiled are targets NRF2 pathway (28,29). Because previous literature suggests that extracellular matrix detachment increases oxidative stress in cancer cells (30–32), hypoxia-mediated induction of NRF2 and Atf4 may play a critical role in priming metastatic cells to survive in high stress conditions, including during matrix detachment. It will be important to assess activation of NRF2 pathway activity in hypoxic metM-Wnt$^{lung}$ cells to determine if this mechanism contributes to Atf4 activation.

Collectively, our results show that non-metastatic and metastatic murine mammary cancer cells utilize different mechanisms to facilitate their survival in stress conditions. Hypoxia drives expression of ATF4 and increases mRNA levels of antioxidant defense genes, and facilitates more drastic reprogramming of glutamine catabolism in metastatic cells. These results warrant further investigation to address the interaction between antioxidant response, ATF4 activation, and the NRF2 signaling pathway in hypoxic metastatic cells, and may help elucidate molecular targets to prevent metastatic progression in cancer patients. Given that the majority of cancer-related deaths are the result of metastatic disease (33), understanding ways to inhibit the steps of metastasis are of great clinical interest.

## 3.6 Acknowledgements

The authors acknowledge the use of the facilities of the Bindley Bioscience Center, a core facility of the NIH-funded Indiana Clinical and Translational Sciences Institute.

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# CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

## 4.1 Summary

One in eight women in the United States will be diagnosed with breast cancer, for a total estimated 279,100 new cases of breast cancer in 2020 (1). It is also predicted that 42,690 women will die of breast cancer in 2020 (1). While the five-year survival rate for women diagnosed with breast cancer is 99% when the cancer is still localized to the primary tumor at time of diagnosis, the five-year survival rate for metastatic disease is only 27% (1). These data are consistent with the observation that metastasis, or the spread of cancer from the primary site to distant organs, accounts for 90% of cancer-related deaths (2), and emphasize the clinical need for strategies to prevent metastasis.

Metabolic reprogramming is a hallmark of cancer (3) and describes the metabolic perturbations that occur in cancer cells compared to their untransformed counterparts. Cancer cells, for example, have increased metabolism of glutamine. Glutamine is the second-most consumed nutrient in cancer cells after glucose, and its metabolism contributes to cell survival through several mechanisms (4). Glutamine can be directly incorporated into nascent peptides during protein synthesis, or can participate in antiport exchange systems to increase entry of other amino acids, such as leucine, into the cell (5). Alternatively, glutamine can be deamidated to glutamate. Similar to glutamine, glutamate can be incorporated into peptides including the antioxidant glutathione, exchanged for extracellular cysteine, or further catabolized to α-ketoglutarate (αKG) (5). Entry of glutamine-derived αKG into the tricarboxylic acid (TCA) cycle is critical to replenish the TCA cycle of intermediates which have been utilized for biosynthetic reactions, such as oxaloacetate which is used for aspartate synthesis. Upon entry into the TCA cycle, αKG is oxidized to produce the coenzymes NADH and $FADH_2$, which fuel ATP production at the electron transport chain. As such, glutamine is a valuable source of carbon and nitrogen for biosynthesis or energy production in proliferating cancer cells. In accordance with this, several studies have demonstrated reliance on glutamine catabolism in cancer cells for continued survival (6).

Hypoxia, or low oxygen tension, significantly modifies metabolism in cancer cells. Hypoxia increases reliance of cancer cells on metabolism of glucose through glycolysis, and actively

suppresses entry of glucose-derived pyruvate into the TCA cycle (7–10). Hypoxia also blocks entry of glutamine into the TCA cycle, and re-directs glutamine-derived αKG through the reverse TCA cycle to citrate to promote fatty acid synthesis (11). By limiting the flow of carbon into and through the forward TCA cycle, hypoxia-induced metabolic reprogramming limits production of cytotoxic reactive oxygen species (ROS) at the electron transport chain and improves cell survival (7,12). Because regions of hypoxia are common features of solid tumors, metabolic reprogramming in response to hypoxia is a necessary cellular adaptation for cancer cell survival and tumor outgrowth.

Changes in nutrient and oxygen availability are both forms of cell stresses. A key feature of proliferating tumor cells is the ability to sense and respond to cell stresses in such a way that preserves cell viability and permits outward expansion of the tumor. In the present work, we have determined the effect of glutamine concentration and hypoxia on metabolic adaptation of non-metastatic M-Wnt compared to metastatic metM-Wnt$^{lung}$ cancer cells. We showed that non-metastatic cells are able to suppress glutamine catabolism in response to high glutamine concentration, which preserves their viability. Metastatic cells, in contrast, do not modify mRNA levels of genes related to glutamine catabolism in response to high glutamine concentration, and display increased sensitivity to the metabolic byproduct ammonium (Chapter 2). While both non-metastatic and metastatic cancer cells are sensitive to hypoxic incubation, metastatic cells rely on activation of activating transcription factor 4 (ATF4) to mediate their survival in detached conditions following a bout of hypoxia (Chapter 3). Thus, metastatic cells demonstrate the ability to activate gene expression to facilitate metastatic processes in response to hypoxia, but lose the ability to downregulate glutamine catabolism compared to their non-metastatic counterparts in normoxia. These data highlight mechanistic vulnerabilities of metastatic cells that may be clinically relevant to prevent or delay cancer progression. However, future research is required to better understand changes in metabolic adaptation as they occur throughout progression.

## 4.2 Future Directions

### 4.2.1 Activation of NRF2 in hypoxia

Given the prevalence of hypoxic regions in solid tumors and the positive relationship between hypoxia and progression (13), it is imperative to understand how tumor cells respond to hypoxia to facilitate their survival. In Chapter 3 we showed that hypoxia suppresses viability of both non-metastatic M-Wnt cells and metastatic metM-Wnt$^{lung}$ cells, but has no effect on survival of cancer cells seeded in detached conditions. Furthermore, we show that hypoxia activates expression of genes that contribute to neutralization of ROS and induces expression of ATF4 in metM-Wnt$^{lung}$ cells. Given that ATF4 is a key protein involved in the integrated stress response (ISR), these observations may suggest that more metastatic cells have higher levels of cell stress than their non-metastatic counterparts in hypoxia. These data are consistent with previous research suggesting that cancer cells have increased accrual of ROS and ROS-mediated cell damage throughout the course of progression (14).

Activation of the ISR is one of several potentially overlapping mechanisms by which metastatic cells respond to oxidative stress in hypoxia. While the activation of genes involved in antioxidant response in hypoxic metM-Wnt$^{lung}$ cells may be caused directly by ATF4-mediated transactivation, it is interesting to note that transactivation may also occur as a result of nuclear factor erythroid 2-related factor 2 (NRF2) pathway activation. NRF2 is an antioxidant response pathway that is stimulated by oxidative stress (15). Activation of the pathway results in binding of NRF2 to antioxidant response elements to induce transcription of genes to eliminate ROS. Interestingly, NRF2 can also induce transcription of Atf4 (16), and ATF4 can similarly induce transcription of Nrf2 (17). Collectively, these data may suggest that increased oxidative stress in hypoxic cells can activate NRF2 and ATF4 through independent pathways, and that each pathway is capable of activating the other, potentially creating a positive feedback loop until cell stress is alleviated (18). It is important to determine the involvement of NRF2 upstream and downstream of ATF4 activation to better understand the interplay of these two pathways. Previous literature has demonstrated that both NRF2 and ATF4 were required for resistance to anoikis in an *in vitro* model of cancer (19), suggesting that therapeutically targeting only one of these two targets could have broad effects on cell survival and progression.

Experiments should be conducted to determine if the NRF2 pathway is active in hypoxia in metM-Wnt$^{lung}$ cells, and if activation contributes to cell survival and metastatic processes. First, a NRF2 reporter assay should be conducted in M-Wnt and metM-Wnt$^{lung}$ cells in normoxia and hypoxia. I hypothesize that NRF2-induced transcription will be strongly induced in hypoxic metM-Wnt$^{lung}$ cells. Next, NRF2 should be inhibited either chemically or genetically to determine if transcriptional upregulation of Atf4 observed in hypoxic metM-Wnt$^{lung}$ cells is dependent on NRF2 activation. I hypothesize that inhibition of NRF2 will partially attenuate hypoxia-mediated Atf4 transcriptional induction, and this partial depletion will also be observed at the protein level. Finally, it is of interest to determine the interplay between ATF4 and NRF2, particularly in the context of survival in low attachment. I propose assessing the effect of NRF2 inhibition on survival in low attachment, and the effect of inhibiting both NRF2 and ATF4 on survival in low attachment. I hypothesize that there will be no difference in the percent decrease in detached survival in cells with only ATF4 depletion compared to cells with ATF4 and NRF2 depletion. These data would suggest that suppressing only one of these two interconnected pathways is sufficient to mediate changes in outcomes associated with metastasis.

### 4.2.2 Activation of autophagy in hypoxia

Metabolic reprogramming in hypoxia is an important mechanism for maintaining cancer cell survival. We showed in Chapter 3 that M-Wnt and metM-Wnt$^{lung}$ cells differentially reprogram their metabolism in response to hypoxia. This observation is consistent with increased mRNA levels of the transaminase Gpt2, whose protein product produces αKG and alanine from glutamate and pyruvate, in hypoxic metM-Wnt$^{lung}$ cells. Gpt2 is an ATF4 target gene, and ATF4 is significantly upregulated in hypoxia in metM-Wnt$^{lung}$ cells. While changes in amino acid metabolism are likely a result of ATF4 induction, ATF4 is also known to strongly promote induction of autophagy to reduce cell stress (13). Induction of autophagy is a useful mechanism to enhance nutrient availability in high cell stress conditions, and may contribute to overall cancer cell survival and progression (14).

Hypoxia can mediate induction of autophagy through several mechanisms, including ATF4 activation. Hypoxia is a unique condition that creates several types of cell stress, including oxidative stress. Hypoxia also creates nutrient stress by rapidly depleting glucose supplies and

endoplasmic reticulum (ER) stress as a result of accumulation of misfolded proteins, which occurs because of the requirement for molecular oxygen to mediate proper formation of disulfide bonds in nascent peptides (15). In accordance with the diverse stress stimuli imparted on cancer cells by hypoxic conditions, multiple systems are in place to respond to the stress to restore cellular homeostasis. In response to cell stress, for example, activates the cellular ISR, and induces the unfolded protein response (UPR), both of which induce expression of the effector protein ATF4. Hypoxia also stabilizes its canonical effector protein, HIF1α, to respond to cell stress. Induction of autophagy is a common outcome of these three major signaling mechanisms. Convergence of cell stress stimuli on induction of autophagy can be beneficial to cancer cells for several reasons. For example, autophagy selectively degrades damaged organelles, alleviating cell stress (14). Selective degradation of mitochondria also limits production of ROS at the electron transport chain, further reducing accumulation of oxidative damage. Finally, autophagy alleviates nutrient stress through recycling of cellular nutrients, thereby decreasing the cellular demand for exogenous glucose in hypoxic tumor cells. Induction of autophagy by hypoxia is particularly important *in vivo*, as impaired vascularization is often accompanied by limited exposure to nutrients (16). Collectively, several cell stresses which arise from hypoxia converge on induction of autophagy to mediate cell survival. We observed induction of ATF4, one of several inducers of autophagy, in hypoxic metM-Wnt$^{lung}$ cells. These data may suggest that ATF4 upregulation is accompanied by activation of autophagy, and may play a critical role in survival in stress conditions.

Future research is required to determine if autophagy is activated in metM-Wnt$^{lung}$ cells in hypoxia, as well as the causes and consequences of induction. In order to determine if autophagy is activated in hypoxic metM-Wnt$^{lung}$ cells, Western blotting should be performed to assess expression of the autophagy-associated proteins Microtubule associated protein 1 light chain 3 (LC3) and p62 (14). Furthermore, the role of autophagy induction in cell survival should be assessed. I hypothesize that autophagy is activated in hypoxic metM-Wnt$^{lung}$ cells, and that its inhibition will reduce cell viability. Understanding the cell mechanisms driving induction of autophagy will also be important in elucidating therapies to reverse this cell-protective response. It will be important to assess the role of the pro-autophagy pathways, including HIF signaling, ISR activation, and UPR activation in response to hypoxia in metastatic compared to non-metastatic cells. I hypothesize that each of the three pathways outlined above will contribute to induction of autophagy in hypoxic metM-Wnt$^{lung}$ cells, and that suppression of any one pathway will not completely diminish

autophagy induction. Finally, determining the effect of autophagy on pro-metastatic processes in non-metastatic and metastatic cells will elucidate the steps of cancer metastasis at which induction of autophagy is required for progression. Given the role that we and others have demonstrated for ATF4 induction in mediating resistance to anoikis, it is important to understand if activation of autophagy contributes to this survival mechanism. Indeed, past work has shown that induction of autophagy mediates resistance to anoikis in cancer cells (17), suggesting that hypoxia-mediated induction of autophagy may play an important role in priming cancer cells for survival in matrix detached conditions.

### 4.2.3 Lipid metabolism in hypoxia

Lipid metabolism changes dramatically in hypoxia compared to normoxia. Previous literature has shown that fatty acid oxidation is inhibited in hypoxia, and that fatty acid synthesis is upregulated. Interestingly, one study showed that synthesis of fatty acids from glutamine through the reductive carboxylation of αKG to citrate is imperative for membrane synthesis and cell survival in hypoxia (11). Other work has shown that hypoxia increases fatty acid uptake, and that these lipids are retained in cytoplasmic lipid droplets (18–20). Some literature has suggested that cells catabolize fatty acids stored in lipid droplets upon re-oxygenation, and that this is an important energy source contributing to cell proliferation in normoxia (20), although less literature is available examining this phenomenon across metastatic progression.

It is thought that metabolic plasticity, or the ability to metabolize diverse nutrients through different pathways, increases as cancer cells become more advanced (21). It is important to understand if this metabolic plasticity extends to cells cycling between hypoxia and normoxia, a common event within solid tumors (22), and how the degree of plasticity or adaptation changes in metastatic compared to non-metastatic cells. Identifying the molecular mechanisms that underlie enhanced plasticity may shed light on treatments to reduce survival of metastatic cells, thereby blocking progression.

In order to assess this question, first, the effect of hypoxia on triacylglycerol accumulation in M-Wnt cells and metM-Wnt$^{lung}$ cells must be assessed. I hypothesize that 48 h incubation in hypoxia will increase triacylglycerol accumulation to an equal extent in both cell lines. Next, it is important to determine fatty acid oxidation in both cell lines upon reoxygenation. I hypothesize that both

cell lines will upregulate fatty acid oxidation in normoxia, and that the upregulation will occur to a greater extent in metM-Wnt$^{lung}$ cells in accordance with their higher overall rate of metabolism (23). Finally, the role of fatty acid oxidation on survival and pro-metastatic processes such as migration should be determined in M-Wnt and metM-Wnt$^{lung}$ cells previously incubated in hypoxia. I hypothesize that use of the fatty acid oxidation inhibitor etomoxir following a bout of hypoxia will suppress viability and migration in both cell lines, but to a greater extent in M-Wnt cells, indicating a higher degree of metabolic plasticity in metM-Wnt$^{lung}$ cells.

### 4.2.4 Glutamine cytotoxicity in metM-Wnt$^{lung}$ cells

In Chapter 2 we showed that metM-Wnt$^{lung}$ cells grown in 4 mM glutamine are less viable than cells in 4 mM glutamine, and that cells in 2 mM glutamine are significantly more sensitive to ammonium chloride treatment than M-Wnt cells. While conversion of glutamine to glutamate through glutaminase is a major source of glutamine production in proliferating cells, use of a glutaminase inhibitor did not increase viability of metM-Wnt$^{lung}$ cells grown in 4 mM glutamine. Thus, it is possible that another mechanism independent of ammonium cytotoxicity reduces viability of metM-Wnt$^{lung}$ cells in 4 mM glutamine. We showed that metM-Wnt$^{lung}$ cells have increased flux of glutamine to glutamate in 4 mM glutamine culture conditions. Increased glutamine catabolism in high glutamine concentrations may contribute to an increase in total ATP production in metM-Wnt$^{lung}$ cells. Importantly, high levels of ATP allosterically inhibit multiple steps of glycolysis (24). Given the reliance of cancer cells on glycolysis for biosynthesis of molecules required for cell proliferation, inhibition of glycolysis secondary to excess ATP production may slow cell proliferation.

In order to test the role of ATP production on glycolysis and viability in metM-Wnt$^{lung}$ cells, first the ratio of ATP/ADP should be tested in metM-Wnt$^{lung}$ cells in 2 mM and 4 mM glutamine concentrations. Given that the $NAD^+$/NADH ratio is significantly increased in 4 mM compared to 2 mM glutamine in metM-Wnt$^{lung}$ cells, I hypothesize that the ATP/ADP ratio correspondingly increases in 4 mM glutamine. Next, it is important to determine the flux of glucose through glycolysis in variable glutamine in metM-Wnt$^{lung}$ cells. I hypothesize that glycolytic flux will be suppressed in 4 mM glutamine compared to 2 mM glutamine in metM-Wnt$^{lung}$ cells. Increasing glucose load or inhibiting ATP production at the electron transport chain may suppress the anti-

proliferative effects of increased glutamine concentrations in metM-Wnt$^{lung}$ cells. For example, I hypothesize that modestly increasing the concentration of glucose in cell culture media from 5 mM to 10 mM may overcome the allosteric inhibition of glycolytic enzymes, thereby improving the cell's ability to use glycolytic intermediates for biosynthetic reactions and restoring viability. Similarly, I hypothesize that suppressing ATP synthase at the electron transport chain will reduce the ATP/ADP ratio, promote higher glycolytic flux, and increase biosynthesis and proliferation.

## 4.3 Conclusion

In this work, we have shown the striking effect that cell stress has on viability and pro-metastatic processes in cancer cells. In particular, we showed that cancer cells become increasingly vulnerable to metabolic stress throughout the course of metastatic progression. Increased metabolism of glutamine in metabolic cells suppressed their viability, emphasizing the high degree of metabolic activity as a potential target to block proliferation of advanced cancer cells. In addition, increased stress in hypoxia induced activation of a stress response pathway only in metastatic cells, again highlighting a potential target to prevent survival specifically of metastatic cells. In sum, this work improves our total understanding of metabolic perturbations that occur in cancer cells, and how these perturbations contribute to cancer progression. Further research in this field will aid in elucidation of molecular targets to slow or prevent the metastasis of cancer cells and save patient lives.

## 4.4 References


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# APPENDIX A. SUPPLEMENTARY DATA

## A.1 Pyruvate carboxylase

Determining expression and activity of the anaplerotic enzyme pyruvate carboxylase (PC) is of particular interest in breast cancer, as previous research has shown a role for this enzyme in breast-to-lung metastasis. We investigated the expression of PC in variable cell culture conditions in M-Wnt and metM-Wnt$^{lung}$ cells and found that PC protein is detectable at three molecular weights (130 kDa, 105 kDa, and 85 kDa), which are subject to regulation by glucose and glutamine concentration, as well as oxygen level (Figure A.1, A.2). Despite its upregulated mRNA level, depleting PC expression had no effect on viability of metM-Wnt$^{lung}$ cells grown in 4 mM glutamine (Figure 3).

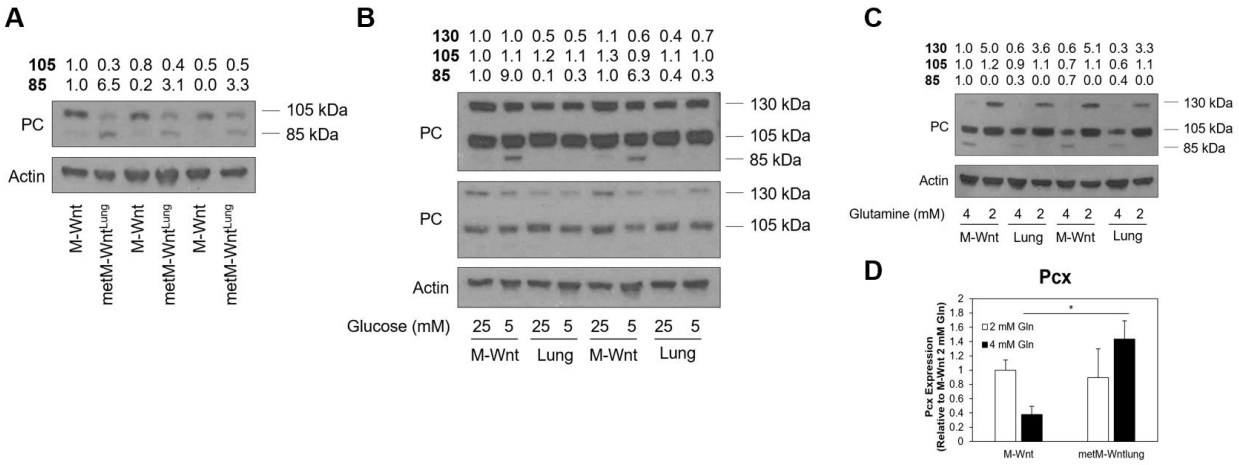


Figure A. 1. Effect of culture conditions on PC expression.

PC protein expression in M-Wnt and metM-Wnt$^{lung}$ cells grown in RPMI with 25 mM glucose and 2 mM glutamine (A); with 5 mM or 25 mM glucose and 4 mM glutamine; with 2 mM or 4 mM glutamine and 5 mM glucose. mRNA levels of PC in 2 mM or 4 mM glutamine and 5 mM glucose (D).

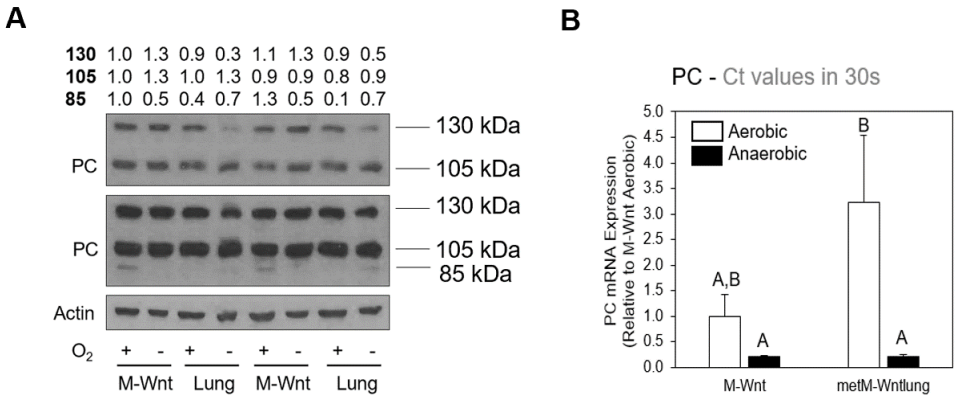


Figure A. 2. Effect of hypoxia on PC expression.

PC protein expression in 5 mM glucose and 4 mM glutamine after cells were cultured in normoxia (+ $O_2$) or hypoxia (- $O_2$) for 48 h (A). Top blot is a short exposure, bottom blot is a long exposure to capture the 85 kDa protein. PC mRNA expression in 5 mM glucose and 4 mM glutamine after cells were cultured in normoxia or hypoxia for 48 h.

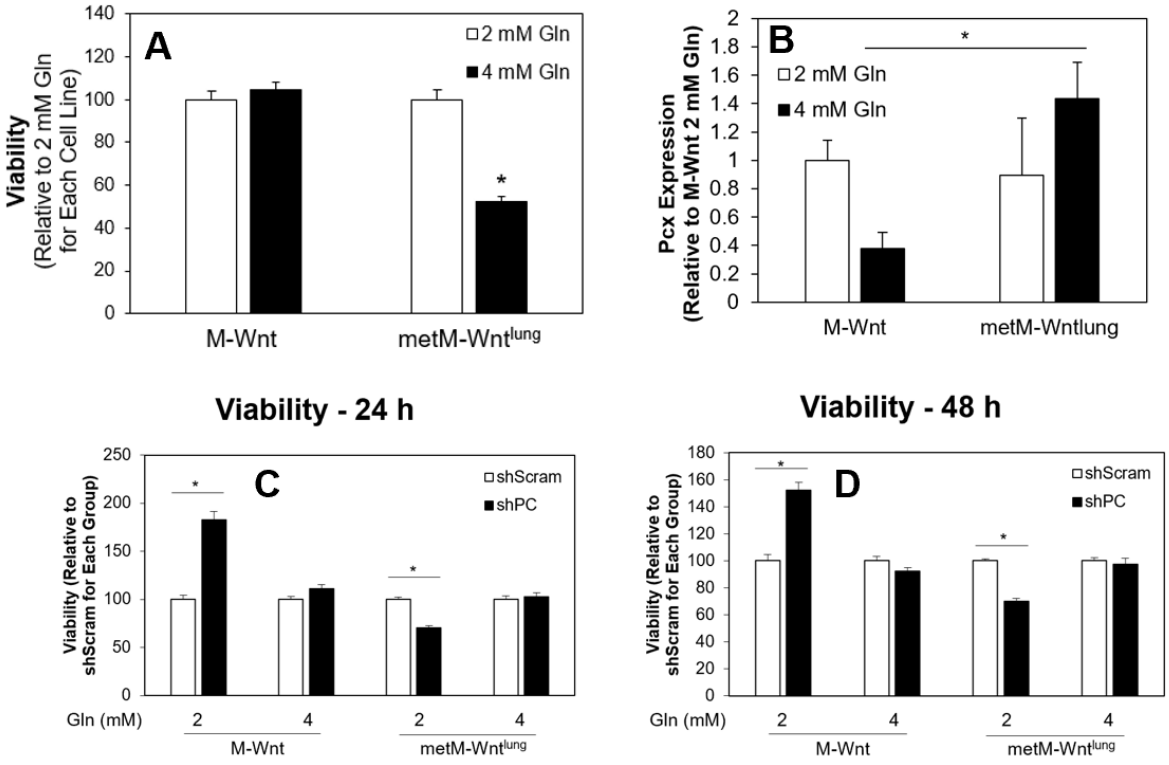


Figure A. 3. Effect of PC depletion.

Viability (A) and PC mRNA expression (B) of M-Wnt and metM-Wnt$^{lung}$ cells constitutively grown in 2 mM or 4 mM glutamine and 5 mM glucose. Effect of PC depletion on viability 24 h (C) and 48 h (D) after seeding.

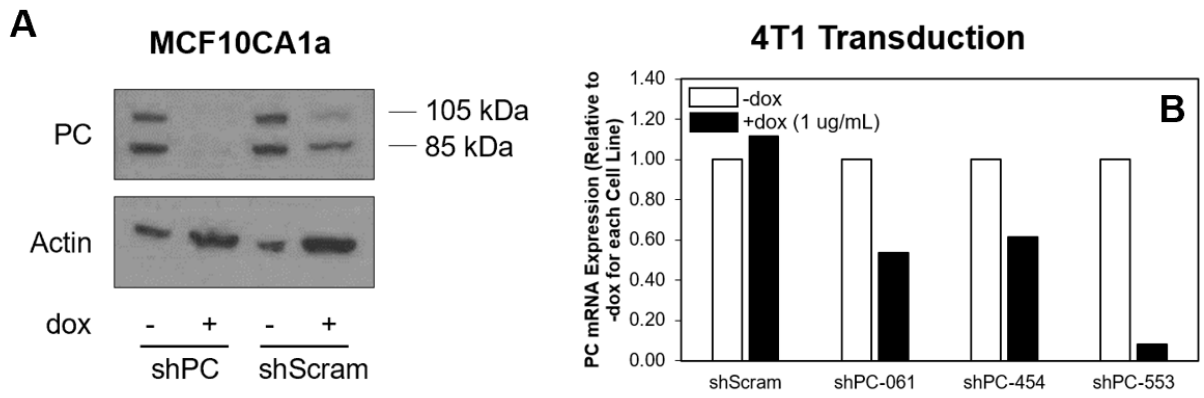


Figure A. 4. Validation of PC depletion.

MCF10CA1a (A) and 4T1 (B) shPC and shScram cells were cultured with or without doxycycline for three days prior to assessing PC expression.

## A.2 1,25 dihydroxyvitamin $D_3$

Previous work from our lab and others have shown anticancer activity of the active metabolite of vitamin D, 1,25 dihydroxyvitamin $D_3$ (1,25$(OH)_2$D). In this work, we show that 1,25$(OH)_2$D treatment, either alone or in combination with hydrogen peroxide or hypoxic incubation, reduces viability of M-Wnt and metM-Wnt$^{lung}$ cells (Figure A.5). We also show that 1,25$(OH)_2$D treatment suppresses expression of pyruvate carboxylase in metM-Wnt$^{lung}$ cells (Figure A.6). Treatment with 1,25$(OH)_2$D reduced survival of M-Wnt and metM-Wnt$^{lung}$ cells grown in detached conditions, and suppressed migration in M-Wnt cells (Figure A.7).

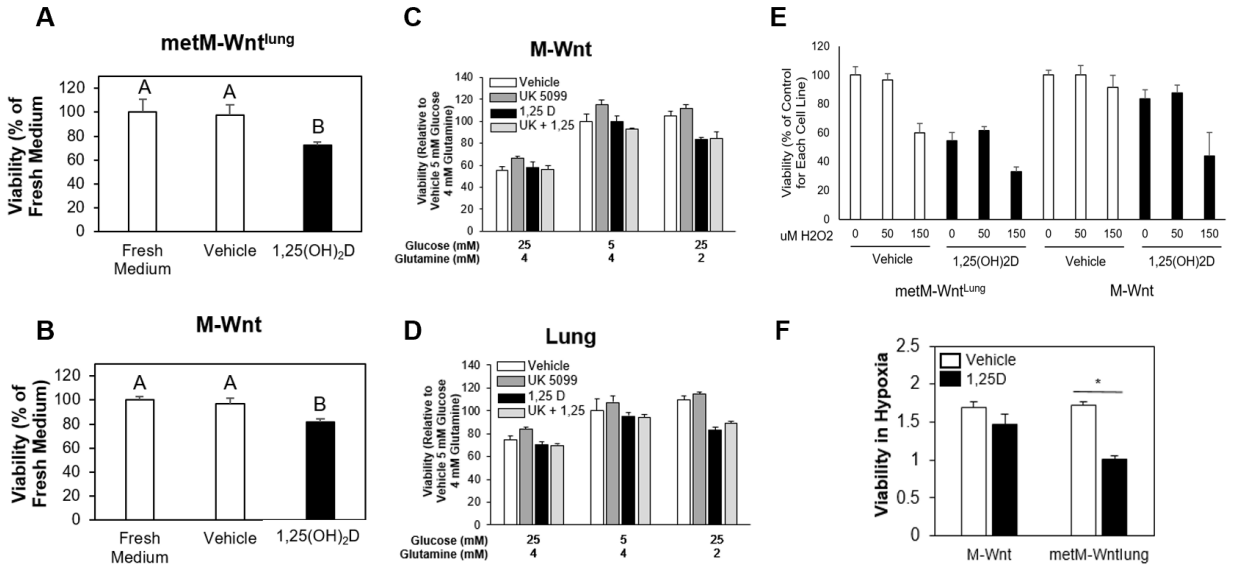


Figure A. 5. Effect of $1,25(OH)_2D$ on viability.

Effect of $1,25(OH)_2D$ on viability was assessed in M-Wnt (A) and metM-Wnt$^{lung}$ cells (B) grown in RPMI. Effect of the combination of $1,25(OH)_2D$ and the monocarboxylate transporter inhibitor UK5099 was assessed in M-Wnt (C) and metM-Wnt$^{lung}$ cells (D) grown in variable nutrient media. Cells were constitutively grown in 25 mM glucose and 4 mM glutamine, seeded into 96 well plates, and media was changed the next day to indicated glucose/glutamine composition with $1,25(OH_2)D$ and/or UK5099. Effect of the combination of $1,25(OH_2)D$ and hydrogen peroxide ($H_2O_2$) was assessed in metM-Wnt$^{lung}$ and M-Wnt cells grown in 5 mM glucose and 4 mM glutamine (E). Effect of $1,25(OH_2)D$ on cells grown in hypoxia in 5 mM glucose and 4 mM glutamine was assessed after 48 h in hypoxia (F).

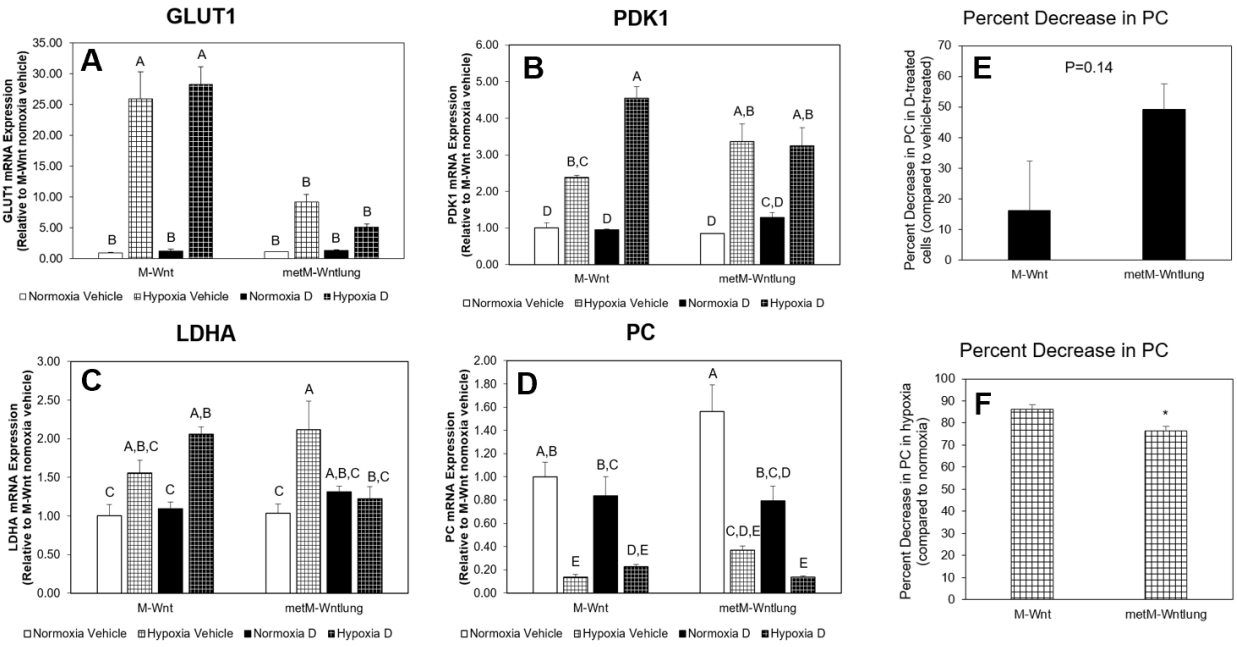


Figure A. 6. Effect of 1,25$(OH)_2$D and/or hypoxia on mRNA levels.

Cells were cultured in 5 mM glucose and 4 mM glutamine and treated with 1,25$(OH)_2$D or vehicle for 3 days. Cells were then transferred to normoxia or hypoxia for an additional 48 h, continuing 1,25$(OH)_2$D or vehicle treatment. Expression of GLUT1 (A), PDK1 (B), LDHA (C), and PC (D) were determined with qRT-PCR. Percent decrease in PC expression with 1,25D treatment (E) or hypoxic incubation (F).

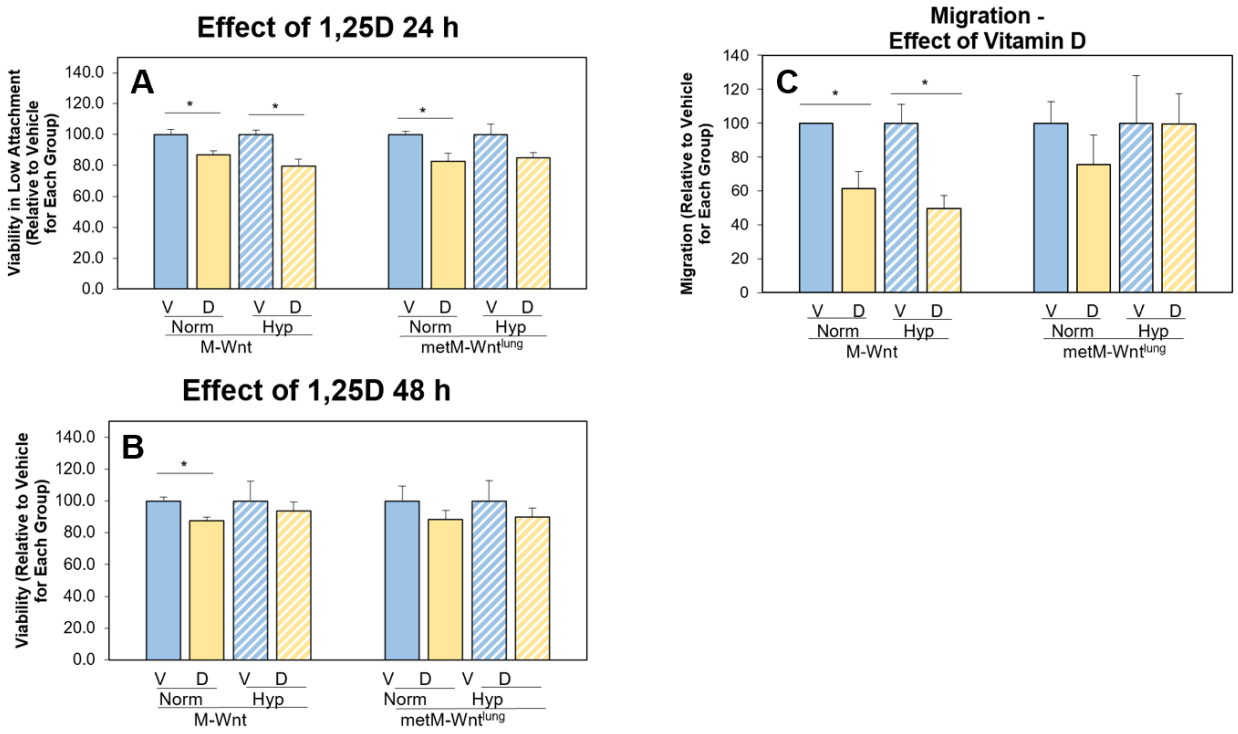


Figure A. 7. Effect of $1,25(OH)_2D$ and/or hypoxia on survival in low attachment and migration.

M-Wnt and metM-Wnt$^{lung}$ cells were constitutively grown in 5 mM glucose and 4 mM glutamine. Cells were pre-treated with vehicle or $1,25(OH)_2D$ for three days. Cells were transferred to hypoxia or normoxia for 48 h, continuing vehicle or $1,25(OH)_2D$ treatment. Cells were removed from hypoxia and seeded into low attachment plates for and viability was measured 24h (A) and 48 h (B) later. Cells were removed from hypoxia and seeded into Transwells to assess migration (C).

## A.3 Variable glutamine

Variable nutrient concentration, including glutamine concentration, is a source of cell stress that may affect survival and processes required for metastatic progression. Increased glutamine concentrations (4 mM) increased expression of angiopoietin-like 4, a protein involved in extravasation of cancer cells (Figure A.8). In addition, 4 mM glutamine concentration suppressed mRNA levels of Sestrin 1, a protein involved in AMPK activation (Figure A.8).

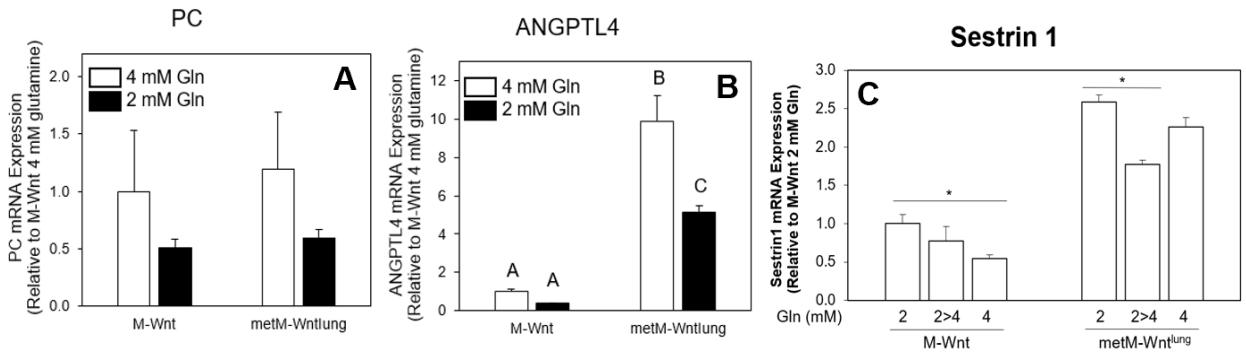


Figure A. 8. Effect of variable glutamine on gene expression.

For (A) and (B), M-Wnt and metM-Wnt$^{lung}$ cells were grown in 25 mM glucose and 4 mM glutamine and seeded into 6 well plates. The next day, media was changed to 25 mM glucose with 2 mM glutamine or maintained at 4 mM glutamine. Cells were cultured for 48 h after changing media, and mRNA expression of PC (A) and angiopoietin-like 4 (B) were determined with qRT-PCR. For (C), cells were constitutively grown in 5 mM glucose and either 2 mM or 4 mM glutamine. Cells were seeded into 6 well plates, and media concentration was either maintained, or cells grown in 2 mM glutamine were switched to 4 mM glutamine (2>4). Cells were incubated for 48 h and expression of Sestrin 1 was determined.

## A.4 Variable glucose

Glucose is a valuable nutrient for cancer cell survival, and its levels can be low in tumors as a result of increased uptake and poor perfusion. Determining the effect of glucose concentration on cell metabolism is important to understanding how cancer cells may adapt to changes in nutrient concentration in the context of the tumor microenvironment. Reducing the glucose concentration in cell culture media to 1 mM improved survival of metM-Wnt$^{lung}$ cells that were treated with hydrogen peroxide compared to 25 mM or 5 mM glucose (Figure A.9). Interestingly, the addition of sodium pyruvate also improved viability of metM-Wnt$^{lung}$ cells treated with high doses of

hydrogen peroxide (Figure A.9). High (25 mM) glucose concentrations also reduce viability of cells compared to 5 mM glucose, and may suppress migration of metM-Wnt$^{lung}$ cells (Figure A.11).

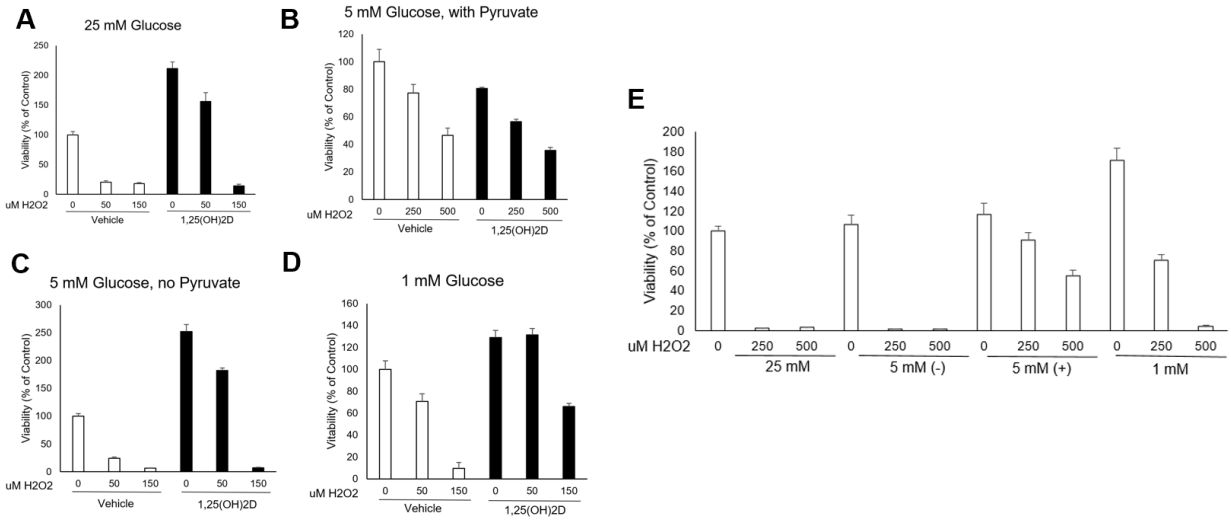


Figure A. 9. Effect of glucose concentration on survival of metM-Wnt$^{lung}$ cells treated with hydrogen peroxide.

metM-Wnt$^{lung}$ cells were constitutively grown in DMEM containing 4 mM glutamine and 25 mM (A), 5 mM with sodium pyruvate (B), 5 mM without sodium pyruvate (C), or 1 mM glucose (D). Cells were pre-treated with vehicle or 1,25$(OH)_2$D for three days, then treated with indicated doses of hydrogen peroxide for 24 h, continuing vehicle or 1,25$(OH)_2$D treatment.

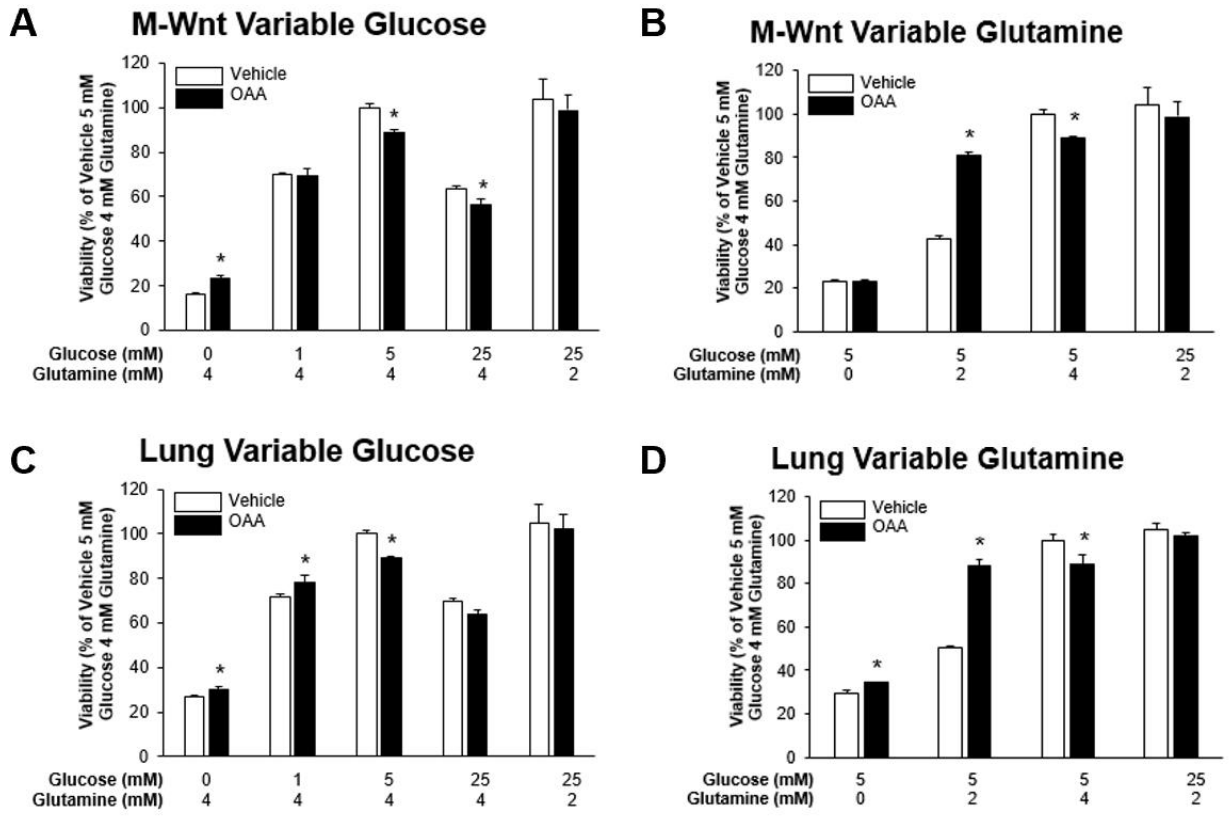


Figure A. 10. Effect of media composition and oxaloacetate (OAA) on viability.

M-Wnt (A,B) and metM-Wnt$^{lung}$ cells (C,D) were constitutively grown in 25 mM glucose and 4 mM glutamine conditions and seeded into 96 well plates. Media was changed to the concentrations indicated on graphs with or without 200 μM oxaloacetate.

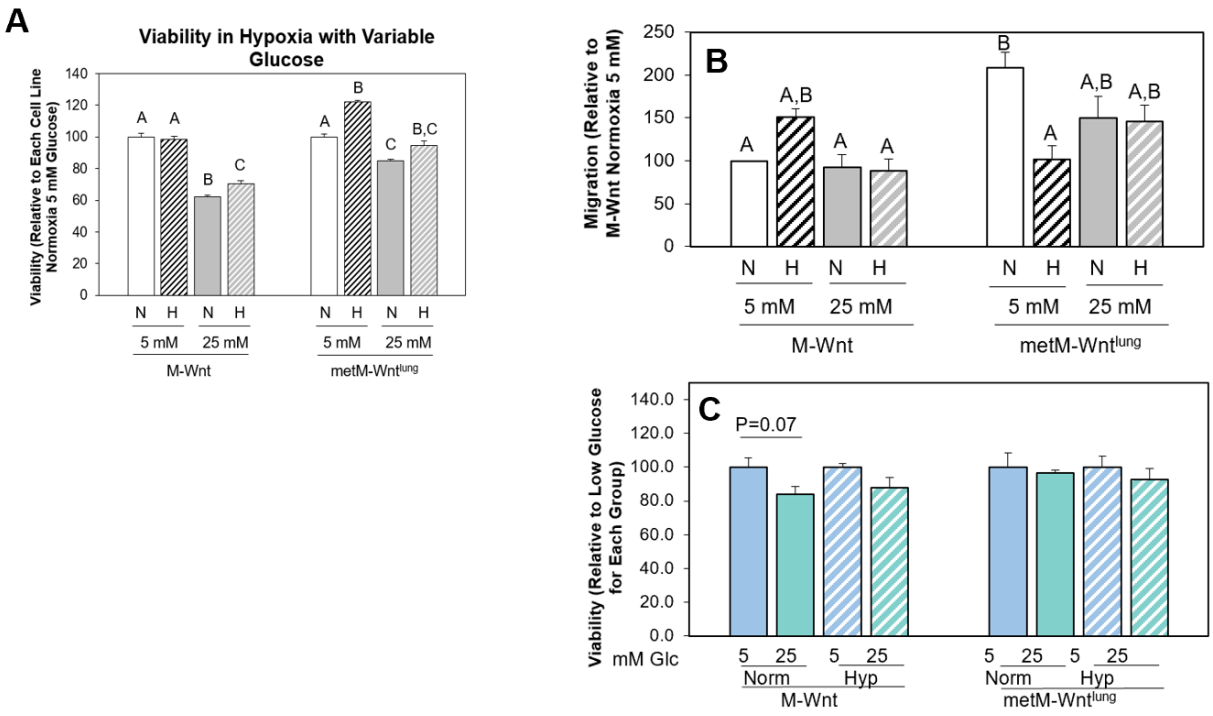


Figure A. 11. Effect of variable glucose and/or hypoxia on viability, migration, and survival in low attachment.

Cells were constitutively grown in media containing 25 mM glucose and 2 mM glutamine or 5 mM glucose and 4 mM glutamine. Cells were incubated in hypoxia or normoxia for 48 h and viability was assessed (A) or cells were seeded into Transwells to assess migration (B) or low attachment plates to assess survival in detached conditions (C).

## A.5 Hypoxia

Hypoxia is a common feature of solid tumors and is associated with increased metastasis and poor patient survival. Hypoxia increases viability of cells grown in 5 mM glucose and 4 mM glutamine (Figure A.12). Inhibiting transaminase activity in 2 mM glutamine suppresses viability of both M-Wnt and metM-Wnt$^{lung}$ cells (Figure A.12). The effect of hypoxia on survival in low attachment and migration was dependent on cell line, glucose concentration, and 1,25$(OH)_2$D treatment (Figure A.13). Hypoxia changes mRNA and protein levels, consistent with a glycolytic phenotype, in cells grown in 5 mM glucose and 4 mM glutamine (Figure A.14, A.15). Hypoxia reduced levels of TCA cycle intermediates in cells grown in 5 mM glucose and 4 mM glutamine (Figure A.17). Hypoxia suppressed flux of glucose to pyruvate and lactate (Figure A.19) and reduced entry of glucose-derived pyruvate into the TCA cycle via pyruvate dehydrogenase or pyruvate carboxylase in cells grown in 5 mM glucose and 2 mM glutamine (Figure A.20). Hypoxia suppressed expression of p62 protein, which may suggest activation of autophagy in cells grown in 5 mM glucose and 2 mM glutamine (Figure A.21).

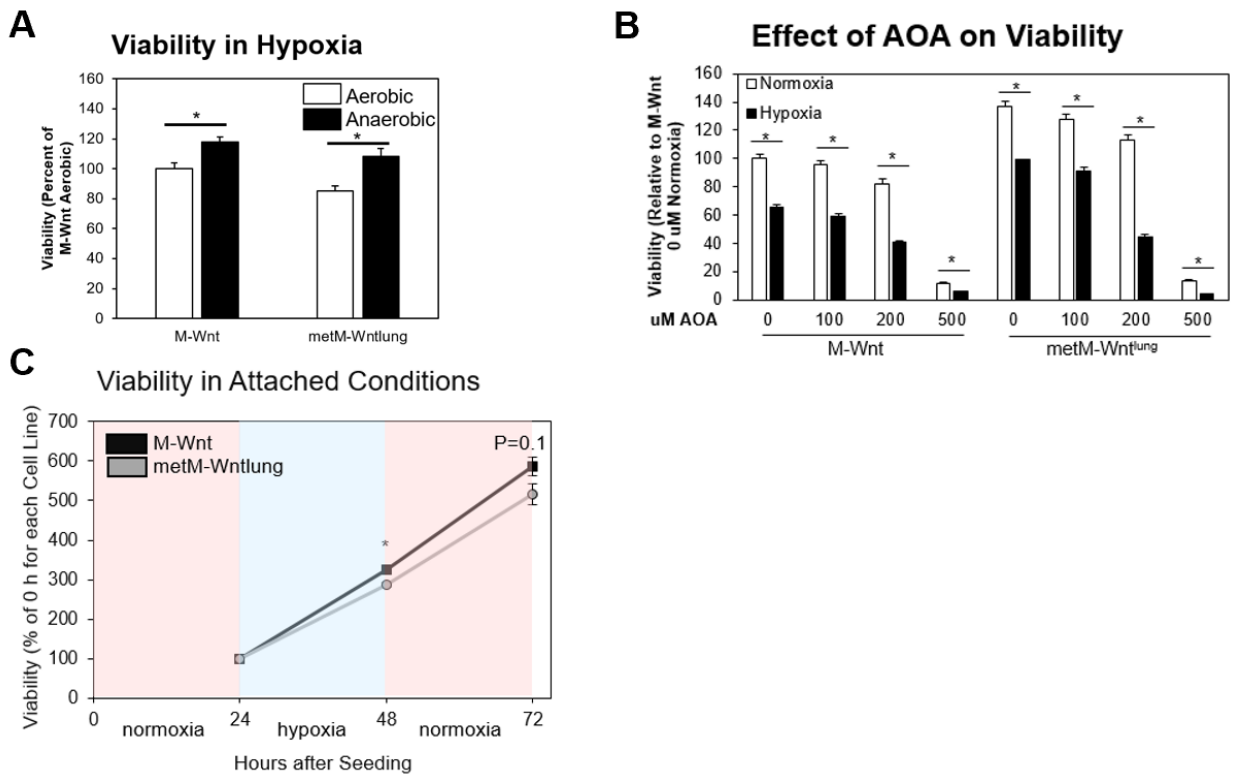


Figure A. 12. Effect of hypoxia on viability.

Cells were grown in 5 mM glucose and 4 mM glutamine and incubated in hypoxia or normoxia for 48 h prior to assessing viability (A). Cells were grown in 5 mM glucose and 2 mM glutamine with or without the transaminase inhibitor aminooxyacetate (AOA) in hypoxia or normoxia for 48 h prior to assessing viability (B). Cells grown in 5 mM glucose and 4 mM glutamine were seeded into 96 well plates and grown in cycling hypoxia for a total of 72 h, as indicated on graph (C).

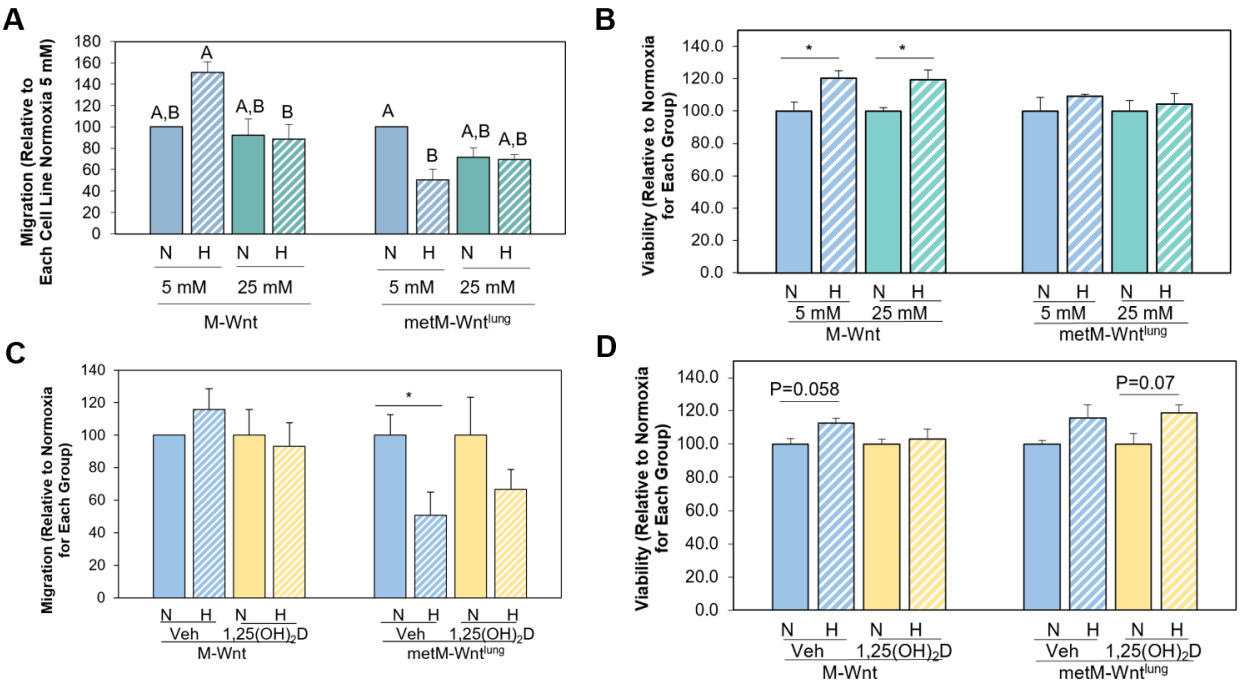


Figure A. 13. Effect of hypoxia on migration and survival in low attachment.

Cells were constitutively grown in either 25 mM glucose and 2 mM glutamine or 5 mM glucose and 4 mM glutamine (A and B), incubated in hypoxia or normoxia for 48 h, and seeded into Transwells to assess migration (A) or into low attachment plates to assess survival in detached conditions (B). Cells were grown in 5 mM glucose and 4 mM glutamine media and pre-treated with vehicle or 1,25(OH)$_2$D for three days prior to incubating in normoxia or hypoxia for 48 h. Following incubation, migration (C) and survival in low attachment (D) were assessed.

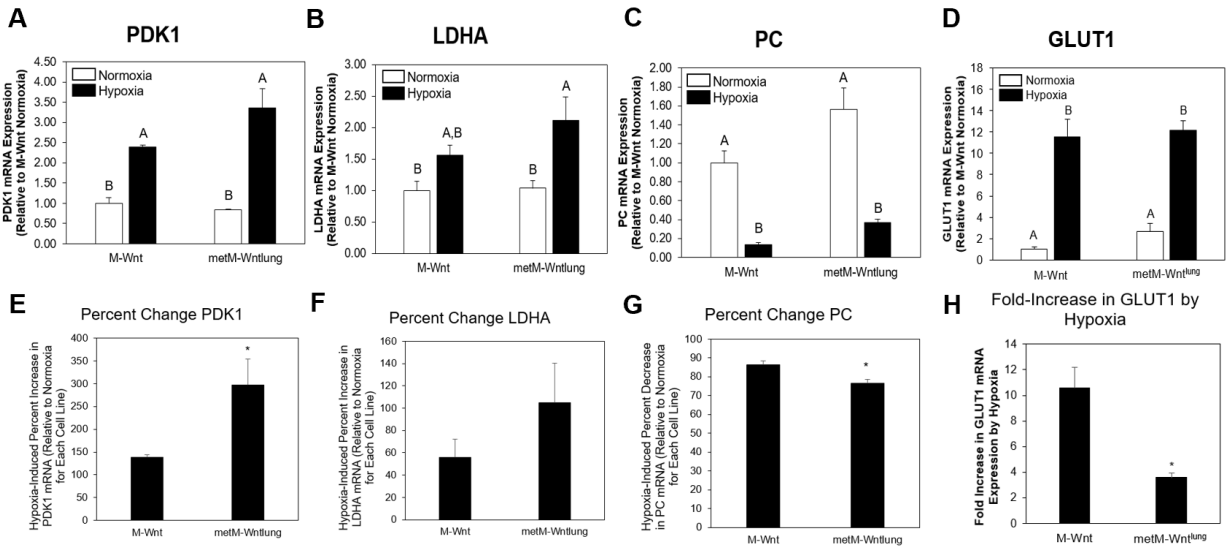


Figure A. 14. mRNA levels in hypoxia.

Cells grown in 5 mM glucose and 4 mM glutamine were incubated in normoxia or hypoxia for 48 h prior to assessment of PDK1 (A), LDHA (B), PC (C), and GLUT1 (D) expression. Percent change in gene expression in hypoxia was assessed (E-H).

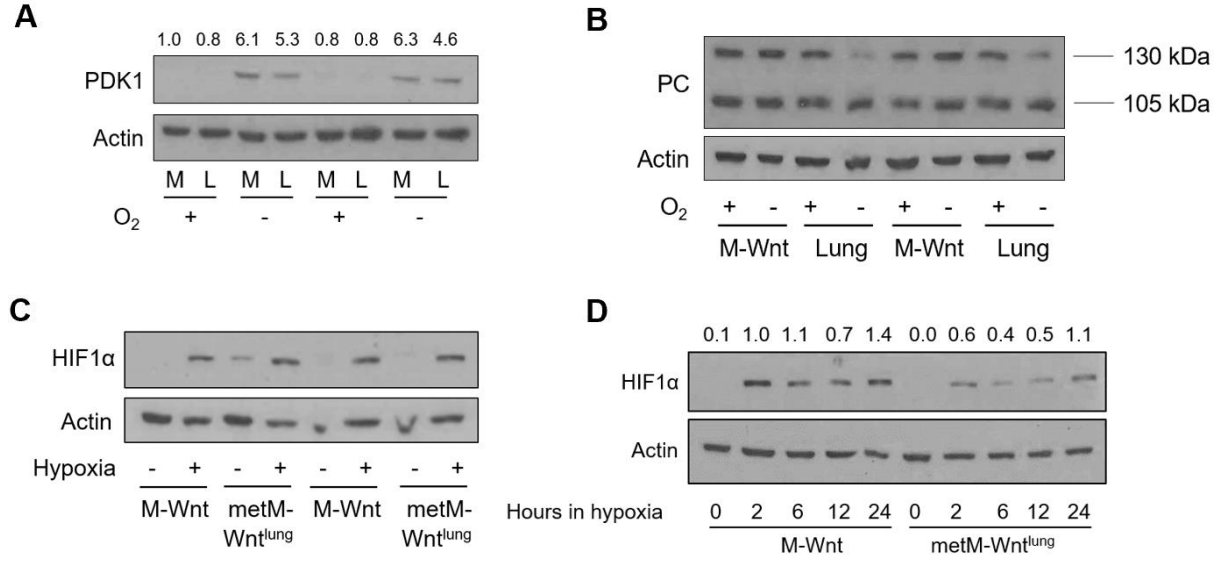


Figure A. 15. Protein levels in hypoxia.

Cells were constitutively grown in 5 mM glucose and 4 mM glutamine and incubated in hypoxia for 48 h before assessing expression of PDK1 (A), PC (B), and HIF1α (C). Cells were incubated in hypoxia for incubated time points and HIF1α protein expression was determined (D).

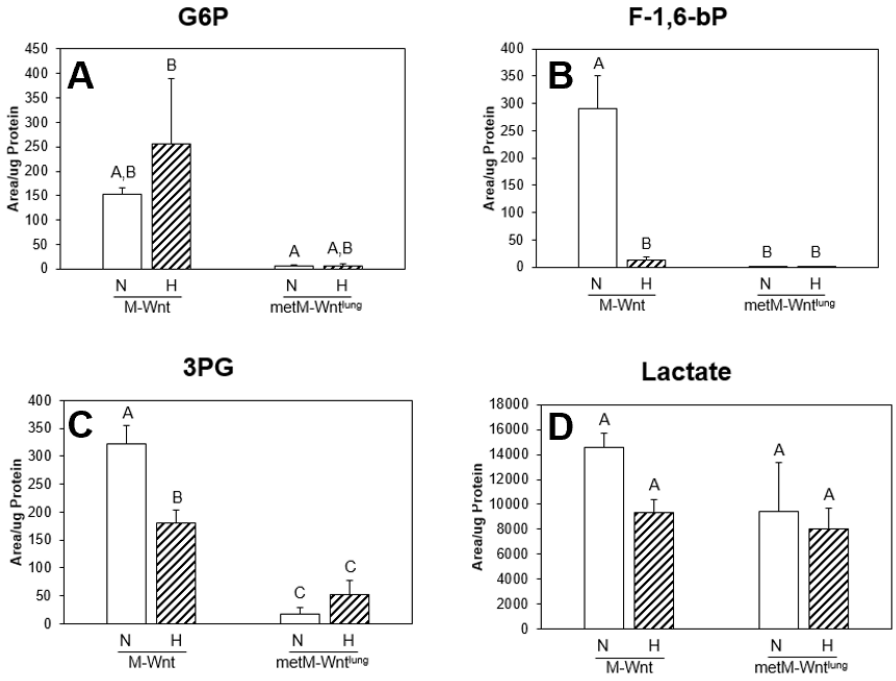


Figure A. 16. Metabolomics in hypoxia.

Cells grown in 5 mM glucose and 4 mM glutamine were incubated in normoxia or hypoxia for 48 h prior to assessing metabolite pool sizes of glycolytic intermediates.

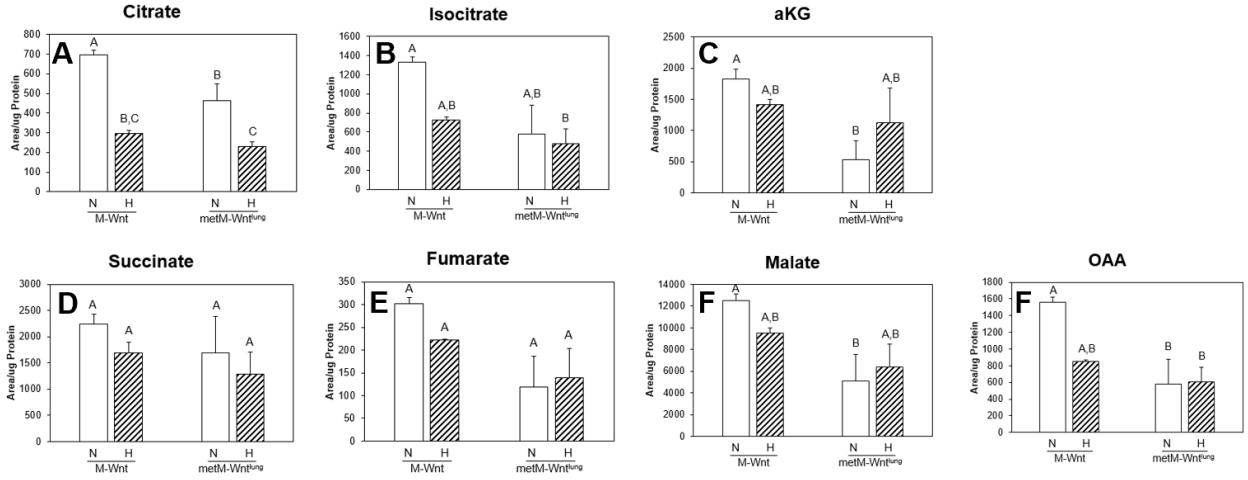


Figure A. 17. Metabolomics in hypoxia.

Cells grown in 5 mM glucose and 4 mM glutamine were incubated in normoxia or hypoxia for 48 h prior to assessing metabolite pool sizes of TCA cycle intermediates.

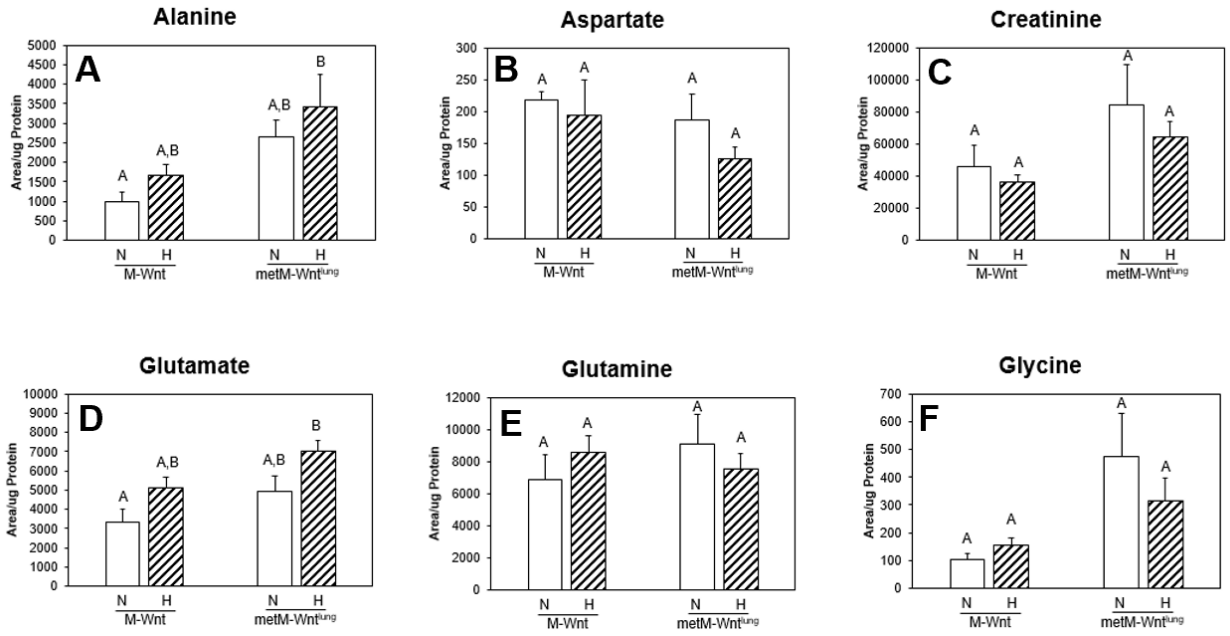


Figure A. 18. Metabolomics in hypoxia.

Cells grown in 5 mM glucose and 4 mM glutamine were incubated in normoxia or hypoxia for 48 h prior to assessing metabolite pool sizes of amino acids intermediates.

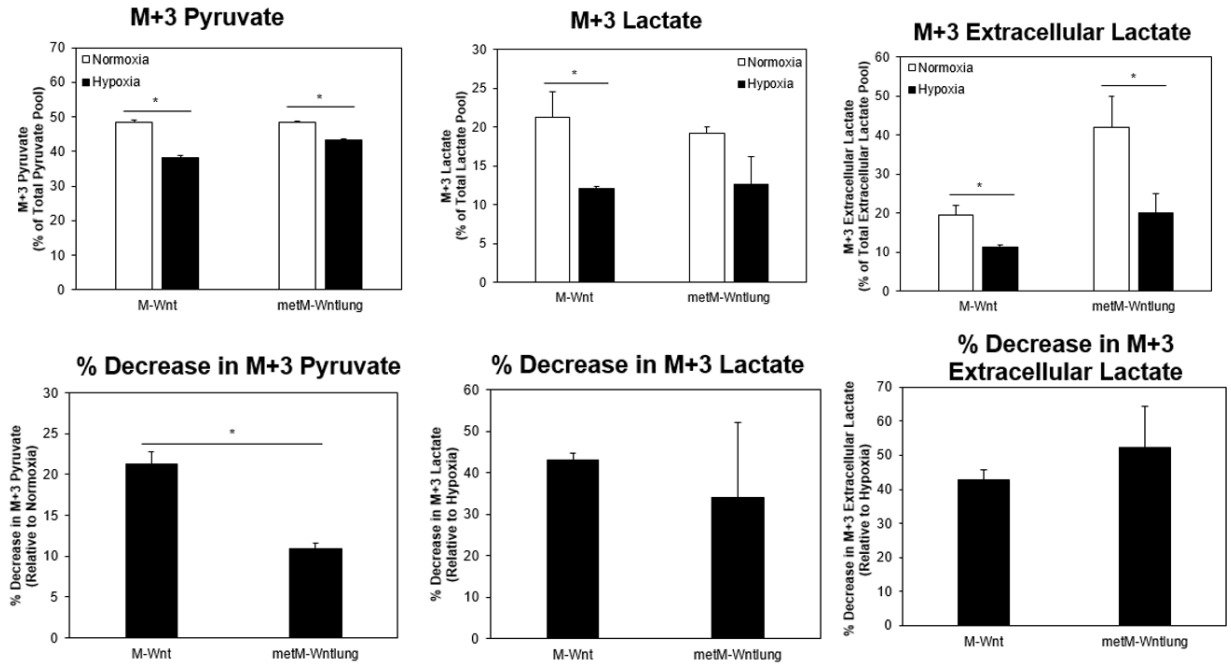


Figure A. 19. Glucose flux in hypoxia.

Cells grown in 5 mM glucose and 2 mM glutamine were incubated in hypoxia or normoxia for 46 h, then media was changed to the 5 mM $^{13}C_6$ labeled glucose and 2 mM unlabeled glutamine. Cells were incubated with labeled glucose for 2 h.

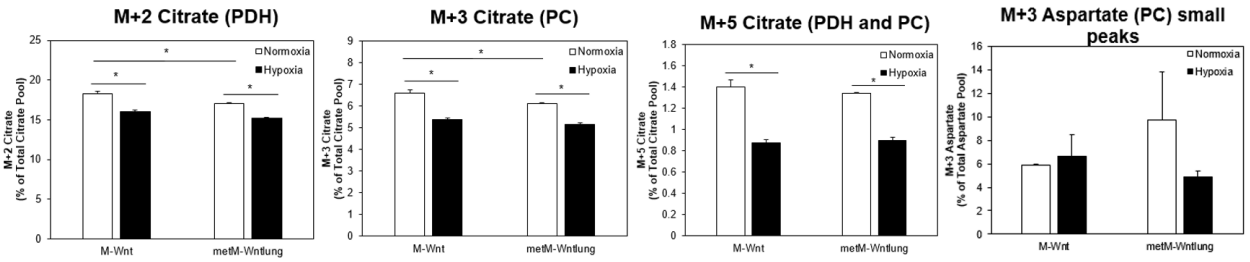


Figure A. 20. Flux into the TCA cycle in hypoxia.

Cells grown in 5 mM glucose and 2 mM glutamine were incubated in hypoxia or normoxia for 46 h, then media was changed to the 5 mM $^{13}C_6$ labeled glucose and 2 mM unlabeled glutamine. Cells were incubated with labeled glucose for 2 h.

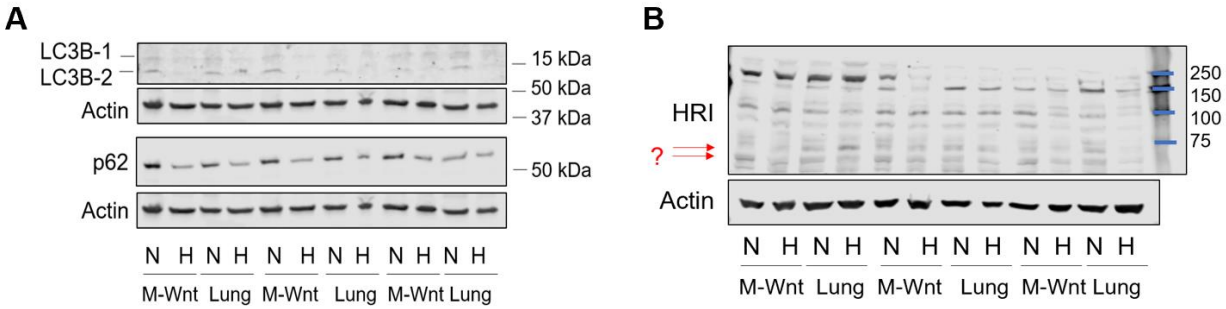


Figure A. 21. Assessment of ATF4-related proteins in hypoxia.

Cells grown in 5 mM glucose and 2 mM glutamine were incubated in hypoxia or normoxia for 48 h prior to assessing expression of the autophagy proteins LC3-B and p62 (A) or the ATF4 kinase HRI (B).

# VITA

**Violet Kiesel**
700 W. State St. West Lafayette IN 47907

**Education**

2017-present Purdue University: West Lafayette, IN
Ph.D. Candidate, Nutrition Science

2014-16 Purdue University: West Lafayette, IN
M.S. Nutrition Science

2009-14 University of Delaware: Newark, DE
B.S. Nutritional Sciences, Dietetics
B.A. Biological Sciences
Minor Anthropology
Honors Program

**Professional Experience**

2017-present Graduate Research Assistant, Purdue University, Department of Nutrition Science
Project: Metabolic stress in non-metastatic and metastatic murine mammary cancer cells
Mentor: Dorothy Teegarden
Preliminary Exam Date: 5 December 2017

2014-16 Graduate Research Assistant, Purdue University, Department of Nutrition Science
Thesis: Diallyl trisulfide modulates Notch pathway components in breast cancer cells
Mentor: Silvia Stan

2013 Undergraduate Research Assistant, University of Delaware, Neuroscience Department
Examination of behavioral changes in rat pups with differential maternal care
Mentor: Tania Roth

2011-14 Student Textbook Manager, Lieberman's University Bookstore, Newark, DE
Textbook purchasing and sales
Supervisors: Michael Dorotheo and Steven Antonis

**Teaching Experience**

| | |
|---|---|
| 2019 | Graduate Teaching Certificate recipient |
| 2018 | Teaching Academy Graduate Teaching Award recipient |
| 2017 | Graduate Lecturer, Purdue University<br>NUTR 330, Diet Selection & Planning Dietary Guidance for Human Health<br>Co-taught one semester with Regan Bailey<br>Online class |
| 2017-18 | Graduate Teaching Assistant, Purdue University<br>NUTR 436, Nutrition Assessment<br>Taught two semesters<br>Instructor: Nana Gletsu-Miller |
| 2015-17 | Graduate Teaching Assistant, Purdue University<br>NUTR 330, Diet Selection & Planning Dietary Guidance for Human Health<br>Taught four semesters<br>Instructors: Barbara Mayfield (2015 – 2016) and Regan Bailey (2016 – 2017) |
| 2015-18 | Graduate Teaching Assistant, Purdue University<br>NUTR 205, Food Science I<br>Taught five semesters<br>Instructors: Charles Santerre (2015 – 2017) and Kimberly Buhman (2017 – 2018) |
| 2014 | Private Tutor, University of Delaware<br>NTDT 401, Micronutrients<br>Instructor: Cheng-Shun Fang |
| 2013 | Undergraduate Teaching Assistant, University of Delaware<br>NTDT 401, Micronutrients<br>Instructor: Cheng-Shun Fang |

**Awards, Honors, and Fellowships**

| | |
|---|---|
| May 2019 | Mary Frances Picciano Dietary Supplement Research Practicum, National Institutes of Health |
| Apr 2019 | Bilsland Fellowship, Purdue University |
| Mar 2019 | Nominated, College of Health and Human Sciences Outstanding Graduate Teaching Award, Purdue University |
| Mar 2019 | First Place Award (Cancer), Health and Disease: Science, Technology, Culture, and Policy Research Poster Session, Purdue University |

| | |
|---|---|
| Mar 2019 | John Milner Nutrition and Cancer Prevention Research Practicum, National Cancer Institute, National Institutes of Health |
| Jan 2019 | Graduate Teacher Certificate, Purdue University |
| Mar 2018 | Teaching Academy Graduate Teaching Award, Purdue University |
| Mar 2018 | Cancer Prevention Internship Program Fellowship, Purdue University |
| Feb 2016 | Interdepartmental Nutrition Program Basic Science poster award, Purdue University |
| May 2014 | Nutrition and Dietetics Panel of Distinguished Seniors Award, Nutritional Sciences, University of Delaware |
| May 2011 | General Honors Award, University of Delaware |

**Publications**

**Kiesel VA**, Sheeley MP, Coleman MF, Kulkoyluoglu Cotul E, Donkin SS, Husrting SD, Wendt MK, Teegarden D. Pyruvate carboxylase and cancer progression. Submitted, *Cancer & Metabolism*.

Bailey RL, **Kiesel VA**, Lobene AJ, Zou P. Redesigning an undergraduate nutrition course through active learning. Under review, *Current Developments in Nutrition*.

**Kiesel VA**, Stan SD, Diallyl trisulfide, a chemopreventive agent from Allium vegetables, inhibits alpha-secretases in breast cancer cells, Biochemical and Biophysical Research Communications, Volume 484, Issue 4, 2017, Pages 833-838

**Conferences and Presentations**

| | |
|---|---|
| Jun 2019 | American Society for Nutrition Poster Session<br>Baltimore, Maryland<br>*Differential Response to 1,25-dihydroxyvitamin D in Metastatic and Non-Metastatic Breast Cancer Cell Lines in Hypoxia*<br>Kiesel VA, Hursting SD, Teegarden D |
| May 2019 | American Institute of Cancer Research Poster Session<br>University of North Carolina Chapel Hill<br>*Differential Response to 1,25-dihydroxyvitamin D in Metastatic and Non-Metastatic Breast Cancer Cell Lines in Hypoxia*<br>Kiesel VA, Hursting SD, Teegarden D |
| Feb 2019 | Health and Disease: Science, Technology, Culture and Policy Research Poster Session<br>Purdue University |

*Differential Response to 1,25-dihydroxyvitamin D in Metastatic and Non-Metastatic Breast Cancer Cell Lines in Hypoxia*
Kiesel VA, Hursting SD, Teegarden D

Jan 2019 Interdepartmental Nutrition Program Poster Session
Purdue University
*Differential Response to 1,25-dihydroxyvitamin D in Metastatic and Non-Metastatic Breast Cancer Cell Lines in Hypoxia*
Kiesel VA, Hursting SD, Teegarden D

Oct 2018 Health and Human Sciences Fall Research Day Poster Session
Purdue University
*Molecular Mechanisms Underlying 1,25-dihydroxyvitamin D Inhibition of Breast to Lung Metastasis*
Kiesel VA, Sheeley M, Wong K, Yum C, Andolino C, Shinde A, Wendt MK, Hursting SD, Teegarden D

Mar 2018 Health and Disease: Science, Technology, Culture and Policy Research Poster Session
Purdue University
*Molecular Mechanisms Underlying 1,25-dihydroxyvitamin D Inhibition of Breast to Lung Metastasis*
Kiesel VA, Sheeley M, Wong K, Yum C, Andolino C, Wilmanski T, Shinde A, Wendt MK, Hursting SD, Teegarden D

Feb 2018 Big Ten Lipids Conference Poster Session
Purdue University
*Molecular Mechanisms Underlying 1,25-dihydroxyvitamin D Inhibition of Breast to Lung Metastasis*
Kiesel VA, Wilmanski T, Shinde A, Wendt MK, Hursting SD, Teegarden D

Feb 2018 Interdepartmental Nutrition Program Poster Session
Purdue University
*Molecular Mechanisms Underlying 1,25-dihydroxyvitamin D Inhibition of Breast to Lung Metastasis*
Kiesel VA, Sheeley M, Yum C, Andolino C, Wilmanski T, Shinde A, Wendt MK, Hursting SD, Teegarden D

Mar 2016 Health and Disease: Science, Technology, Culture and Policy Research Poster Session
Purdue University
*Diallyl Trisulfide Modulates Notch Signaling Pathway in Breast Cancer Cells*

Feb 2016 Interdepartmental Nutrition Program Poster Session
Purdue University
*Diallyl Trisulfide Modulates Notch Signaling Pathway in Breast Cancer Cells*

**Travel Grants**

2019 Compton Graduate Research Travel Award, funded $500
2019 Compton Training Travel Award, funded $500
2019 Purdue Graduate Student Government Travel Award, funded $500

**Professional Memberships**

2019 – Present American Society for Nutrition
Student Graduate Member