

THE ROLE OF HYPOXIA-REGULATED RHOB EXPRESSION IN
BREAST CANCER TUMORIGENESIS AND METASTASIS

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Abstract

Although Rho GTPases RhoA, RhoB, and RhoC share more than 85% amino acid sequence identity, they may play very distinct roles in tumor progression. RhoA and RhoC have been suggested in many studies to contribute positively to tumor progression, but the role of RhoB in cancer still remains unclear. RhoB contains a unique C-terminal region that undergoes specific post-translational modifications affecting its localization and function. In contrast to RhoA and RhoC, RhoB not only localizes at the plasma membrane, but also on endosomes, multivesicular bodies and has even been reported in the nucleus. Recent studies have shown that small GTPases such as RhoA, Rac1 and Cdc42 are induced *in vitro* during hypoxia. Hypoxia within a solid tumor arises from the increase in oxygen consumption of rapidly proliferating cancer cells, and a decrease in oxygen availability due to structurally and functionally abnormal blood vessels that form within these tumors. Although RhoA and RhoC are well characterized and have been extensively studied under hypoxia, whether and how hypoxia regulates RhoB remains elusive.

We investigated the effect of hypoxia on the expression of RhoB and the mechanism and significance of RhoB expression in breast cancer. We found that hypoxia significantly upregulated the mRNA and protein expression of RhoB in a HIF-1 independent manner in SUM159 and MDA-MB-231 cell lines. CRISPR-mediated reduction in RhoB expression caused a marked decrease in HIF-1 α expression in an AKT-dependent manner, but did not show the reverse for RhoB overexpression. Therefore, we conclude that RhoB is necessary, but not sufficient for HIF-1 induction under hypoxia. Additionally, we observed that loss of RhoB in 3D cellular environments

led to a small, but significant, decrease in cell motility and proliferation. Furthermore, orthotopic implantation of RhoB knockdown cells into immune-deficient mice contributed to a slower tumor growth and decreased metastasis compared to the wild type and non-targeted controls. Our work suggests that RhoB may play an oncogenic role in breast cancer potentially by increasing HIF-1 α . This work warrants additional studies to determine whether targeting RhoB could have implications in the treatment of breast cancer.

Advisors: Dr. Daniele Gilkes and Dr. Denis Wirtz

Committee: Dr. Daniele Gilkes and Dr. Denis Wirtz

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Definition of Symbols

O₂ Diatomic Oxygen

pO₂ Partial pressure of Oxygen

List of Acronyms

CRISPR	Clustered Regularly Interspaced Palindromic Repeats
CTCs	Circulating Tumor Cells
DNA	Deoxyribose Nucleic Acid
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
FTI	Farnesyltransferase Inhibitor
GAP	GTPase-activating protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GEF	Guanine Nucleotide Exchange Factor
HDAC	Histone Deacetylase
HER2	Human Epidermal Growth Factor Receptor 2
HIFs	Hypoxia-inducible Factors
HRE	Hypoxia Response Element
IHC	Immunohistochemistry
IL-6	Interleukin-6
IL-8	Interleukin-8
mTOR	Mechanistic target of rapamycin
NTC	Non-targeted control
ODD	Oxygen-Dependent Degradation Domain
p-Erk	Phospho-Erk
p-Akt	Phospho-Akt
PHD	Prolyl Hydroxylase

PIP ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PI3K	Phosphotidylinositide 3-kinase
PR	Progesterone Receptor
qRT-PCR	Quantitative Real-time Reverse Transcription Polymerase Chain Reaction
SE	Standard Error
SEM	Standard Error of the Mean

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Introduction

1.1 Breast Cancer Overview

Breast cancer is the most common cancer that occurs in women. In 2017, more than 250,000 new cases are expected to be diagnosed among women, equating to about 30% of all cancers¹. This means that 1 in 8 women will develop invasive breast cancer during her lifetime. Additionally, breast cancer is still the 2nd leading cause of cancer-related deaths for women in the United States (Fig. 1). Although the number of deaths have decreased by 34% since 1990, in 2017, more than 40,000 women are still predicted to die from breast cancer¹. A woman's risk of breast cancer nearly doubles if she has a first-degree relative who has also been diagnosed. About 5-10% of breast cancers can be linked to inherited gene mutations, most commonly in the BRCA1 and BRCA2 genes². Upon diagnosis, the patient's treatment options are determined by the presence or absence of three key receptors: ER, PR and HER2, as well as clinical staging based on size, lymph node involvement and tumor histology. Despite all of these evaluations, there are still no techniques of definitively identifying patients who will relapse or whose tumor will metastasize³. Consequently, there is still very little we can do to improve the prognoses for these types of patients.

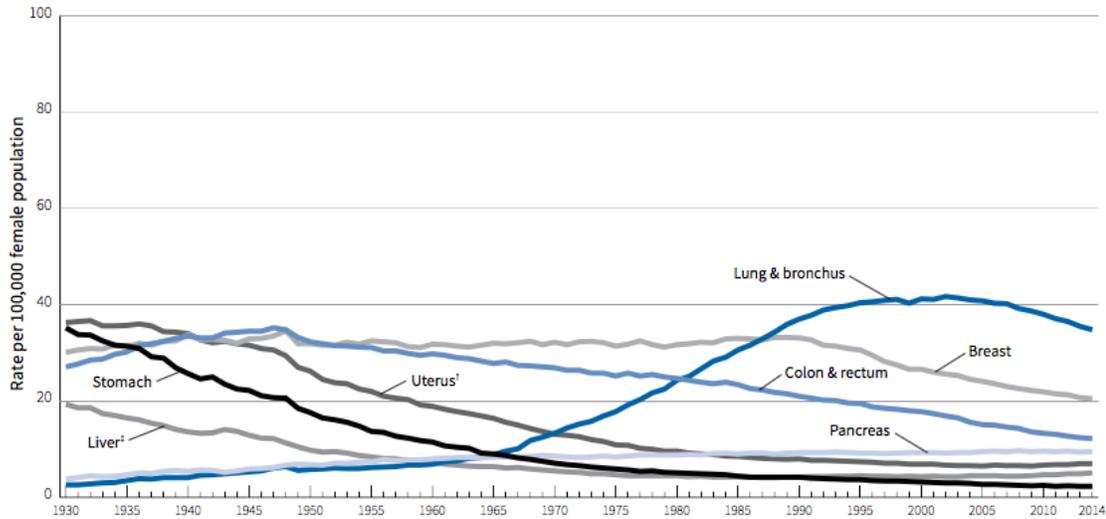


Figure 1: Trends in age-adjusted cancer death rates by site, females, US, 1930-2014¹. According to the American Cancer Society, in 2016, breast cancer remains the second leading cause of death among women in the United States.

1.1.1 Tumorigenesis

Breast cancer arises when changes at the cellular, genetic, and epigenetic levels develop and abnormal cell division begins to occur. Normal cells are transformed into cancer cells and begin to proliferate rapidly and may even form a malignant mass.

Usually, cell proliferation is balanced with apoptosis to maintain the integrity of tissues and organs. However, some cells develop mutations in their DNA that disrupt this process thereby upsetting this balance between cell growth and death. Mutations can include those that inactivate DNA repair genes, create an oncogene as well as inactivating many tumor suppressor genes. A series of several mutations to certain classes of genes is required before a normal cell will transform into a cancer cell⁴.

There is no single cause of cancer development. Variants of inherited genes may predispose individuals to cancer. Additionally, environmental factors such as carcinogens and radiation also cause mutations to DNA that may play a role in tumorigenesis. Besides

these, random mistakes in normal DNA replication could also result in cancer causing mutations that are ultimately uncontrollable.

1.1.2 Metastatic process

Metastasis is the least understood process in tumorigenesis. Of the deaths attributed to cancer, 90% are due to metastasis, and treatments that prevent or cure metastasis remain elusive⁵. In most instances, primary tumors are successfully removed by surgery or treated with radiation. However, once the tumor cells begin to disseminate to other organs in the body, it is very difficult to treat and in most cases, leads to death.

Metastasis consists of a series of rate-limiting steps. In order for the initiation process to begin, the tumor cells must acquire a motile phenotype, which is mediated by a process called the epithelial-mesenchymal transition (EMT). The cell can then detach from the primary, vascularized tumor, penetrate the surrounding tissue, enter nearby blood vessels (intravasation) and circulate in the vascular system (Fig. 2)⁶. Successful migration depends on the survival of circulating tumor cells (CTCs) in the blood circulation. Some of these cells eventually adhere to blood vessel walls and are able to extravasate and migrate into the local tissue, where they can form a secondary tumor in places like the lungs, liver, brain and bones. There has been an increasing amount of evidence that the oxygen (O₂) content of tumor tissue is an important determinant of metastasis.

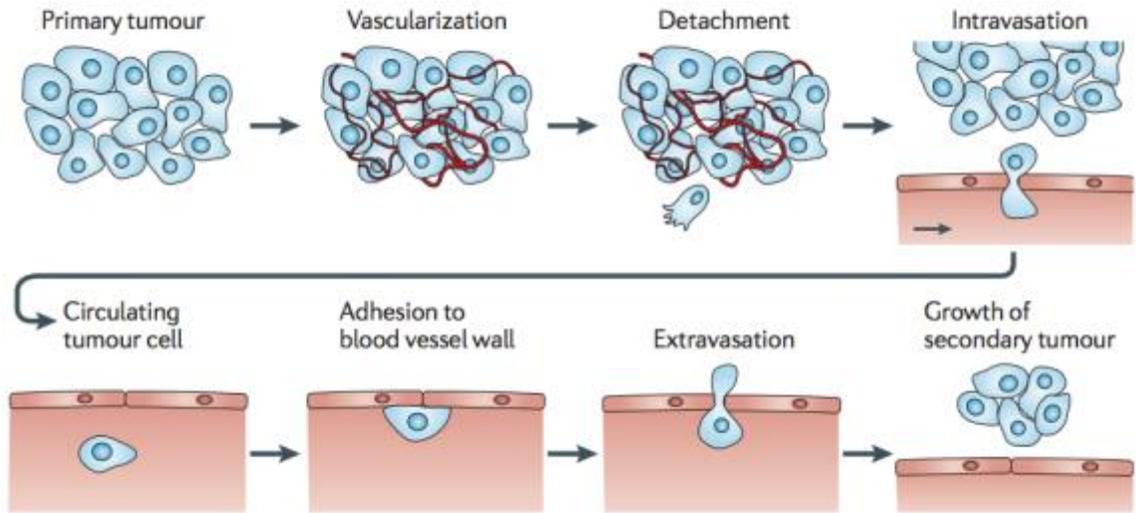


Figure 2: The metastatic process⁶. In a series of steps that comprise the metastatic process, cancer cells migrate or flow through very different microenvironments, including the stroma, the blood vessel endothelium, the vascular system and the tissue at a secondary site.

1.2 Hypoxia

1.2.1 Hypoxia and breast cancer

About 25-40% of invasive breast cancers exhibit hypoxic regions⁷. On average, the partial pressure of oxygen (pO_2) in normal human breast tissue is 65 mmHg (~8.5%), whereas human breast cancers have a median pO_2 of 10 mmHg (~1/3%). Patients that have a primary tumor with pO_2 of less than 10 mmHg are at an increased risk of metastasis and mortality, independent of any other factors⁸.

Human cells require an adequate and continuous supply of oxygen for use as the terminal electron acceptor in the process of mitochondrial respiration that generates ATP, which is then used to power most biochemical reactions in the body⁹. Rapid cancer cell proliferation, combined with structural and function abnormalities in tumor blood vessels, results in regions within solid tumors that have reduced oxygen availability⁵. To adapt to the hypoxic microenvironment, cancer cells acquire different physiological responses

mediated by HIFs. During this process, hypoxic cells obtain invasive and metastatic properties as well as resistance to chemotherapy and radiation therapy, which together constitute the lethal cancer phenotype^{7,9}.

1.2.2 Hypoxia Inducible Factors

The most well-characterized hypoxia response pathway is mediated by hypoxia-inducible factor-1 (HIF-1). HIFs facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating the expression of genes that are involved in many cellular processes, including glucose uptake and metabolism, angiogenesis, erythropoiesis, cell proliferation, and apoptosis¹⁰. HIF-1 is a heterodimer with a HIF-1 β subunit that is constitutively expressed and an oxygen-dependent HIF-1 α subunit.

In an O₂-rich environment (Fig. 3), prolyl hydroxylases (PHDs) modify the Pro402 and Pro564 residues of HIF-1 α and allow the VHL tumor suppressor protein to bind to HIF-1 α , which leads to its ubiquitylation and proteasomal degradation. Under low oxygen conditions (Fig. 4), this degradation is inhibited and HIF-1 α stabilizes and dimerizes with its beta subunit to bind with the hypoxia response element (HRE). By interacting with its coactivator, HIF-1 activates transcription of target genes, which aid in increase O₂ delivery or facilitate metabolic adaptation to hypoxia and consequently tumor progression. Similar to HIF-1 α , HIF-2 α is also regulated by oxygen-dependent hydroxylation (Fig.4). HIF-1 α and HIF-2 α are structurally similar in DNA binding and dimerization domains, but differ in their transactivation domains¹¹. HIF-2 α also dimerizes with HIF-1 β under low oxygen conditions, but is expressed in a cell-restricted manner

and plays important roles in erythropoiesis, vascularization, and pulmonary development¹².

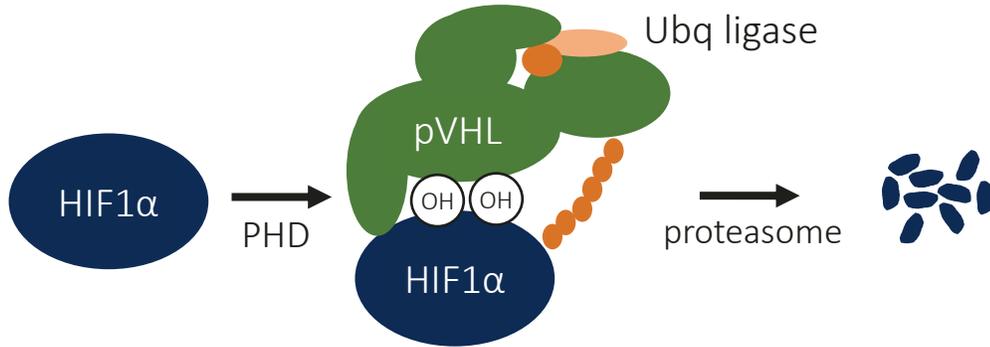


Figure 3: Normal (20% O₂) oxygen conditions. Under normoxic conditions, two proline residues within the ODD of the HIF- α subunit are modified by the PHDs, allowing for HIF-recognition by a protein-ubiquitin ligase complex containing pVHL, and leading to HIF- α degradation by the proteasome.

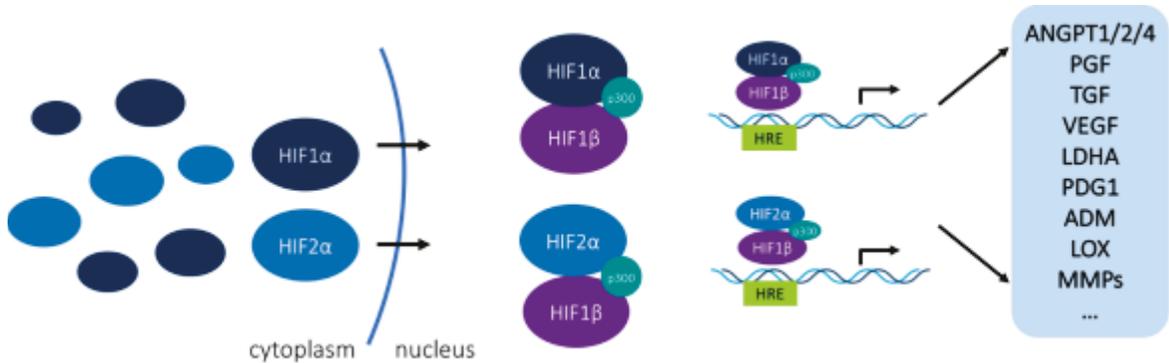


Figure 4: Low (1% O₂) oxygen conditions. When oxygen levels decrease (hypoxia), prolyl hydroxylation are blocked. HIF- α accumulates and interacts with the HIF- β subunit and the transcriptional complex, thereby promoting transcription of downstream HIF target genes that include the HIF prolyl hydroxylase.

1.2.3 HIFs in Cancer Progression

Tumorigenesis involves a number of alterations in cell physiology that contributes to malignant growth. Importantly, HIFs have been found to promote key steps in

tumorigenesis, including angiogenesis, metabolism, proliferation, metastasis and differentiation¹⁰. Data from clinical studies that are complemented with mice models, have demonstrated that HIF-1 has a key role in primary tumor growth and vascularization⁸. These studies showed that lung metastasis was severely decreased in HIF-1 α knock-out mice. HIF-1 α is known to be overexpressed in many cancer types and is associated with poor prognosis in breast, brain, oropharynx, cervix, ovary and uterus cancers¹¹. Interestingly, HIF-1 α is more highly expressed in metastases (69%) than with the actual primary tumor (29%)¹¹. This could be one of the main reasons why metastasis results in such a poor patient outcome.

HIF-1 also regulates the expression of genes that encode proteins with key roles in cancer biology. This occurs when HIF-1 α and HIF-2 α bind and activate the transcription of many target genes with cis-acting hypoxia response elements that contain the consensus binding site 5'-RCGTG-3'¹³. These genes induced by hypoxia encode proteins that regulate angiogenesis, cell survival, chemotherapy and radiation resistance, genetic instability, immortalization, immune evasion, invasion and metastasis, proliferation, metabolism and pH regulation, and stem cell maintenance¹³.

1.3 Rho GTPases

1.3.1 Overview of Rho GTPases

Rho proteins are small molecules (~21 kDa) that belong to the Ras superfamily and function as binary switches in a wide variety of signaling pathways¹⁴. They consist of a family of 20 intracellular signaling molecules that are most well-known for their role in regulating the actin cytoskeleton (Fig. 5). However, they are also known to activate

pathways that regulate gene transcription, vesicle trafficking and cytoskeletal reorganization, which are all processes that regulate growth, differentiation, adhesion, and migration of cells. In order to stimulate these pathways, the small GTPases must be in their active, GTP-bound conformation. Only in this state are they able to bind effector proteins and transduce signals from membrane receptors such as cytokine and growth factor receptors, integrins and G-protein coupled receptors¹⁵.

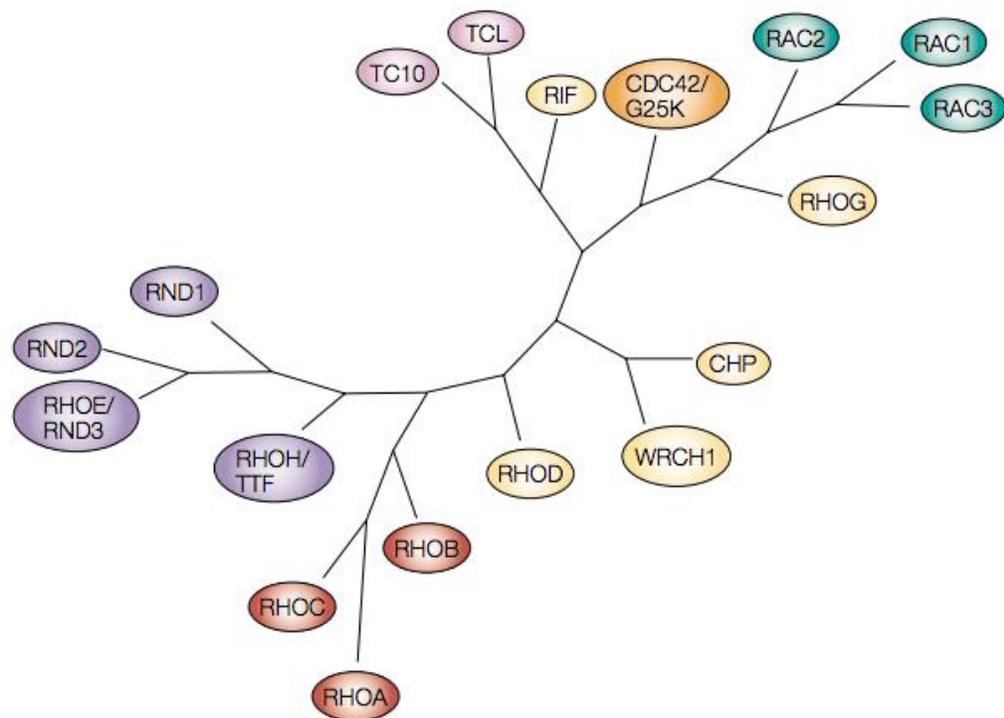


Figure 5: The Rho-protein family¹⁶. The Rho family of proteins contain around 20 small GTPases that can be subdivided on the basis of functional, biochemical and sequence data.

1.3.2 Rho GTPase Structure and Regulation

A structural feature that distinguishes the Rho proteins from other small GTPases is the Rho insert domain located between a β strand and an α helix within the small

GTPase domain¹⁴. Typically, Rho proteins are 190-250 residues long and consist of only the GTPase domain and short terminal C-terminal extensions. They all contain the sequence motifs characteristic of all GTP-binding proteins, bind to GDP and GTP with high affinity. Additionally, most of the members undergo C-terminal post-translational modifications by isoprenoid lipids, which in turn regulate their subcellular location and function¹⁴.

The activity of Rho GTPases in response to receptor stimulation is tightly controlled in order to stimulate, locally and temporally, specific downstream signaling pathways in cells¹⁵. There are three general classes of regulators of Rho signaling including GEFs, GAPs and GDIs. Figure 5 depicts the general mechanism of Rho proteins where GEFs activate Rho proteins by allowing the catalytic exchange of GDP to GTP, GAPs control their ability to hydrolyze the GTP back to GDP thereby inactivating them, and GDI proteins act as an anchor allowing tight control of Rho activation.

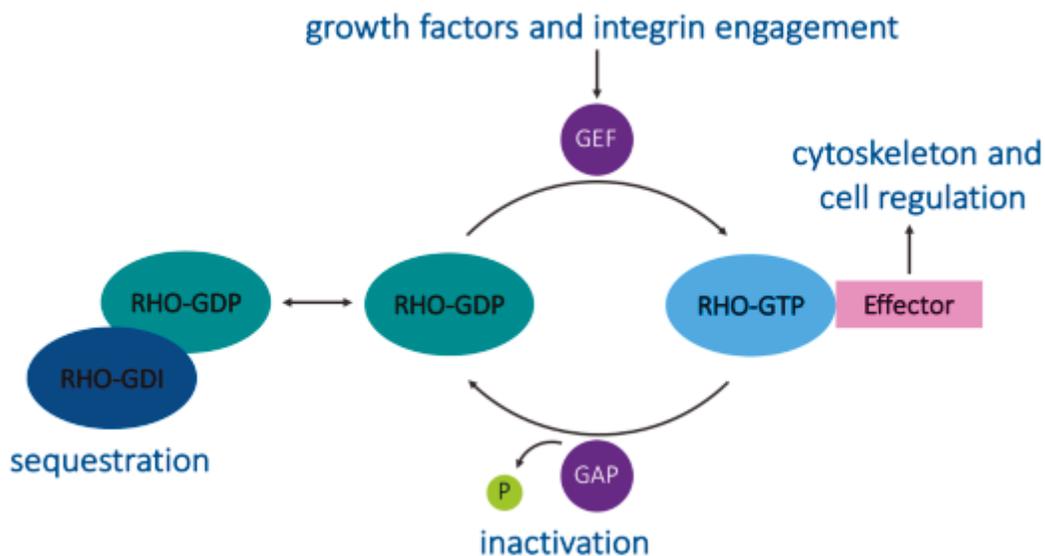


Figure 6: General mechanism of Rho proteins. Rho proteins are activated by the cyclic activity of GEFs, which activate Rho-GDP by adding a phosphate making it Rho-GTP and GAPs, which inactivate them by taking off a phosphate.

GTP-bound Rho proteins interact with many different effector molecules that influence their activity and localization. The regulation of these effector proteins is what eventually leads to changes in cell behavior. Many effector proteins are kinases, which phosphorylate cellular targets to control the behavior of the cell. The best-characterized effector kinases are the p21-activated kinases, which makes sense since they bind to active Cdc42 and Rac1 as well as the ROCKs, which bind active RhoA¹⁶. Another type of effector molecule are scaffold proteins, which operate through protein-protein interactions to control cellular functions.

1.3.3 Rho Proteins and Cancer

Ras proteins are mutated in 30% of human cancers with different origins, which hold the possibility that the same might be true for the Rho GTPase family. As of now though, no mutations have been reported in the Rho family. Since it is already established that Rho proteins have roles in the regulation of polarization, migration, proliferation and survival of cells, it was expected that a mutational activation or inactivation would be the cause of tumor formation and progression^{14,15,16-19}. However, since no mutations have been found, deregulation of Rho GTPase signaling could instead occur at the level of expression or activation of Rho GTPases, accomplished by the level of expression or activation of their regulators or downstream effectors¹⁵. *In vitro* studies have shown that Rho-GEFs are more potent oncogenes than GTPase-defective Rho proteins, and that a fast GTP-GDP cycling mutant of Cdc42 has a greater transforming capacity than a GTPase-defective mutant²⁰. The correlation between Rho-protein overexpression and clinical

outcome suggests the potential use of Rho-protein expression levels as prognostic indicators¹⁶.

Even in normal, healthy cells, Rho GTPases are known for their involvement in cell morphology and motility. Cdc42 has a big role in extending the filopodia, which senses different signals and establishes the directionality of movement. Rac then assembles lamellipodia at the leading edge of the cell and RhoA generates a contractile force to push the cell forward. Besides having a deregulated proliferation process, tumor cells also have modified morphological features that allow them to cross through tissue boundaries and develop metastatic sites throughout the body. Given the role of Rho proteins in normal cell migration and their deregulation in cancer cells, it is likely that their role in the invasive phenotype of tumor cells is significant. As shown in Figure 7, many studies have revealed that each Rho GTPase has distinct roles in tumor progression.

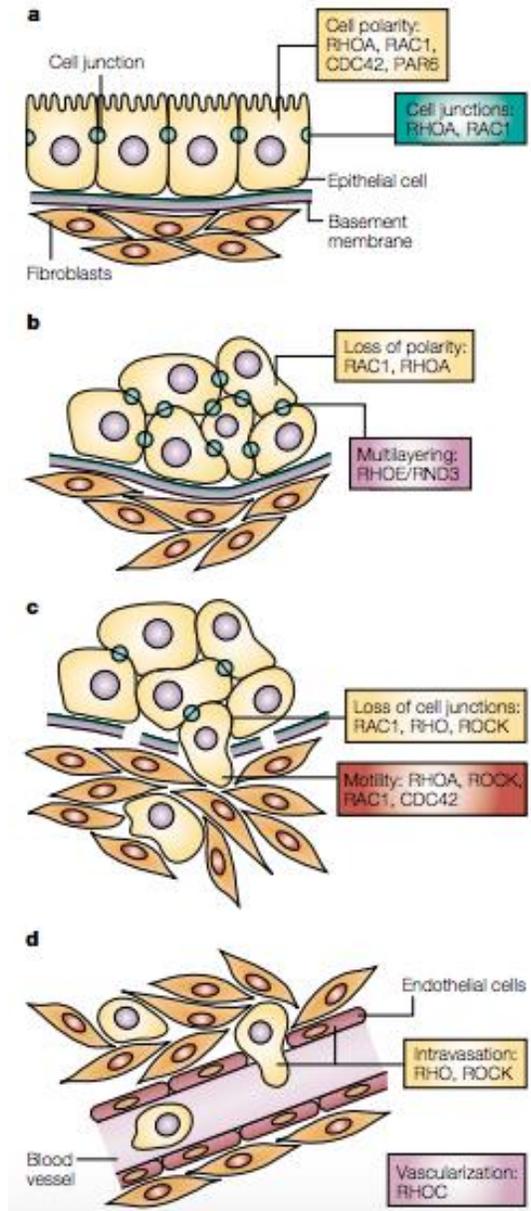


Figure 7: Involvement of Rho proteins at different stages of tumor progression¹⁶. Rho proteins each have a distinct role in tumorigenesis, which allow the cells to proliferate and eventually become malignant.

1.3.4 RhoB in Cancer

Although Rho GTPases RhoA, RhoB, and RhoC share more than 85% amino acid sequence identity, they may play very distinct roles in tumor progression¹⁸. RhoA and

RhoC have been suggested in many studies to contribute positively to tumor progression, but the role of RhoB in cancer still remains unclear²¹⁻²³. RhoB has several additional features that are distinct among Rho proteins. Unlike most small GTPases, which are relatively stable, RhoB is turned over quickly, its synthesis rapidly upregulated by various growth and stress stimuli¹⁷. RhoB contains a unique C-terminal region that undergoes specific post-translational modifications affecting its localization and function¹⁸. In contrast to RhoA and RhoC, RhoB not only localizes at the plasma membrane, but also on endosomes, multivesicular bodies and has even been reported in the nucleus¹⁹. All of these characteristics ultimately contribute to the regulation of proliferation, survival, invasion and angiogenic capacity in some way by RhoB.

RhoB was first described to contribute to fibroblast transformation downstream of the Ras onco-protein, but more recently it has been described as contributing negatively to tumor growth¹⁹. There has been much controversy between different groups as to whether RhoB is a tumor promotor or suppressor. However, since there is evidence of RhoB being both overexpressed and downregulated in different cancers, it is highly likely that RhoB functions in a contextual manner, responding to specific signals in the tumor microenvironment.

1.4 Interleukin-8 in Cancer

IL-8 (CXCL8) is a proinflammatory cytokine that belongs to the CXC superfamily and was initially described as a neutrophil chemoattractant²⁴. It is involved in many intracellular signaling pathways (Fig. 8) and its expression has been shown to be regulated by many different stimuli including inflammatory signals, environmental

stresses like hypoxia, and steroid hormones. The biological effects of IL-8 are mediated through the binding of IL-8 to two cell-surface G protein-coupled receptors, CXCR1 and CXCR2²⁵. The expression of IL-8 receptors on cancer cells, endothelial cells, neutrophils, and tumor-associated macrophages indicates that the IL-8 secretion from cancer cells may have a profound effect on the tumor microenvironment²⁵. Recent studies have shown that a variety of human tumor cells constitutively secrete IL-8. Additionally, IL-8 may promote the growth of several different types of cancer including non-small cell lung cancer, colorectal cancer, breast cancer, ovarian cancer, and may even correlate with their metastatic potential²⁴. Targeting CXCR1/2 signaling has already been proven effective in *in vivo* models of breast cancer as well as primary invasive and metastatic breast cancers, which has quickly initiated the use of inhibitors to target these receptors in clinical trials.

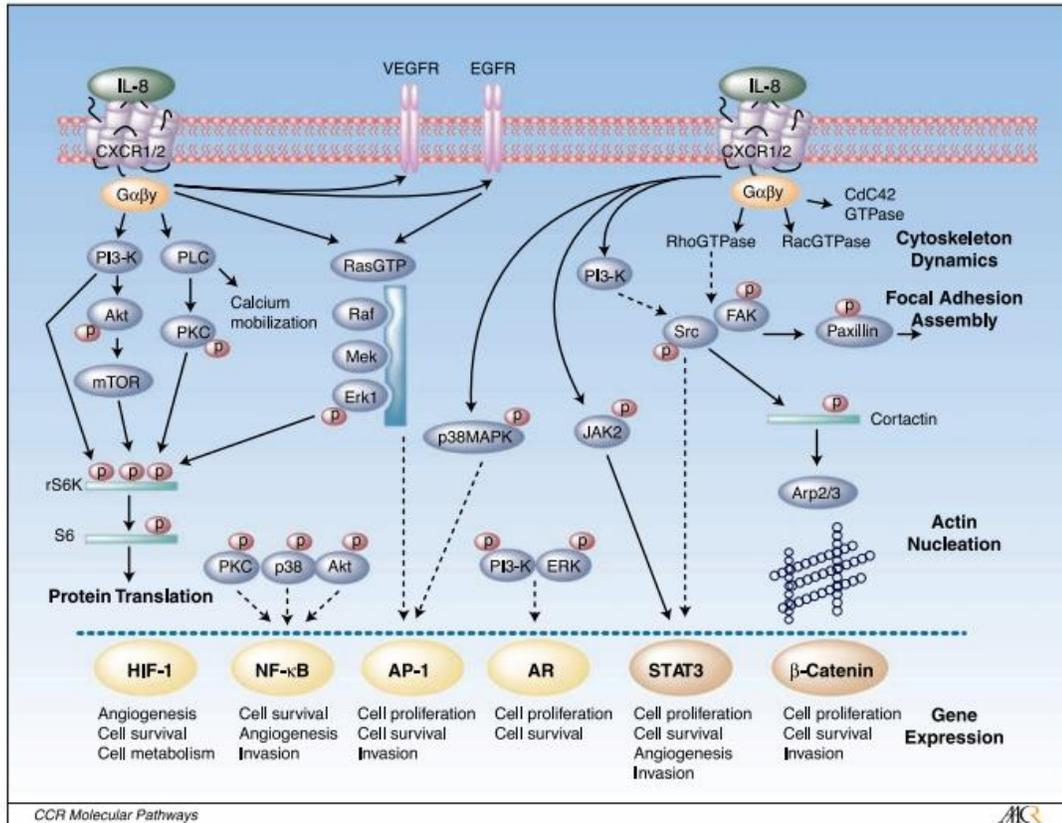


Figure 8: Characterized IL-8 signaling pathways²⁵. IL-8 signaling is involved in many different pathways that are influence in all aspects of the cell cycle.

Phosphatidylinositol-3 kinase (PI3K) is one of the principle effectors of IL-8 promoted chemotaxis of neutrophils, which results in increased phosphorylation of its substrate Akt. Increased Akt expression and activity have been identified in many types of cancer and its role in mediating cell survival, angiogenesis, and cell migration have been well-established as a crucial therapeutic target in cancer²⁵. Activation of Akt by IL-8 signaling has already been shown in many different cancer cell lines.

1.5 PI3K/Akt Pathway in Cancer

PI3Ks are heterodimeric lipid kinases that are composed of a regulatory and catalytic subunit that are encoded by different genes²⁶. Akt is a serine/threonine specific kinase that is known to play an essential role in multiple cellular processes, such as cell growth, migration, survival, metabolism, angiogenesis, proliferation and others, which are all known to contribute to tumor progression²⁷. Activation of Akt is involved in many pathways (Fig. 9) and plays a key role in fundamental cellular functions by phosphorylating a variety of substrates. The abnormal activation of the PI3K/Akt pathway has been shown in many studies as an essential step toward the initiation and maintenance of tumors²⁸. Besides tumor initiation, it has also been found that disruption of this pathway also plays a role in the potential response of a tumor to cancer treatment.

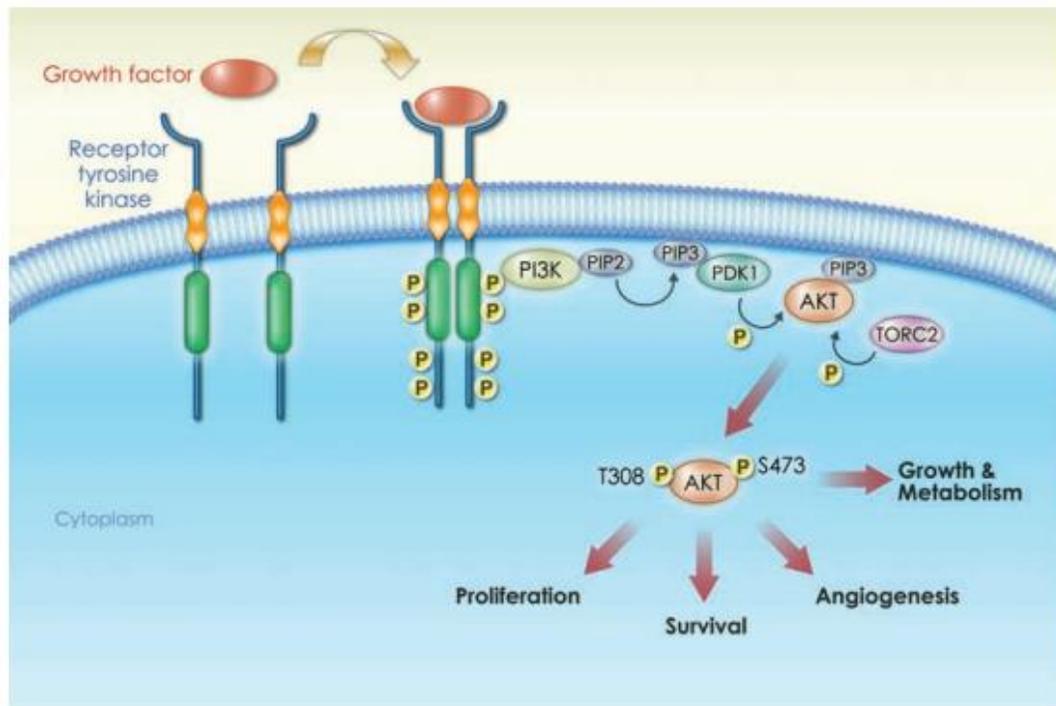


Figure 9: Schematic representation of the PI3K/Akt pathway and its main components²⁸. Akt is known to contribute to cell growth and metabolism, proliferation, survival as well as angiogenesis.

PI3Ks are activated receptor tyrosine kinases, and the deregulation of their function has been implicated in several human cancers. Akt is activated by recruitment to the plasma membrane through direct contact with PIP₃ and phosphorylation at Thr308 and Ser473. Thr308 is phosphorylated by the 3-phosphoinositide-dependent protein kinase PDK1, whereas Ser473 is phosphorylated by a molecularly unidentified kinase, PDK2²⁶. This PI3K/Akt has shown to be able to be activated by the low-molecular-weight GTP/GDP binding GTPase Ras, which is found oncogenically mutated in 30% of all human cancers. The available clinical evidence of PI3K-pathway deregulation in many cancers and the identification of downstream kinases such as Akt, mTOR, PDK1 and ILK, that are involved in mediated the effects of PI3K, provide potential targets for small-molecule therapies.

1.6 Motivation

It was previously published that HIFs activate RhoA and ROCK1 expression and signaling in breast cancer cells, which promoted an invasive phenotype characterized by enhanced migration and contractility under hypoxic but not control cell culture conditions¹². RhoA and RhoC have been implicated in tumor progression with roles in almost every step in the tumor progression process, but the role of RhoB still remains unclear^{23,22}. Recent studies have also shown that small GTPases such as RhoA, Rac1 and Cdc42 are induced *in vitro* during hypoxia. Additionally, RhoA and RhoC have been well-characterized and extensively studied under hypoxia, however whether and how hypoxia regulates RhoB remains elusive^{29,21}. Since RhoB has only been minimally studied and belongs to the same family as RhoA, RhoC, Rac 1 and ROCK1, we decided to investigate

the role of hypoxia-regulated RhoB expression in breast cancer progression to understand whether targeting RhoB in cancer patients with hypoxic tumors would be a beneficial treatment therapy.

Materials and Methods

2.1 Cell Culture

MDA-MB-231, MDA-MB-175 and MDA-MB-468 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS, Corning) and 1% penicillin-streptomycin (Invitrogen). SUM159 cells were kindly provided by the Sukumar lab and were cultured in Ham's F12 medium supplemented with 5% (v/v) FBS, 1% penicillin-streptomycin and 5% Insulin/Hydrocortisone. Cells were maintained in a humidified environment at 37°C and 5% CO₂ during culture and live cell imaging. Hypoxic cells were maintained at 37°C in a modular incubator chamber (Billups-Rothenberg) flushed with a gas mixture containing 1% O₂, 5% CO₂, and 94% N₂. Phospho-Akt inhibitors (Wortmannin and LY294002) and phospho-Erk inhibitors (UO126 and PD98059) were obtained from Cell Signaling Technology (Danvers, MA). Concentrations used were 1 μM, 50 μM, 10 μM and 50 μM for Wortmannin, LY294002, UO126 and PD98059, respectively.

2.2 Knockdown by CRISPR/Cas9

LentiCRISPR v2 plasmid used for generating a CRISPR-Cas9 endonuclease was a gift from Feng Zhang (Broad Institute, Massachusetts Institute of Technology, Cambridge, MA, obtained via Addgene (Addgene plasmid #52961)). HIF-1 α /2 α and RhoB knockout by CRISPR/Cas9 was performed as previously described with slight modifications³⁰. Insert oligonucleotides that include a guide RNA sequence were designed as shown in Table 1. After annealing, these oligos were inserted into the BsmBI

cloning site. After bacterial transformation and DNA purification, all plasmid constructs were confirmed by nucleotide sequencing. The LentiCRISPR v2 plasmids and a non-targeted control were briefly co-transfected with 4 μ g PsPAX2 and 1 μ g pMD2.G into a 10 cm dish of 293T cells using PolyJet™ transfection reagent (SigmaGen Laboratories, Rockville, MD) according to the manufacturer’s instructions. Media was refreshed 16-24h following initial transfection. Filtered viral supernatant was collected 48h post media change and was added to SUM159 and MDA-MB-231 cells. Puromycin (0.5 μ g/mL) was added to the medium of cells transduced for selection. After selection, cells were expanded and used for experiments.

gRNA	Forward	Reverse
NTC2	CACCGCACACGGTCCATACATACA	AAACTGTATGTATGGACCGTGGTGC
RhoB-3	CACCGCACATAGTTCTCGAAGACGG	AAACCCGTCTTCGAGAACTATGTGC
RhoB-4	CACCGCACCGTCTTCGAGAACTATG	AAACCATAGTTCTCGAAGACGGTGC
HIF-1α (site 1)	CACCGCCATCAGCTATTTGCGTGTG	AAACCACACGCAAATAGCTGATGGC
HIF-1α (site 2)	CACCGTGTGAGTTCGCATCTTGATA	AAACTATCAAGATGCGAACTCACAC
HIF-2α (site 1)	CCACGCAAGGCCTCCATCATGCGAC	AAACGTCGCATGATGGAGGCCTTGC
HIF-2α (site 2)	CCACGACGAATCTCCTCATGGTTCG	AAACGCGACCATGAGGAGATTCGTC

Table 1: Insert oligonucleotide sequences used for CRISPR/Cas-9 gene knockout

2.3 Overexpression by Gateway Cloning

R77-E279 Hs.RHOB (provided by Dominic Esposito, NCI, Addgene plasmid #70563) was subcloned into Gateway destination vector pLenti CMV/TO Puro Dest

(670-1) (Campeau *et al*, 2009, Addgene plasmid #17293) via LR recombination reaction. After bacterial transformation and DNA purification, all plasmid constructs were confirmed by nucleotide sequencing. This expression plasmid was transfected into 293T cells using PolyJet™ transfection reagent (SignaGen) according to the manufacturer's instructions. Filtered viral supernatant collected 24 h posttransfection was added to SUM159 cells. Puromycin (0.5 µg/mL) was added to the medium of cells transduced for selection. After selection, cells were expanded and used for experiments.

2.4 Reverse transcription (RT) and qPCR

Total RNA was extracted using TRI Reagent (Zymo Research, Irvine, CA) and the Direct-zol™ RNA Mini Prep Plus kit (Zymo Research) according to the manufacturer's instructions. One microgram of total RNA was used for first-strand DNA synthesis with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). qPCR was performed using human specific primers and iTaq SYBR Green Universal Master Mix (Bio-Rad Laboratories). The expression of each target mRNA relative to 18S rRNA was calculated based on the threshold cycle (Ct) as $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{\text{target}} - Ct_{18S}$ and $\Delta(\Delta Ct) = \Delta Ct_{\text{test}} - \Delta Ct_{\text{control}}$. Primer sequences are shown in Table 2.

Primer	Forward	Reverse
RhoB	GAGAACATCCCCGAGAAGTG	CTTCCTTGGTCTTGGCAGG
P4HA1	CCCTGAGACTGGAAAATTGACCACAGC	GGGGTTCATACTGTCCTCCA ACTCCA
CA-IX	GGATCTACCTACTGTTGAGGCT	CATAGCGCGAATGACTCTGGT
HK2	CCAGTTCATTCACATCATCAG	CTTACACGAGGTCACATAGC
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTT CAGGTTG
IL-8	AGGTGCAGTTTTGCCAAGGAG	GCAGACTAGGGTTGCCAGATTTA
18S hs	GAGGATGAGGTGGAACGTGT	AGAAGTGACGCAGCCCTCTA
18S m+h	CGGCGACGACCCATTCGAAC	GAATCGAACCCCTGATTCCCCGTC

Table 2: Primer sequences used for real-time qPCR

2.5 Western Blotting

Cells were lysed in IGEPAL CA-630 buffer (150mM NaCl, 1% IGEPAL CA-630, 50 mM Tris-HCl, pH 8.0 and protease inhibitors) for 10 min on ice, centrifuged for 10 min at 13,000 rpm at 4°C and the insoluble debris were discarded. Whole cell lysates were fractionated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated for 1 hour with 5% milk in TBS-T (Tris-buffered saline and 0.1% Tween-20) and then incubated overnight with primary antibodies diluted in blocking buffer. Antibodies against the following proteins were used: HIF-1 α (BD Biosciences, San Jose, CA), HIF-2 α (Novus Biologicals, Littleton, CO), RhoB (Santa Cruz Biotechnology, Dallas, TX), Actin (ProteinTech, Rosemont, IL), Phospho-Akt and Akt (Cell Signaling, Danvers, MA). The membrane was then washed and incubated with the corresponding HRP-conjugated secondary antibody (Azure Biosystems, Dublin, CA)

for 2 hours. After washing, the chemiluminescence signal was detected on an AZURE C300 using ECL (PerkinElmer, Waltham, MA).

2.6 Animal Studies

2.6.1 Orthotopic Implantation

Female 5- to 7-week-old NOD-SCID (Charles Rivers Laboratories, Wilmington, MA) mice were used according to protocol approved by the Johns Hopkins University Animal Care and Use Committee. Mice were anesthetized, and 3×10^6 SUM159 cells or 2×10^6 MDA-MB-231 cells resuspended in a 50:50 PBS: Matrigel solution were injected into the mammary fat pad (process shown in Fig. 10). Tumors were measured in three dimensions (a, b, and c), and volume (V) was calculated as $V = abc \times 0.52$. Tumors, ipsilateral axillary lymph nodes, and lungs were harvested, formalin fixed, paraffin embedded and used for IHC staining. Agarose inflated lung sections were stained with hematoxylin and eosin to detect metastatic foci. Tumor tissue was used to isolate RNA for qPCR to quantify various gene expressions. Lung tissue was used to isolate genomic DNA for qPCR to quantify human HK2 and mouse 18s rRNA gene sequences.

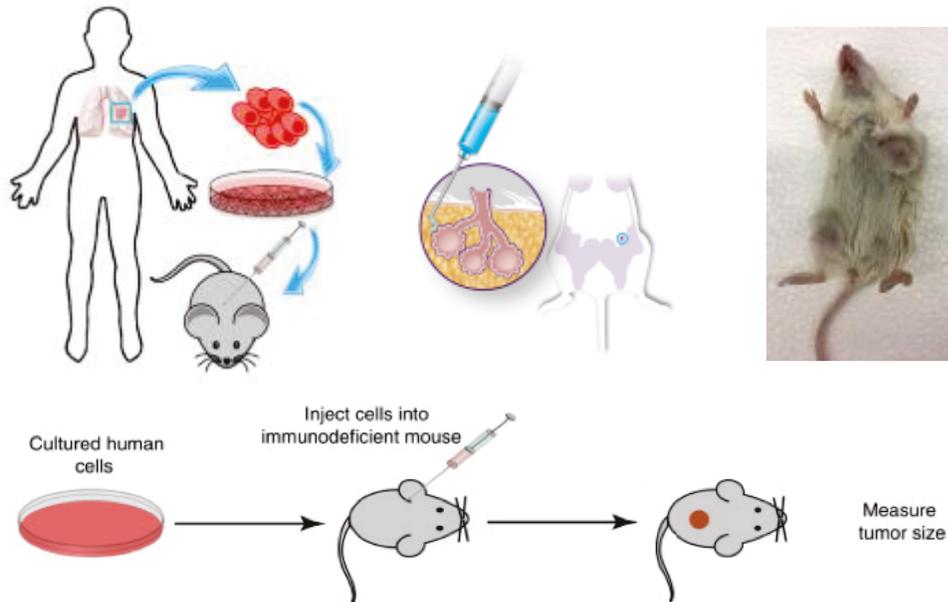


Figure 10: Schematic of in vivo experimental process. Human cells were expanded and collected for injection into the MFP of NOD-SCID mice. Tumor volume was measured over the course of 1-1.5 months.

2.6.2 Lung Tissue Preparation, Genomic DNA Extraction

Lungs were digested with lysis buffer and proteinase K at 55°C overnight, and genomic DNA was extracted according to manufacturer’s instructions using the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). Two hundred nanograms of genomic DNA was used for qPCR to quantify human HK2 and mouse 18S rDNA sequences.

2.7 Immunohistochemistry

Tumors were fixed in 10% formalin and were embedded in paraffin. Sections were dewaxed with xylene and rehydrated with graded ethanol, followed by antigen retrieval using citrate-EDTA buffer (10 mM Citric Acid (pH 6.1), 2mM EDTA and

0.05% Tween 20) by heating at 85°C for 40 min and subsequently cooling at room temperature for 30 min.

Immunohistochemistry was performed using the LSAB+ System HRP kit (Dako, Agilent Technologies, Santa Clara, CA) and RhoB antibody (Santa Cruz Biotechnology). Briefly, the slides were incubated in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity; washed sequentially with H₂O and PBS-T for 5 min; blocked in 1% BSA/PBS for 30 min; washed twice with PBS; and incubated with RhoB antibody at a 1:500 dilution in 1% BSA/PBS for 1 hour. After three PBS-T washes, slides were incubated with Biotinylated Linker for 15 min, washed again with PBS-T and incubated with Streptavidin-HRP for 15 min. After another PBS-T wash, slides were incubated with substrate-chromogen solution for around 30 seconds and rinsed gently with water. Sections were counterstained with Mayer's hematoxylin for 5 min; rinsed with water; dipped briefly in 0.1% sodium bicarbonate and rinsed again with water. The slides were then dehydrated with an increasing gradient of ethanol (70%, 80%, 95%, 100%), followed by xylene and mounted with Paramount agent.

2.8 Motility Assays

2.8.1 3D Cell Migration Assay

Collagen matrices were prepared with soluble rat tail type I collagen in acetic acid (Corning) to achieve a final concentration of 1 mg/mL collagen. 1M NaOH was then added to normalize the pH to about 7.0. Remaining volume filled with a 1:1 ratio of reconstitution buffer [0.2 HEPES (Sigma-Aldrich), 0.26 M NaHCO₃ (Sigma-Aldrich), and water as solvent] and culture medium. All matrices were plated in 24-well culture

plates and collagen gels were left to solidify for 1 hour in an incubator at 5% CO₂ and 37°C. Immediately following solidification, 500µl of cell culture medium was added on top of the gel.

Cells were incubated for 1 hour before time-lapse movies were acquired. Cell movements over time were imaged using Biotek's Lionheart F automated microscope at 10X. Images were taken every 5 minutes for 13.5 hours. Cells in the time-lapse movies were tracked using MetaMorph software to calculate x- and y-coordinates at each time interval.

2.8.2 APRW modeling for motility analysis

APRW model analysis was performed as described in detail using MatLab (code available)³¹. 3D cell trajectory data were used to statistically profile cell migration using the mean squared displacement (MSD) which can be obtained from $[x(t), y(t)]$ coordinates of cells with time (t). $MSD(\tau) = [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2$ where $\tau = 5 \text{ min} * \text{frame number}$. Values of persistence and speed are obtained from APRW model fitting and expressed as speed (S) and persistence (P) of cells, which can be used to calculate total cell diffusivity (D_{tot}). $D_{tot} = (S_p^2 P_p + S_{np}^2 P_{np})/4$ where both speed (S) and persistence (P) are calculated along both the primary and nonprimary axes.

2.9 PrestoBlue Proliferation Assay

To generate the standard curve of proliferation rate of SUM159 cells, different numbers of cells (1000, 5000, 10000, 50000, 100000, and 200000) were seeded in 24-well plates and culture overnight. Each well was then incubated with 100 µl PrestoBlue

reagent (Invitrogen) for 2 hours with 5% CO₂ at 37°C. 200 µl of cell culture media with PrestoBlue reagent were collected in 96-well plates. The absorbance was measured using the Cytation 5 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT).

3000 cells were seeded in 24-well plates and cultured for 4 days. Cells were incubated with 100 µl PrestoBlue reagent for 2 hours with 5% CO₂ at 37°C and 200 µl cell culture media and PrestoBlue reagent mixtures were collected in 96-well plates. Absorbance was measure as described above.

2.10 ELISA

To measure IL-8 secretion, IL-8 levels in culture supernatants were determined by using an ELISA kit (Abcam Human IL-8 ELISA kit, Cambridge, MA) according to manufacturer's instructions. A monoclonal antibody specific for IL-8 was coated onto the wells of the strips provided. Samples, including standards of known IL-8 concentrations, controls as well as unknowns were pipetted into the wells. During the first incubation, the standards and samples were incubated with a biotinylated monoclonal antibody specific for IL-8. After washing, Streptavidin-HRP was added and incubated, which allowed the biotinylated antibody to bind. The wells were washed again and a TMB substrate solution was added, which acts on the bound enzyme to induce a color change. The intensity of this colored product is directional proportional to the concentration of IL-8 present in the samples. A curve of the absorbance versus the concentration of IL-8 in the standard wells was plotted. By comparing the absorbance of the samples with the standard curve, the concentrations of the unknown samples were calculated.

2.11 Statistical Analysis

The mean values \pm standard error of mean (SEM) were calculated and plotted using GraphPad Prism (6, San Diego, CA) software. When appropriate, statistical analysis were performed to compare means, namely two-tailed unpaired t-tests, one-way and two-way ANOVA followed by Bonferroni post-tests to determine statistical significance, which is indicated in the graphs as *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Results and Discussion

3.1 Hypoxia induces RhoB expression on the RNA and protein level

To test whether RhoB was induced under hypoxic conditions, the gene expressions and protein levels of RhoB in three breast cancer cell lines, MDA-MB-175, SUM159, and MDA-MB-231, were compared under both normal tissue culture conditions (20% O₂) and hypoxic conditions (1% O₂). It was observed that in all three of these cell lines RhoB mRNA and protein expression was induced under hypoxia (Fig. 11). Because MDA-MB-231 cells have a lower basal level of RhoB, a 10X higher exposure was used to image this blot. An immunoblot of SUM159 and MDA-MB-231 cells was performed to assess the levels of HIF-1 α , HIF-2 α and RhoB protein over a 48-hour time period (Fig. 12). Like previously seen, HIF-1 α peaks at 4 hours and then decreases to a steady state level following 24 hours of continuous exposure. RhoB increases gradually under hypoxic stimulation. The graphs of fold change vs. time under hypoxia are a quantitative way of assessing induction levels based on densitometry of the corresponding western blots.

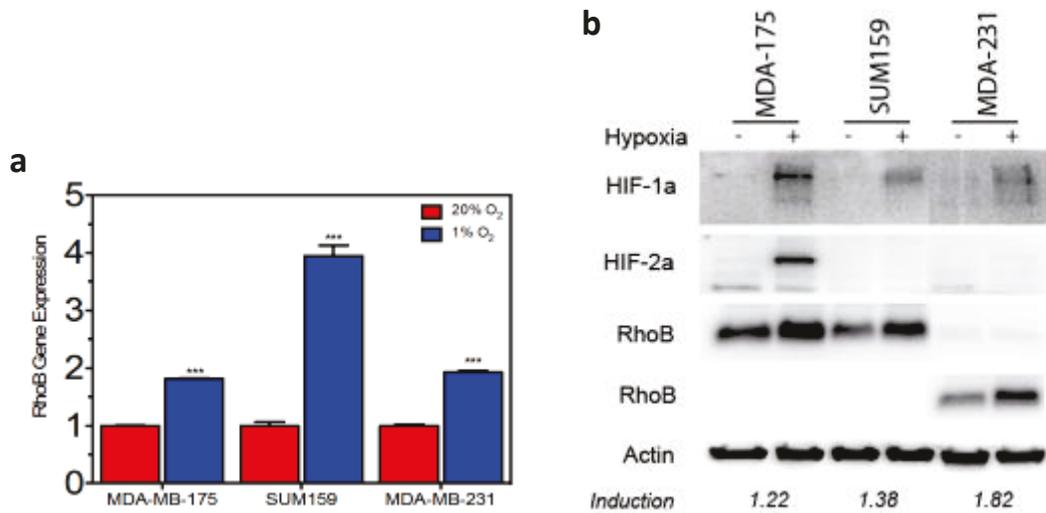


Figure 11: Hypoxia significantly upregulated RhoB expression at the mRNA and protein level. a) Real time-PCR b) and immunoblot of RhoB fold change under hypoxia in MDA-MB-175, SUM159 and MDA-MB-231 cells normalized to the 20% expression.

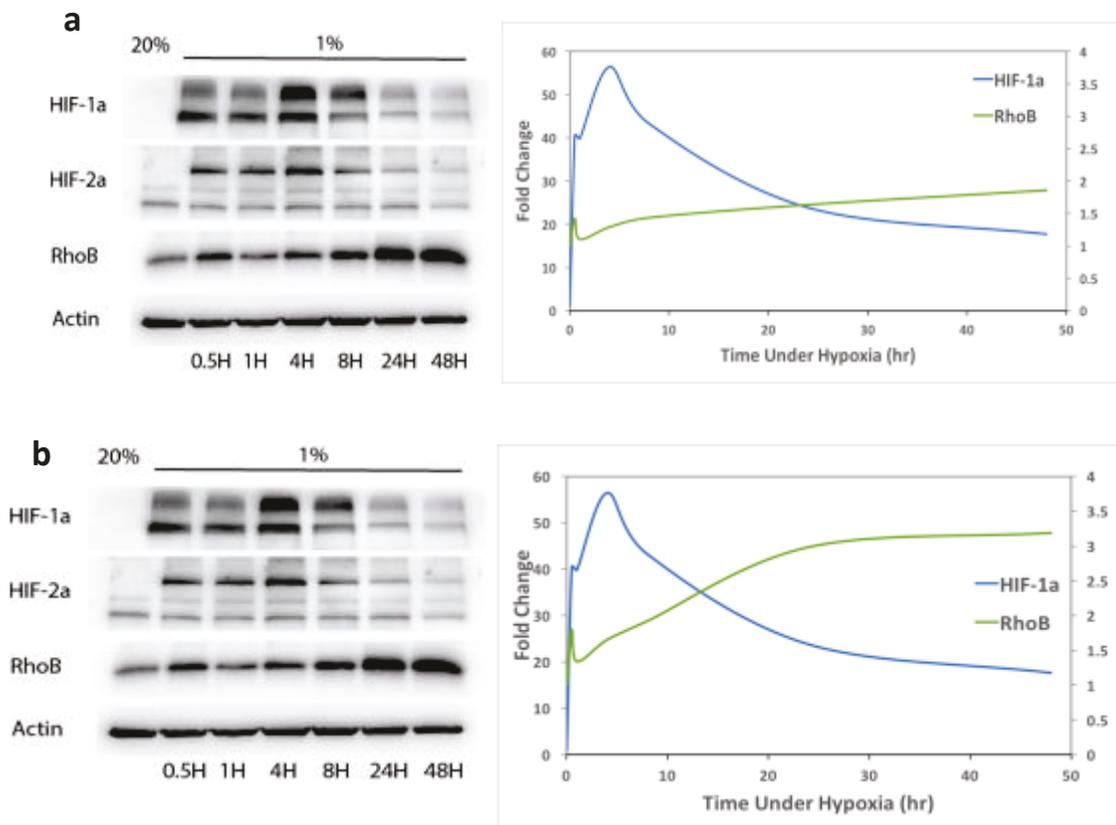


Figure 12: Time course of HIF-1 α and RhoB. Immunoblot of a) SUM159 and b) MDA-MB-231 cells to assess the levels of HIF-1 α , HIF-2 α , and RhoB protein over a 48-hour time period. Left axis depicts the fold change for HIF-1 α expression and the right axis shows RhoB expression fold change.

3.2 Hypoxia-induced RhoB occurs in a HIF-1 α independent manner

In order to find the mechanism by which hypoxia regulates RhoB, we began to look at possible upstream targets of RhoB. HIF-1 is known to regulate most genes under hypoxia, therefore HIF-1 α and HIF-2 α CRISPR-mediated knockdowns were used to check for RhoB mRNA and protein expression under 20% and 1% O₂. If RhoB was downstream of HIF-1 α /2 α , its expression would be affected by the HIF-1 α /2 α knockdowns. However, both RhoB mRNA and protein expression did not decrease for these knockdown cell lines under hypoxic conditions when compared to the wild type (WT) and non-targeted controls (NTC2) (Fig. 13a, c, d, f). To verify that HIF-1 α was knocked down, PCR was also run on P4HA1, which is a downstream target of HIF-1 α . As shown in Figure 13b, e, the P4HA1 gene expression does not get induced in the HIF-1 α knockdowns as it does in the others, revealing successful knockdown. These results illustrate that RhoB is induced under hypoxia in a HIF-1 α and HIF-2 α independent manner.

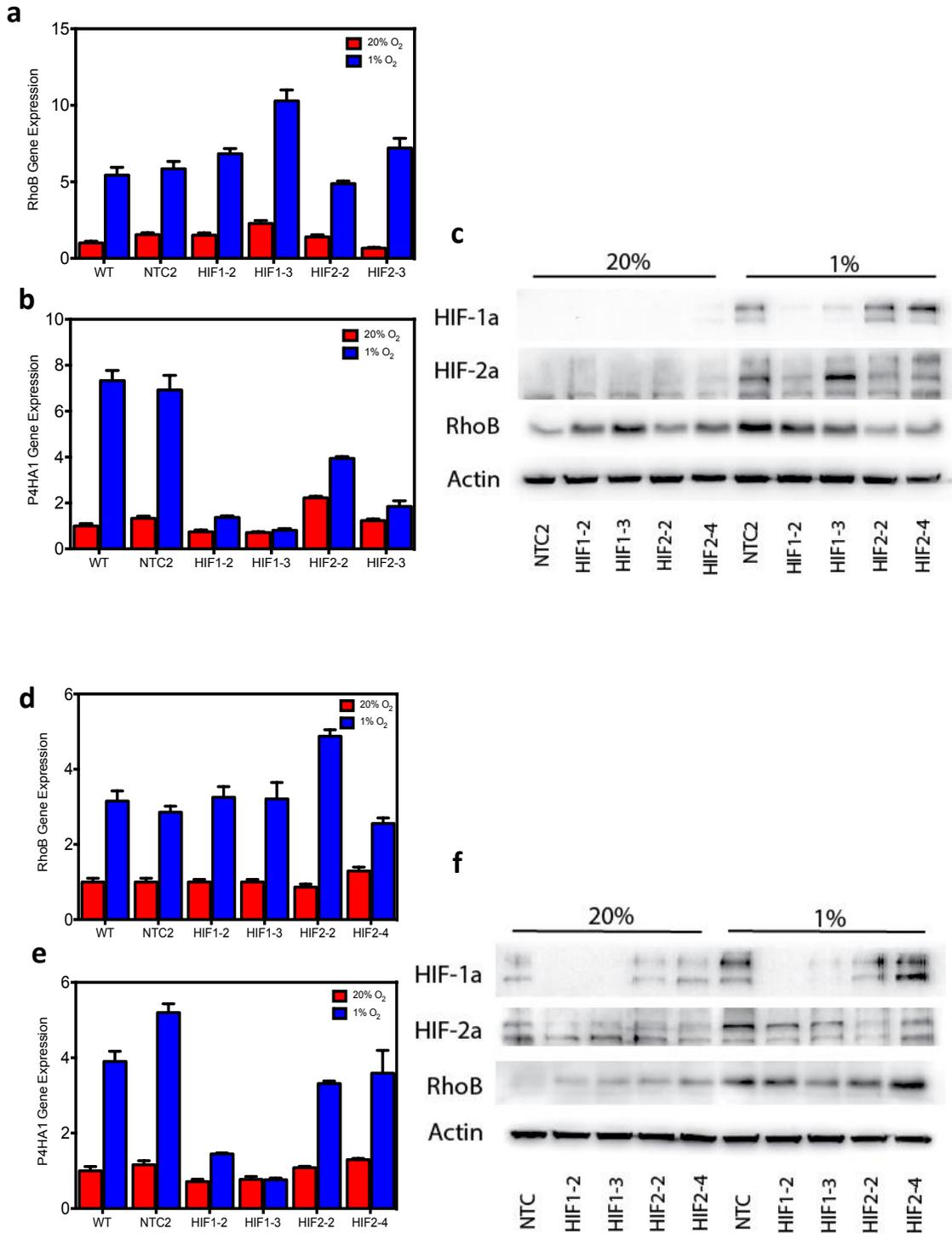


Figure 13: Hypoxia-induced RhoB expression is HIF-1 α /2 α independent. SUM159 cells expressing a CRISPR gDNA targeting HIF-1 α and HIF-2 α did not change RhoB expression at the a) mRNA c) or protein level under hypoxia. b) P4HA1 gene expression was checked to verify knockdown of HIF-1 α . d-f) MDA-MB-231 cells showing the same results as a-c.

3.3 CRISPR-mediated reduction of RhoB causes a decrease in HIF-1 α expression

To examine the functionality of RhoB, we created RhoB knockdown clones using CRISPR in both SUM159 and MDA-MB-231 cell lines. Before running any experiments, it is important to test the efficiency of the knockdowns. To ensure RhoB expression was reduced, we ran a western blot with the WT and NTC2 controls and compared the RhoB levels with the two RhoB knockdowns (RhoB-3 and RhoB-4) under 20% and 1% O₂. Figure 14 shows two good clones with a significantly reduced RhoB expression. Interestingly, when RhoB was depleted, a significant decrease in HIF-1 α and HIF-2 α expression was also observed (Fig. 14c, d). To see if this was also true on the RNA level, quantitative PCR analysis was performed on P4HA1 and CA-IX, both downstream targets of HIF-1 α (Fig. 14a, b). As expected, there are also notable decreases in these gene expressions under hypoxia when RhoB is knocked down. These results were consistent with those seen in the MDA-MB-231 cells (Fig. 14d). Based on these observations, RhoB seems to regulate HIF-1 α and HIF-2 α expression under hypoxia.

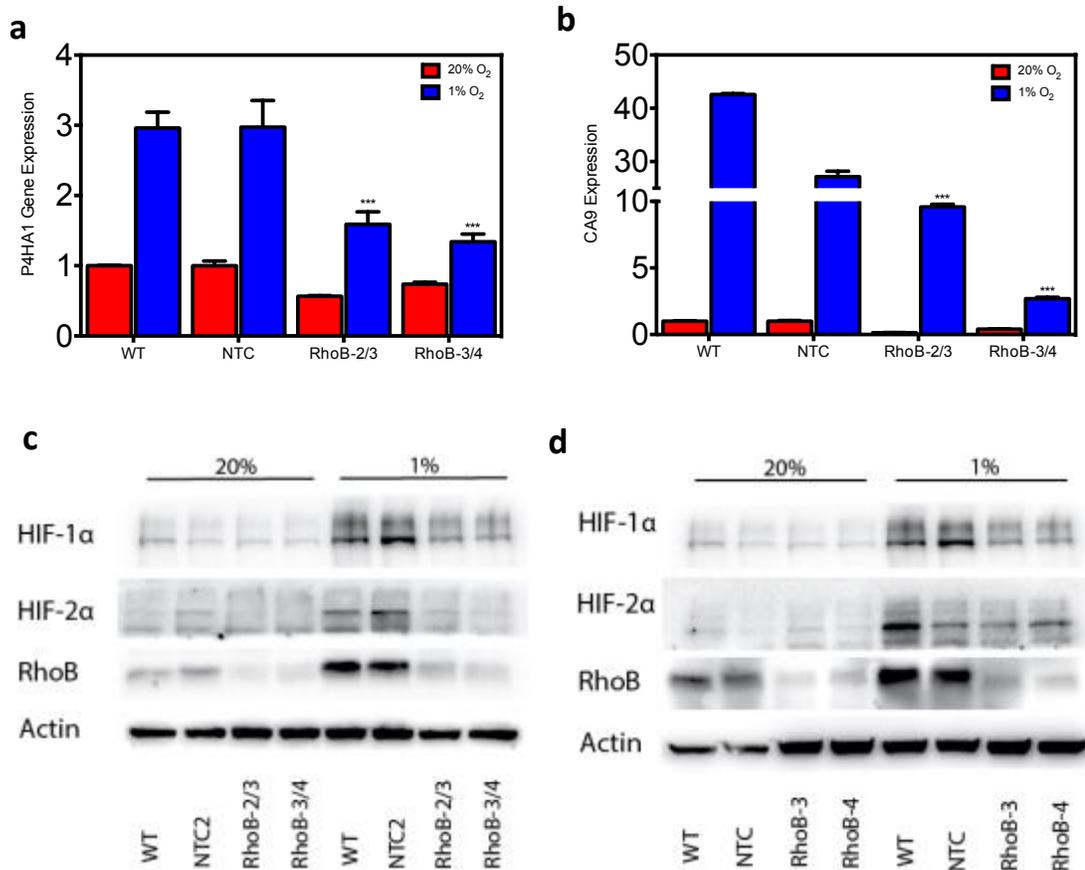


Figure 14: CRISPR-mediated reduction of RhoB expression caused a decrease in HIF-1 α expression under hypoxia. a, b) P4HA1 and CA-IX mRNA levels were analyzed by qPCR in SUM159 cells expressing a CRISPR gDNA targeting RhoB under 20% and 1% O₂ for 24H. c) Immunoblot assays were also performed on c) SUM159 and d) MDA-MB-231 lysates to assess levels of HIF-1 α , HIF-2 α and RhoB exposed to 20% or 1% O₂ for 48H.

If a decrease in RhoB expression simultaneously caused a reduced HIF-1 α level, the reverse should be seen when RhoB is overexpressed. To investigate this hypothesis, SUM159 cells were transduced by a vector overexpressing RhoB. To validate the overexpressed cell line, a western blot was run for HIF-1 α and RhoB protein levels under both normoxic and hypoxic conditions (Fig. 15a). Instead of seeing an increase in HIF-1 α expression as expected, there is actually a slight decrease with the overexpressed RhoB cell lines. To verify this, gene expression of HIF-1 α downstream targets, P4HA1 and CA-

IX, were analyzed by real time-PCR (Fig. 15b, c). From these graphs, it is clear that the results match those from the western. The gene expression of P4HA1 and CA-IX are not overexpressed with an increased RhoB level. After all of this, we can conclude that RhoB is necessary, but not sufficient for HIF-1 induction under hypoxia.

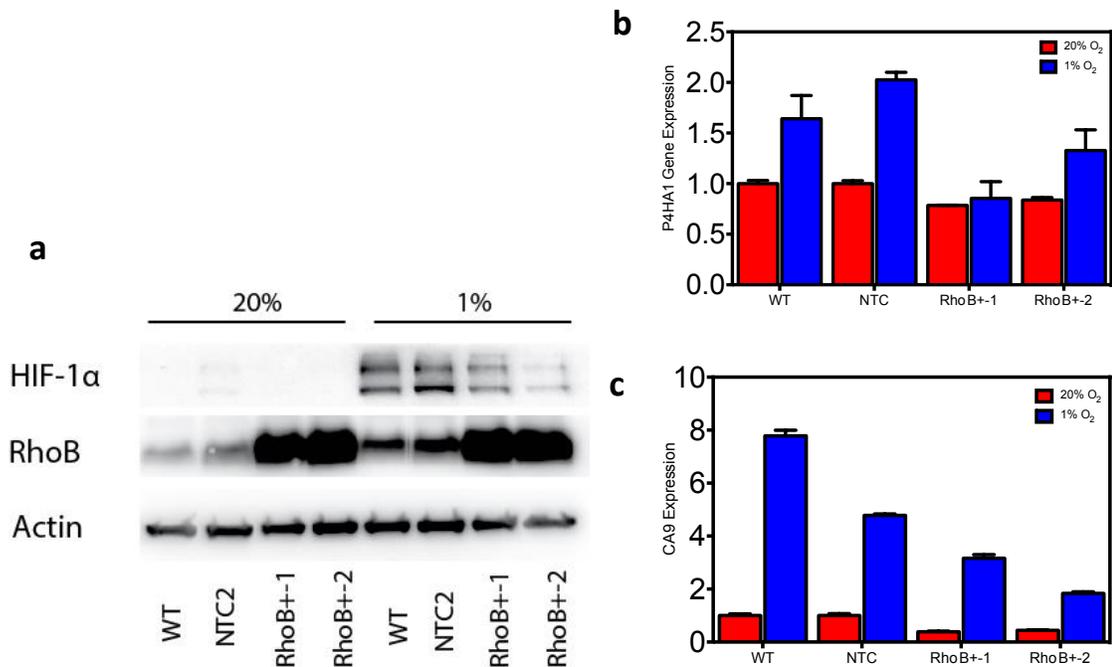


Figure 15: RhoB overexpression does not lead to an increase in HIF-1α. a) Immunoblot assay was performed on SUM159 subclones containing a vector overexpressing RhoB to assess levels of HIF-1α, HIF-2α and RhoB exposed to 20% or 1% O₂ for 48H. b, c) P4HA1 and CA-IX mRNA levels were analyzed by qPCR in the same subclones under 20% and 1% for 24H.

3.4 Loss of RhoB leads to a decrease in cell motility and proliferation in 3D environments

RhoB is known to contribute to enhanced morphology and cell motility, two crucial components in the process of tumor progression. Because of this, we embedded MDA-MB-231 wild type, non-targeted control and both RhoB knockdown cell lines into

a 3D collagen matrix and tracked their movement over a 16-hour time period. By doing this, our goal was to understand the role of RhoB in cancer cell motility and how it is involved in the metastatic cascade. With the x, y coordinates obtained from Metamorph, the average cell velocities (Fig. 16a) as well as the total cell diffusivities (Fig. 16b) were calculated as previously described²². From this, a small, but significant decrease in the velocity and a larger reduction in total cell diffusivity was noted. By plotting the 3D trajectories of each cell, we could visualize their movement with respect to each condition (Fig. 16c). It is clear that the RhoB knockdown cells had a much less spread out migration profile than the wild type and non-targeted controls. Because total cell diffusivity is calculated using both speed (S) and persistence (P), it appears that although the knockdowns did have a slightly slower average velocity, it is really the decreased persistence that drives the reduction in total cell diffusivity and the trajectory differences.

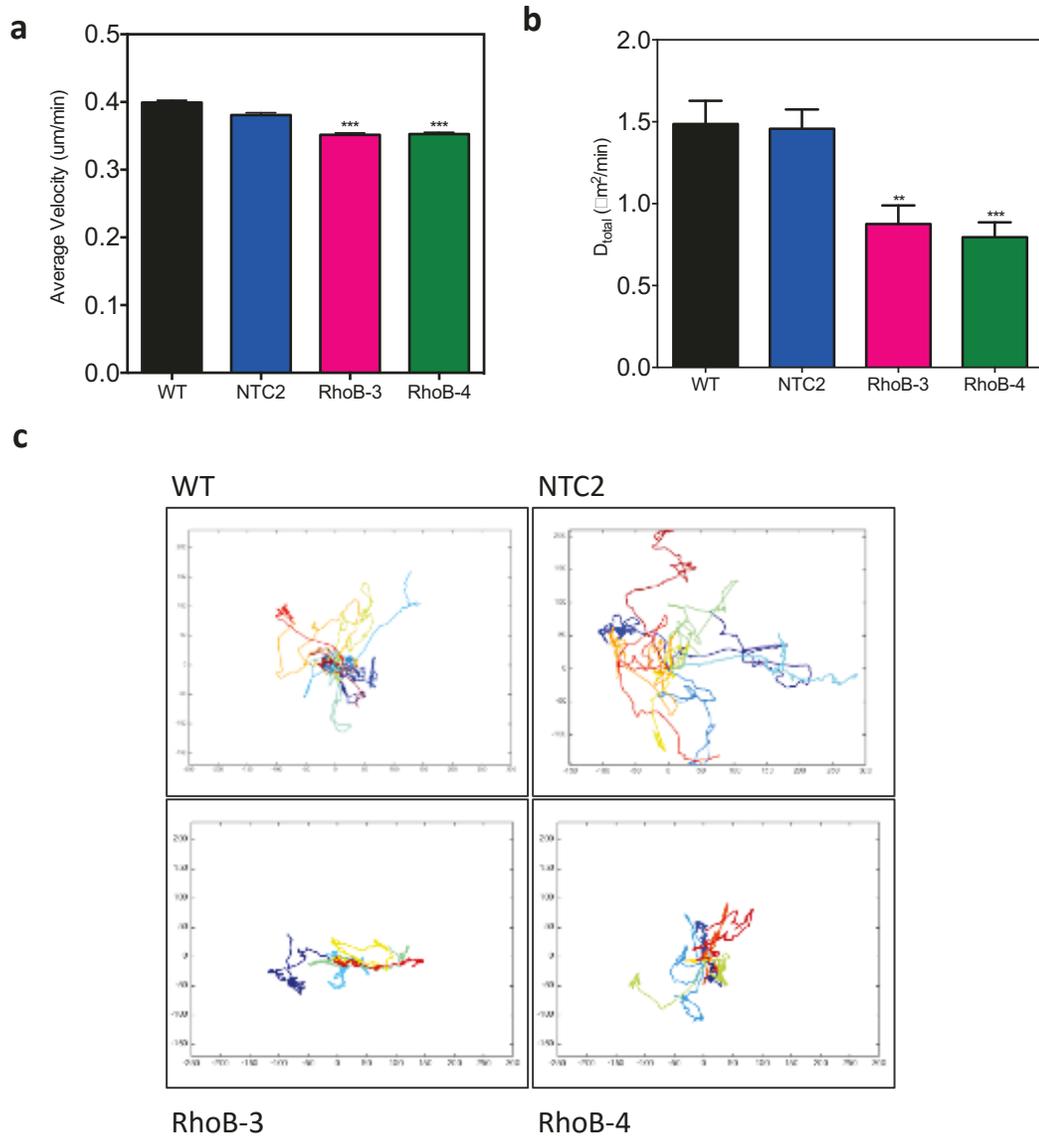


Figure 16: RhoB contributes to a faster cell motility in 3D environments. a) Average cell velocities and b) total cell diffusivity of migrating cells are calculated using x,y coordinates obtained from Metamorph tracking software. c) Cell trajectories (n=75-100) are plotted using x,y coordinates obtained at 5-min intervals over a 13h time course.

To test the effect of downregulation of RhoB in breast cancer cell proliferation, we compared the growth of parental SUM159 cells with SUM159 cells expressing a CRISPR gDNA targeting RhoB or a non-target control (NTC2) on 3D collagen matrices.

There was a small but statistically significant reduction in cell proliferation in the RhoB-3 and RhoB-4 knockdown cells when compared to a wild-type or NTC2 cells (Fig. 17).

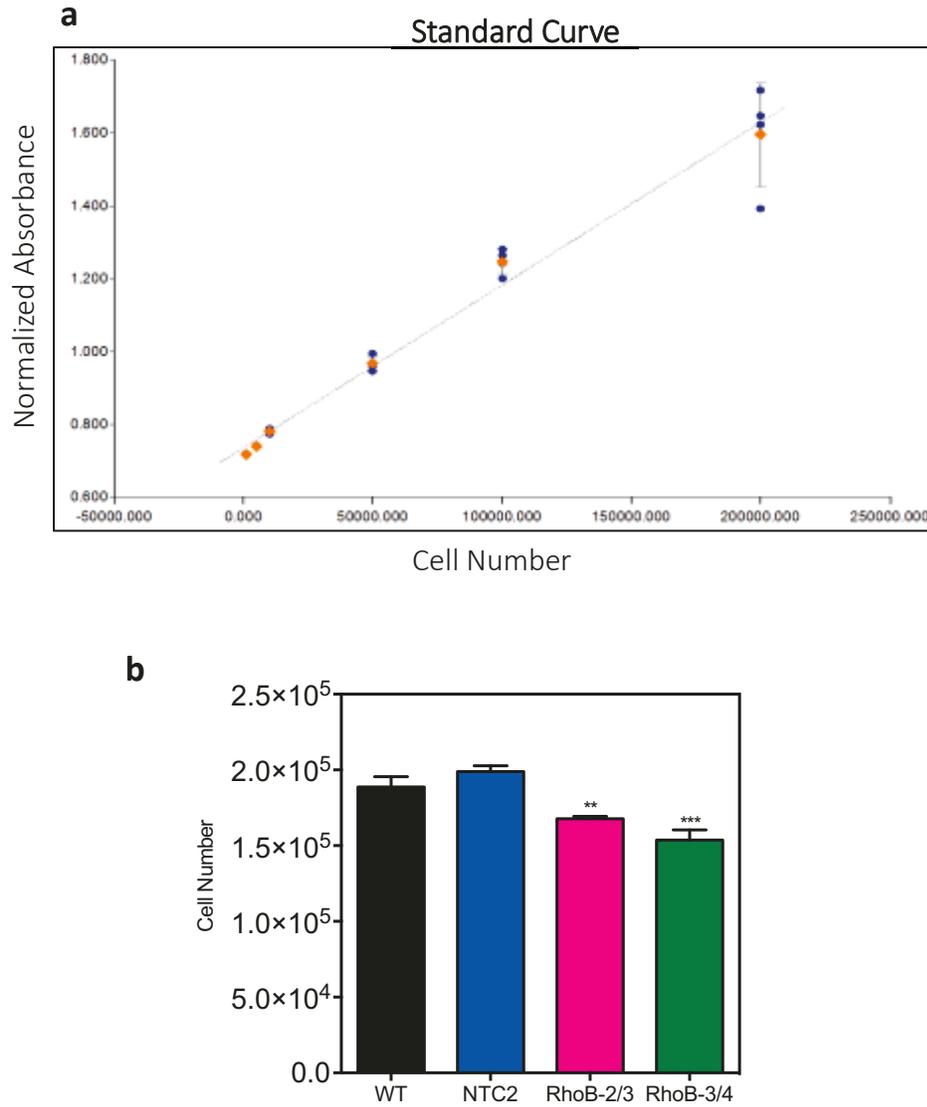
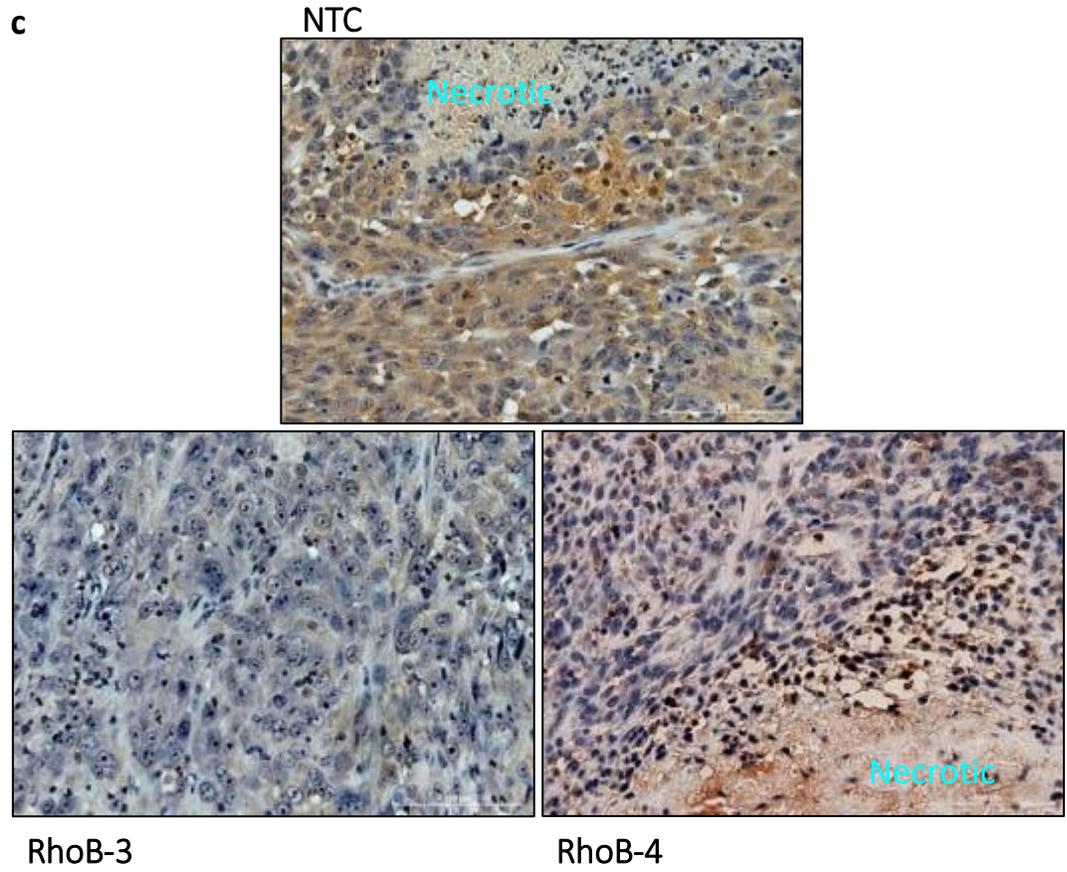
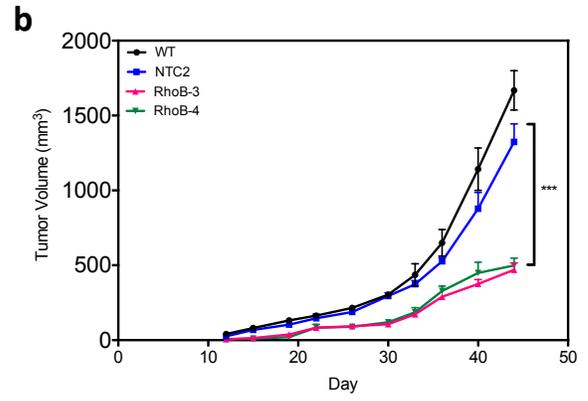
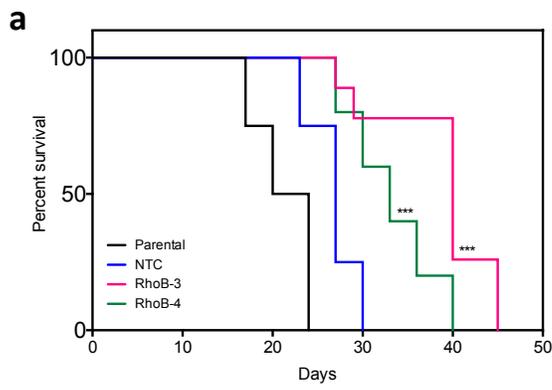


Figure 17: RhoB contributes to a faster cell proliferation in 3D environments. a) Standard curve used to correlate absorbance with actual cell number. b) Cell count as a measure of proliferation after a 4-day culture period, compared to the standard curve.

3.5 RhoB contributes to a slower tumor growth and decreased metastasis in vivo

To explore the role of RhoB in vivo and how it affects tumor growth and metastasis, we orthotopically implanted 3×10^6 SUM159 and 2×10^6 MDA-MB-231 into the mammary fat pad of NOD-SCID mice. Tumor growth was measured over the course of 1-1.5 months and volume was compared between the WT, NTC2, RhoB-3 and RhoB-4. The time it took for the tumor volume to reach 1000 mm^3 was used to construct a Kaplan-Meier analysis curve (Fig. 18a). Although the RhoB-3 knockdown showed slightly better results than the RhoB-4, the knockdowns clearly have a slower tumor growth than the parental controls. Previously, it was found that a similar result was seen with the HIF-1 α and HIF-2 α knockdowns in mice⁸. Based on these observations, we show that RhoB knockdown cells show slower tumor growth in mice, similar to that of the HIF-1 α .

Tumor sections were subjected to IHC using an antibody against RhoB to verify these tumors still had a reduced RhoB expression (Fig. 18c). When compared to the NTC, there is barely any RhoB staining in the RhoB-3 and a small amount around in the necrotic regions in the RhoB-4 subclone. Tumor tissue was also saved for qPCR analysis of human RhoB content (Fig. 18d), which quantitatively shows what is seen in the immunohistochemistry staining. P4HA1 gene expression (Fig. 18e) was also analyzed via qPCR to correlate with our in vitro results that a decrease in RhoB downregulates HIF-1 α downstream targets. These conclusions are also consistent with our tumor growth curves in that RhoB-3 has a more reduced RhoB expression, which corresponds with a slower tumor growth and less P4HA1 mRNA levels.



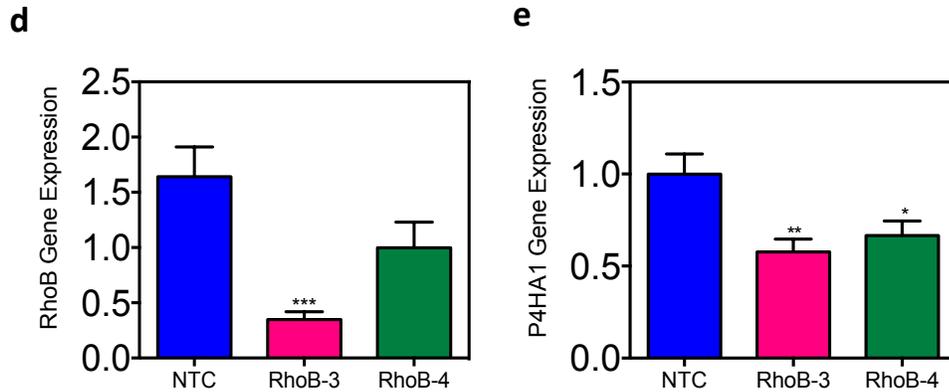
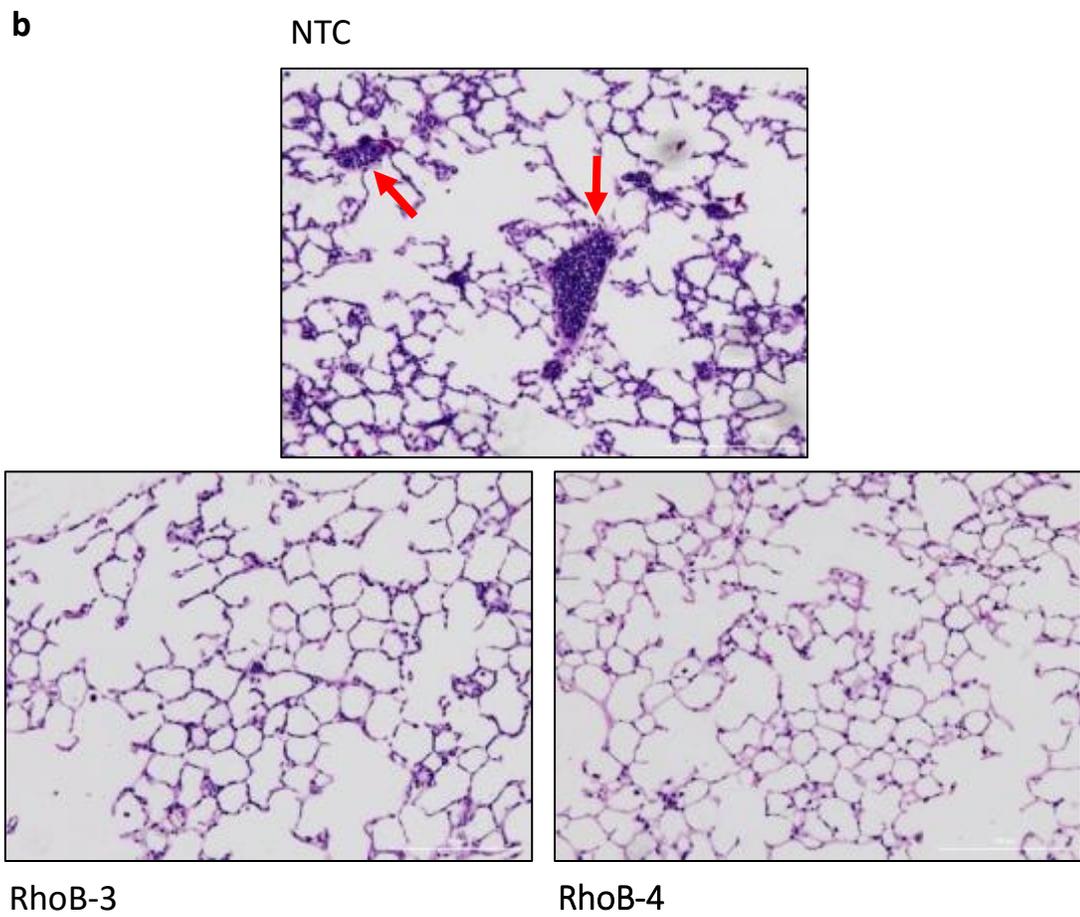
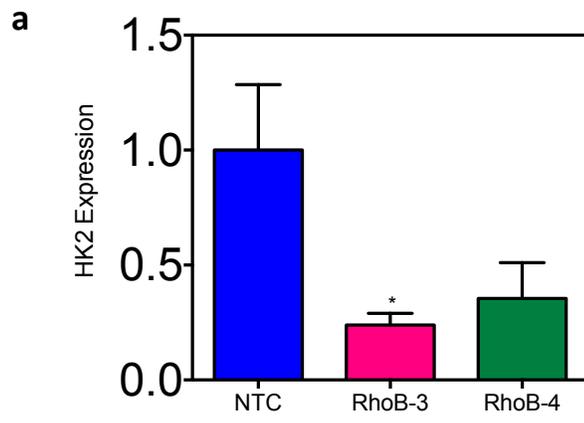


Figure 18: RhoB promotes tumor growth of SUM159 and MDA-MB-231 cells. a) The indicated subclones of SUM159 were injected into the MFP of NOD-SCID mice and tumor volume was plotted as a Kaplan-Meier analysis of percent survival ($V=a*b*c*0.52$, 1 being $>1000 \text{ mm}^3$) b) The indicated subclones of MDA-MB-231 were injected into the MFP of NOD-SCID mice and tumor volume was measured in three dimensions a, b, c ($V=a*b*c*0.52$). c) Tumor sections (scale bar = $100 \mu\text{m}$) were subjected to IHC using an antibody against RhoB. d) Human RhoB content in tumors were quantified by qPCR. e) P4HA1 gene expression was also quantified by qPCR.

Since both migration and invasion are two critical steps in the metastatic cascade, we compared the overall metastatic burden resulting from control tumors or tumors lacking RhoB. To do this, we isolated genomic DNA from the mouse lung for analysis of human DNA by quantitative real-time PCR (Fig. 19a). Inhibition of RhoB expression in tumors significantly decreased lung metastasis. Lung metastasis was also assessed histologically by hematoxylin and eosin (H&E) staining (Fig. 19b). The number of metastatic foci per field of view in each section confirmed the qPCR results. We also assessed breast cancer cell infiltration of the ipsilateral axillary lymph node by H&E staining. The lymph nodes of the mice bearing control tumors were enlarged and completely invaded with cancer cells, whereas a more normal follicular structure and size were maintained in mice bearing RhoB knockdown tumors (Fig. 19c).



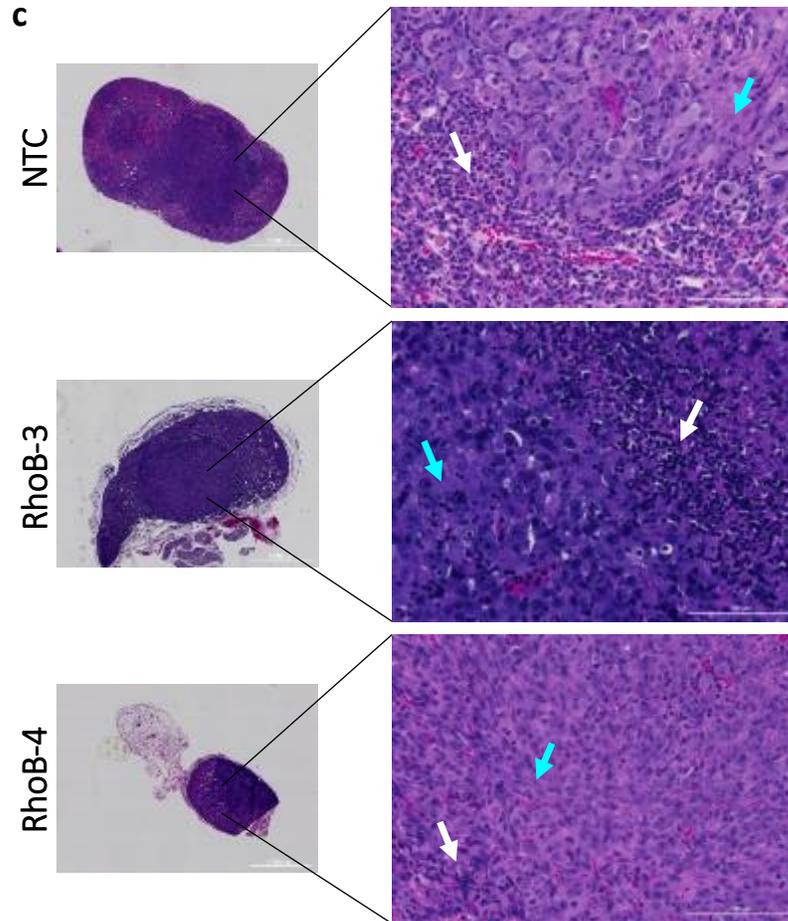


Figure 19: RhoB promotes metastasis of SUM159 and MDA-MB-231 cells a) Lung sections (scale bar = 200 μ m) were stained with hematoxylin and eosin to identify metastatic foci. b) Human genomic DNA content in lungs were quantified by qRT-PCR for human HK2 gene sequences. c) Lymph node sections (scale bar = 1000 μ m) were stained with H&E to identify metastatic foci. White arrows indicate lymph cells and blue arrows indicate the invading tumor cells.

3.6 Inhibition of IL-8R results in a decreased RhoB expression

From previous work, we investigated the effect of blocking IL-6R and IL-8R with inhibitors, Tocilizumab and Reparixin respectively, on 3D cell motility and in vivo tumor growth and metastatic potential. We found that using these inhibitors in MDA-MB-231 cells resulted in a reduced RhoB mRNA expression as well both in vivo (Fig. 20a) and in vitro (Fig. 20b).

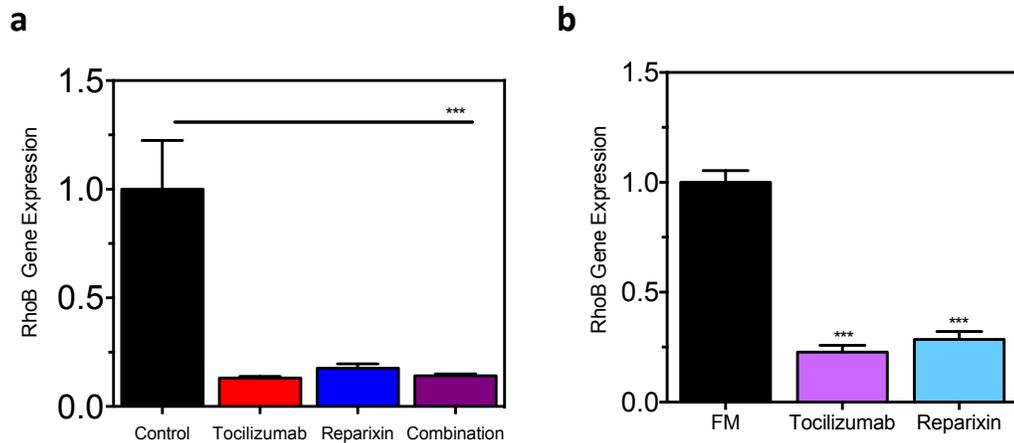


Figure 20: IL-8 receptor inhibition decreases RhoB expression both in vivo and in vitro. a) RhoB gene expression in MDA-MB-231 tumors were analyzed via qRT-PCR where mice were dosed with either saline (control), Tocilizumab (IL-6R inhibitor), Reparixin (IL-8R inhibitor) or a combination of both. b) RhoB gene expression in MDA-MB-231 cells analyzed by qRT-PCR under the same conditions except in 3D collagen matrices.

We hypothesized that IL-6 and/or IL-8 might be regulators of RhoB expression and could potentially be induced by hypoxic conditions. To test this hypothesis MDA-MB-175, SUM159, MDA-MB-468 and MDA-MB-231 cells were exposed to 20% or 1% O₂ for 24H. IL-6 and IL-8 mRNA expression was evaluated using qRT-PCR. Increased gene expressions were seen for IL-8, but not IL-6 when exposed to hypoxia (Fig. 21 a, b). Since IL-6 was not enhanced under hypoxic conditions at the RNA level, only IL-8 was used for further experiments. IL-8 is a secreted protein. ELISA assays are commonly used to assess the level of secreted proteins and therefore was used to measure the concentration IL-8 in the conditioned media of MDA-MB-175, MDA-MB-231 and SUM159 cells under both hypoxic and normoxic conditions. SUM159 HIF-1 α /2 α knockdowns were used to evaluate the role of HIF-1/2 α in IL-8 induction as we hypothesized that the secreted IL-8 levels should not change in the presence or absence of HIF-1 α , but would still be enhanced under hypoxic conditions. However, instead of

seeing an increase in the amount of produced IL-8 under 1% O₂, there was actually a decrease in every sample tested (Fig. 21c). This could be due to several factors. It is possible that IL-8 protein is not upregulated under hypoxia and, in turn, affecting RhoB in a separate, hypoxia-independent pathway. It is very clear that RhoB is influenced in some way by the IL-6 and IL-8 receptors, however most likely by a different mechanism. Alternatively, IL-8 and its receptor, CXCR2 are known to have a autocrine signaling feedback loop that is essential for eliciting the tumorigenicity of triple-negative breast cancer cells³². It is possible that under hypoxia, the cell is taking up IL-8 at a faster rate than it is being produced. This could explain a decrease in secreted IL-8 levels but an increase in IL-8 mRNA levels under hypoxia. Future experiments that examine the IL-8/CXCR2 signaling cascade will help us examine the interplay between hypoxia and IL-8 downstream signaling events.

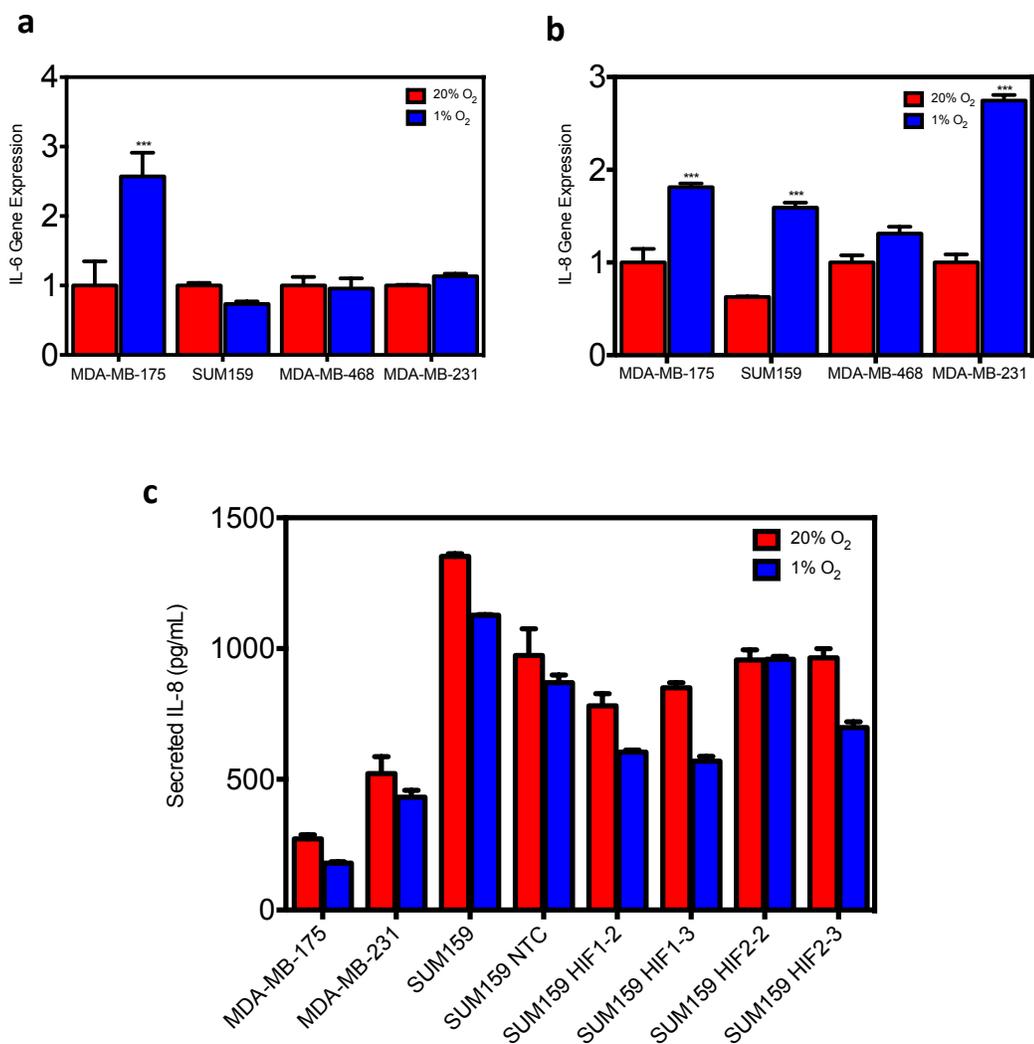


Figure 21: IL-8 mRNA is induced under hypoxia, but not the secreted IL-8. a) IL-6 and b) IL-8 mRNA levels were analyzed by qRT-PCR in MDA-MB-175, SUM159, MDA-MB-468 and MDA-MB-231 breast cancer cell lines exposed to 20% or 1% O₂ for 24H. c) Production of IL-8 protein by human breast cancer cells under 20% and 1% O₂ was determined by ELISA.

3.7 RhoB affects HIF-1 α expression in an Akt-dependent manner

Many published reports suggest that PI3K and Akt could play a major role in regulating the stabilization of HIF-1 α in hypoxia^{33,34}, although the PI3K/Akt signaling pathway by hypoxia seems to be cell type-specific. Some authors have also found that PI3K/Akt is activated by hypoxia³⁵. Additionally, in both tumor cells and stromal

endothelial cells, RhoB function has been linked to the regulation of PI3K/Akt survival pathways³⁶. In order to see which of these pathways were true in our system, we first treated SUM159 cells with phospho-Akt inhibitors (Wortmannin and LY294002) and phospho-Erk inhibitors (UO126 and PD98059) as controls. When SUM159 cells were treated with p-Akt, but not p-Erk inhibitors, the HIF-1 α expression decreased under hypoxia (Fig. 22a). Inhibiting p-Akt does not affect RhoB expression (Fig. 22a). Next, we hypothesized that pAkt may require RhoB. To assess the contribution of RhoB to phospho-Akt, we assessed the level of phosphorylated Akt in the RhoB knockdown cells under 20% and 1% O₂ conditions (Fig. 22b). Interestingly, when RhoB expression was abrogated, p-Akt expression was significantly reduced. These results illustrate that under hypoxia, RhoB regulates HIF-1 α in a mechanism that is dependent upon Akt phosphorylation.

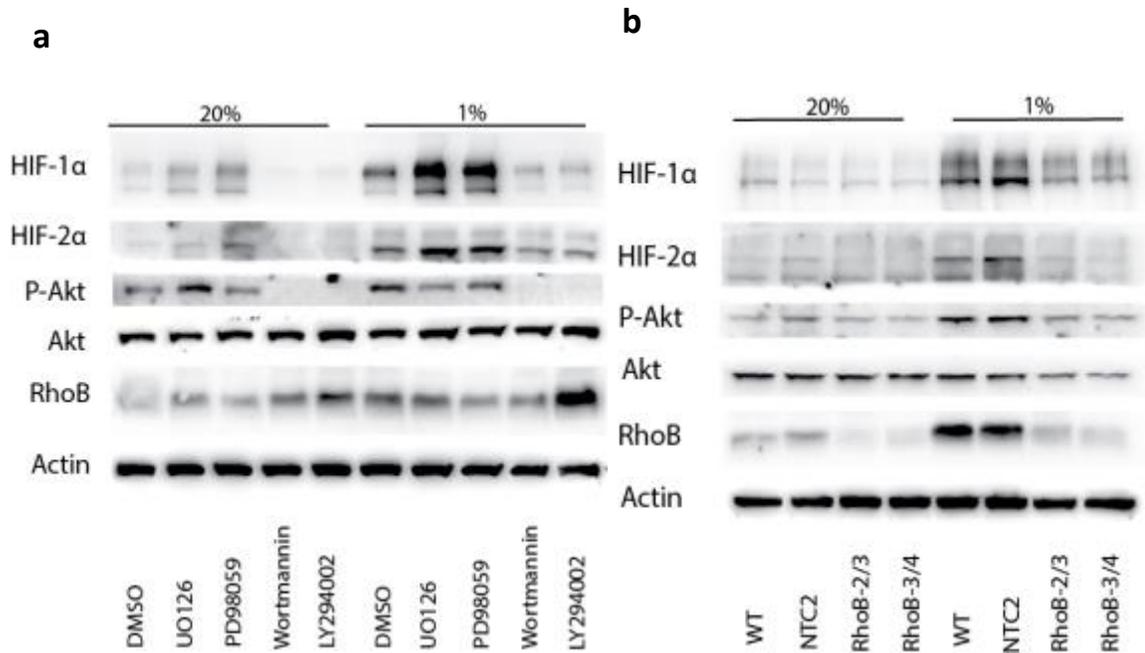


Figure 22: RhoB leads to AKT phosphorylation. a) Immunoblot of SUM159 cells treated for 1H prior to hypoxic stimulation with either DMSO, p-ERK inhibitor (UO126 and PD98059) or p-AKT inhibitor (Wortmannin and LY294002) to assess levels of HIF-1α, HIF-2α, phospho-AKT, AKT and RhoB under 20% and 1% O₂ for 4H. b) Immunoblot of RhoB knockdown subclones to assess levels of HIF-1α, HIF-2α, phosphor-Akt, Akt and RhoB under 20% and 1% O₂ for 48H.

Conclusion

The study described has highlighted a novel role for RhoB in the regulation of a new pathway controlled by hypoxia in breast cancer cells, SUM159 and MDA-MB-231. We found that hypoxia upregulates RhoB expression, which in turn initiates the phosphorylation of Akt and thereby increases HIF-1 α expression (Fig. 23). We also demonstrated that RhoB contributes to an increased cell motility, total cell diffusivity and proliferation in a 3D setting. Correlating these results with an in vivo environment, we illustrate that RhoB promotes tumor growth and metastasis to the lungs and lymph nodes. Our work suggests that RhoB may play an oncogenic role in breast cancer, potentially by increasing HIF-1 α under hypoxic conditions. This work warrants additional studies to determine whether targeting RhoB could have implications in the treatment of breast cancer, especially in patients with hypoxic tumors.

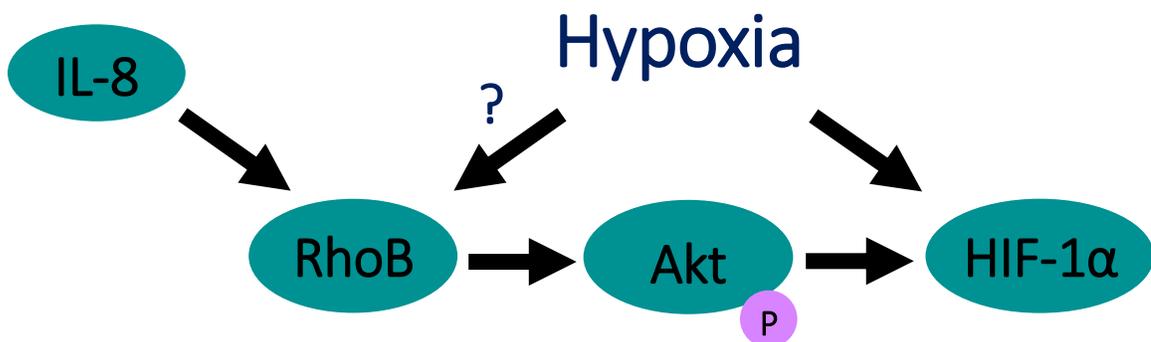


Figure 23: The current hypoxia \rightarrow RhoB \rightarrow AKT \rightarrow HIF-1 α pathway. Hypoxia upregulates RhoB expression, which in turn, initiates the phosphorylation of Akt and thereby increasing HIF-1 α expression.

Future Work

Further research will be done to identify the exact mechanism by which hypoxia upregulates RhoB. As of now, there are no available studies that explain this connection in breast cancer. Being able to establish this link could help to justify whether targeting RhoB with therapeutic agents such as FTIs, antagonists of Ras-dependent neoplastic transformation, or HDAC inhibitors would be beneficial. Additionally, in order to better quantify metastatic potential in our *in vivo* model, we will stain the lymph nodes with human vimentin and count metastatic foci. We are also in the process of completing another *in vivo* experiment using a MDA-MB-231 model to compare with the SUM159 results.

References

1. American Cancer Society. Cancer Facts & Figures 2016. *Cancer Facts Fig. 2016* 1–9 (2016). doi:10.1097/01.NNR.0000289503.22414.79
2. Godet, I. & Gilkes, D. M. BRCA1 and BRCA2 mutations and treatment strategies for breast cancer. *Integr. Cancer Sci. Ther.* **4**, 5–7 (2017).
3. Gilkes, D. M. & Semenza, G. L. Role of hypoxia-inducible factors in breast cancer metastasis. *Future Oncol.* **9**, 1623–36 (2013).
4. Wood, L. D. *et al.* The genomic landscapes of human breast and colorectal cancers. *Science* **318**, 1108–13 (2007).
5. Gilkes, D. M., Semenza, G. L. & Wirtz, D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nat. Rev. Cancer* **14**, 430–9 (2014).
6. Wirtz, D., Konstantopoulos, K. & Searson, P. C. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat. Rev. Cancer* **11**, 512–22 (2011).
7. Liu, Z., Semenza, G. L. & Zhang, H. Hypoxia-inducible factor 1 and breast cancer metastasis. *J. Zhejiang Univ. Sci. B* **16**, 32–43 (2015).
8. Zhang, H. *et al.* HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. *Oncogene* **31**, 1757–1770 (2012).
9. Semenza, G. L. *et al.* Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol. Sci.* **33**, 207–14 (2012).
10. Rankin, E. B. & Giaccia, A. J. The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ.* **15**, 678–85 (2008).
11. Lu, X. & Kang, Y. Hypoxia and hypoxia-inducible factors: Master regulators of metastasis. *Clinical Cancer Research* **16**, 5928–5935 (2010).
12. Gilkes, D. M. *et al.* Hypoxia-inducible factors mediate coordinated RhoA-ROCK1 expression and signaling in breast cancer cells. *Proc Natl Acad Sci U S A.* **111**, E384-93 (2014).
13. Semenza, G. L. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* **29**, 625–634 (2009).
14. Parri, M. & Chiarugi, P. Rac and Rho GTPases in cancer cell motility control. *Cell Commun. Signal.* **8**, 23 (2010).
15. Ellenbroek, S. I. J. & Collard, J. G. Rho GTPases: Functions and association with cancer. *Clin. Exp. Metastasis* **24**, 657–672 (2007).
16. Sahai, E. & Marshall, C. J. Rho-Gtpases and Cancer. *Nat. Rev. Cancer* **2**, 133–142 (2002).
17. Prendergast, G. C. Actin' up: RhoB in cancer and apoptosis. *Nat. Rev. Cancer* **1**, 162–168 (2001).
18. Wang, L., Yang, L., Luo, Y. & Zheng, Y. A novel strategy for specifically down-regulating individual Rho GTPase activity in tumor cells. *J. Biol. Chem.* **278**, 44617–25 (2003).
19. Vega, F. M. & Ridley, A. J. Rho GTPases in cancer cell biology. *FEBS Letters* **582**, 2093–2101 (2008).
20. Lin, R., Bagrodia, S., Cerione, R. & Manor, D. A novel Cdc42Hs mutant induces cellular transformation. *Curr. Biol.* **7**, 794–7 (1997).

21. Horiuchi, A. *et al.* Up-regulation of small GTPases, RhoA and RhoC, is associated with tumor progression in ovarian carcinoma. *Lab. Invest.* **83**, 861–870 (2003).
22. Van Golen, K. L., Wu, Z. F., Xiao Tan Qiao, Li Wei Bao & Merajver, S. D. RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. *Cancer Res.* **60**, 5832–5838 (2000).
23. Pillé, J.-Y. *et al.* Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. *Mol. Ther.* **11**, 267–74 (2005).
24. Shi, Q. *et al.* Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. *Clin. Cancer Res.* **5**, 3711–3721 (1999).
25. Waugh, D. J. J. & Wilson, C. The interleukin-8 pathway in cancer. *Clinical Cancer Research* **14**, 6735–6741 (2008).
26. Vivanco, I. & Sawyers, C. L. The phosphatidylinositol 3-Kinase-AKT pathway in human cancer. *Nat Rev Cancer* **2**, 489–501 (2002).
27. Osaki, M., Oshimura, M. & Ito, H. PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis* **9**, 667–676 (2004).
28. Garcia-Echeverria, C. & Sellers, W. R. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. *Oncogene* **27**, 5511–5526 (2008).
29. Wojciak-Stothard, B., Tsang, L. Y. F., Paleolog, E., Hall, S. M. & Haworth, S. G. Rac1 and RhoA as regulators of endothelial phenotype and barrier function in hypoxia-induced neonatal pulmonary hypertension. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290**, L1173–L1182 (2006).
30. Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat. Methods* **11**, 783–784 (2014).
31. Wu, P.-H., Giri, A. & Wirtz, D. Statistical analysis of cell migration in 3D using the anisotropic persistent random walk model. *Nat. Protoc.* **10**, 517–27 (2015).
32. Hartman, Z. C. *et al.* Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. *Cancer Res.* **73**, 3470–3480 (2013).
33. Mazure, N. M., Chen, E. Y., Laderoute, K. R. & Giaccia, A. J. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras- transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood* **90**, 3322–3331 (1997).
34. Zhong, H. *et al.* Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.* **60**, 1541–5 (2000).
35. Mottet, D. *et al.* Regulation of hypoxia-inducible factor-1 protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3 pathway in HepG2 cells. *J. Biol. Chem.* **278**, 31277–31285 (2003).
36. Kazerounian, S. *et al.* RhoB differentially controls akt function in tumor cells and stromal endothelial cells during breast tumorigenesis. *Cancer Res.* **73**, 50–61 (2013).

Curriculum Vitae

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EDUCATION

The Johns Hopkins University	Baltimore, MD
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RESEARCH EXPERIENCE

Graduate Research Assistant, Johns Hopkins Medical Institute Baltimore, MD
PI: Dr. Daniele Gilkes, Department of Oncology 10/2015 – Present

Project 1: The role of hypoxia-regulated RhoB expression in breast cancer progression

- Investigating the effects of hypoxia on RhoB and figuring out the role of RhoB in a breast cancer setting

Project 2: Oxygenation potential of high-molecular weight polymerized human hemoglobin

- Investigating the delivery of hemoglobin to tumors in conjunction with chemotherapy to oxygenate the tumors

Project 3: Hypoxia selectively enhances integrin $\alpha 5$ expression in breast cancer to promote metastasis (published)

- Found that inhibition of ITGA5 expression prevents metastasis as well as several pathways that converge to promote ITGA5 during tumor progression

Undergraduate Research Assistant, Johns Hopkins University Baltimore, MD
PI: Dr. Denis Wirtz, Department of ChemBE 08/2014 – 01/2016

Project 1: Synergistic IL6 and IL8 paracrine signaling pathway infers a strategy to inhibit tumor cell migration (accepted)

- Identified a novel synergistic mechanism between Interleukin 6 and Interleukin 8 that enhances cancer cell motility as metastatic cells proliferate and increase local density

Project 2: The influence of hypoxia on the functional coupling of cancer cell proliferation and migration

- Investigating the effects of a hypoxic environment to cancer cell proliferation and migration

Research Assistant, University of California, Irvine, School of Medicine Irvine, CA

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05/2014 – 08/2014

Project 1: Reactivation of mutant p53 with small molecules

- Investigated the restoration of p53 tumor suppressor functions by small drug-like molecules and the exact mechanisms of recovery while studying the interactions these reactivating compounds have on the p53 gene containing single missense point mutations

TEACHING EXPERIENCE

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08/2016-12/2016

PUBLICATIONS

- **Ju, Julia A.**, Godet, Inês, Ye, I Chae, Byun, Jungmin, Jayatilaka, Hasini, Lee, Sun Joo, Xiang, Lisha, Samanta Debangshu, Lee, Meng Horng, Wu, Pei-Hsun, Wirtz, Denis, Semenza, Gregg L., Gilkes, Daniele M. "Hypoxia selectively enhances integrin receptor expression to promote metastasis." *Molecular Cancer Research* (2017): molcanres-0338.
- Jayatilaka, H., Tyle, P., Chen, J., Kwak, M., **Ju, J.**, Kim, H.J., Lee, J., Wu, P., Gilkes, D., Fan, R., Wirtz, D. "Synergistic IL6 and IL8 paracrine signaling pathway infers a strategy to inhibit tumor cell migration." *Nature Communications* (2017).

DISTINGUISHMENTS

Francis J. Fisher Research Award, The Johns Hopkins University (2016)

Awarded to an undergraduate student who is excelling academically and engaged in basic or applied cancer research

Provost Undergraduate Research Award (PURA), The Johns Hopkins University (2015)

Awarded to undergraduate students who conduct independent research

Dean's List, The Johns Hopkins University (Fall 2015, Spring 2015/16)

Awarded to undergraduate students who earn at least 14 credits with a term GPA of 3.5 or above