Genetic Deletion or Pharmacologic Inhibition of PIM1 Impairs Breast Cancer Growth and Metastasis

Patrick Eugene Faughnan

A Dissertation for the Degree of Doctor of Philosophy in Biochemistry and Molecular Genetics
at the University of Virginia

Dedication

This dissertation is dedicated in loving memory to my grandfather John Hrustich for his unwavering encouragement, love, and support.

Acknowledgements

My Ph.D. has been a long journey and I would not have been able to complete it without the help and support of so many along the way.

First, I would like to thank my Ph.D. advisor, Dr. Golam Mohi. Thank you for all of your help and support throughout my Ph.D. We have had a long journey together that started in Syracuse, NY at SUNY Upstate Medical University. I have learned a tremendous amount from you and know I am fully prepared for whatever comes next. I am very grateful to have been able to work on such an interesting project. I worked on a project that was not something our lab normally worked in, but Dr. Mohi was very supportive in building this new area of research in our lab.

I would next like to thank my family. Without their constant love, support and encouragement, I'm not sure I would have ever finished. My parents have aways been supportive in all of my endeavors, not matter what they were. The life lessons they instilled along the way have helped bring me to where I am today. My brothers, Donnie and Greg have also been very encouraging and the two people I generally went to first when things weren't going well. I would also like to thank my several aunts, uncles, and cousins who have supported me along the way, especially Aunt Mary-Ellen, my fellow academic in the family, and Uncle Greg.

Next, I would like to thank my committee members, Dr. Sanchita Bhatnagar, Dr. Dan Gioeli, Dr. Kevin Janes, and Dr. Marty Mayo for serving in my thesis committee. I have appreciated the advice and guidance they have provided me.

I would also like to thank the former and current members of the Mohi Lab. Dr. Fatou Jobe, thank you for all your help and guidance, especially when I was new in the lab. Dr. Dipmoy Nath, thank you for all of your help in anything lab related and being a great friend. Dr. Avik

Dutta, thank you for all of your help and encouragement over the years, and for teaching me a lot about working efficiently. Matthew Stuver, thank you for being a great friend, our talks about football, and your ability to make me laugh, even if it was at your expense. Dr. Yue Yang, thank you for all of your help in the lab over the years, especially with my flow experiments. Bao Le, thank you for not only being a great lab mate, and your help with anything R related, but also for being a great roommate. Dr. Ferdous Ur-Rahman, for always being able to make me laugh, your help running my flow experiments, and your guidance have all been greatly appreciated. Dr. Chandrajeet Singh, thank you for all of your hard work and help with the breast cancer project. Dr. Abbas Salar and Dr. Mohammed Abu Sayem, thank you for all of your guidance over the years, and a special thank you for keeping me well fed.

I would also like to thank the BMG department and the University of Virginia for providing me with the resources and a wonderful work environment needed to carry out my research. After moving to UVA, everyone we encountered was very helpful and collaborative with my project. I would especially like to thank Nancy Rush, Carolyn Smith, Sonya Churchman, and Hellen Norfleet-Shiflett. On top of keeping the department running, thank you for all of your help and just being a friendly face when I was stressed. I would also like to thank our core research facilities, including the Pinn Hall animal care staff, the Flow Cytometry Core, Advanced Imaging Core, and Research Histology core. They have been a great resource we utilized throughout my Ph.D.

Lastly, I want to thank my friends for all of their help and support throughout my Ph.D. Getting a Ph.D. can be very stressful at times, and my friends really helped me stay levelheaded throughout my time here. I would especially like to thank Kevin Cox, thank you for all of the dinners you invited me over for and for being my first friend in Charlottesville. I would also like

to thank Kevin Heald, for always listening to me complain when things weren't going well, and for always being there whenever I needed him to be. Our hours spent in a discord chat have really helped me stay positive.

Dissertation Abstract

Genetic Deletion or Pharmacologic Inhibition of PIM1 Impairs Breast Cancer Growth and Metastasis

Patrick Faughnan

Sponsor: Golam Mohi, Ph.D.

Metastatic breast cancers often do not respond to currently available therapies, highlighting a need for new drug targets. Expression of PIM1 is upregulated in a significant percentage of breast cancers. PIM1 expression is also significantly increased in metastatic breast cancers compared to non-metastatic breast cancer patients. We hypothesized that PIM1 plays an important role in progression and metastasis of breast cancer. Knockdown/deletion of PIM1 significantly inhibited the proliferation, migration, and invasion of several breast cancer cell lines. To assess the in vivo role of PIM1 in breast cancer progression and metastasis, we crossed PIM1 knockout mice with MMTV-PyMT metastatic breast cancer mouse model. We found that deletion of PIM1 significantly inhibited tumor growth and reduced lung metastasis in these mice. Pharmacologic inhibition of PIM1 kinase using TP-3654 significantly inhibited cell proliferation, migration, and invasion in breast cancer cells, and reduced breast tumor growth and lung metastasis in MMTV-PyMT mice. We also found that PIM1 deletion or inhibition decreased EMT markers SLUG and Vimentin and pro-metastatic marker c-MET. RNA-seq analysis showed genes related to translation, proliferation, EMT and metastasis pathways were affected by PIM1 depletion or inhibition. Together, these results suggest that PIM1 plays an important role in the progression and metastasis of breast cancer.

List of Abbreviations

Abbreviation	Description
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ADH	Atypical Ductal Hyperplasia
Akt	Protein Kinase B
ALH	Atypical Lobular Hyperplasia
ALK	Anaplastic lymphoma kinase
APAF1	Adapter Protein Apoptotic Protease Activating Factor
AR	Androgen Receptor
AREG	Amphiregulin
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3-Related Protein
BC	Breast Cancer
BCRP	Breast Cancer Resistance Protein
BL1	Basal-Like 1
BL2	Basal-Like 2
BMX	Cytoplasmic tyrosine-protein kinase BMX
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
BTC	Betacellulin
CARD Domain	Caspase Recruitment Domain
CD	Cluster of differentiation
CDK	Cyclin Dependent Kinase
cDNA	Complementary deoxyribonucleic acid
c-Myc	Cellular Myelocytomatosis
CP	Cisplatin
CXCL12	C-X-C motif chemokine 12

CXCR4	C-X-C chemokine receptor type 4
DAPI	4′,6-diamidino-2-phenylindole
DCIS	Ductal Carcinoma in Situ
DCT	Docetaxel
DED	Death-Inducing Domain
DISC	Death Inducing Signal Complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DR3	Death Receptor 3
DUSP1	Dual Specificity Phosphatase 1
E2F	E2 Factor
E47	E2A immunoglobulin enhancer-binding factors E12/E47
ECM	Extra-Cellular Matrix
EGF	Epidermal Growth Factor
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFR	Epidermal Growth Factor Receptor
eIF4E	Eukaryotic translation initiation factor 4E
EMT	Epithelial to Mesenchymal Transition
EPI	Epigen
ER	Estrogen Receptor
ERE	Estrogen Response Element
EREG	epiregulin
ERK	Extracellular Signal-Related Kinases
FADD	FAS-associated death domain
Fas	APO-1
FBS	Fetal Bovine Serum

FEA	Flat epithelial atypia
FGFRs	Fibroblast Growth Factor Receptor
FLT3	FMS-like tyrosine kinase 3
FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
FOXC1	Forkhead Box C1
GSEA	Gene Set Enrichment Analysis
H&E	hematoxylin and eosin
HB-EGF	heparin-binding EGF-like growth factor
HER1	Human Epidermal Growth Factor Receptor 1
HER2	Human Epidermal Growth Factor Receptor 2
HER3	Human Epidermal Growth Factor Receptor 3
HR+	Hormone Receptor Positive
HSP	Heat Shock Protein
IDC	Invasive Ductal Carcinoma
IGF1R	Insulin Like Growth Factor 1 Receptor
IL	Interleukin
ILC	Invasive Lobular Carcinoma
IM	Immunomodulatory
INF	Interferon
IP	Intraperitoneal injection
ITD	Internal Tandem Duplication
JAK	Janus Kinase
KD	Knockdown
KO	Knockout
LCIS	Lobular Carcinoma in Situ
LDL	Low-Density Lipoprotein
M	Mesenchymal
MAPK	Mitogen-activated protein kinases

Met	tyrosine-protein kinase Met
MM	Multiple Myeloma
MME	Membrane Metallo endopeptidase
MMPs	Matrix metalloproteinases
MMTV	Mouse Mammary Tumor Virus
MOMP	Mitochondrial Outer Membrane Permeabilization
mRNA	Messenger ribonucleic acid
MSL	Mesenchymal Stem-Like
mTOR	Mammalian target of rapamycin
NGF	Nerve Growth Factor
NK	Natural Killer
ns	Not Significant
p70-s6k	Ribosomal protein S6 kinase beta 1
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCA	Principle Component Analysis
PD-1	Programmed Cell Death Protein-1
PDGFR	Platelet-Derived Growth Factor Receptor
PD-L1	Programmed Death Ligand 1
PgP	P-Glycoprotein
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PIM	Proviral Integration Site of Moloney Proviral Integration Site of Moloney Murine Leukemia
PLK1	Polo-Like Kinase 1
PR	Progesterone Receptor
PyMT	Polyoma Middle T Antigen
qRT-PCR	Quantitative Real-time polymerase chain reaction
Ras	Rat Sarcoma Virus

Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RNA-Seq	RNA-Sequencing
ROS	Reactive Oxygen Species
RTKs	Receptor Tyrosine Kinases
Ser	Serine
SERMs	Selective Estrogen Receptor Modulators
sgRNA	Short Guide RNA
SH3	SRC homology 3
shRNA	Short Harpin RNA
Slug	Zinc finger protein SNAI2
SMAD	Small Mothers Against Decapentaplegic
Snail	Zinc finger protein SNAI1
SOCS	Suppressor of Cytokine Signaling
STAT	signal transducer and activator of transcription
TAM	Tumor Associated Macrophage
TCGA	The Cancer Genome Atlas
TGFa	transforming growth factor α
TGFBR	TGF-beta type I/II serine/threonine kinase receptor
TGF-β	Transforming Growth Factor Beta
TH1	T Helper Cells Type 1
TH2	T Helper Cells Type 2
Thr	Threonine
TILs	Tumor-Infiltrating Lymphocytes
TN	Triple Negative
TNBC	Triple Negative Breast Cancer
TNFR1	TNF Receptor 1
TNF-α	Tumor necrosis factor-alpha

TP	TP3654
TP53	Tumor Protein P53
TRADD	TNF Receptor associated death domain
TRAIL	Apo2-L
TRAIL Receptor	TNF-Related Apoptosis Inducing Ligand Receptor 1
Twist	Twist Family BHLH Transcription Factor
Vim	Vimentin
Wnt	Wingless-Type MMTV Integration Site Family
WT	Wild type
Zeb	Zinc Finger E-Box Binding Homeobox

Table of Contents

List of Figures

List of Tables

Chapter 1

Overview of Breast Cancer

Breast cancer is the most frequently diagnosed cancer in women, with an estimated 287,850 new cases in 2022 (1). It is also the second leading cause of cancer-related deaths in women with an estimated 43,250 deaths in 2022 (1). Breast cancer is a very heterogeneous disease that has been extensively studied to characterize the distinct cellular, molecular, and tissue level changes that occur throughout the progression from normal breast tissue to invasive carcinoma. Several different models have been proposed that attempt to characterize these changes. Ductal and lobular carcinomas make up 80% and 10% of all carcinomas respectively (1-2).

Ductal dysplasia can be broken into 5 distinct steps. Starting with normal cells which progress to flat epithelial atypia (FEA), to atypical ductal hyperplasia (ADH), to ductal carcinoma in situ (DCIS) and then finally into luminal invasive ductal carcinoma (IDC) (3).

Similarly, lobular dysplasia can be divided into normal lobular cells, atypical lobular hyperplasia (ALH), lobular carcinoma in situ (LCIS), and invasive lobular carcinoma (ILC) (3). Each of these histological grades have distinct morphological, cellular, and molecular characteristics that are used to assign what stage of dysplasia is present.

Normal breast tissues are composed of two main structures: ducts and lobules. A normal breast has between 10-12 branching ducts before forming into a terminal ductal lobular unit. (Figure 1) (4-5). These structures are made up by two different types of epithelial cells, luminal epithelial cells and myoepithelial cells (5). Epithelial cells line the ducts and lobules, whereas

myoepithelial cells are contractile cells that function to move milk from the glands, through the ducts, and out of the breast through an opening at the nipple (5).

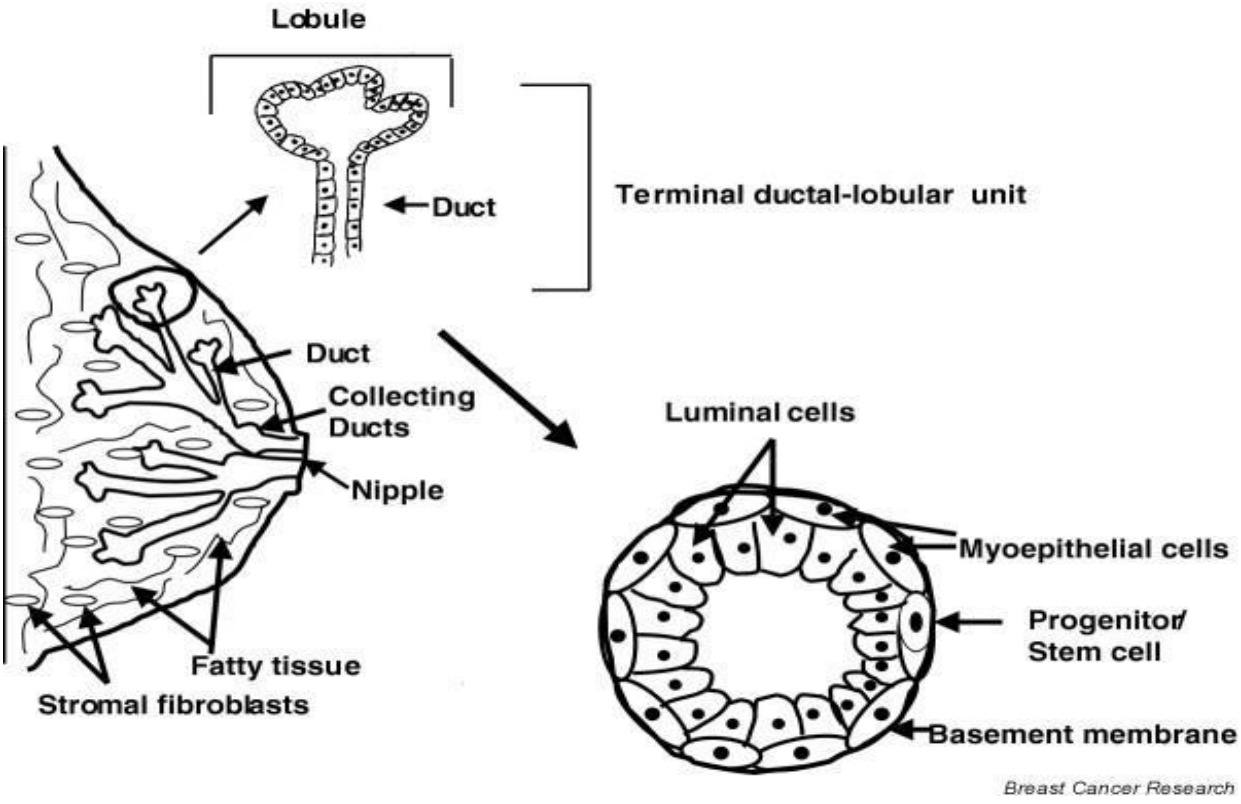

Figure 1: Structure of a normal mammary gland. Mammary glands consist of branched ducts that terminate at milk producing lobules. The ducts and lobules are made up of two main cell types myoepithelial and luminal epithelial cells (4).

Flat epithelial atypia is defined as a neoplastic alteration of the terminal duct or lobular units characterized by the replacement of native luminal epithelium by ductal cells containing low grade cytologic atypia (6). The atypical cells maintain a flat pattern of growth without evidence of atypical cellular architecture (6). The cells are frequently columnar in shape but can also be cuboidal. The major identifying feature of FEA is an increase in the growth of epithelial cells

which pseudo stratify the duct or lobular structure, while maintaining a flat pattern of growth (6). FEA is most frequently identified by cytologic analysis; FEA is comprised of cells with enlarged hyperchromatic nuclei, increased cytoplasm which tend to be eosinophilic in color. Despite these cytologic changes, the nuclei remain evenly spaced, which gives an appearance of ordered proliferation.

There are several genetic and molecular alterations that can lead to FEA. The most common include expression of estrogen receptor (ER) and progesterone receptor (PR), increased expression of cyclin D1, a decrease in the tumor suppressor protein 14-3-3, loss of chromosome 16q, positive immunohistochemical staining for keratin 19, and a lack of HER2 expression (3). Due to these genetic alterations, FEA is believed to be an early step in the evolution of low-grade ductal carcinomas.

Atypical ductal hyperplasia (ADH) is defined as a clonal proliferation of ductal cells, that contains some, but not all the features of ductal carcinoma in situ (5). The cells in ADH resemble those of DCIS, however, they only partially fill the ducts. These cells tend to be regularly spaced, and still maintain a monomorphic pattern of proliferation. Chromosomal abnormalities such as a loss of chromosome 16q and a gain of 17p are also observed in ADH subtype (3-5).

Ductal carcinoma in situ (DCIS) is defined as a malignant proliferation of epithelial cells that occurs in the duct or lobules. Its major distinguishing morphological feature from ADH is that the neoplasm has completely filled in the acinar structure of the duct (5). It is important to note that the classification between DCIS and LCIS is not where the carcinoma occurs, but rather the appearance of the cells that make up the tumor. An additional major hallmark of DCIS is that it

has yet to spread through the basement membrane (5). At this stage there are often other receptors that are upregulated that will be discussed later in this paper.

DCIS is thought to be a direct step in the evolution of IDC. The major difference between DCIS and IDC is that in IDC there is a loss of myoepithelial cells and carcinoma has spread through the basement membrane (3-5). A common feature of IDC is loss of E-cadherin (3). E-cadherin serves as a cell-cell adhesion molecule (7). Loss of this adhesion allows the invasive carcinoma cells to spread through the basement membrane (5).

IDCs can further be broken into either low-grade or high-grade IDCs. Low grade IDCs tend to have fewer genetic alterations when compared to high-grade IDCs. (5) Some common alterations of low-grade IDC include loss of chromosome 16q, and gains of 1q, 8q, and 16p (3,5). High grade IDCs contain frequent gains of 8q, 16p, 17q, and 20q and frequent loss of 1p, 8p, 11q 13q, and 18q (3). A third intermediate grade of IDCs is also commonly found, which contains a combination of the previously described chromosomal aberrations (3). Previously it has been hypothesized that low-grade IDCs will eventually progress to high-grade IDCs (5). However, because these chromosomal alterations are so frequently found in distinct groups, logically one can assume that low-grade IDCs do not progress into high-grade IDCs, rather, previous genetic alterations determine whether an IDC is high or low grade (6).

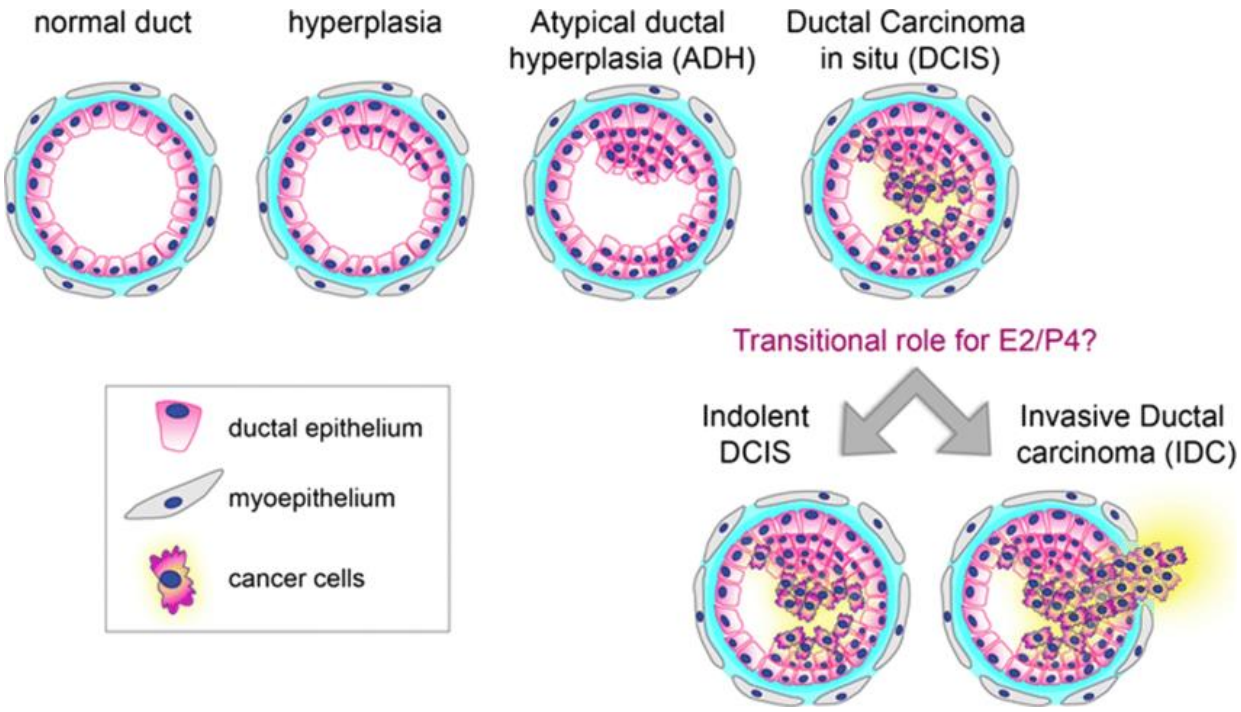

Figure 2: Graphical representation of the several different stages of ductal dysplasia. These include a normal biopsy, Hyperplasia, Atypical Ductal Hyperplasia (ADH), Ductal Carcinoma in situ (DCIS), and Invasive Ductal Carcinoma (IDC) (7).

Lobular carcinomas follow a similar histological progression, however there are a few key differences. Lobular neoplasia is not as linear of an evolution as certain ductal carcinomas. While there are similar precursors to LCIS, they are more used to assess the risk of developing LCIS, rather than at what stage the disease is in. This is largely supported by an equal chance of developing LCIS in either breast, not just the breast that contains lobular neoplasias.

The earliest detectable dysplasia involving lobular cells is atypical lobular hyperplasia. Atypical lobular hyperplasia can be identified by cells that are identical to those that make up LCIS; however, they have not filled more than 50% of the acini within a lobule (5). Like ADH, loss of

chromosome 16q and gain of chromosome 17p are common in ALH (3,5). The major difference from ADH is that in ALH there has already been the loss of E-cadherin (5).

Lobular carcinoma in situ is defined as a clonal proliferation of cells within lobules and that grow in a dis-cohesive fashion, usually due to loss of E-cadherin (5). The major identifying histologic feature of LCIS is that the lobular cells are greatly expanded; however, this expansion of cells does not distort the lobular architecture of effected areas (5). The proliferation of these cells does fill these structures; however, the architecture itself is not disturbed and can still be recognized during histological analysis.

Invasive lobular carcinoma is morphologically characterized by small round cells that have a smaller cytoplasm portion of the cell when compared to IDC (3,5). Similarly, the architecture of the lobule tends to remain unchanged in ILC (8). The major identifying histological feature that differentiates LCIS form ILC is the presence of infiltrating, discohesive cells which lack tubule formation (5).

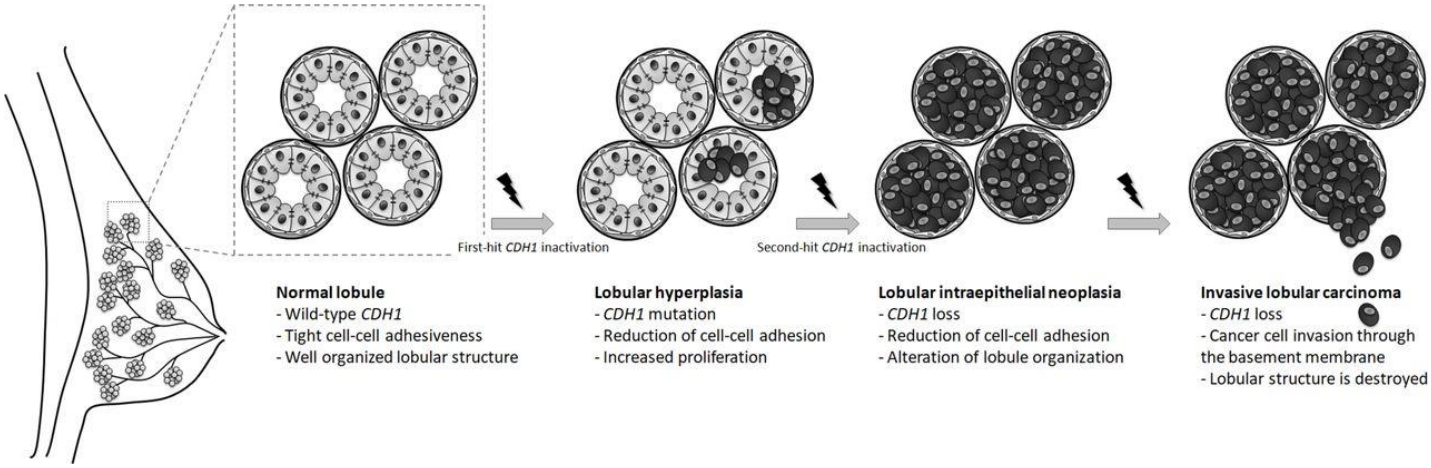

Figure 3: Schematic overview of the several different stages of lobular dysplasia. These include a normal biopsy, Atypical Lobular Hyperplasia (ALH), Lobular Carcinoma in situ (LCIS), and Invasive Lobular Carcinoma (ILC) (8).

Molecular subtypes of breast cancer

There are two main ways that invasive breast cancers are classed, by PAM50 molecular subtype and by hormone/HER2 receptor status. Both classifications are used as prognostic markers as well as guides as how to direct treatments for different types of breast cancer. Molecular subsets were proposed by Perou et al. through use of microarray analysis to identify clusters of tumors that have similar cellular and molecular patterns (9). This analysis produced 4 distinct molecular subgroups: Luminal A, Luminal B, HER2+, and Basal-like.

Approximately 30% to 40% of all invasive breast cancers are classified as luminal A. Luminal A breast cancers are characterized as having high estrogen receptor expression, no expression of HER2, with low expression of Ki-67 (9). Ki-67 is a protein that is expressed in all stages of the cell cycle except G0. Expression of Ki-67 is routinely used as a marker for cellular division (10). The general principle being that tumors that have fewer Ki-67 positive cells are growing slower, and therefore have a better prognosis (10).

The other luminal subtype, luminal B makes up approximately 20 to 30% of all invasive breast cancers (9). This molecular subtype is characterized by moderate expression of estrogen and progesterone receptor, lack of HER2 expression, and high expression of Ki-67. (10) Luminal B breast cancers have amplifications of genes associated with cell proliferation and thus tend to proliferate at a faster rate than luminal A (10). Luminal B breast cancers are usually responsive to endocrine therapies and are somewhat responsive to chemotherapy (10). Additionally, some luminal B breast cancers also have amplification of HER2 (10). An additional marker for both luminal A and luminal B subtypes are expression of cytokeratins 8 and 18 (5,9,10).

The HER2+ molecular subtype of breast carcinoma is classified by IHC staining that is negative for estrogen and progesterone receptor, but positive for HER2 (9). These carcinomas are usually a high grade, more likely to have spread to the axillary lymph node and have a high rate of reoccurrence (10). The HER2 molecular subtype has high Ki-67 expression and tends to respond to chemotherapy, as well as the directed treatments for HER2 (10,13).

Basal breast carcinomas are identified by their lack of expression of ER, PR, or HER2 (9). Additionally, Basal - like breast carcinomas express keratins 5, 6, and 17 (11). Basal - like carcinomas tend to be higher tumor grade, have a strong association with BRCA1 mutations, and often have a high Ki-67 score (10,11). Additionally, this molecular subtype is associated with higher risk of recurrence, and better response to chemotherapy when compared to the other subtypes (11). It is important to note that not all TNBCs are basal-like, and not all basal-like breast carcinomas are triple negative, meaning that all carcinomas cannot be identified by only these sub-groups (10).

Breast Cancer Receptor Subtypes

Breast cancers have been classified based on over-expression of different receptors. The three main receptors used to classify breast cancer include estrogen receptor, progesterone receptor and human epidermal growth factor 2 receptors (HER2). Based on the expression of these receptors breast carcinomas can be grouped into three different categories: ER/PR positive, HER2 positive, and triple negative.

Estrogen receptor is a nuclear protein that contains a DNA biding domain as well as multiple phosphorylation sites which regulate activity (12). Estrogen receptor functions by binding to regions of DNA called estrogen response elements, located in the promoter region of the target genes (12-13). This binding activates transcription of target genes. Estrogen receptors also indirectly regulate transcription through protein-protein interactions with additional transcription factors in the nucleus (12-13). Over 700 genes have been identified that are regulated either directly or indirectly by estrogen receptor (14). Similarly, progesterone receptor also acts as a transcription factor (12-13). Progesterone receptor expression is primarily regulated by ER, which is why the two are commonly co-expressed in breast cancers (14).

The female sex hormones, estrogens are made up of 4 different hormones: 1) estrone, 2) estradiol, 3) estriol, and 4) estrerol (15). Estrogens are primarily synthesized in the ovaries but are also produced in adipose tissue and the adrenal glands (15-16). Estrogens are primarily synthesized from low-density lipoprotein-cholesterol (LDL) (15-16). Estrogen signaling controls a broad variety of physiological functions such as regulation of the menstrual cycle, bone density brain function, reproduction, development of the breast tissue and sexual organs (15-16). Estrogen has also been shown to play an important role in inflammation (17). Estrogens bind to estrogen receptors, which causes ER dimerization and translocation to the nucleus (18). Once in the nucleus dimerized ER binds to DNA at specific promoters to activate transcription of different genes known as estrogen response elements (EREs) (18). Over 700 genes have been identified as estrogen response elements, these genes promote breast cancer cell growth and metastasis (19).

A cancer is classified as ER or PR positive if it has greater than 1% or more of tumor cells that have positive immunohistochemical nuclear staining for estrogen receptor (5). ER/PR positive

breast cancers are associated with the best prognosis. (20) This is in part due to directed treatments that can either inhibit hormone synthesis or receptor antagonists such as tamoxifen (21). Approximately 70% of all breast cancers are ER/PR+ (22).

HER2 is a transmembrane tyrosine kinase receptor that mediates cell proliferation and survival (23). It is activated by extracellular signals such as epidermal growth factor (23). After ligand binding, HER2 can either homo or heterodimerize with HER1, HER2, or HER3 receptors (24). Upon dimerization, the HER proteins trans phosphorylate tyrosine residues on the intracellular portion of the protein (24). This allows binding of its downstream targets which include PI3K and Ras (24). These signal transducers are commonly misregulated in cancer (25-26). HER2+ cancers tend to be more aggressive than ER/PR+ cancers and are associated with a median prognosis (23). This is in part due to the advent of HER2 antagonists such as lapatinib or Herceptin (27). HER2+ make up approximately 20% of breast cancers (28).

Triple negative breast cancers are a subset of breast cancers that do not express ER, PR or HER2 receptors (5). These cancers are often the most aggressive and associated with the worst prognosis (20). TNBCs make up a very heterogenous subset of cancer which in part makes them more difficult to treat; no directed treatments are currently approved for TNBC (22).

TNBC is a very heterogenous disease; because there are several different genetic alterations that can lead to TNBC. Lehman et al. attempted to further separate these cancers into six different molecular subtypes (29). These subtypes include Basal-like one (BL1), Basal-like two (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor. These subtypes were generated through gene expression analysis of 21 different data sets that include 587 TNBC samples (29).

The BL1 subtype contains alterations in DNA damage repair mechanisms such as BRCA1/2 and ATR as well as amplification of genes that activate cellular division such as MYC, NRAS, and PLK1 (29). This subtype was also found to have a higher expression of Ki-67, indicating an increased proliferation rate of this subtype (29-30).

BL2 TNBCs have amplifications in several different growth factors including EGF, MET, NGF, Wnt, and IGF1R. This subtype also contains genetic alterations that alter the metabolism of the cell, which tend to push the cell towards a more glycolysis-based metabolism (29). Finally, this molecular subtype of TNBCs were found to have expression of myoepithelial markers TP63 and MME (29).

The IM subtype of TNBCs were found to have enrichment of immune signal transduction pathways. Such pathways include NK cell pathway, TH1/TH2, and the B-cell receptor signaling pathway. Additionally, this subtype shows enhancement of the JAK/STAT signaling pathway. This subtype has been associated with a better prognosis than the others (29).

Both the mesenchymal (M) and mesenchymal stem-like (MSL) subtypes express genes involved in motility, extracellular matrix (ECM), cell differentiation, and genes associated with the epithelial to mesenchymal transition. The major difference between these two subtypes is that mesenchymal TNBCs have higher expression of genes associated with cellular proliferation. The major cell signaling pathways enriched in MSL and M TNBCs are Wnt, ALK, TGF-B, and ERK signaling (29).

Lastly, the LAR subtype showed increased gene enrichment in steroid synthesis pathways and an increase in expression of androgen receptor (AR), as well as an increase of AR's downstream

targets. This subtype also contains enrichment of genes related to steroid synthesis as well as estrogen metabolism, despite being estrogen receptor negative (29,31).

Table 1: Potential directed treatments based on specific TNBC sub-group assignment (29).

Subtype	Molecular changes	Potential drug regimen
BL1	BRCA1/2 Mutations EGFR Amplification High rate of proliferation	PARP inhibitors EGFR inhibitors Chemotherapy
BL2	Wnt signaling Higher rate of proliferation EGF signaling	Salinomycin Chemotherapy PI3K inhibitors
IM	JAK/STAT signaling enriched Cytokine signaling enriched Antigen presentation	JAK2 inhibitors PD-1 inhibitor Chemotherapy
M	Motility signaling enriched Cell differentiation pathways WNT signaling enriched	Chemotherapy Salinomyacin TGF-B inhibitors
MSL	Motility signaling enriched Cell differentiation pathways WNT signaling enriched Slow Growing	Salinomyacin TGF-B inhibitors No Chemotherapy

LAR	Enriched steroid synthesis Increased AR	Androgen Antagonists Endocrine therapy

Despite the great amount of tumor heterogeneity, the current standard treatment for all TNBCs is chemotherapy. This has proven largely ineffective, with a median survival of only 13 months (32). Thus, there is an unmet need to identify additional new therapeutic targets in order to improve patient outcomes.

Current Therapies

There are several different factors that are used to determine the course of treatment for breast cancer patients. Current criteria include what stage the cancer is at the time of diagnosis, overexpression of different receptors previously outlined, pre/post-menopausal status, mutation of particular genes, and how fast the cancer is growing (33). Depending on these factors the best course of treatment is determined. Current therapies include surgery, radiation, chemotherapy, endocrine therapy, immunotherapy, and directed therapies; The most commonly used treatments are surgery and radiation therapy (34).

Breast cancer stage at the time of diagnosis is a critical factor for deciding the best course of treatment. Breast cancer stage can be classified into stages 0-IV depending on how far the cancer has progressed; each stage has different criteria that are used to identify an individual breast cancer's stage (35). Stage 0 breast cancers are classified as a malignant population of cells, which are localized to the lobule or duct, but have not spread through the basement membrane (33-34). Stage I breast cancers are defined as tumors that are smaller than 2 cm,

which have not spread to adjacent lymph nodes (33-34). The main therapies for stage I breast cancers are surgery and radiation (33-34). If stage I breast cancers are HR+ then following surgery and radiation, patients may also receive hormone therapy for at least 5 years. In some cases, if tumors are larger than 0.5 cm chemotherapy can also be used (33-34). Stage II breast cancers are defined as invasive carcinomas less than 5 cm, which have spread to 3 or less of the axillary lymph nodes (33-34). Stage III breast cancers are defined as invasive carcinomas that are 5 cm or less, which have spread to 4 or more axillary lymph nodes, invasive carcinomas that are over 5 cm without spreading to lymph nodes, or any invasive carcinoma that has spread to 10 or more lymph nodes (33-34). Stage III breast cancers are classified as locally advanced (33-34). Stage IV breast cancers are classified as invasive carcinomas that have spread to distant metastatic sites (33-34). Each stage has varying survival rates, which are summarized in Table 2.

Table 2: 5-year Survival Rates of Breast Cancer by Stage at time of Diagnosis (33-34).

Stage	Classification	5-year survival rate
0	In situ	100
I	Early invasive	99.1
II	Early invasive	83.6
III	Locally advanced	57
IV	Metastatic	23.4

Stage 0 cancers are generally DCIS or LCIS; aberrant growths of cells that have not yet become invasive. LCIS generally does not progress to an increased risk of invasive carcinomas, so treatment for these generally only includes tamoxifen for HR+ breast cancers (36). In addition, it is also recommended that patients receive yearly mammography imaging to check for new tumors (36). Conversely, DCIS does progress to invasive cancers more frequently, so the treatments are much different (37). Breast-conserving surgery coupled with radiation are generally recommended for the treatment of DCIS (34-35). Full mastectomy without follow-up radiation, and tamoxifen for HR+ cancers are also used as treatment options (34-35).

Stage I and Stage II breast cancers are considered early-stage invasive carcinomas. Treatment of stage I or stage II breast cancers generally starts with has mastectomy, however, in recent years breast-conserving surgery has become more common for patients (34-35). For patients that receive breast-conserving surgery, radiation treatment is then recommended because it has been found to reduce recurrence and improved patient survival rates (34). Patients with stage I/II breast cancers also receive adjuvant therapies after surgery and radiation (38). Treatment options include systemic chemotherapy, endocrine therapy, or specific therapies depending on the receptor status of the individual cancer (34-35).

Chemotherapy options for breast cancer include an anthracycline such as doxorubicin or epirubicin, and a taxane, including docetaxel or paclitaxel (34). Anthracyclines are a drug derived from certain types of Streptomyces bacteria (39). They work by causing DNA, resulting in cell death (39). Taxanes are a class of diterpenes identified from plants (40). Taxanes bind to microtubule protein B-tubulin, preventing its polymerization, a necessary function for cell division to occur (40). These chemotherapies can be used before or after surgery or radiation depending on the stage. These differences are outlined further in this section.

Endocrine therapies include aromatase inhibitors, gonadotropin-releasing hormone agonist, or selective estrogen receptor modulators (SERMs) (34-35). Both of these therapies are used for HR+ breast cancers and work by inhibiting estrogen and progesterone signaling. Gonadotropin-releasing hormone agonists, such as Goserelin, prevent the ovaries from producing estrogen and progesterone (41). Similarly, aromatase inhibitors, such as anastrozole or letrozole reduce estrogen and progesterone in the body (42-44). Aromatase converts androgens to estrogen, and blocking it lowers the conversion of these hormones into estrogen (45). SERMs such as tamoxifen work by directly inhibiting the estrogen receptor in the cancer cells, preventing its downstream signaling cascade (46). In addition to these drugs that target the estrogen receptor or estrogen production in the body, CDK4/6 inhibitors, such as palbociclib, ribociclib, and abemaciclib also have been approved recently to treat HR+ breast cancers (47-49). These drugs are often used as a second line of treatment, if patients do not respond well to initial treatment (49). These treatments are mainly used to treat HR+ breast cancers.

A final directed therapy that has been developed is targeting ERBB2. In patients with HER2+ breast cancers, after surgery and radiation treatment patients often receive adjuvant HER2+ inhibitor treatment (50). Trastuzumab is a humanized monoclonal antibody that inhibits ERBB2 and prevents its dimerization (50). Small molecule ERBB2 inhibitors, such as lapatinib also have been developed (51). Lapatinib reversibly binds to the cytoplasmic ATP-binding site of ERBB2, blocking receptor phosphorylation and activation (51). These treatments are used for stage I and II breast cancers that are HER2+.

For stage III locally advanced breast cancers many patients receive neo-adjuvant chemotherapy treatment in order to shrink tumors prior to surgery and radiation (34-35). Chemotherapies typically given include an anthracycline and taxane (34-35). Recent clinical trials have also

included neo-adjuvant treatment with tamoxifen for HR+ breast cancers or trastuzumab for HER2+ breast cancers but found little benefit to these (52-53). Following surgery/radiation treatment, patients receive these drugs as adjuvant therapy and showed improved patient responses (34-35).

Stage IV metastatic breast cancers are treated largely with palliative care in order to make patients more comfortable, but success rates remain low for treating the disease once it has metastasized (34-35). Patients with HR+ breast cancers often receive radiation therapy followed with endocrine therapy or chemotherapy which can slow the progression of the disease (46). Many patients however, only elect for endocrine therapy because side effects from chemotherapy are much more severe (34). Patients with HER2+ breast cancers often receive a combination of chemotherapy and trastuzumab (50). Lastly, TNBC patients, where no directed treatments are available, receive a combination of chemotherapy including an anthracycline, a taxane, and an alkylating agent such as carboplatin or cisplatin (54). Anthracyclines such as doxorubicin or epirubicin inhibit topoisomerase, resulting in DNA damage and ROS production (39). Carboplatin and cisplatin cause intra and inter strand cross linking of DNA molecules, which results in inhibited DNA synthesis, preventing cell division (55). Taxanes block beta tubulin de-polymerization, locking the cell cytoskeleton (40). The polymerization and depolymerization of beta tubulin are necessary for dividing cells to separate properly (40).

Breast Cancer Metastasis

A major clinical challenge in the treatment of breast cancer is metastasis. Metastasis is defined as the spread of cancer cells from primary tumors to form new secondary tumors, which are like

those of the primary tumor (56). Metastatic breast cancer does not respond to the same treatments as primary tumors, and care is often only palliative for these patients (56). This identifies a great need for additional treatments that can prevent metastasis or that specifically target the metastatic cells themselves. Breast cancer itself is not often lethal, however, the lethality from breast cancer occurs when it metastasizes to other vital organs (56). Metastasis is a multifaceted process that can be broken into the following steps: first, local tumor cells must infiltrate adjacent tissues, followed by intravasation, invasion through blood vessels and into the circulatory system (57). Tumor cells must then survive in circulation, infiltrate back through blood vessels (extravasation), and finally proliferate in distant sites (57).

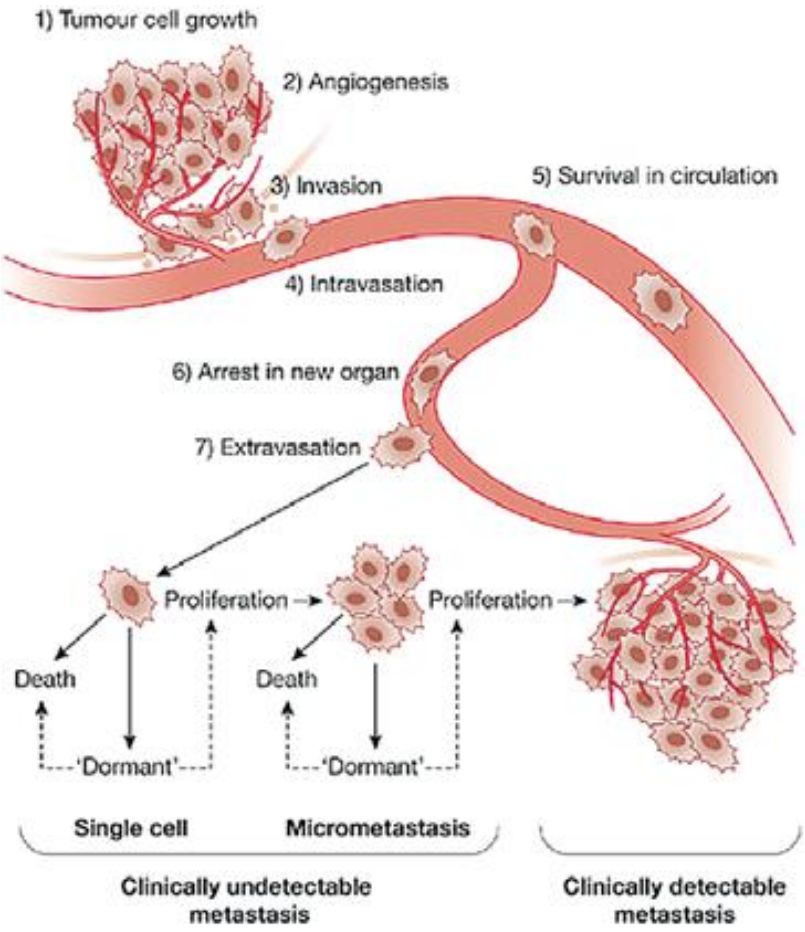

Figure 5. Overview of the steps of breast cancer metastasis. Breast cancer metastasis is made up of multiple steps starting with tumor growth. Following primary tumor growth, breast cancers gain access to blood vessels from angiogenesis. Invasive cells then move through the basement membrane through intravasation. The cells must then survive in circulation until they arrive at a distant site where they leave the blood (extravasation) followed by growth at the distant site (57).

One of the ways cancer cells survive this process is through a series of changes in gene expression known as the epithelial to mesenchymal transition (EMT), which causes cells to upregulate several mesenchymal genes (58). EMT is characterized by loss of cell-to-cell adhesions, gain of mesenchymal properties, distinct changes in gene expression, and an increase in cell motility/invasiveness (59). Several different markers of EMT have been identified including upregulation of transcription factors Snail/Slug, Twist, Zeb1/2, and E47, loss of E-

cadherin, upregulation of N-cadherin, and upregulation of different MMPs (60). The changes in expression of the EMT transcription factor signaling is driven by several different oncogenic signaling pathways including TGF-β/SMAD, PI3K, RAS/MAPK, Wnt, hypoxia, and TNF-α (61-65).

Transforming growth factor-β (TGF-β) is often identified as one of the primary activating pathways of EMT (66). Early in cancer development, a tumor suppressor role of TGF-β has been identified, where TGF-β signaling promotes apoptosis and growth arrest, so TGF-β signaling is often decreased (66). During tumor progression however, TGF-β signaling becomes upregulated or maintained (66). TGF-β binds to serine/threonine receptors TGFBRI and TGFBRII (67). Once bound to the receptor, TGFBRII trans-phosphorylates TGFBRI, TGFBRI then phosphorylates its downstream targets Smad2 and Smad3 (67). Phosphorylated Smad2/3 then forms a complex with Smad4 where it then translocates to the nucleus, and actives transcription of genes related to EMT such as Snail, Slug, and Twist (68). In addition, TGF-β has been shown to act independent of Smad signaling to activate PI3K, Akt, and MAPK (67).

Several different RTKs have been implicated as promoting EMT, including epidermal growth factor receptors (EGFRs), platelet-derived growth factor receptors (PDGFRs), and fibroblast growth factor receptors (FGFRs) (69). RTKs are transmembrane proteins, which after ligand binding undergo conformational changes, resulting in activation and phosphorylation of downstream targets (70). These downstream targets include Ras/MAPK, JAK/STAT, and PI3K, all of which have been identified as promoting EMT (70).

The EGFR family of receptors consist of EGRF1, HER2, EGFR3, and EGFR4 (71). As the name suggests, EGFRs primary ligand is epidermal growth factor (EGF) (71). Several other ligands that bind to EGFRs have also been identified including transforming growth factor α

(TGFα), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AREG), epiregulin (EREG), and epigen (EPI) (71). After ligand binding EGFRs form either homo or heterodimers, then trans-auto-phosphorylate causing a conformational change, which results in recruitment of downstream signaling proteins or adapter proteins (71). In the context of breast cancer EMT, EGFR downstream signaling has been shown to activate PI3K, Ras, and MAPK (72). EGFR signaling has also been implicated in breast cancer metastasis specifically to the brain (73).

PI3K has been shown to increase epithelial cell proliferation (74). PI3K has also been found to promote EMT in conjunction with TGF-β (68). PI3K also acts as a signaling intermediate of several different growth factor and integrin receptors (74). Ras/MAPK signaling has been reported to promote EMT and metastasis by phosphorylating Twist1 at serine68, which stabilizes the protein, thus leading to increased expression and activity (75).

Another major signaling pathway that has been reported to have a significant role in breast cancer metastasis is the CXCR4/CXCL12 signaling axis (76-77). CXCR4 is a transmembrane chemokine receptor containing seven transmembrane helical regions connected by six extra cellular loops (78). CXCR4 binds the ligand CXCL12 (78). CXCR4 is highly expressed on the surface of breast cancer cells (79). CXCL12 is a chemokine, that acts as a chemoattractant (78). Unlike CXCR4, CXCL12, is expressed at its highest levels in organs that breast cancers commonly metastasize, including the lungs, brain, and bone marrow (79). CXCL12 is released by stromal cells, including fibroblasts and endothelial cells as well as macrophages (78). CXCL12 binding to CXCR4 activates PI3K/Akt, NF-kB, STAT3, and Src/ERK signaling (78). When CXCL12 binds to CXCR4 CXCL12 binding to CXCR4 causes actin polymerization

resulting pseudopodia formation, promoting migration/invasion, leading to metastasis to distant organs (78).

The Wnt signaling pathway has also been identified as playing an important role in EMT and metastasis (80). There have been 19 different Wnt genes identified in the human genome, all of which produce secreted lipoglycoproteins (81). Wnt signaling couples to different receptors activating various downstream pathways, consisting of both canonical and non-canonical Wnt signaling (80). Canonical Wnt signaling promotes breast cancer cellular proliferation and cancer cell stemness (80). Non-canonical Wnt-PCP and Wnt-Ca2+ signaling however have been found to promote breast cancer metastasis (80).

These different molecular mechanisms result in metastasis in an organotrophic manner; breast cancers tend to metastasize to specific organs, depending on which pathways are activated in an individual cancer (82). These different pathways are not upregulated across all breast cancers but depending on which are activated determines which organ the primary tumors metastasize to. The different pathways described and the organs they promote metastasis to is summarized below in table 3.

Table 3: Molecular Mediators as Breast Cancer Metastasis Activators (82).

Metastasis activators	Role	Potential therapeutic strategy
Growth factors		
TGF-β	Bone metastasis	TGF-β pan-neutralizing mAbs: 1D11, 2G7 along with doxorubicin treatment
PDGF	Bone and lung metastasis	PDGF mAbs
IL-1	Bone and lung metastasis	Combinatorial therapy of mAbs - PDR001, CJM112, Ilaris and EGFR tyr kinase inhibitor EGF816

Metastasis activators	Role	Potential therapeutic strategy
BMP	Induce tumorigenesis, metastasis, and invasion	Soluble decoy receptors of the ligand-binding domains of BMP receptors
CXCL1	Lung metastasis	CXCl1 mAbs
Receptors		
PDGFRβ	Lung metastasis	PDGFRβ mAb: IMC-2C5
EGFR	Brain metastasis	Panitumumab with 5-fluorouracil, epidoxorubicin, and cyclophosphamide followed by docetaxel
CXCR4	Lung and liver metastasis	AMD3100 or Plerixafor
Integrins	αvβ3 - lung metastasis αvβ5 - liver metastasis	Integrin signaling specific antibodies, peptides, peptidomimetics, and other antagonists
Notch	Bone metastasis	miR-34a along with Paclitaxel and Adriamycin
Transcription factors		
TWIST1	Lung's metastasis	Harmine, inhibitor of TWIST1
ID1	Lung's metastasis	Small molecule inhibitors against ID1
ECM proteins		
VCAM-1	Lung & bone metastasis	Targeting integrins - VLA-4/α4β1 with mAbs to inhibit interaction with VCAM-1
VCAN	Lung metastasis	Knockdown of versican
Other factors		
PDK1	Liver metastasis	Small molecule inhibitors against PDK1
COX-2	Brain metastasis	Non-steroidal anti-inflammatory drugs (NSAIDs)
miRNAs	miR-19a and miR-141 - brain metastasis	Oligonucleotides targeting mature miRNA
Wnt/β-catenin	Lung metastasis	PORCN inhibitors, WNT ligand antagonists, and FZD antagonists/mAbs

Most of these different pathways converge on activation of transcription factors Slug, Snail, and Twist (83). They result in several changes in the cells which promote metastasis. These changes are summarized in figure 4 below (83). Slug, Snail, and Twist have been shown to promote stem-like behavior of cells, drug resistance, metastasis, and immunosuppression (83).

One of the downstream targets of Slug, Snail, and Twist is Zeb1. Zeb1 expression has been found to repress E-cadherin expression and induce a cancer-stem-cell differentiation state (84). These cells are identified by flow cytometry by $CD44^{high}/CD24^{low}$ (85). It has been suggested that this population of stem cells is induced by EMT and are necessary for dissemination as part of metastasis (85). Additionally, CSCs play an important role in generation of secondary tumors due to their self-renewal ability (86). These several changes resulting from EMT promote breast cancer metastasis.

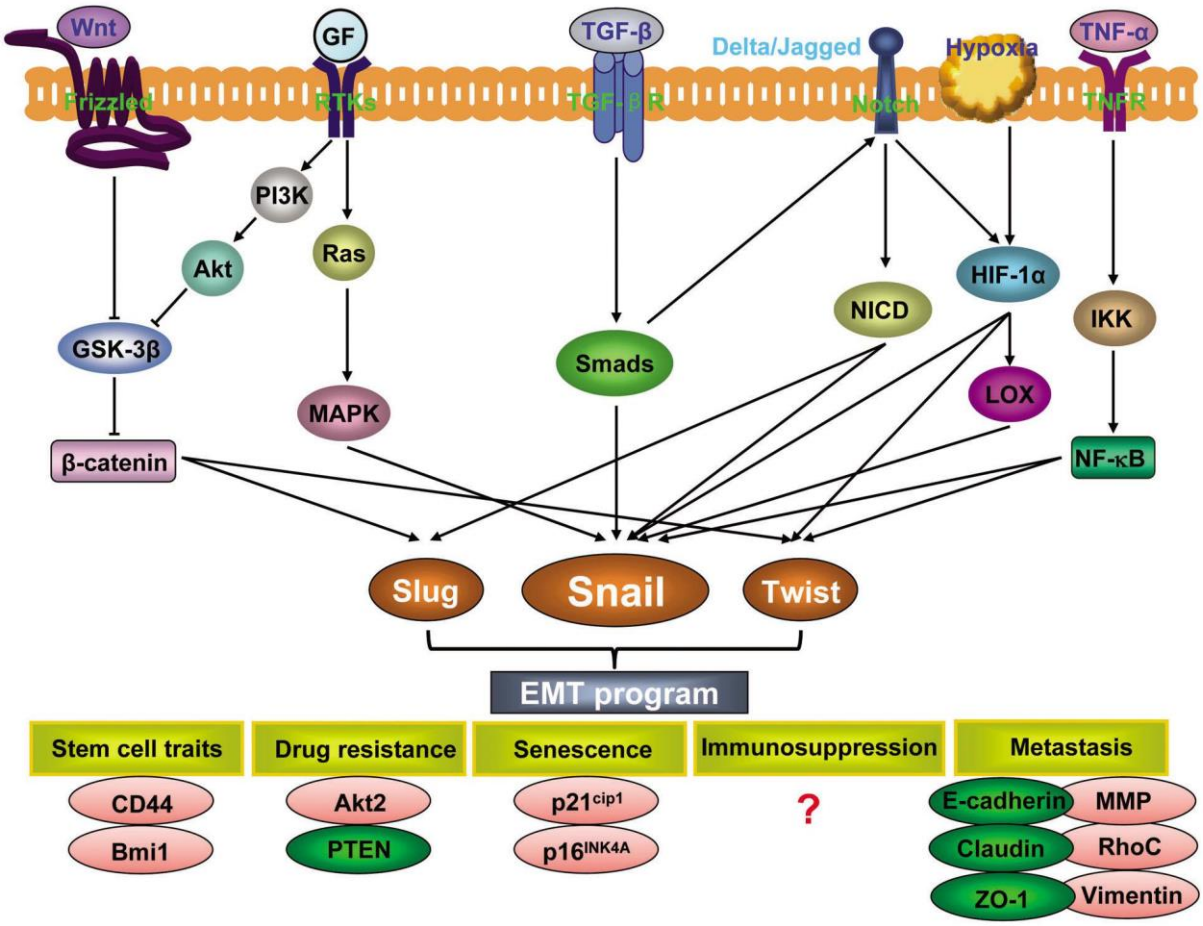

Figure 6. Overview of EMT signaling. Several different signaling pathways can be activated which result in an increase in expression of transcription factors Slug, Snail, and Twist. These transcription factors activate expression of genes related to cell stemness, drug resistance, senescence, immunosuppression, and metastasis (83).

The first step in metastasis is invasion of tumor cells into the local surrounding tissues. First, the cells must breakdown local cell to cell adhesions; epithelial cells are anchored to neighboring cells through a variety of different interactions. A predominant cell-cell adhesion of epithelial cells is E-Cadherin (87). E-Cadherin consists of 5 extracellular domains, which form extracellular junctions from cell to cell using homophilic interactions (87). The intracellular portion of E-cadherin bonds to catenin proteins alpha, beta and gamma, which link E-cadherin to

the actin cytoskeleton of cells (87). In metastatic breast cancers, E-cadherin is commonly lost or mutated to lose its function, which breaks down cell to cell anchoring. Additionally, with the loss of E-cadherin, cancer cells often upregulate N-cadherin (89). N-cadherin has been shown to enhance the migratory and invasive capacity of tumor cells (90).

Once cell to cell anchorage is lost, cells then invade into the local stromal cells after breaking down the local extra cellular matrix (ECM). The ECM is a network of proteins that surround, support and give structure to cells and tissues of the body. Depending on the tissue, the composition of the ECM varies greatly. In the mammary gland, the ECM is comprised of collagen fibers, laminin, fibronectin, and proteoglycans (91). These molecules play an important role in regulating epithelial cell growth, embryonic branching and morphogenesis (92). In order to invade into local tissues, the ECM must first be broken down. In breast cancer this is often carried out by metalloproteinases (MMPs). There are currently over 20 different MMPs that have been identified in humans (93). MMPs are secreted from cells and remain inactive until being cleaved by extracellular proteases (93). Specifically, MMP-2, MMP-9, and MMP-11 have been identified as playing important roles in breast cancer progression and metastasis (94). MMP-2 and MMP-9 are collagenases, which primarily degrade collagen IV in the ECM (95). MMP-11 is a stromelysin, which breaks down the non-collagen connective tissues of the ECM, including fibronectin, laminin, and proteoglycans (95). MMP-11 has been shown to play an important role in early tumor invasion, but also prevents apoptosis (95).

After breaking free from neighboring cells, and invading into local tissues, tumor cells then invade into the circulatory system through a process known as intravasation (57). ECM degradation forms small channels through the basement membrane through which invasive cells move through with ameboid like movement (57). These invasive cells then push through the

pores in the ECM. Some reports have suggested that mesenchymal cells gain additional motility and primarily make up the invasive cells (96).

Once entering the blood stream, cancer cells must then survive in circulation (57). Under normal conditions, if epithelial cells become detached, they undergo the process of anoikis (97). Cells that have gone through EMT, gain many features of stem cells, one of which being anchorage independent growth (98). After the cells enter the bloodstream, they travel around the body until they extravate, and establish new secondary tumors at distant sites (57). Once tumors have metastasized, they do not respond to treatments as well, and patient mortality risk is greatly increased (34-35).

An additional feature of tumor progression promoted by EMT is immune evasion. Immune evasion is a balance of immune suppression and inflammation, both processes vital to tumor progression and metastasis. EMT gene signatures are associated with increased expression of several different immune inhibitory receptors and ligands including PD-1 and PD-L1 (99-100).

PD-1 is a 55-kDa transmembrane protein normally expressed on the surface of activated t cells, natural killer cells, macrophages, dendritic cells, and monocytes (102). Notch signaling, a common pathway involved in the activation of EMT also results in increased expression of PD-1 (103). In addition, c-FOS has also been shown to increase PD-1 expression (104). PD-1 binds to ligand PD-L1 resulting in impaired immune response (101).

PD-L1 is a 33-kDa transmembrane glycoprotein normally expressed on the surface of macrophages, activated T cells, B cells, and dendritic cells (105). PD-L1 can also be induced by inflammation in epithelial cells (106).

PD-L1 expression is frequently increased on the surface of cancer cells(106). In TNBC Vimentin expression and loss of E-cadherin are correlated with upregulation of PD-L1 (107). There are multiple mechanisms which result in increased PDL1 expression on the surface of tumors including inflammation which results in increased production of INF-γ (108). This increase in INF-γ leads to increased expression of PD-L1 on the surface of tumor cells (108). In addition, loss of PTEN and PIK3CA mutations have been found to increase expression of PDL1 (107). Loss of PTEN is common in both HR+ and TNBCs, with as many as 35% of TNBCs having loss of PTEN (107). PIK3CA mutations have also been identified in approximately 40% of all breast tumors (107).

Binding of PD-L1 to PD-1 suppresses anti-tumor immune responses by inhibiting production of cytokines, promoting apoptosis in T cells, and inhibiting cytotoxic T cell activation (104). TNBCs have been reported to have a high number of tumor-infiltrating lymphocytes (TILs), suggesting immunotherapy may be beneficial for TNBC treatment (107). Recently, atezolizumab has been FDA approved for the treatment of locally advanced and metastatic TNBCs expressing PD-L1 in combination with chemotherapy (109). However, atezolizumab as a monotherapy provided little benefit to TNBC patients (109). When used in combination with chemotherapy there has been some improvement in patient outcomes, but additional work must be done to further improve patient responses.

Drug-Resistance in Breast Cancer

Another major clinical challenge persistent in breast cancer is drug-resistance. Approximately 45-50% of breast cancer patients will develop some form of drug resistance (110). There are two main theories to explain how drug resistance arises in cancer, inherent resistance and acquired resistance (112). Inherent resistance postulates that tumors are made up of a heterogenous population of cells, and only certain populations of cells will be affected by a particular drug regimen (112). After treatment, the cells that remain are only the cells that are resistant to the treatment given (112). This produces more aggressive tumors that will not respond to subsequent treatment. Acquired resistance suggest that drug treatment causes changes in gene expression, which results in cancers that are resistant to the treatment they are receiving (112). More recently, with lineage tracing experiments, a newer model suggesting a mixture of both theories are true: tumors contain heterogenous populations of cells, some of which are genetically "primed" to become resistant (112-113). After treatment these cells undergo changes in gene expression, which results in drug-resistant cancers. There are several different mechanisms that cancers use to become drug resistant. These include target modulation, drug efflux, reduced drug uptake, drug modification/inactivation, or pathway modification (112). Drug-resistance is a common problem in the treatment of breast cancers and finding new ways to overcome drug-resistance is a critical need in breast cancers (114-115).

EMT also plays an important role in breast cancer drug resistance. Approximately 45-50% of all breast cancers will become resistant (110). The mechanisms of EMT contributing to drug resistance are not well understood, however evidence has been reported that it does play a significant role. Expression of Twist, Snail, or Slug have been reported to contribute to drug resistance (116). Increased Twist expression has been reported to increase AKT-2 expression,

which leads to paclitaxel resistance in breast cancer cell lines (117). Slug and Snail have been shown to inhibit apoptosis, and lead to resistance to doxorubicin (118). The mesenchymal subtype of TNBCs described previously, which are enriched for cancer stemness, are highly resistant to chemotherapies and are associated with worse overall survival (29). In MCF-7 cells, aberrant EGFR signaling has been identified as contributing to tamoxifen resistance (119). EMT's contribution to drug-resistance is still not fully understood, however it does significantly contribute to drug-resistance (120).

References

1. Tavassoli F. A DP. *World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Breast and Female Genital Organs*. WHO: Geneva, 2003; 9–19.

2. Arpino, G., Bardou, V. J., Clark, G. M., & Elledge, R. M. (2004). Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. *Breast Cancer Research*, *6*(3), R149–R156. http://doi.org/10.1186/bcr767

3. Bombonati, A., & Sgroi, D. C. (2011). The molecular pathology of breast cancer progression. The Journal of Pathology, 223(2), 308-318. doi:10.1002/path.2808

4. Dimri, G., Band, H., & Band, V. (2005). Mammary epithelial cell transformation: insights from cell culture and mouse models. *Breast Cancer Research*, *7*(4), 171–179. http://doi.org/10.1186/bcr1275

5. Kumar, V., Abbas, A. K., Fausto, N., Robbins, S. L., & Cotran, R. S. (2005). *Robbins and Cotran pathologic basis of disease*. Philadelphia: Elsevier Saunders.

6. Progression from ductal carcinoma *in situ* to invasive breast cancer: Revisited Cowell, Catherine F. et al. Molecular Oncology , Volume 7 , Issue 5 , 859 - 869

7. Villanueva H, Grimm S, Dhamne S, Rajapakshe K, Visbal A, Davis CM, Ehli EA, Hartig SM, Coarfa C, Edwards DP. The Emerging Roles of Steroid Hormone Receptors in Ductal Carcinoma in Situ (DCIS) of the Breast. J Mammary Gland Biol Neoplasia. 2018 Dec;23(4):237-248. doi: 10.1007/s10911-018-9416-0. Epub 2018 Oct 18. Erratum in: J Mammary Gland Biol Neoplasia. 2018 Nov 21;: PMID: 30338425; PMCID: PMC6244884.

8. Corso G, Figueiredo J, La Vecchia C, Veronesi P, Pravettoni G, Macis D, Karam R, Lo Gullo R, Provenzano E, Toesca A, Mazzocco K, Carneiro F, Seruca R, Melo S, Schmitt F, Roviello F, De Scalzi AM, Intra M, Feroce I, De Camilli E, Villardita MG, Trentin C, De Lorenzi F, Bonanni B, Galimberti V. Hereditary lobular breast cancer with an emphasis on E-cadherin genetic defect. J Med Genet. 2018 Jul;55(7):431-441. doi: 10.1136/jmedgenet-2018-105337. Epub 2018 Jun 21. PMID: 29929997.

9. Peppercorn, J., Perou, C. M., & Carey, L. A. (2008). Molecular subtypes in breast cancer evaluation and management: Divide and conquer. Cancer Investigation, 26(1), 1-10. doi:10.1080/07357900701784238

10. Tang P FAU - Tse, Gary,M., & Tse, G. M. (0730). Immunohistochemical surrogates for molecular classification of breast carcinoma: A 2015 update

11. Masuda, S. (2012). Breast cancer pathology: The impact of molecular taxonomy on morphological taxonomy. Pathology International, 62(5), 295-302. doi:10.1111/j.1440-1827.2012.02790.x

12. Björnström, L., & Sjöberg, M. (2005). Mechanisms of estrogen receptor signaling: Convergence of genomic and nongenomic actions on target genes. Molecular Endocrinology, 19(4), 833-842. doi:10.1210/me.2004-0486

13. Lin, C.-Y., Ström, A., Vega, V. B., Li Kong, S., Li Yeo, A., Thomsen, J. S., … Liu, E. T. (2004). Discovery of estrogen receptor α target genes and response elements in breast tumor cells. *Genome Biology, 5*(9), R66. http://doi.org/10.1186/gb-2004-5-9-r66

14. Klinge CM. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. 2001 Jul 15;29(14):2905-19. doi: 10.1093/nar/29.14.2905. PMID: 11452016; PMCID: PMC55815.

15. Fuentes N, Silveyra P. Estrogen receptor signaling mechanisms. Adv Protein Chem Struct Biol. 2019;116:135-170. doi: 10.1016/bs.apcsb.2019.01.001. Epub 2019 Feb 4. PMID: 31036290; PMCID: PMC6533072.

16. Hilton HN, Clarke CL, Graham JD. Estrogen and progesterone signalling in the normal breast and its implications for cancer development. Mol Cell Endocrinol. 2018 May 5;466:2-14. doi: 10.1016/j.mce.2017.08.011. Epub 2017 Aug 26. PMID: 28851667.

17. Marquardt RM, Kim TH, Shin JH, Jeong JW. Progesterone and Estrogen Signaling in the Endometrium: What Goes Wrong in Endometriosis? Int J Mol Sci. 2019 Aug 5;20(15):3822. doi: 10.3390/ijms20153822. PMID: 31387263; PMCID: PMC6695957.

18. Fuentes N, Silveyra P. Estrogen receptor signaling mechanisms. Adv Protein Chem Struct Biol. 2019;116:135-170. doi:10.1016/bs.apcsb.2019.01.001

19. Tang S, Han H, Bajic VB. ERGDB: Estrogen Responsive Genes Database. Nucleic Acids Res. 2004;32(Database issue):D533-D536. doi:10.1093/nar/gkh083

20. Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Mortality - All COD, Aggregated With State, Total U.S. (1969-2019) <Katrina/Rita Population Adjustment>, National Cancer Institute, DCCPS, Surveillance Research Program, released April 2021. Underlying mortality data provided by NCHS (www.cdc.gov/nchs)

21. Cuzick J, Sestak I, Cawthorn S, et al. Tamoxifen for prevention of breast cancer: extended long-term follow-up of the IBIS-I breast cancer prevention trial. Lancet Oncol. 2015;16(1):67-75. doi:10.1016/S1470-2045(14)71171-4

22. Yeo, B., Turner, N. C., & Jones, A. (2014). An update on the medical management of breast cancer. Bmj, 348

23. Outcome of patients with early-stage breast cancer treated with doxorubicin-based adjuvant chemotherapy as a function of HER2 and TOP2A status. (PMID: 19620488) Tubbs R. … Hayes D.F.*(J. Clin. Oncol. 2009)*

24. Moasser, M. M. (2007). The oncogene HER2; Its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene*, *26*(45), 6469–6487. http://doi.org/10.1038/sj.onc.1210477

25. Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X. Targeting PI3K in cancer: mechanisms and advances in clinical trials. Mol Cancer. 2019;18(1):26. Published 2019 Feb 19. doi:10.1186/s12943-019-0954-x

26. Prior IA, Hood FE, Hartley JL. The Frequency of Ras Mutations in Cancer. Cancer Res. 2020;80(14):2969-2974. doi:10.1158/0008-5472.CAN-19-3682

27. Kunte S, Abraham J, Montero AJ. Novel HER2-targeted therapies for HER2-positive metastatic breast cancer. Cancer. 2020;126(19):4278-4288. doi:10.1002/cncr.33102

28. Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Populations - Total U.S. (1969-2020) <Katrina/Rita Adjustment> - Linked To County Attributes - Total U.S., 1969-2020 Counties, National Cancer Institute, DCCPS, Surveillance Research Program, released January 2022.

29. Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of Clinical Investigation*, *121*(7), 2750–2767. http://doi.org/10.1172/JCI45014

30. Bianchini, G., Balko, J. M., Mayer, I. A., Sanders, M. E., & Gianni, L. (2016). Triple-negative breast cancer: Challenges and opportunities of a heterogeneous disease. Nat Rev Clin Oncol, 13(11), 674-690.

31. Gasparini P, Fassan M, Cascione L, Guler G, Balci S, et al. (2014) Androgen Receptor Status Is a Prognostic Marker in Non-Basal Triple Negative Breast Cancers and Determines Novel Therapeutic Options. PLoS ONE 9(2): e88525. doi: 10.1371/journal.pone.0088525

32. André, F., & Zielinski, C. C. (2012). Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. Annals of Oncology, 23(suppl 6), vi46-vi51. doi:10.1093/annonc/mds195

33. Waks AG, Winer EP. Breast Cancer Treatment: A Review. JAMA. 2019;321(3):288-300. doi:10.1001/jama.2018.19323

34. Trayes KP, Cokenakes SEH. Breast Cancer Treatment. Am Fam Physician. 2021;104(2):171-178.

35. Gradishar WJ, Anderson BO, Abraham J, et al. Breast Cancer, Version 3.2020, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw. 2020;18(4):452-478. doi:10.6004/jnccn.2020.0016

36. Alexandre M, Maran-Gonzalez A, Viala M, et al. Decision of Adjuvant Systemic Treatment in HR+ HER2- Early Invasive Breast Cancer: Which Biomarkers Could Help?. Cancer Manag Res. 2019;11:10353-10373. Published 2019 Dec 11. doi:10.2147/CMAR.S221676

37. Cowell CF, Weigelt B, Sakr RA, et al. Progression from ductal carcinoma in situ to invasive breast cancer: revisited. Mol Oncol. 2013;7(5):859-869. doi:10.1016/j.molonc.2013.07.005

38. Jatoi I, Anderson WF, Jeong JH, Redmond CK. Breast cancer adjuvant therapy: time to consider its time-dependent effects [published correction appears in J Clin Oncol. 2011 Oct 10;29(29);3948]. J Clin Oncol. 2011;29(17):2301-2304. doi:10.1

39. Martins-Teixeira MB, Carvalho I. Antitumour Anthracyclines: Progress and Perspectives. ChemMedChem. 2020;15(11):933-948. doi:10.1002/cmdc.202000131200/JCO.2010.32.3550

40. Mosca L, Ilari A, Fazi F, Assaraf YG, Colotti G. Taxanes in cancer treatment: Activity, chemoresistance and its overcoming. Drug Resist Updat. 2021;54:100742. doi:10.1016/j.drup.2020.100742

41. Lambertini M, Moore HCF, Leonard RCF, et al. Gonadotropin-Releasing Hormone Agonists During Chemotherapy for Preservation of Ovarian Function and Fertility in Premenopausal Patients With Early Breast Cancer: A Systematic Review and Meta-Analysis of Individual Patient-Level Data. J Clin Oncol. 2018;36(19):1981-1990. doi:10.1200/JCO.2018.78.0858

42. Patel HK, Bihani T. Selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) in cancer treatment. Pharmacol Ther. 2018;186:1-24. doi:10.1016/j.pharmthera.2017.12.012

43. Kharb R, Haider K, Neha K, Yar MS. Aromatase inhibitors: Role in postmenopausal breast cancer. Arch Pharm (Weinheim). 2020;353(8):e2000081. doi:10.1002/ardp.202000081

44. Sood A, Lang DK, Kaur R, Saini B, Arora S. Relevance of Aromatase Inhibitors in Breast Cancer Treatment. Curr Top Med Chem. 2021;21(15):1319-1336. doi:10.2174/1568026621666210701143445

45. Molehin D, Rasha F, Rahman RL, Pruitt K. Regulation of aromatase in cancer. Mol Cell Biochem. 2021;476(6):2449-2464. doi:10.1007/s11010-021-04099-0

46. Thorat MA, Balasubramanian R. Breast cancer prevention in high-risk women. Best Pract Res Clin Obstet Gynaecol. 2020;65:18-31. doi:10.1016/j.bpobgyn.2019.11.006\

47. Rugo HS, Lerebours F, Ciruelos E, et al. Alpelisib plus fulvestrant in PIK3CA-mutated, hormone receptor-positive advanced breast cancer after a CDK4/6 inhibitor (BYLieve): one cohort of a phase 2, multicentre, open-label, non-comparative study [published correction appears in Lancet Oncol. 2021 May;22(5):e184]. Lancet Oncol. 2021;22(4):489-498. doi:10.1016/S1470-2045(21)00034-6

48. Piezzo M, Cocco S, Caputo R, et al. Targeting Cell Cycle in Breast Cancer: CDK4/6 Inhibitors. Int J Mol Sci. 2020;21(18):6479. Published 2020 Sep 4. doi:10.3390/ijms21186479

49. Cristofanilli M, Rugo HS, Im SA, et al. Overall Survival with Palbociclib and Fulvestrant in Women with HR+/HER2- ABC: Updated Exploratory Analyses of PALOMA-3, a Double-blind, Phase III Randomized Study. Clin Cancer Res. 2022;28(16):3433-3442. doi:10.1158/1078-0432.CCR-22-0305

50. Cesca MG, Vian L, Cristóvão-Ferreira S, Pondé N, de Azambuja E. HER2-positive advanced breast cancer treatment in 2020. Cancer Treat Rev. 2020;88:102033. doi:10.1016/j.ctrv.2020.102033

51. Xuhong JC, Qi XW, Zhang Y, Jiang J. Mechanism, safety and efficacy of three tyrosine kinase inhibitors lapatinib, neratinib and pyrotinib in HER2-positive breast cancer. Am J Cancer Res. 2019;9(10):2103-2119. Published 2019 Oct 1.

52. Kanjanapan Y, Lok SW, Gibbs P, et al. Impact of prior (neo)adjuvant trastuzumab (NAT) exposure on the efficacy of HER2-targeted therapy for metastatic breast cancer. Breast Cancer Res Treat. 2020;184(1):87-95. doi:10.1007/s10549-020-05825-w

53. Shi ZQ, Qiu PF, Liu YB, et al. Neo-adjuvant chemotherapy and axillary de-escalation management for patients with clinically node-negative breast cancer. Breast J. 2019;25(6):1154-1159. doi:10.1111/tbj.13422

54. Bergin ART, Loi S. Triple-negative breast cancer: recent treatment advances. F1000Res. 2019;8:F1000 Faculty Rev-1342. Published 2019 Aug 2. doi:10.12688/f1000research.18888.1

55. Ghosh S. Cisplatin: The first metal based anticancer drug. Bioorg Chem. 2019;88:102925. doi:10.1016/j.bioorg.2019.102925

56. Kim MY. Breast Cancer Metastasis. Adv Exp Med Biol. 2021;1187:183-204. doi:10.1007/978-981-32-9620-6_9

57. Al-Mahmood S, Sapiezynski J, Garbuzenko OB, Minko T. Metastatic and triple-negative breast cancer: challenges and treatment options. Drug Deliv Transl Res. 2018;8(5):1483-1507. doi:10.1007/s13346-018-0551-3

58. Lüönd F, Sugiyama N, Bill R, et al. Distinct contributions of partial and full EMT to breast cancer malignancy. Dev Cell. 2021;56(23):3203-3221.e11. doi:10.1016/j.devcel.2021.11.006

59. Saitoh M. Involvement of partial EMT in cancer progression. J Biochem. 2018;164(4):257-264. doi:10.1093/jb/mvy047

60. Matysiak M, Kapka-Skrzypczak L, Jodłowska-Jędrych B, Kruszewski M. EMT promoting transcription factors as prognostic markers in human breast cancer. Arch Gynecol Obstet. 2017;295(4):817-825. doi:10.1007/s00404-017-4304-1

61. Palamaris K, Felekouras E, Sakellariou S. Epithelial to Mesenchymal Transition: Key Regulator of Pancreatic Ductal Adenocarcinoma Progression and Chemoresistance. Cancers (Basel). 2021;13(21):5532. Published 2021 Nov 4. doi:10.3390/cancers13215532

62. Xu W, Yang Z, Lu N. A new role for the PI3K/Akt signaling pathway in the epithelial-mesenchymal transition. Cell Adh Migr. 2015;9(4):317-324. doi:10.1080/19336918.2015.1016686

63. Okada T, Sinha S, Esposito I, et al. The Rho GTPase Rnd1 suppresses mammary tumorigenesis and EMT by restraining Ras-MAPK signalling [published correction appears in Nat Cell Biol. 2019 Apr;21(4):534]. Nat Cell Biol. 2015;17(1):81-94. doi:10.1038/ncb3082

64. Yang S, Liu Y, Li MY, et al. FOXP3 promotes tumor growth and metastasis by activating Wnt/β-catenin signaling pathway and EMT in non-small cell lung cancer. Mol Cancer. 2017;16(1):124. Published 2017 Jul 17. doi:10.1186/s12943-017-0700-1

65. Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Sci Signal. 2014;7(344):re8. Published 2014 Sep 23. doi:10.1126/scisignal.2005189

66. David CJ, Huang YH, Chen M, et al. TGF-β Tumor Suppression through a Lethal EMT. Cell. 2016;164(5):1015-1030. doi:10.1016/j.cell.2016.01.009

67. Tzavlaki K, Moustakas A. TGF-β Signaling. Biomolecules. 2020;10(3):487. Published 2020 Mar 23. doi:10.3390/biom10030487

68. Luo K. Signaling Cross Talk between TGF-β/Smad and Other Signaling Pathways. Cold Spring Harb Perspect Biol. 2017;9(1):a022137. Published 2017 Jan 3. doi:10.1101/cshperspect.a022137

69. Buyuk B, Jin S, Ye K. Epithelial-to-Mesenchymal Transition Signaling Pathways Responsible for Breast Cancer Metastasis. Cell Mol Bioeng. 2021;15(1):1-13. Published 2021 Sep 2. doi:10.1007/s12195-021-00694-9

70. Butti R, Das S, Gunasekaran VP, Yadav AS, Kumar D, Kundu GC. Receptor tyrosine kinases (RTKs) in breast cancer: signaling, therapeutic implications and challenges. Mol Cancer. 2018;17(1):34. Published 2018 Feb 19. doi:10.1186/s12943-018-0797-x

71. Sigismund S, Avanzato D, Lanzetti L. Emerging functions of the EGFR in cancer. Mol Oncol. 2018;12(1):3-20. doi:10.1002/1878-0261.12155

72. Voon DC, Wang H, Koo JK, et al. EMT-induced stemness and tumorigenicity are fueled by the EGFR/Ras pathway. PLoS One. 2013;8(8):e70427. Published 2013 Aug 12. doi:10.1371/journal.pone.0070427

73. Sirkisoon SR, Carpenter RL, Rimkus T, Miller L, Metheny-Barlow L, Lo HW. EGFR and HER2 signaling in breast cancer brain metastasis. Front Biosci (Elite Ed). 2016;8(2):245-263. Published 2016 Jan 1. doi:10.2741/E765

74. Xie Y, Shi X, Sheng K, et al. PI3K/Akt signaling transduction pathway, erythropoiesis and glycolysis in hypoxia (Review). Mol Med Rep. 2019;19(2):783-791. doi:10.3892/mmr.2018.9713

75. Hong J, Zhou J, Fu J, et al. Phosphorylation of serine 68 of Twist1 by MAPKs stabilizes Twist1 protein and promotes breast cancer cell invasiveness. Cancer Res. 2011;71(11):3980-3990. doi:10.1158/0008-5472.CAN-10-2914

76. Zielińska KA, Katanaev VL. The Signaling Duo CXCL12 and CXCR4: Chemokine Fuel for Breast Cancer Tumorigenesis. Cancers (Basel). 2020;12(10):3071. Published 2020 Oct 21. doi:10.3390/cancers12103071

77. Chen IX, Chauhan VP, Posada J, et al. Blocking CXCR4 alleviates desmoplasia, increases T-lymphocyte infiltration, and improves immunotherapy in metastatic breast cancer. Proc Natl Acad Sci U S A. 2019;116(10):4558-4566. doi:10.1073/pnas.1815515116

78. Pozzobon T, Goldoni G, Viola A, Molon B. CXCR4 signaling in health and disease. Immunol Lett. 2016;177:6-15. doi:10.1016/j.imlet.2016.06.006

79. Xu C, Zhao H, Chen H, Yao Q. CXCR4 in breast cancer: oncogenic role and therapeutic targeting. Drug Des Devel Ther. 2015;9:4953-4964. Published 2015 Aug 28. doi:10.2147/DDDT.S84932

80. Duchartre Y, Kim YM, Kahn M. The Wnt signaling pathway in cancer. Crit Rev Oncol Hematol. 2016;99:141-149. doi:10.1016/j.critrevonc.2015.12.005

81. Nusse R, Clevers H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. Cell. 2017;169(6):985-999. doi:10.1016/j.cell.2017.05.016

82. Yeeravalli R, Das A. Molecular mediators of breast cancer metastasis. Hematol Oncol Stem Cell Ther. 2021;14(4):275-289. doi:10.1016/j.hemonc.2021.02.002

83. Polireddy, K., Chen, Q. (2016). Cancer of the Pancreas: Molecular Pathways and Current Advancement in Treatment. Journal of Cancer, 7(11), 1497-1514. https://doi.org/10.7150/jca.14922.

84. Schulte J, Weidig M, Balzer P, et al. Expression of the E-cadherin repressors Snail, Slug and Zeb1 in urothelial carcinoma of the urinary bladder: relation to stromal fibroblast activation and invasive behaviour of carcinoma cells. Histochem Cell Biol. 2012;138(6):847-860. doi:10.1007/s00418-012-0998-0

85. Quaglino E, Conti L, Cavallo F. Breast cancer stem cell antigens as targets for immunotherapy. Semin Immunol. 2020;47:101386. doi:10.1016/j.smim.2020.101386

86. Butti R, Gunasekaran VP, Kumar TVS, Banerjee P, Kundu GC. Breast cancer stem cells: Biology and therapeutic implications. Int J Biochem Cell Biol. 2019;107:38-52. doi:10.1016/j.biocel.2018.12.001

87. Mendonsa AM, Na TY, Gumbiner BM. E-cadherin in contact inhibition and cancer. Oncogene. 2018;37(35):4769-4780. doi:10.1038/s41388-018-0304-2

88. Padmanaban V, Krol I, Suhail Y, et al. E-cadherin is required for metastasis in multiple models of breast cancer. Nature. 2019;573(7774):439-444. doi:10.1038/s41586-019-1526-3

89. Corso G, Figueiredo J, De Angelis SP, et al. E-cadherin deregulation in breast cancer. J Cell Mol Med. 2020;24(11):5930-5936. doi:10.1111/jcmm.15140

90. Cao ZQ, Wang Z, Leng P. Aberrant N-cadherin expression in cancer. Biomed Pharmacother. 2019;118:109320. doi:10.1016/j.biopha.2019.109320

91. Insua-Rodríguez J, Oskarsson T. The extracellular matrix in breast cancer. Adv Drug Deliv Rev. 2016;97:41-55. doi:10.1016/j.addr.2015.12.017

92. Zhu J, Xiong G, Trinkle C, Xu R. Integrated extracellular matrix signaling in mammary gland development and breast cancer progression. Histol Histopathol. 2014;29(9):1083-1092. doi:10.14670/HH-29.1083

93. Kapoor C, Vaidya S, Wadhwan V; Hitesh, Kaur G, Pathak A. Seesaw of matrix metalloproteinases (MMPs). J Cancer Res Ther. 2016;12(1):28-35. doi:10.4103/0973-1482.157337

94. Dong H, Diao H, Zhao Y, et al. Overexpression of matrix metalloproteinase-9 in breast cancer cell lines remarkably increases the cell malignancy largely via activation of transforming growth factor beta/SMAD signalling. Cell Prolif. 2019;52(5):e12633. doi:10.1111/cpr.12633

95. Radisky ES, Radisky DC. Matrix metalloproteinases as breast cancer drivers and therapeutic targets. Front Biosci (Landmark Ed). 2015;20(7):1144-1163. Published 2015 Jun 1. doi:10.2741/4364

96. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition [published correction appears in Science. 2019 Jan 25;363(6425):]. Science. 2013;339(6119):580-584. doi:10.1126/science.1228522

97. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta. 2013;1833(12):3481-3498. doi:10.1016/j.bbamcr.2013.06.026

98. Liu X, Chipurupalli S, Jiang P, et al. ErbB2/Her2-dependent downregulation of a cell death-promoting protein BLNK in breast cancer cells is required for 3D breast tumor growth. Cell Death Dis. 2022;13(8):687. Published 2022 Aug 6. doi:10.1038/s41419-022-05117-9

99. Jiang Y, Zhan H. Communication between EMT and PD-L1 signaling: New insights into tumor immune evasion. Cancer Lett. 2020;468:72-81. doi:10.1016/j.canlet.2019.10.013

100. Tsuchiya H, Shiota G. Immune evasion by cancer stem cells. Regen Ther. 2021;17:20-33. Published 2021 Mar 11. doi:10.1016/j.reth.2021.02.006

101. Arasanz H, Gato-Cañas M, Zuazo M, et al. PD1 signal transduction pathways in T cells. Oncotarget. 2017;8(31):51936-51945. Published 2017 Apr 19. doi:10.18632/oncotarget.17232

102. Ghosh C, Luong G, Sun Y. A snapshot of the PD-1/PD-L1 pathway. J Cancer. 2021;12(9):2735-2746. Published 2021 Mar 5. doi:10.7150/jca.57334

103. Yang Z, Qi Y, Lai N, et al. Notch1 signaling in melanoma cells promoted tumor-induced immunosuppression via upregulation of TGF-β1. J Exp Clin Cancer Res. 2018;37(1):1. Published 2018 Jan 4. doi:10.1186/s13046-017-0664-4

104. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. Am J Cancer Res. 2020;10(3):727-742. Published 2020 Mar 1.

105. Wu Y, Chen W, Xu ZP, Gu W. PD-L1 Distribution and Perspective for Cancer Immunotherapy-Blockade, Knockdown, or Inhibition. Front Immunol. 2019;10:2022. Published 2019 Aug 27. doi:10.3389/fimmu.2019.02022

106. Sun C, Mezzadra R, Schumacher TN. Regulation and Function of the PD-L1 Checkpoint. Immunity. 2018;48(3):434-452. doi:10.1016/j.immuni.2018.03.014

107. Mittendorf EA, Philips AV, Meric-Bernstam F, et al. PD-L1 expression in triple-negative breast cancer. Cancer Immunol Res. 2014;2(4):361-370. doi:10.1158/2326-6066.CIR-13-0127

108. Garcia-Diaz A, Shin DS, Moreno BH, et al. Interferon Receptor Signaling Pathways Regulating PD-L1 and PD-L2 Expression [published correction appears in Cell Rep. 2019 Dec 10;29(11):3766]. Cell Rep. 2017;19(6):1189-1201. doi:10.1016/j.celrep.2017.04.031

109. Kwapisz D. Pembrolizumab and atezolizumab in triple-negative breast cancer. Cancer Immunol Immunother. 2021;70(3):607-617. doi:10.1007/s00262-020-02736-z

110. Fahad Ullah M. Breast Cancer: Current Perspectives on the Disease Status. Adv Exp Med Biol. 2019;1152:51-64. doi:10.1007/978-3-030-20301-6_4

111. Haider T, Pandey V, Banjare N, Gupta PN, Soni V. Drug resistance in cancer: mechanisms and tackling strategies. Pharmacol Rep. 2020;72(5):1125-1151. doi:10.1007/s43440-020-00138-7

112. Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med. 2002;53:615-627. doi:10.1146/annurev.med.53.082901.103929

113. Nussinov R, Tsai CJ, Jang H. Anticancer drug resistance: An update and perspective. Drug Resist Updat. 2021;59:100796. doi:10.1016/j.drup.2021.100796

114. Nedeljković M, Damjanović A. Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer-How We Can Rise to the Challenge. Cells. 2019;8(9):957. Published 2019 Aug 22. doi:10.3390/cells8090957

115. Bai X, Ni J, Beretov J, Graham P, Li Y. Cancer stem cell in breast cancer therapeutic resistance. Cancer Treat Rev. 2018;69:152-163. doi:10.1016/j.ctrv.2018.07.004

116. Hashemi M, Arani HZ, Orouei S, et al. EMT mechanism in breast cancer metastasis and drug resistance: Revisiting molecular interactions and biological functions. Biomed Pharmacother. 2022;155:113774. doi:10.1016/j.biopha.2022.113774

117. Ning Y, Zhang W, Hanna DL, et al. Clinical relevance of EMT and stem-like gene expression in circulating tumor cells of metastatic colorectal cancer patients. Pharmacogenomics J. 2018;18(1):29-34. doi:10.1038/tpj.2016.62

118. Huang J, Li H, Ren G. Epithelial-mesenchymal transition and drug resistance in breast cancer (Review). Int J Oncol. 2015;47(3):840-848. doi:10.3892/ijo.2015.3084

119. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. Cancer Res. 2007;67(3):1352-1360. doi:10.1158/0008-5472.CAN-06-1020

120. Du B, Shim JS. Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. Molecules. 2016;21(7):965. Published 2016 Jul 22. doi:10.3390/molecules21070965

Chapter 2: PIM Kinases

PIM Kinase family members

PIM kinases are a family of serine/threonine kinases consisting of 3 family members, PIM1, PIM2, and PIM3. (1) PIM1 is located on chromosome 17 and has 2 isoforms, a 33 kDa isoform and a 44 kDa isoform due to an alternative transcriptional start site (2). Both isoforms contain the same kinase domain and are considered to functionally bind the same targets. The short isoform exists primarily in the cytoplasm and in the nucleus, the long isoform of PIM1 however contains an additional PXXP domain at the N-terminal end which binds to the SRC homology 3 (SH3) domain of BMX tyrosine kinase, causing it to localize to the plasma membrane (3). At the membrane, the long isoform interacts with membrane associated proteins more frequently than the short isoform. PIM2 is located on the X chromosome and has 3 isoforms, 34 kDa, 37 kDa, and 40 kDa isoforms due to multiple alternative transcriptional start sites (3). PIM3, located on chromosome 15, consists of a single 34 kDa isoform. All three PIM family members have a highly conserved kinase domain. PIM1 and PIM3 have a 71% homology in amino acid sequence, and PIM1 and PIM2 have a 61% homology (4).

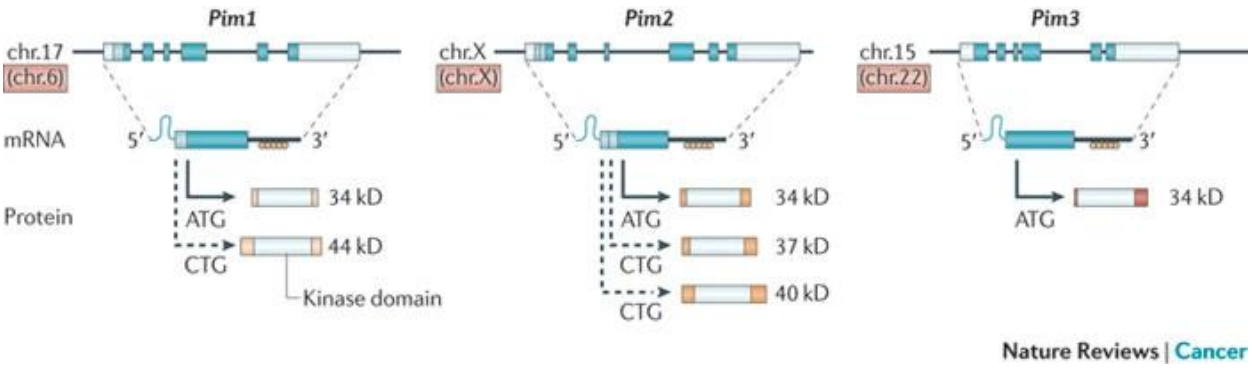

Figure 1. Schematic diagram of PIM kinases gene structure, mRNA, and protein isoforms (1).

PIM kinases are expressed ubiquitously throughout the body; however, their expression varies in different tissues. PIM1 is highly expressed in hematopoietic cells, gastric, and prostate cells, PIM2 is highly expressed in the brain and lymphoid tissues, and PIM3 is primarily expressed in the breast, kidney, and brain (5-9). Interestingly, PIM kinases are different from other kinases because they lack regulatory domains and therefore, their catalytic activity is often correlated with expression (10).

Regulation of PIM Kinases

PIM kinases are primarily regulated at the transcriptional level. Several different tyrosine kinase receptors have been identified that regulate the expression of the PIM genes. These include Epidermal growth factor receptor (EGFR) and FMS-like tyrosine kinase 3 (FLT3) (11-12). PIM kinases are downstream of the JAK/STAT pathway (13). The JAK family of tyrosine kinases is made up of JAK1, JAK2, JAK3, and TYK2 (14). Activation of JAKs following binding of

cytokines to their cognate receptors results in phosphorylation/activation of signal transducers and activator of transcription (STATs) (15). STATs can also be activated by EGFR and SRC kinase (16). Specifically, STAT3 and STAT5 have been found to bind to the PIM1 promoter, increasing its transcription (17-18). Several different cytokines and cellular conditions have been found to regulate the transcription and translation of PIM kinases, including hypoxia, ROS, and DNA damage (19-21).

PIM kinases are regulated at both the transcriptional and translational levels. PIM kinases are also regulated by its mRNA stability. PIM mRNA transcripts contain several unstable AUUU(A) sequences in their 3'UTR, which lead to them being unstable and having a relatively short half-life, allowing PIM kinases to be regulated by transcription effectively (22). These transcripts also require cap-dependent translation to be made into a protein, adding an additional level of regulation (23).

PIM kinases are also regulated at the protein level. Similar to the transcript, PIM kinases also have a short protein half-life, of only around 5 minutes (24). The proteins are rapidly targeted by ubiquitin E3 ligases, and rapidly degraded by ubiquitin meditated mechanisms (25). HSP90 and HSP70 have also been suggested to regulate PIM kinase protein stability (26-27). If a PIM protein is not ubiquitinated, it binds to HSP90, resulting in increased protein stability, and a longer protein half-life (26). If PIM proteins are however ubiquitinated, they bind to HSP70, suggesting that HSP70 plays a role in PIM protein degradation (27).

Instead of being regulated by post-translational modifications, they are principally regulated at the transcriptional and translational level. A post-translational modification that affects the stability of PIM1 has however been identified. BMX phosphorylates PIM1 at tyrosine 218, which increases PIM1 activity and stability, however this phosphorylation is not required for PIM1 to be active, and thus is not considered the primary method of regulation (28).

In summary, PIM kinases are regulated by several different regulatory pathways, at the transcriptional, translational, and protein levels. These different pathways are summarized below (Fig 2).

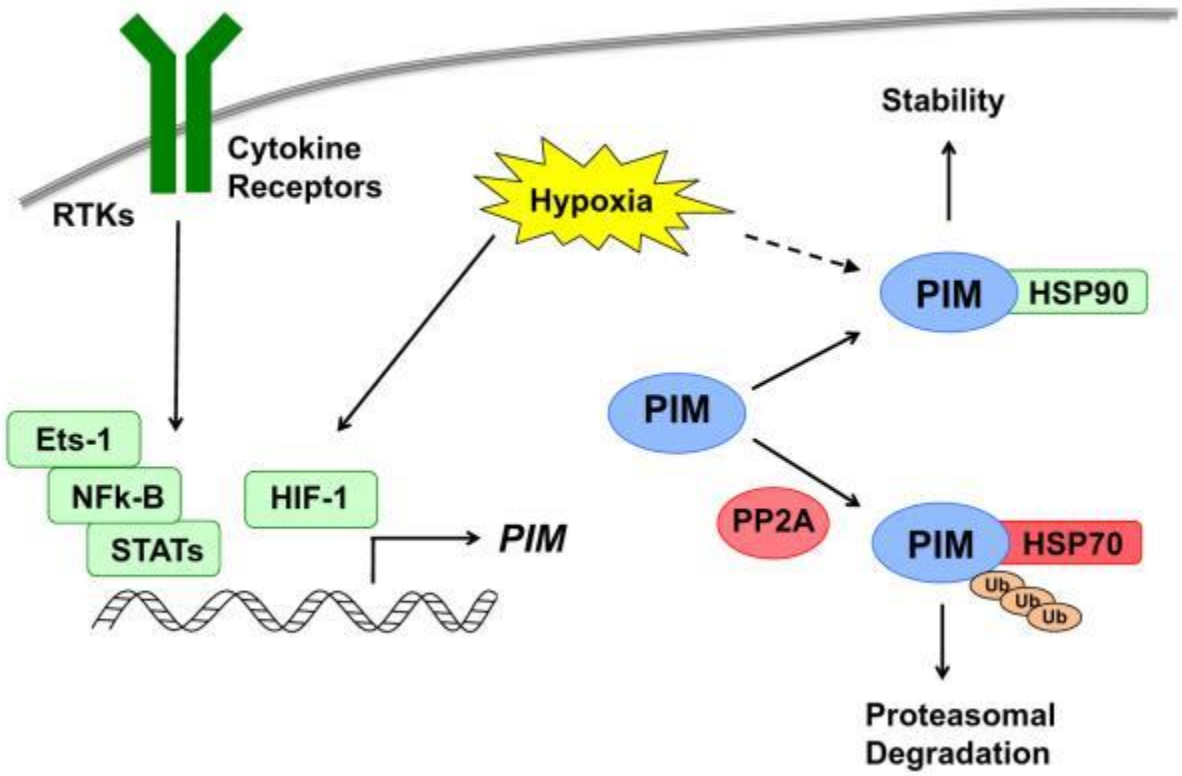

Figure 2: Regulation of PIM kinase expression. PIM transcription is controlled by RTK, which after ligand binding activate several different signaling pathways including JAK/STAT, NF-kB, and Ets-1. Hypoxia has also been shown to induce PIM expression through HIF-1 signaling. At the protein level, PIM kinases are regulated by ubiquitination. HSP70 binds to ubiquitinated PIM, leading to rapid degradation by the proteosome. Conversely, HSP90 binding stabilizes PIM kinases (29).

PIM Kinases Promote Cell Growth and Division

PIM kinases play critical roles in promoting cell cycle, inhibiting apoptosis, promoting cap-dependent translation and cell growth. As a kinase, it does this by phosphorylating specific proteins at either a serine or threonine residue. A PIM recognition sequence has been identified as: K/R-X-X-X-**S/T**-X (30). This is very similar to the motif that is recognized by Akt, R-X-R-X-X-S/T, and as a result there is an overlap between PIM and Akt signaling pathways (31).

One pathway that PIM overlaps with Akt to regulate is mTORC1 signaling to control cell growth and division. Mammalian target of rapamycin (mTOR) is serine threonine kinase that functions in two complexes mTORC1 and mTORC2(32). PIM kinases have been found to directly interact with mTORC1 as well as additional targets of it up and downstream (29). mTORC1 is complex of comprised of mTOR, Raptor, PRAS40, and mLST8, that regulates translation, growth, proliferation, and metabolism (32). Both PIM and Akt kinases directly phosphorylate PRAS40 at Thr246, a negative regulator of mTOR signaling, leading to its dissociation from the complex, increasing mTOR signaling (33-35).

Previous studies have provided evidence that PIM kinases regulate phosphorylation of 4EBP1 and p70S6K via both direct and indirect mechanisms (36-37). mTORC1 phosphorylates 4EBP1, which causes it to dissociate from eIF4E, resulting in increased dissociation cap dependent translation (32). PIM kinases have also been found to directly phosphorylate 4EBP1 at Thr37, resulting in further activation of cap-dependent translation (38). p70s6K has also been shown to be affected by PIM kinase inhibition. p70s6K is activated by mTORC1, which results in its phosphorylation of the S6 ribosomal protein, causing increased protein synthesis and cell growth. PIM kinases also act upstream of mTORC1 (36). Specifically, it has been found that PIM2 phosphorylates TSC2 at SER1798(39). TSC2 functions to inhibit mTORC1, but when in order

to do so it must first form a complex with TSC1 (40). Phosphorylation of TSC2 prevents this association, resulting in activation of mTORC1 signaling (40). TSC2 is also targeted by Akt at different residues, indicating an overlapping effect of Akt and PIM on mTORC1 upstream signaling (40). These pathways make up the major signaling cascades of PIM kinases that promote cell growth.

PIM kinases effect on the cell cycle

PIM kinases also play a vital role in regulating the cell cycle. The cell cycle is a very tightly controlled balance of signaling that can lead to cell proliferation, senescence or apoptosis. Cells progress through the different stages of the cell cycle due to signals passed between cyclin and cyclin-dependent kinase (CDK) signals (41). These signals direct the cell through specific events ending in mitosis, resulting in 2 daughter cells (41). The stages of the cell cycle are G1, S, G2, and M phases (41). In the G1 phase, the cell prepares to synthesize new DNA (41). This is followed by S-phase where the cell synthesizes a copy of its DNA, resulting in a 2N DNA content (41). After S-phase. The cell goes through another gap phase in G2, prior to progressing to M phase or mitosis (41). Cells that are not undergoing the cell cycle are in the G0 phase (41).

The cell cycle has several levels of regulation, two such negative cell cycle regulators are p21 and p27 (44-45). Before a cell divide there are several checkpoints that must be passed. One such checkpoint is DNA damage. DNA damage activates p53, which activates expression of p21 (45). Previous studies have also indicated that DNA damage has also been shown to induce expression of PIM kinases (*). p27 on the other hand, has relatively stable mRNA expression and is primarily regulated at the protein level (45). There are several different phosphorylation sites on p27 that can affect its protein expression. These include Thr187 which can be phosphorylated by CyclinE-CDK2 complexes, which leads to ubiquitin mediated proteosomal

degradation (47). Both p21 and p27 are potent CDK inhibitors, which prevent cells from progressing through the cell cycle (45).

Previous studies have demonstrated that PIM kinases directly phosphorylate the negative cell cycle regulators p21 and p27 (48-50). Under normal cellular conditions, p21 and p27 localize to the nucleus, where they function to prevent cyclin dependent kinase from phosphorylating their targets (45). Phosphorylation of p21 and p27 causes them to be sequestered by the cytoplasm where they then bind to 14-3-3 protein and are targeted for ubiquitin mediated degradation (45,47). With high PIM expression, can drive down the protein expression of both p21 and p27, resulting in the cell entering the cell cycle, even if all the normal checkpoints of the cell cycle have not been met (47-49).

PIM Kinases Inhibit Apoptosis

PIM kinases also play an important role in regulating apoptosis. Apoptosis is defined as a controlled cell death that occurs as a normal part of an organism's growth or development (51). Apoptosis results in cell shrinkage, nuclear fragmentation, chromatin condensation, and protease activation (51). Apoptosis is a tightly regulated process controlled by multiple signaling pathways (52).

One pathway involved in regulating apoptosis is BAD signaling. BAD is a pro-apoptotic protein that normally binds to anti-apoptotic proteins BCL-2/XL (53). This binding prevents BCL-2 and BCL-XL proteins from inhibiting BAX and BAK dimerization, resulting in mitochondrial outer membrane permeabilization (53). BAD can be phosphorylated by Akt at Ser112 to inhibit apoptosis (54-55). Phosphorylation of BAD prevents it from binding to BCL-2 family proteins,

and results in BAD being sequestered by in they cytoplasm where it is then bound to 14-3-3 protein and degraded (59).

It has also been reported that PIM kinases are able to phosphorylate BAD inhibiting apoptosis (53-55). Previous reports have shown that hypoxia induced PIM expression resulted in reduced mitochondrial permeabilization and decreased activation of caspase 9 (55). Under normal cellular conditions, PIM kinases are able to prevent apoptosis after a cellular stress, this likely evolved because it is energetically more efficient to fix a damaged cell rather than undergo apoptosis and make a new cell to replace the damaged cell.

PIM kinases in cancer

PIM kinases promote cell growth, lead progression through the cell cycle, inhibit apoptosis, and promote EMT/metastasis, which have made them of particular interest in cancer. PIM kinases have been found to be overexpressed in a variety of different cancers (56-63). Increased expression of PIM1 has been found in several different cancers including prostate cancer, and both lymphoid and myeloid leukemia and lymphomas (64-66). PIM1 expression is also correlated with tumor aggressiveness and resistance to chemotherapy in these settings (67-72). PIM1 and PIM3 have also been implicated in lymphoproliferative diseases associated with Epstein-Barr virus or Kaposi sarcoma associated herpes virus (71-72).

PIM2 is also overexpressed in a wide variety of leukemias and solid tumors. PIM2 expression has been found to be increased in acute lymphoblastic leukemia and chronic lymphocytic leukemias (73-74). In these settings, PIM2 plays an important role in neoplastic cell migration (73-74). PIM2 has also been found to be increased in acute myeloid leukemia (AML), where PIM2 phosphorylation of 4E-BP1 has been shown to promote cancer progression and

tumorigenesis (75-77). Multiple myeloma cells also overexpress PIM2 (78). In multiple myeloma, PIM2 expression is increased in both the MM cells and the surrounding stromal cells (78). PIM2 in MM promotes tumor progression and represses the DNA-damage response pathway by phosphorylation of ATR (78). It has also been reported in MM cells that PIM2 mediated phosphorylation of TSC2 at Ser1798, resulting in mTORC1 activation (79). Lastly, PIM2 has also been found to be overexpressed in liver and ovarian solid tumors (80-81). In these settings progression through the cell cycle and inhibition of apoptosis has been shown to play an important role in cancer progression.

PIM3 has also been found to be overexpressed in cancers, but to a lesser extent than PIM1 or PIM2. PIM3 expression is increased in ovarian cancer and significantly correlated with worse overall survival (81). PIM3 is also highly expressed in hepato-cellular carcinomas when compared to normal hepatocytes (84). Lastly, PIM3 expression has been found to be amplified in pancreatic and colon cancers when compared to normal cells (83, 85-86).

PIM Kinases Promote EMT and Metastasis

PIM kinases have been found to play a significant role in the metastasis of multiple different cancers. The mechanisms by which PIM kinases promote metastasis is still being worked on, however there are multiple different reports stating that PIM kinases promote EMT and metastasis (87-89). It has been reported that PIM1 phosphorylates CXCR4 at Ser339, which stabilizes the protein in the membrane, leading to increased CXCR4 activity (90-92). This was sufficient to increase prostate cancer cells metastasis in PC3 cell xenografts (93). Additionally, in prostate cancer cells when stroma cells were examined it was found that they had higher PIM1

expression in patients with metastatic cancer compared to non-metastatic prostate cancers (94). PIM kinases are also important in collagen deposition, which is important for metastasis of multiple different cancers, including breast and prostate cancers (95-96).

It has been suggested that the primary way PIM kinases promote metastasis is by promoting EMT. As mentioned previously, PIM kinases phosphorylate EiF4B at Ser-406 (97-98). One of the downstream effects of this is upregulation of c-MET (97). C-met is an important inducer of cancer metastasis and promotes EMT (97). Additionally, it has been reported that PIM kinases phosphorylate Notch, leading to increased Notch signaling (99). As outlined in chapter 1, Notch signaling is an important mediator of EMT, and activating Notch signaling will promote both EMT and metastasis.

PIM Kinases Promote Drug Resistance

Drug-resistance is a common problem in treating cancers. PIM kinases have been suggested to contribute to drug resistance. The mechanisms involving PIM kinases in drug-resistance include phosphorylation of P-glycoprotein (PgP), breast cancer resistant protein (BCRP), BMX, and fms-like tyrosine kinase (FLT3) (100-103). PIM kinases have also been reported to inhibit p53 mediated apoptosis (104). Additionally, it has also been reported that PIM kinases phosphorylate eIF4B which can also contribute to drug resistance (105). Primarily, PIM1 has been identified as inducing drug-resistance, however there are also reports that PIM2 may also contribute to drug resistance (106-108).

Both BCRP and Pgp are efflux channels that leads to multiple drug resistance from the drugs being pumped out of the cell (100-104). The long isoform of PIM1 (44 kDa) has been found to

directly phosphorylate both BCRP Thr362 at and Pgp at Ser683 (101). The long isoform localizes to the membrane, where it encounters these proteins (101). BCRP phosphorylation promotes its multimerization and translocation to the membrane (100). Phosphorylation of Pgp on the other hand prevents the 150 kDa isoform from proteasomal degradation, leading to increased surface expression (101).

PIM kinases have also been found to found to phosphorylate BMX kinase which can also lead to drug resistance (102). Specifically, in prostate cancer cells, the long isoform of PIM1 competes with p53 to bind BMX (102). P53 normally inhibits BMX kinase, however when it is phosphorylated, this inhibition is no longer present, leading to BMX promoting cell survival ultimately leading to drug resistance (102).

PIM1 also phosphorylates FLT3 (103). FLT3 is a receptor tyrosine kinase, which in cancer, is commonly mutated by internal tandem duplication (ITD) (103). FLT3-ITD containing cancers are resistant to FLT3 inhibition (103). FLT3-ITD mutations can often lead to over activation of STAT5, which in turn increases PIM1 expression (103). PIM1 additionally phosphorylates FLT3-ITD at Ser935, which leads to increased FLT3-ITD stability (103). This increased stability promotes aberrant FLT3-ITD signaling, leading to further resistance to resistance to kinase inhibitor drugs (103).

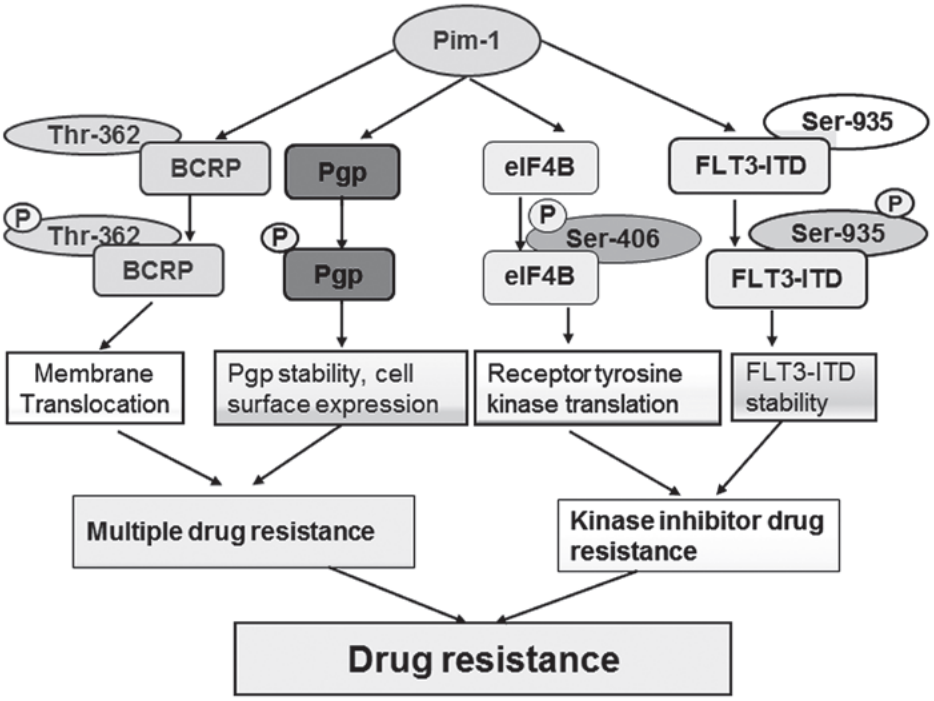

Figure 3: Outline of PIM1 signaling contributing to drug-resistance. PIM1 phosphorylates BCRP and Pgp, stabilizing drug efflux and leading to multiple drug resistance. PIM1 also phosphorylates eIF4B and FLT3-ITD, both of which contribute to kinase inhibitor drug-resistance (109).

PIM Kinase Inhibitors

Small molecule inhibitors targeting PIM kinases have been developed. SGI-1776 is a first-generation pan-PIM kinase inhibitor that showed promise in pre-clinical studies; however, it failed in clinical trials due to dose-limiting cardiotoxicity (110). SGI-1776 can also inhibit the potassium channel hERG, leading to cardiac QT prolongation and cardio toxicity (111). AZD-1208 is another pan-PIM kinase inhibitor that showed promise in preclinical testing for treatment of multiple cancers, however it failed in clinical trials for AML and advanced solid tumors due to lack of efficacy and high frequency of adverse events (112). TP-3654 is a second-generation pan PIM kinase inhibitor, which has greater specificity towards PIM1 than PIM2 and PIM3 (113). TP-3654 has shown efficacy in preclinical testing in multiple cancer types (114-117). Clinical trials for TP3654 are currently being carried out in myeloproliferative neoplasms and advanced-stage solid tumors (NCT04176198 and NCT03715504). Preliminary data from Phase I dose escalation and safety studies showed TP3654 is well-tolerated in humans and exhibited promising response in patients with myelofibrosis.

References

1. Nawijn MC, Alendar A, Berns A. For better or for worse: the role of Pim oncogenes in tumorigenesis. Nat Rev Cancer. 2011;11(1):23-34. doi:10.1038/nrc2986

2. Saris CJ, Domen J, Berns A. The Pim-1 oncogene encodes two related protein–serine/threonine kinases by alternative initiation at AUG and CUG. EMBO J. 1991; 10:655–664. [PubMed: 1825810]

3. Xie Y, Xu K, Dai B, Guo Z, Jiang T, Chen H, Qiu Y. The 44 kDa Pim-1 kinase directly interacts with tyrosine kinase Etk/BMX and protects human prostate cancer cells from apoptosis induced by chemotherapeutic drugs. Oncogene. 2006; 25:70–78. [PubMed: 16186805]

4. Baytel D, Shalom S, Madgar I, Weissenberg R, Don J. The human Pim-2 proto-oncogene and its testicular expression. Biochim Biophys Acta. 1998; 1442:274–285. [PubMed: 9804974]

5. Cohen AM, Grinblat B, Bessler H, Kristt D, Kremer A, Schwartz A, Halperin M, Shalom S, Merkel D, Don J. Increased expression of the hPim-2 gene in human chronic lymphocytic leukemia and non-Hodgkin lymphoma. Leuk Lymphoma. 2004; 45:951–955. [PubMed: 15291354]

6. Mikkers H, Nawijn M, Allen J, Brouwers C, Verhoeven E, Jonkers J, Berns A. Mice deficient for all PIM kinases display reduced body size and impaired responses to hematopoietic growth factors. Mol Cell Biol. 2004; 24:6104–6115. [PubMed: 15199164]

7. Bachmann M, Kosan C, Xing PX, Montenarh M, Hoffmann I, Moroy T. The oncogenic serine/threonine kinase Pim-1 directly phosphorylates and activates the G2/M specific phosphatase Cdc25C. Int J Biochem Cell Biol. 2006; 38:430–443. [PubMed: 16356754]

8. Cibull TL, Jones TD, Li L, Eble JN, Ann Baldridge L, Malott SR, Luo Y, Cheng L. Overexpression of Pim-1 during progression of prostatic adenocarcinoma. J Clin Pathol. 2006; 59:285–288. [PubMed: 16505280]

9. Feldman JD, Vician L, Crispino M, Tocco G, Baudry M, Herschman HR. Seizure activity induces PIM-1 expression in brain. J Neurosci Res. 1998; 53:502–509. [PubMed: 9710271]

10. Qian KC, Studts J, Wang L, Barringer K, Kronkaitis A, Peng C, Baptiste A, LaFrance R, Mische S, Farmer B. Expression, purification, crystallization and preliminary crystallographic analysis of human Pim-1 kinase. Acta Crystallogr Sect F: Struct Biol Cryst Commun. 2005; 61:96–99

11. Kim KT, Baird K, Ahn JY, Meltzer P, Lilly M, Levis M, Small D. Pim-1 is up-regulated by constitutively activated FLT3 and plays a role in FLT3-mediated cell survival. Blood. 2005; 105:1759–1767. [PubMed: 15498859]

12. Quesnelle KM, Boehm AL, Grandis JR. STAT-mediated EGFR signaling in cancer. J Cell Biochem. 2007; 102:311–319. [PubMed: 17661350]

13. Wernig G, Gonneville JR, Crowley BJ, Rodrigues MS, Reddy MM, Hudon HE, Walz C, Reiter A, Podar K, Royer Y, Constantinescu SN, Tomasson MH, Griffin JD, Gilliland DG, Sattler M. The Jak2V617F oncogene associated with myeloproliferative diseases requires a functional FERM domain for transformation and for expression of the Myc and Pim proto-oncogenes. Blood. 2008; 111:3751–3759. [PubMed: 18216297]

14. Verma, A., Kambhampati, S., Parmar, S. et al. Jak family of kinases in cancer. Cancer Metastasis Rev 22, 423–434 (2003). https://doi.org/10.1023/A:1023805715476

15. Xin P, Xu X, Deng C, et al. The role of JAK/STAT signaling pathway and its inhibitors in diseases. Int Immunopharmacol. 2020;80:106210. doi:10.1016/j.intimp.2020.106210

16. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264(5164):1415-1421. doi:10.1126/science.8197455

17. Tolomeo M, Cascio A. The Multifaced Role of STAT3 in Cancer and Its Implication for Anticancer Therapy. Int J Mol Sci. 2021;22(2):603. Published 2021 Jan 9. doi:10.3390/ijms22020603

18. Sonkin D, Palmer M, Rong X, et al. The identification and characterization of a STAT5 gene signature in hematologic malignancies. Cancer Biomark. 2015;15(1):79-87. doi:10.3233/CBM-140434

19. Hu YL, Passegué E, Fong S, Largman C, Lawrence HJ. Evidence that the Pim1 kinase gene is a direct target of HOXA9. Blood. 2007;109(11):4732-4738. doi:10.1182/blood-2006-08-043356

20. Chauhan SS, Toth RK, Jensen CC, Casillas AL, Kashatus DF, Warfel NA. PIM kinases alter mitochondrial dynamics and chemosensitivity in lung cancer. Oncogene. 2020;39(12):2597-2611. doi:10.1038/s41388-020-1168-9

21. Zhang Y, Parsanejad M, Huang E, et al. Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage. J Neurochem. 2010;112(2):497-510. doi:10.1111/j.1471-4159.2009.06476.x

22. Wingett D, Reeves R, Magnuson NS. Stability changes in pim-1 proto-oncogene mRNA after mitogen stimulation of normal lymphocytes. J Immunol. 1991;147(10):3653-3659.

23. Peters TL, Li L, Tula-Sanchez AA, Pongtornpipat P, Schatz JH. Control of translational activation by PIM kinase in activated B-cell diffuse large B-cell lymphoma confers sensitivity to inhibition by PIM447. Oncotarget. 2016;7(39):63362-63373. doi:10.18632/oncotarget.11457

24. Ma J, Arnold HK, Lilly MB, Sears RC, Kraft AS. Negative regulation of Pim-1 protein kinase levels by the B56beta subunit of PP2A. Oncogene. 2007;26(35):5145-5153. doi:10.1038/sj.onc.1210323

25. Linn DE, Yang X, Xie Y, et al. Differential regulation of androgen receptor by PIM-1 kinases via phosphorylation-dependent recruitment of distinct ubiquitin E3 ligases. J Biol Chem. 2012;287(27):22959-22968. doi:10.1074/jbc.M111.338350

26. Mizuno K, Shirogane T, Shinohara A, Iwamatsu A, Hibi M, Hirano T. Regulation of Pim-1 by Hsp90. Biochem Biophys Res Commun. 2001;281(3):663-669. doi:10.1006/bbrc.2001.4405

27. Shay KP, Wang Z, Xing PX, McKenzie IF, Magnuson NS. Pim-1 kinase stability is regulated by heat shock proteins and the ubiquitin-proteasome pathway. Mol Cancer Res. 2005;3(3):170-181. doi:10.1158/1541-7786.MCR-04-0192

28. Xie Y, Xu K, Dai B, et al. The 44 kDa Pim-1 kinase directly interacts with tyrosine kinase Etk/BMX and protects human prostate cancer cells from apoptosis induced by chemotherapeutic drugs. Oncogene. 2006;25(1):70-78. doi:10.1038/sj.onc.1209058

29. Warfel NA, Kraft AS. PIM kinase (and Akt) biology and signaling in tumors. Pharmacol Ther. 2015;151:41-49. doi:10.1016/j.pharmthera.2015.03.001

30. Friedmann M, Nissen MS, Hoover DS, Reeves R, Magnuson NS. Characterization of the proto-oncogene Pim-1: kinase activity and substrate recognition sequence. Arch Biochem Biophys. 1992; 298:594–601. [PubMed: 1416988]

31. Rust HL, Thompson PR. Kinase consensus sequences: a breeding ground for crosstalk. ACS Chem Biol. 2011;6(9):881-892. doi:10.1021/cb200171d

32. Popova NV, Jücker M. The Role of mTOR Signaling as a Therapeutic Target in Cancer. Int J Mol Sci. 2021;22(4):1743. Published 2021 Feb 9. doi:10.3390/ijms22041743

33. Zhang F, Beharry ZM, Harris TE, et al. PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells. Cancer Biol Ther. 2009;8(9):846-853. doi:10.4161/cbt.8.9.8210

34. Kim W, Youn H, Seong KM, et al. PIM1-activated PRAS40 regulates radioresistance in non-small cell lung cancer cells through interplay with FOXO3a, 14-3-3 and protein phosphatases. Radiat Res. 2011;176(5):539-552. doi:10.1667/rr2609.1

35. Malla R, Ashby CR Jr, Narayanan NK, Narayanan B, Faridi JS, Tiwari AK. Proline-rich AKT substrate of 40-kDa (PRAS40) in the pathophysiology of cancer. Biochem Biophys Res Commun. 2015;463(3):161-166. doi:10.1016/j.bbrc.2015.05.041

36. Keeton EK, McEachern K, Dillman KS, et al. AZD1208, a potent and selective pan-Pim kinase inhibitor, demonstrates efficacy in preclinical models of acute myeloid leukemia. *Blood*. 2014;123(6):905-913. doi:10.1182/blood-2013-04-495366

37. Cervantes-Gomez F, Stellrecht CM, Ayres ML, Keating MJ, Wierda WG, Gandhi V. PIM kinase inhibitor, AZD1208, inhibits protein translation and induces autophagy in primary chronic lymphocytic leukemia cells. Oncotarget. 2019;10(29):2793-2809. Published 2019 Apr 19. doi:10.18632/oncotarget.26876

38. Yang Q, Chen LS, Neelapu SS, Miranda RN, Medeiros LJ, Gandhi V. Transcription and translation are primary targets of Pim kinase inhibitor SGI-1776 in mantle cell lymphoma. Blood. 2012;120(17):3491-3500. doi:10.1182/blood-2012-02-412643

39. Lu J, Zavorotinskaya T, Dai Y, et al. Pim2 is required for maintaining multiple myeloma cell growth through modulating TSC2 phosphorylation. Blood. 2013;122(9):1610-1620. doi:10.1182/blood-2013-01-481457

40. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002;4(9):648-657. doi:10.1038/ncb839

41. Matthews HK, Bertoli C, de Bruin RAM. Cell cycle control in cancer. Nat Rev Mol Cell Biol. 2022;23(1):74-88. doi:10.1038/s41580-021-00404-3

44. Bretones G, Delgado MD, León J. Myc and cell cycle control. Biochim Biophys Acta. 2015;1849(5):506-516. doi:10.1016/j.bbagrm.2014.03.013

45. Abukhdeir AM, Park BH. P21 and p27: roles in carcinogenesis and drug resistance. Expert Rev Mol Med. 2008;10:e19. Published 2008 Jul 1. doi:10.1017/S1462399408000744

46. Zhang, Y., Parsanejad, M., Huang, E., Qu, D., Aleyasin, H., Rousseaux, M.W.C., Gonzalez, Y.R., Cregan, S.P., Slack, R.S. and Park, D.S. (2010), Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage. Journal of Neurochemistry, 112: 497-510. https://doi.org/10.1111/j.1471-4159.2009.06476.x

47. Hnit SS, Xie C, Yao M, et al. p27(Kip1) signaling: Transcriptional and post-translational regulation. Int J Biochem Cell Biol. 2015;68:9-14. doi:10.1016/j.biocel.2015.08.005

48. Zhang X, Song M, Kundu JK, Lee MH, Liu ZZ. PIM Kinase as an Executional Target in Cancer. J Cancer Prev. 2018;23(3):109-116. doi:10.15430/JCP.2018.23.3.109

49. Banerjee S, Lu J, Cai Q, Sun Z, Jha HC, Robertson ES. EBNA3C augments Pim-1 mediated phosphorylation and degradation of p21 to promote B-cell proliferation. PLoS Pathog. 2014;10(8):e1004304. Published 2014 Aug 14. doi:10.1371/journal.ppat.1004304

50. Lou L, Wang Y, Cui J, Yan X, Xue L, Li Y. Differential expression of Pim-3, c-Myc, and p-p27 proteins in adenocarcinomas of the gastric cardia and distal stomach. Tumour Biol. 2014;35(5):5029-5036. doi:10.1007/s13277-014-1664-z

51. D'Arcy MS. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. Cell Biol Int. 2019;43(6):582-592. doi:10.1002/cbin.11137

52. Kashyap D, Garg VK, Goel N. Intrinsic and extrinsic pathways of apoptosis: Role in cancer development and prognosis. Adv Protein Chem Struct Biol. 2021;125:73-120. doi:10.1016/bs.apcsb.2021.01.003

53. Bui NL, Pandey V, Zhu T, Ma L, Basappa, Lobie PE. Bad phosphorylation as a target of inhibition in oncology. Cancer Lett. 2018;415:177-186. doi:10.1016/j.canlet.2017.11.017

54. Liu C, Chen K, Wang H, et al. Gastrin Attenuates Renal Ischemia/Reperfusion Injury by a PI3K/Akt/Bad-Mediated Anti-apoptosis Signaling. Front Pharmacol. 2020;11:540479. Published 2020 Nov 6. doi:10.3389/fphar.2020.540479

55. Shah MA, Kang JB, Kim MO, Koh PO. Chlorogenic acid alleviates the reduction of Akt and Bad phosphorylation and of phospho-Bad and 14-3-3 binding in an animal model of stroke [published online ahead of print, 2022 Sep 30]. J Vet Sci. 2022;10.4142/jvs.22200. doi:10.4142/jvs.22200

56. Liu B, Wang Z, Li HY, Zhang B, Ping B, Li YY. Pim-3 promotes human pancreatic cancer growth by regulating tumor vasculogenesis. Oncol Rep. 2014;31(6):2625-2634. doi:10.3892/or.2014.3158

57. Watanabe D, Nogami A, Okada K, Akiyama H, Umezawa Y, Miura O. FLT3-ITD Activates RSK1 to Enhance Proliferation and Survival of AML Cells by Activating mTORC1 and eIF4B Cooperatively with PIM or PI3K and by Inhibiting Bad and BIM. Cancers (Basel). 2019;11(12):1827. Published 2019 Nov 20. doi:10.3390/cancers11121827

58. Alsubaie M, Matou-Nasri S, Aljedai A, et al. In vitro assessment of the efficiency of the PIM-1 kinase pharmacological inhibitor as a potential treatment for Burkitt's lymphoma. Oncol Lett. 2021;22(2):622. doi:10.3892/ol.2021.12883

59. Blanco FF, Jimbo M, Wulfkuhle J, et al. The mRNA-binding protein HuR promotes hypoxia-induced chemoresistance through posttranscriptional regulation of the proto-oncogene PIM1 in pancreatic cancer cells. Oncogene. 2016;35(19):2529-2541. doi:10.1038/onc.2015.325

60. Wu J, Chu E, Kang Y. PIM Kinases in Multiple Myeloma. Cancers (Basel). 2021;13(17):4304. Published 2021 Aug 26. doi:10.3390/cancers13174304

61. Chauhan SS, Toth RK, Jensen CC, Casillas AL, Kashatus DF, Warfel NA. PIM kinases alter mitochondrial dynamics and chemosensitivity in lung cancer. Oncogene. 2020;39(12):2597-2611. doi:10.1038/s41388-020-1168-9

62. Xu J, Zhang T, Wang T, You L, Zhao Y. PIM kinases: an overview in tumors and recent advances in pancreatic cancer. Future Oncol. 2014;10(5):865-876. doi:10.2217/fon.13.229

63. Narlik-Grassow M, Blanco-Aparicio C, Carnero A. The PIM family of serine/threonine kinases in cancer. Med Res Rev. 2014;34(1):136-159. doi:10.1002/med.21284

64. Xie Y, Bayakhmetov S. PIM1 kinase as a promise of targeted therapy in prostate cancer stem cells. Mol Clin Oncol. 2016;4(1):13-17. doi:10.3892/mco.2015.673

65. Mosquera Orgueira A, Ferreiro Ferro R, Díaz Arias JÁ, et al. Detection of new drivers of frequent B-cell lymphoid neoplasms using an integrated analysis of whole genomes. PLoS One. 2021;16(5):e0248886. Published 2021 May 4. doi:10.1371/journal.pone.0248886

66. Cuesta-Domínguez Á, León-Rico D, Álvarez L, et al. BCR-JAK2 drives a myeloproliferative neoplasm in transplanted mice. J Pathol. 2015;236(2):219-228. doi:10.1002/path.4513

67. Holder SL, Abdulkadir SA. PIM1 kinase as a target in prostate cancer: roles in tumorigenesis, castration resistance, and docetaxel resistance. Curr Cancer Drug Targets. 2014;14(2):105-114. doi:10.2174/1568009613666131126113854

68. Eerola SK, Santio NM, Rinne S, et al. Phosphorylation of NFATC1 at PIM1 target sites is essential for its ability to promote prostate cancer cell migration and invasion. Cell Commun Signal. 2019;17(1):148. Published 2019 Nov 15. doi:10.1186/s12964-019-0463-y

69. Eguchi M, Minami Y, Kuzume A, Chi S. Mechanisms Underlying Resistance to FLT3 Inhibitors in Acute Myeloid Leukemia. Biomedicines. 2020;8(8):245. Published 2020 Jul 24. doi:10.3390/biomedicines8080245

70. Li Q, Sun RX, Ou Y, Luo HM, Wu Y. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2019;27(3):664-672. doi:10.19746/j.cnki.issn.1009-2137.2019.03.006

71. Cheng F, Weidner-Glunde M, Varjosalo M, et al. KSHV reactivation from latency requires Pim-1 and Pim-3 kinases to inactivate the latency-associated nuclear antigen LANA. PLoS Pathog. 2009;5(3):e1000324. doi:10.1371/journal.ppat.1000324

72. Rainio EM, Ahlfors H, Carter KL, et al. Pim kinases are upregulated during Epstein-Barr virus infection and enhance EBNA2 activity. Virology. 2005;333(2):201-206. doi:10.1016/j.virol.2005.01.001

73. Kapelko-Słowik K, Dybko J, Grzymajło K, et al. Expression of the PIM2 gene is associated with more aggressive clinical course in patients with chronic lymphocytic leukemia. Adv Clin Exp Med. 2019;28(3):385-390. doi:10.17219/acem/90771

74. Kapelko-Slowik K, Owczarek TB, Grzymajlo K, et al. Elevated PIM2 gene expression is associated with poor survival of patients with acute myeloid leukemia. Leuk Lymphoma. 2016;57(9):2140-2149. doi:10.3109/10428194.2015.1124991

75. Liu Z, Tian M, Ding K, Liu H, Wang Y, Fu R. High expression of PIM2 induces HSC proliferation in myelodysplastic syndromes via the IDH1/HIF1-α signaling pathway. Oncol Lett. 2019;17(6):5395-5402. doi:10.3892/ol.2019.10256

76. Hospital MA, Jacquel A, Mazed F, et al. RSK2 is a new Pim2 target with pro-survival functions in FLT3-ITD-positive acute myeloid leukemia. Leukemia. 2018;32(3):597-605. doi:10.1038/leu.2017.284

77. Kapelko-Slowik K, Owczarek TB, Grzymajlo K, et al. Elevated PIM2 gene expression is associated with poor survival of patients with acute myeloid leukemia. Leuk Lymphoma. 2016;57(9):2140-2149. doi:10.3109/10428194.2015.1124991

78. Ramachandran J, Santo L, Siu KT, Panaroni C, Raje N. Pim2 is important for regulating DNA damage response in multiple myeloma cells. Blood Cancer J. 2016;6(8):e462. Published 2016 Aug 26. doi:10.1038/bcj.2016.73

79. Nair JR, Caserta J, Belko K, et al. Novel inhibition of PIM2 kinase has significant anti-tumor efficacy in multiple myeloma. Leukemia. 2017;31(8):1715-1726. doi:10.1038/leu.2016.379

80. Musiani D, Hammond DE, Cirillo L, et al. PIM2 kinase is induced by cisplatin in ovarian cancer cells and limits drug efficacy. J Proteome Res. 2014;13(11):4970-4982. doi:10.1021/pr500651n

81. Tang X, Cao T, Zhu Y, et al. PIM2 promotes hepatocellular carcinoma tumorigenesis and progression through activating NF-κB signaling pathway. Cell Death Dis. 2020;11(7):510. Published 2020 Jul 2. doi:10.1038/s41419-020-2700-0

82. Zhuang H, Zhao MY, Hei KW, et al. Aberrant expression of pim-3 promotes proliferation and migration of ovarian cancer cells. Asian Pac J Cancer Prev. 2015;16(8):3325-3331. doi:10.7314/apjcp.2015.16.8.3325

83. Li, T., Wang, Z., Hou, Y.f., Li, Y.y. (2017). Pim-3 Regulates Stemness of Pancreatic Cancer Cells via Activating STAT3 Signaling Pathway. Journal of Cancer, 8(9), 1530-1541. https://doi.org/10.7150/jca.18628.

84. Marayati R, Stafman LL, Williams AP, et al. CRISPR/Cas9-mediated knockout of PIM3 suppresses tumorigenesis and cancer cell stemness in human hepatoblastoma cells. Cancer Gene Ther. 2022;29(5):558-572. doi:10.1038/s41417-021-00334-4

85. Nakano H, Hasegawa T, Saito N, et al. Design and synthesis of an in vivo-efficacious PIM3 kinase inhibitor as a candidate anti-pancreatic cancer agent. Bioorg Med Chem Lett. 2015;25(24):5687-5693. doi:10.1016/j.bmcl.2015.10.098

86. Zhou Y, Zhou YN, Liu SX, Wang J, Ji R, Yan X. Effects of PIM3 in prognosis of colon cancer. Clin Transl Oncol. 2021;23(10):2163-2170. doi:10.1007/s12094-021-02624-7

87. Gao X, Liu X, Lu Y, et al. PIM1 is responsible for IL-6-induced breast cancer cell EMT and stemness via c-myc activation. Breast Cancer. 2019;26(5):663-671. doi:10.1007/s12282-019-00966-3\

88. Zhao B, Liu L, Mao J, Zhang Z, Wang Q, Li Q. PIM1 mediates epithelial-mesenchymal transition by targeting Smads and c-Myc in the nucleus and potentiates clear-cell renal-cell carcinoma oncogenesis [published correction appears in Cell Death Dis. 2021 Mar 4;12(3):246]. Cell Death Dis. 2018;9(3):307. Published 2018 Feb 22. doi:10.1038/s41419-018-0348-9

88. Marayati R, Julson J, Bownes LV, et al. PIM3 kinase promotes tumor metastasis in hepatoblastoma by upregulating cell surface expression of chemokine receptor cxcr4. Clin Exp Metastasis. 2022;39(6):899-912. doi:10.1007/s10585-022-10186-3

89. Santio NM, Koskinen PJ. PIM kinases: From survival factors to regulators of cell motility. Int J Biochem Cell Biol. 2017;93:74-85. doi:10.1016/j.biocel.2017.10.016

90. Brault L, Rovó A, Decker S, Dierks C, Tzankov A, Schwaller J. CXCR4-SERINE339 regulates cellular adhesion, retention and mobilization, and is a marker for poor prognosis in acute myeloid leukemia. Leukemia. 2014;28(3):566-576. doi:10.1038/leu.2013.201

92. Białopiotrowicz E, Górniak P, Noyszewska-Kania M, et al. Microenvironment-induced PIM kinases promote CXCR4-triggered mTOR pathway required for chronic lymphocytic leukaemia cell migration. J Cell Mol Med. 2018;22(7):3548-3559. doi:10.1111/jcmm.13632

93. Ye, C., Zhang, C., Huang, H., Yang, B., Xiao, G., Kong, D., ... & Sun, Y. (2018). The natural compound myricetin effectively represses the malignant progression of prostate cancer by inhibiting PIM1 and disrupting the PIM1/CXCR4 interaction. Cellular Physiology and Biochemistry, 48(3), 1230-1244.

94. Santio, N. M., Salmela, M., Arola, H., Eerola, S. K., Heino, J., Rainio, E. M., & Koskinen, P. J. (2016). The PIM1 kinase promotes prostate cancer cell migration and adhesion via multiple signalling pathways. Experimental cell research, 342(2), 113-124.

95. Zemskova, M. Y., Song, J. H., Cen, B., Cerda-Infante, J., Montecinos, V. P., & Kraft, A. S. (2015). Regulation of prostate stromal fibroblasts by the PIM1 protein kinase. Cellular signalling, 27(1), 135-146.

96. Chaudhary, P. (2019). Mechanism of Extracellular Matrix Induced Tumor Survival and Drug Resistance in Castration-Resistant Prostate Cancer (Doctoral dissertation, The University of Arizona).

97. Cao, L., Wang, F., Li, S., Wang, X., Huang, D., & Jiang, R. (2019). PIM1 kinase promotes cell proliferation, metastasis and tumor growth of lung adenocarcinoma by potentiating the c-MET signaling pathway. Cancer letters, 444, 116-126.

98. Yang, J., Wang, J., Chen, K., Guo, G., Xi, R., Rothman, P. B., ... & Chen, J. L. (2013). eIF4B phosphorylation by pim kinases plays a critical role in cellular transformation by Abl oncogenes. Cancer research, 73(15), 4898-4908.

99. Santio, N. M., Landor, S. K. J., Vahtera, L., Ylä-Pelto, J., Paloniemi, E., Imanishi, S. Y., ... & Koskinen, P. J. (2016). Phosphorylation of Notch1 by Pim kinases promotes oncogenic signaling in breast and prostate cancer cells. Oncotarget, 7(28), 43220.

100. Xie, Y., Burcu, M., Linn, D. E., Qiu, Y., & Baer, M. R. (2010). Pim-1 kinase protects P-glycoprotein from degradation and enables its glycosylation and cell surface expression. Molecular pharmacology, 78(2), 310-318.

101. Xie, Y., Xu, K., Linn, D. E., Yang, X., Guo, Z., Shimelis, H., ... & Qiu, Y. (2008). The 44-kDa Pim-1 kinase phosphorylates BCRP/ABCG2 and thereby promotes its multimerization and drug-resistant activity in human prostate cancer cells. Journal of Biological Chemistry, 283(6), 3349-3356.

102. Xie, Y., Xu, K., Dai, B., Guo, Z., Jiang, T., Chen, H., & Qiu, Y. (2006). The 44 kDa Pim-1 kinase directly interacts with tyrosine kinase Etk/BMX and protects human prostate cancer cells from apoptosis induced by chemotherapeutic drugs. Oncogene, 25(1), 70-78.

103. Natarajan, K., Xie, Y., Burcu, M., Linn, D. E., Qiu, Y., & Baer, M. R. (2013). Pim-1 kinase phosphorylates and stabilizes 130 kDa FLT3 and promotes aberrant STAT5 signaling in acute myeloid leukemia with FLT3 internal tandem duplication. PloS one, 8(9), e74653.

104. Zemskova, M., Lilly, M. B., Lin, Y. W., Song, J. H., & Kraft, A. S. (2010). p53-Dependent Induction of Prostate Cancer Cell Senescence by the PIM1 Protein Kinase Senescence Regulated by PIM1. Molecular Cancer Research, 8(8), 1126-1141.

105. An, N., Xiong, Y., LaRue, A. C., Kraft, A. S., & Cen, B. (2015). Activation of pim kinases is sufficient to promote resistance to MET small-molecule inhibitors. Cancer research, 75(24), 5318-5328.

106. Lu, C., Qiao, P., Sun, Y., Ren, C., & Yu, Z. (2021). Positive regulation of PFKFB3 by PIM2 promotes glycolysis and paclitaxel resistance in breast cancer. Clinical and Translational Medicine, 11(4), e400.

107. Musiani, D., Hammond, D. E., Cirillo, L., Erriquez, J., Olivero, M., Clague, M. J., & Di Renzo, M. F. (2014). PIM2 kinase is induced by cisplatin in ovarian cancer cells and limits drug efficacy. Journal of proteome research, 13(11), 4970-4982.

108. Isaac, M., Siu, A., & Jongstra, J. (2011). The oncogenic PIM kinase family regulates drug resistance through multiple mechanisms. Drug Resistance Updates, 14(4-5), 203-211.

109. Tursynbay Y, Zhang J, Li Z, et al. Pim-1 kinase as cancer drug target: An update. Biomed Rep. 2016;4(2):140-146. doi:10.3892/br.2015.561

110. Chen, L. S., Redkar, S., Taverna, P., Cortes, J. E., & Gandhi, V. (2011). Mechanisms of cytotoxicity to Pim kinase inhibitor, SGI-1776, in acute myeloid leukemia. Blood, The Journal of the American Society of Hematology, 118(3), 693-702.

111. Batra, V., Maris, J. M., Kang, M. H., Reynolds, C. P., Houghton, P. J., Alexander, D., ... & Smith, M. A. (2012). Initial testing (stage 1) of SGI-1776, a PIM1 kinase inhibitor, by the pediatric preclinical testing program. Pediatric blood & cancer, 59(4), 749-752.

112. Cortes J, Tamura K, DeAngelo DJ, et al. Phase I studies of AZD1208, a proviral integration Moloney virus kinase inhibitor in solid and haematological cancers. Br J Cancer. 2018;118(11):1425-1433. doi:10.1038/s41416-018-0082-1

113. Foulks JM, Carpenter KJ, Luo B, et al. A small-molecule inhibitor of PIM kinases as a potential treatment for urothelial carcinomas. Neoplasia. 2014;16(5):403-412. doi:10.1016/j.neo.2014.05.004

114. Dutta A, Nath D, Yang Y, et al. Genetic ablation of Pim1 or pharmacologic inhibition with TP-3654 ameliorates myelofibrosis in murine models. Leukemia. 2022;36(3):746-759. doi:10.1038/s41375-021-01464-2

115. Whillock AL, Mambetsariev N, Lin WW, Stunz LL, Bishop GA. TRAF3 regulates the oncogenic proteins Pim2 and c-Myc to restrain survival in normal and malignant B cells [published correction appears in Sci Rep. 2019 Nov 20;9(1):17502]. Sci Rep. 2019;9(1):12884. Published 2019 Sep 9. doi:10.1038/s41598-019-49390-9

116. Wu CP, Li YQ, Chi YC, Huang YH, Hung TH, Wu YS. The Second-Generation PIM Kinase Inhibitor TP-3654 Resensitizes ABCG2-Overexpressing Multidrug-Resistant Cancer Cells to Cytotoxic Anticancer Drugs. Int J Mol Sci. 2021;22(17):9440. Published 2021 Aug 30. doi:10.3390/ijms22179440

117. Lampron MC, Vitry G, Nadeau V, et al. PIM1 (Moloney Murine Leukemia Provirus Integration Site) Inhibition Decreases the Nonhomologous End-Joining DNA Damage Repair Signaling Pathway in Pulmonary Hypertension. Arterioscler Thromb Vasc Biol. 2020;40(3):783-801. doi:10.1161/ATVBAHA.119.313763

Chapter 3

Genetic Deletion or Pharmacologic Inhibition of PIM1 Impairs Breast Cancer Growth and Metastasis

Abstract

Metastatic breast cancers often do not respond to currently available therapies, highlighting a need for new drug targets. Expression of PIM1 is upregulated in a significant percentage of breast cancers. PIM1 expression is also significantly increased in metastatic breast cancers compared to non-metastatic breast cancer patients. We hypothesized that PIM1 plays an important role in progression and metastasis of breast cancer. Knockdown/deletion of PIM1 significantly inhibited the proliferation, migration, and invasion of several breast cancer cell lines. To assess the in vivo role of PIM1 in breast cancer progression and metastasis, we crossed PIM1 knockout mice with MMTV-PyMT metastatic breast cancer mouse model. We found that deletion of PIM1 significantly inhibited tumor growth and reduced lung metastasis in these mice. Pharmacologic inhibition of PIM1 kinase using TP-3654 significantly inhibited cell proliferation, migration, and invasion in breast cancer cells, and reduced breast tumor growth and lung metastasis in MMTV-PyMT mice. We also found that PIM1 deletion or inhibition decreased EMT markers SLUG and Vimentin and pro-metastatic marker c-MET. RNA-seq analysis showed genes related to translation, proliferation, EMT and metastasis pathways were affected by PIM1 depletion or inhibition. Together, these results suggest that PIM1 plays an important role in the progression and metastasis of breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer in women, with an estimated 287,850 new cases in 2022. (1) Breast cancer is also the second leading cause of cancer related deaths in women with an estimated 43,250 deaths in 2022. (1) Despite these numbers the overall 5-year survival rate for localized breast cancer is 99.1%. (1) The 5-year overall survival however drops dramatically to 30% if the patient has distant metastasis detected at the time of diagnosis. (1) Metastatic breast cancer does not respond to the same treatments as primary tumors, and care is often only palliative for these patients. This identifies a great need for additional treatments that can prevent metastasis or that specifically target the metastatic cells themselves.

Metastasis is a multifaceted process that can be broken into the following steps: first, local tumor cells must infiltrate adjacent tissues, followed by intravasation, invasion through blood vessels and into the circulatory system. (2) Tumor cells must then survive in circulation, infiltrate back through blood vessels (extravasation), and finally proliferate in distant sites. (3) In order to survive this process, cancer cells must undergo epithelial to mesenchymal transition (EMT), which causes cells to upregulate several mesenchymal genes. (4) EMT upregulates several different transcription factors including SNAIL and SLUG, which causes several other changes in gene expression. (5) These include an upregulation of vimentin, as well as several different matrix metalloproteinases (MMPs). (6-7) These signaling pathways provide several different points which metastasis can be targeted.

PIM kinases are a family of serine/threonine kinases consisting of 3 family members, PIM1, PIM2, and PIM3. (11) These family members are expressed ubiquitously throughout the body; however, expression of each family member differs depending on the tissue. (12) PIM kinases have been shown to increase cell division, growth, survival, and have also been implicated in

drug resistance. (13-16) More importantly, PIM kinases have also been shown to promote cell stemness, EMT, and metastasis in several other cancer settings. (8-9, 17-20) Recently, PIM1 has also been identified as a potential therapeutic target in TNBC. (21-22) However, the contribution of PIM1 in breast cancer metastasis has remained unknown.

In this study, we investigated the role of PIM1 in breast cancer growth and metastasis. We have found that PIM1 is amplified or overexpressed in a high percentage of breast cancers. We also found that PIM1 expression is significantly higher in metastatic breast cancers compared to non-metastatic breast cancers. We found that genetic deletion of PIM1 or inhibition with TP3654 significantly decreased cell growth, migration, and invasion *in vitro*, and decreased tumor growth as well as significantly reduced lung metastasis in a MMTV-PyMT mouse model of metastatic breast cancer. Overall, these results suggest PIM1 plays a key role not only in breast cancer growth, but also metastasis. Thus, inhibition of PIM1 might be useful for treatment of breast cancer.

Materials and Methods

Cell culture

Human MDA-mb-231, HCC-1806, HS578t, BT-20, MDA-MB-468, T47D, MCF-7, ZR-75-1, BT-474, SKBR3, MCF10A, and HMEC cells were purchased from ATCC. MDA-231, MCF-7, BT-474, and SKBR3 cells were maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin, and minimum essential ammino acids. 4T1, HCC-1806, and ZR-75-1 cells were maintained in RPMI supplemented with 10% FBS, penicillin/streptomycin, and minimum essential ammino, acids. Cells were tested for mycoplasma every 6 months. Cell culture conditions and seeding numbers are summarized below in table 1.

Table 1. Cell culture conditions and seeding densities.

Name	Tissue	MEDIA	Seeding Density (Cells/cm2)	6 Well	12 Well	24 Well	96 Well
4T1-Luc	Breast	RPMI + 10% FBS	1.0E+4	1.0E+5	4.0E+4	2.0E+4	3.0E+3
BT20	Breast	MEM Earles + 10%FBS	1.0E+4	1.0E+5	4.0E+4	2.0E+4	3.0E+3
BT474	Breast	DMEM + 10% FBS	3.0E+4	3.0E+5	1.2E+5	6.0E+4	9.0E+3
HCC1187	Breast	RPMI + 10% FBS	5.0E+4	5.0E+5	2.0E+5	1.0E+5	1.5E+4
HCC1599	Breast	RPMI + 10% FBS	2.0E+4	2.0E+5	8.0E+4	4.0E+4	6.0E+3
HCC1937	Breast	RPMI + 10% FBS	2.0E+4	2.0E+5	8.0E+4	4.0E+4	6.0E+3
HCC38	Breast	RPMI + 10% FBS	1.0E+4	1.0E+5	4.0E+4	2.0E+4	3.0E+3
HS578T	Breast	DMEM + 10% FBS + 10 µg/mL insulin	1.0E+4	1.0E+5	4.0E+4	2.0E+4	3.0E+3
MCF-10A	Breast	MEGM + BPE, Hydrocortisone, hEGF, and Insulin	5.0E+4	5.0E+5	2.0E+5	1.0E+5	1.5E+4
MCF-12A	Breast	MEGM + BPE, Hydrocortisone, hEGF, and Insulin	5.0E+4	5.0E+5	2.0E+5	1.0E+5	1.5E+4
MCF-7	Breast	DMEM + 10% FBS	4.0E+4	4.0E+5	1.6E+5	8.0E+4	1.2E+4
MDA-231	Breast	DMEM + 10% FBS	1.0E+4	1.0E+5	4.0E+4	2.0E+4	3.0E+3
MDA-468	Breast	DMEM + 10% FBS	1.0E+4	1.0E+5	4.0E+4	2.0E+4	3.0E+3
SKBR3	Breast	DMEM + 10% FBS	2.0E+4	2.0E+5	8.0E+4	4.0E+4	6.0E+3
ZR-75	Breast	RPMI + 10% FBS	1.0E+4	1.0E+5	4.0E+4	2.0E+4	3.0E+3

Transfection and viral infections

Viruses were generated by transfecting HEK-293T cells with PEI using plasmids containing corresponding shRNA or sgRNA plasmids. psPAX2 and pMDG.2 plasmids were used as packaging vectors. For infections, cells were seeded and allowed to adhere overnight, the following day cells were infected with lentivirus. After 48 hours of infection cells were selected with 2ug/mL puromycin for 72 hours. After puromycin selection cells were seeded and allowed

to grow for 3 days in base media +2% FBS. After 72 hours viable cells were counted using trypan blue exclusion.

Colony formation assay

MDA-231 or HCC-1806 cells were seeded (1,000 cells/well (6-well plates)) and allowed to grow for 7-14 days, at which time cells were fixed with methanol stained with crystal violet. To quantify, the number of colonies per well were counted.

Mammosphere assay

MDA-231 and HCC-18006 cells were plated at a density of 10,000 cells per well in 4 mL of 3D tumorsphere XF media on ultra-low-attachment plates. Cells were allowed to grow for 7-10 days. Total number of Mamospheres were then quantified by allowing spheres to gather at the bottom of a 15 mL tube by gravity sedimentation, followed by resuspension in 200 μL of PBS. Mamospheres were quantified using OrganoSeg software (citation).

Invasion/migration assays

For wound healing assays cells were plated and allowed to grow into a confluent layer, a scratch was then created using a 10 μL pipette tip. The cells were then imaged at 6-hour intervals until the gap was closed. Migration was quantified using ImageJ.

For migration assays, 1,000 cells were plated onto the top layer of corning transwell cell culture inserts. The cells were then incubated for 13 hours. Unmigrated cells were removed with a

cotton swab and cells that had migrated to the bottom of the insert were fixed with methanol and then stained with crystal violet. To quantify 5 images were taken and the number of cells in each image were counted. For invasion assays a similar protocol was used, but we utilized corning biocoat transwell inserts.

Inhibitor Treatments

TP3654 was dissolved in DMSO. Cells were seeded and allowed to adhere overnight. The following day TP3654 was added at indicated concentrations. Drug and media were changed daily, and cells were allowed to grow for 72 hours. Viable cells were then quantified using presto-blue HS.

PyMT Mice

MMTV-PyMT mice were purchased from Jackson labs. PIM1 and PIM2 whole body knockout mice were kindly provided by Dr. Anton Burns. PyMT were crossed with PIM1 KO mice to generate PIM1 KO PyMT mice. Mice were analyzed at 2-week intervals from 8-14 weeks of age. Tumor growth and mouse weight were monitored every 4 days. 2.0 mm tumor width and 10% weight loss were used as humane endpoints. Mice were euthanized and lung and tumors were fixed in formalin. These tissues were then sectioned, and H&E staining was performed to evaluate tumor progression and lung metastasis. Lung metastasis was quantified by counting the number of metastases per section.

For in vivo drug treatments TP3654 was dissolved in PBS+10% Tween 20. Mice were given 200 mg/kg TP3654 via oral gavage daily starting when mice were 6 weeks old. The mice were treated for 6 weeks and then euthanized. Tumor burden was monitored weekly by measuring tumor length and width. Tumor volume was calculated using the equation volume=length*width*width/2. The sum of tumor volumes for each mouse was used to calculate the total tumor burden. Mouse lungs and tumors were fixed in formalin and evaluated for tumor progression and lung metastasis after H&E staining.

NSG Xenografts

8-week-old NSG mice were purchased from Charles River. Mice were injected with 1x106 cells suspended in a 50:50 mixture of PBS and Matrigel. Treatment was initiated after tumors reached 0.5 cm in width. Mice were grouped into four treatment groups: Vehicle (control), 200 mg/kg TP3654 given orally daily, 2mg/kg doxorubicin administered by ip. Injection once per week, and a combination of TP3654 and doxorubicin. Tumor growth was monitored every 4 days using digital calipers. Mice were weighed once per week. After 5 weeks of treatment the tumor, lungs, brain, liver, kidney, and heart and spleen were fixed in formalin. H&E staining of tissues was then performed to analyze metastasis.

Flow Cytometry

Mouse tumor cells were cut into small pieces using a razor blade. The tumors were then transferred into DMEM/F12 +10% FBS + DNase I and Collagenase and incubated for 1 hour at 37C. Cells were then passed through a 70 μm filter. Cells were then pelleted and resuspended in 20 mL of RBC lysis buffer for 10 min on ice. Following RBC lysis cells were resuspended in

base media ficol separation was performed. The cells were then resuspended in FACS buffer and stained for 1 hour on ice. After staining cells were run on the Cytek aurora cytometer.

RT-qPCR

Total RNA was extracted using the Qiagen RNA-easy kit. 500 ng or RNA was then converted to cDNA using the Qiagen QuantiTect Reverse Transcription Kit. RT-qPCR was performed using SYBR Green and run on a quant studio 3. Primers can be found in table 1. 18S, HPRT1, or GAPDH were used as endogenous controls.

Western Blot

Total cell lysates were obtained using RIPA lysis. Protein concentration was determined using a Bradford assay. 50 ug of protein were loaded into each well. Antibodies and conditions used for each blot can be found below in table 2.

Patient Data

TCGA Breast cancer data from Firehouse Legacy and Pan Cancer Atlas studies were analyzed using cbioportal. Samples were checked for copy number gain and mRNA overexpression. A cutoff of log fold change of 1.5 was used for mRNA overexpression.

RNA-Seq

MDA-231 cells were used for RNA-Seq analysis. Control cells, PIM1 deleted cells, and cells treated for 48 hours with 2.0 μM TP3654 were used for sequencing. Total RNA was extracted from cells using RNeasy Mini kit (Qiagen). RNA sequencing was performed using NextSeq 500

High Output Kit and NextSeq 500 sequencing instrument (Illumina). RNA-seq data alignment was performed using UCSC hg38 reference genome with Bowtie2. The read counts and differential analysis were done using GenomicAlignments and DESeq2. P-adjusted value of <0.05 with a fold change of 1.5 was considered as significant change in gene expression.

Statistics

Results are expressed as mean +/- SEM. Statistical significance was determined using GraphPad prism 9. Student's t test and one-way anova were primarily used, appropriate tests are provided in figure legends. $P < 0.05$ was considered statistically significant. P values were marked with asterisks on figures (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.).

Results

PIM1 expression is increased in breast cancer patient samples

Analysis of TCGA data revealed over-expression or gene amplification of PIM1 in ~10% of breast cancer patients (Figs 1A-B). PIM2 and PIM3 were also misregulated but at a lower percentage (Figs 1A, B). Additionally, PIM1 expression was also significantly increased in metastatic breast cancer patients compared to patients with only primary tumors (Fig 1C). PIM2 and PIM3 expression was not significantly altered in metastatic breast cancer (Fig 1D, E).

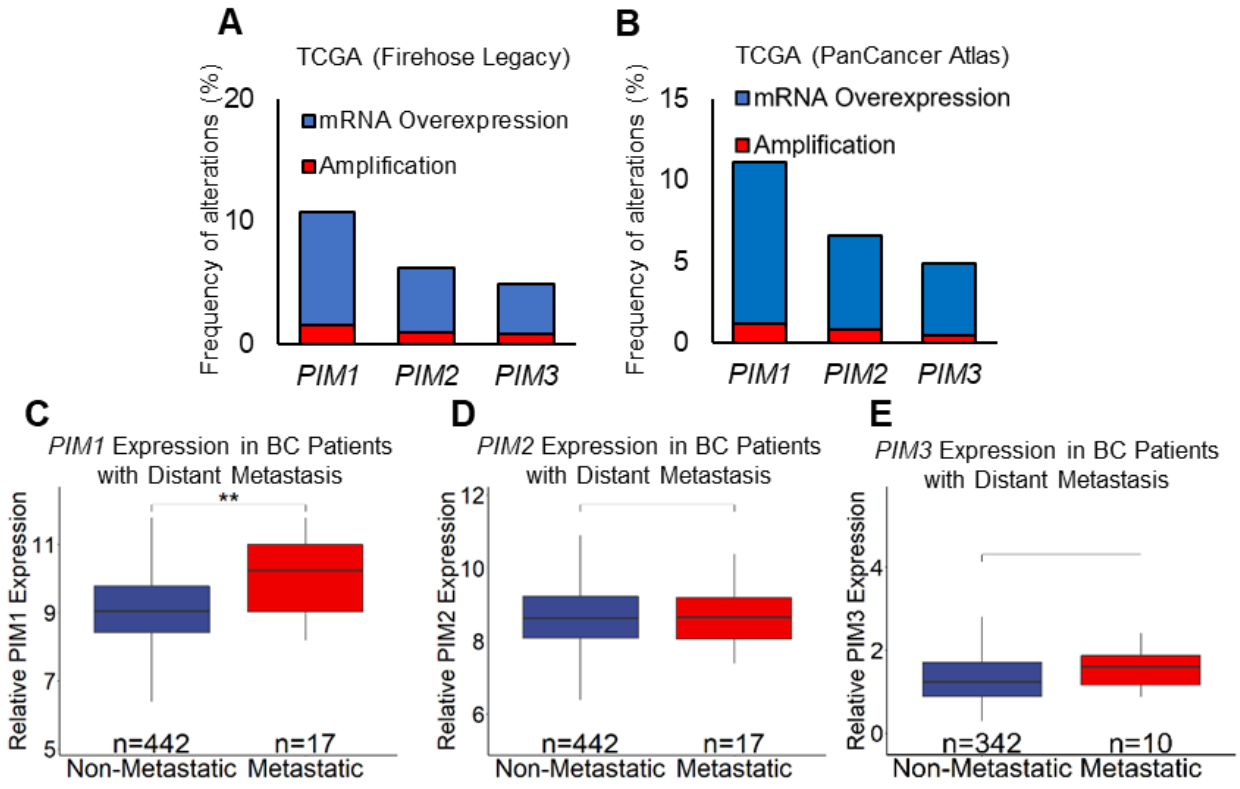

Figure. 1. PIM1 Expression is increased in breast cancer patients. Analysis of the gene expression data from (A) TCGA (Firehose legacy) and (B) TCGA (PanCancer Atlas) breast cancer data sets show PIM1 expression is increased in 10% of breast cancer patients. A Z-score of 1.5 was used for mRNA overexpression. (C) Further analysis of TCGA data showed that patients with metastatic breast cancer had significantly higher PIM1 expression. D-E) PIM2 and PIM3 expression was not significantly different in metastatic breast cancer patients to non-metastatic (*P<0.05, **P<0.01, ***P<0.001).

We further evaluated PIM expression in breast cancers with different receptor status. We evaluated both TCGA data and data from Yau et al. 2010. TCGA analysis reveled that both PIM1 and PIM2 expression was significantly higher in TNBC tumors compared to HR+ or HER2+ breast cancers. (Fig 2 A-B). Data was also analyzed from Yau's 2010 breast cancer research publication. Similar to TCGA data, TNBC patients had significantly higher PIM1 expression when compared to HR+ or HER2+ breast cancers (Fig 2 C). Unlike TCGA data however, TNBC patients in this study only had significantly higher PIM2 expression when

compared to HR+ patients (Fig 2 D). Our data analysis also reveled that HER2+ breast cancer patients from this data set also had significantly higher expression of PIM2 when compared to HR+ breast cancer patients. We did not find any significant alterations of PIM3 expression by receptor status.

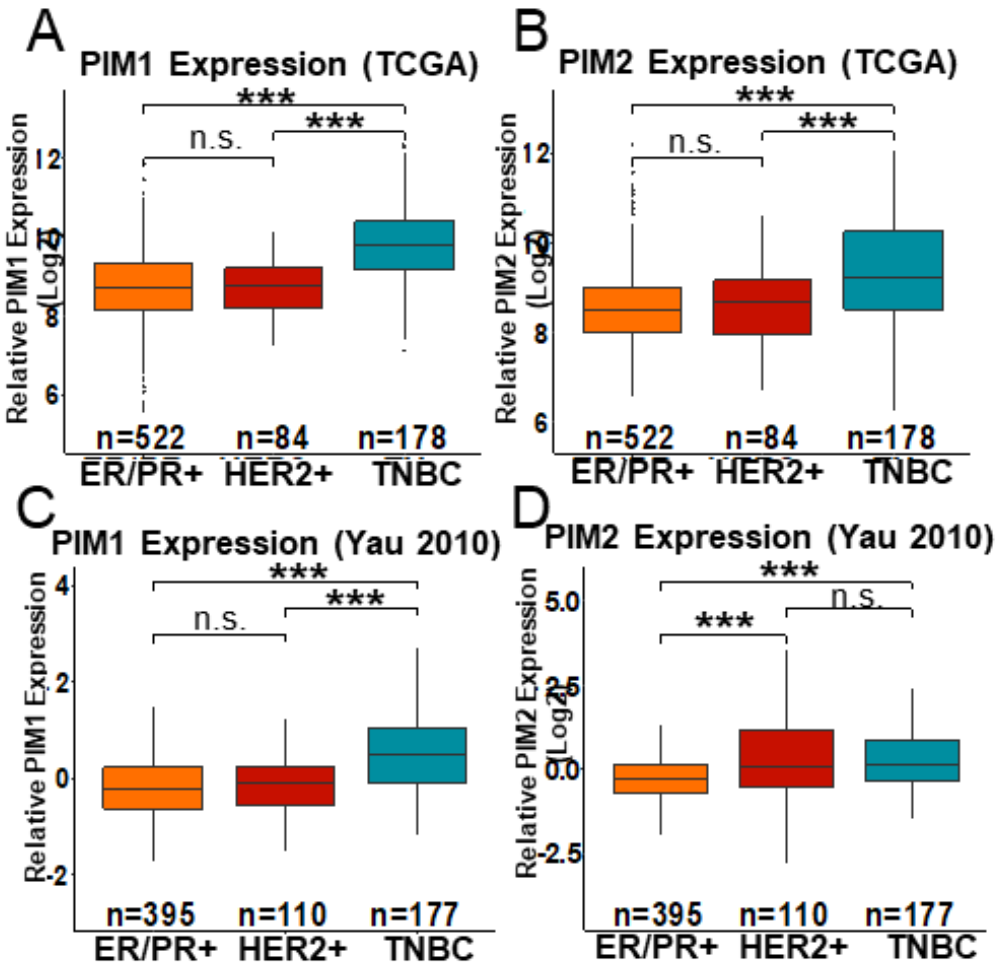

Figure. 2. PIM1 and PIM2 expression is significantly upregulated in triple-negative breast cancer (TNBC). Analysis of the gene expression data from TCGA breast cancer data set (A-B) and Yau et al. Breast Cancer Res. 2010 data set (C-D) show significantly increased expression of PIM1 and PIM2 in triple-negative breast cancers (TNBC) compared to ER/PR+ and Her2+ breast cancers. *P<0.05, **P<0.01, ***P<0.001.

Next, we evaluated if PIM expression level had any correlation with the survival of breast cancer patients. We found that patients with a high level of PIM1 expression had significantly shorter survival rates in patients with triple negative breast cancer (Fig 3A). In contrast, PIM2 and PIM3 expression level did not show significant association with shortened survival in TNBC (Fig 3B-C). PIM1 expression, however, was not correlated with survival in HER2+ or HR+ breast

cancers (Figs 3D, G). PIM2 and PIM3 expression were not associated with significantly shortened survival of HR+ breast cancer patients (Figs 3E, F), however high expression of either PIM2 or PIM3 in HER2+ breast cancer patients were significantly correlated with longer patient survival (Figs 3 H-I). Overall, these results suggest that PIM1 expression may have prognostic value in breast cancer, particularly in TNBC.

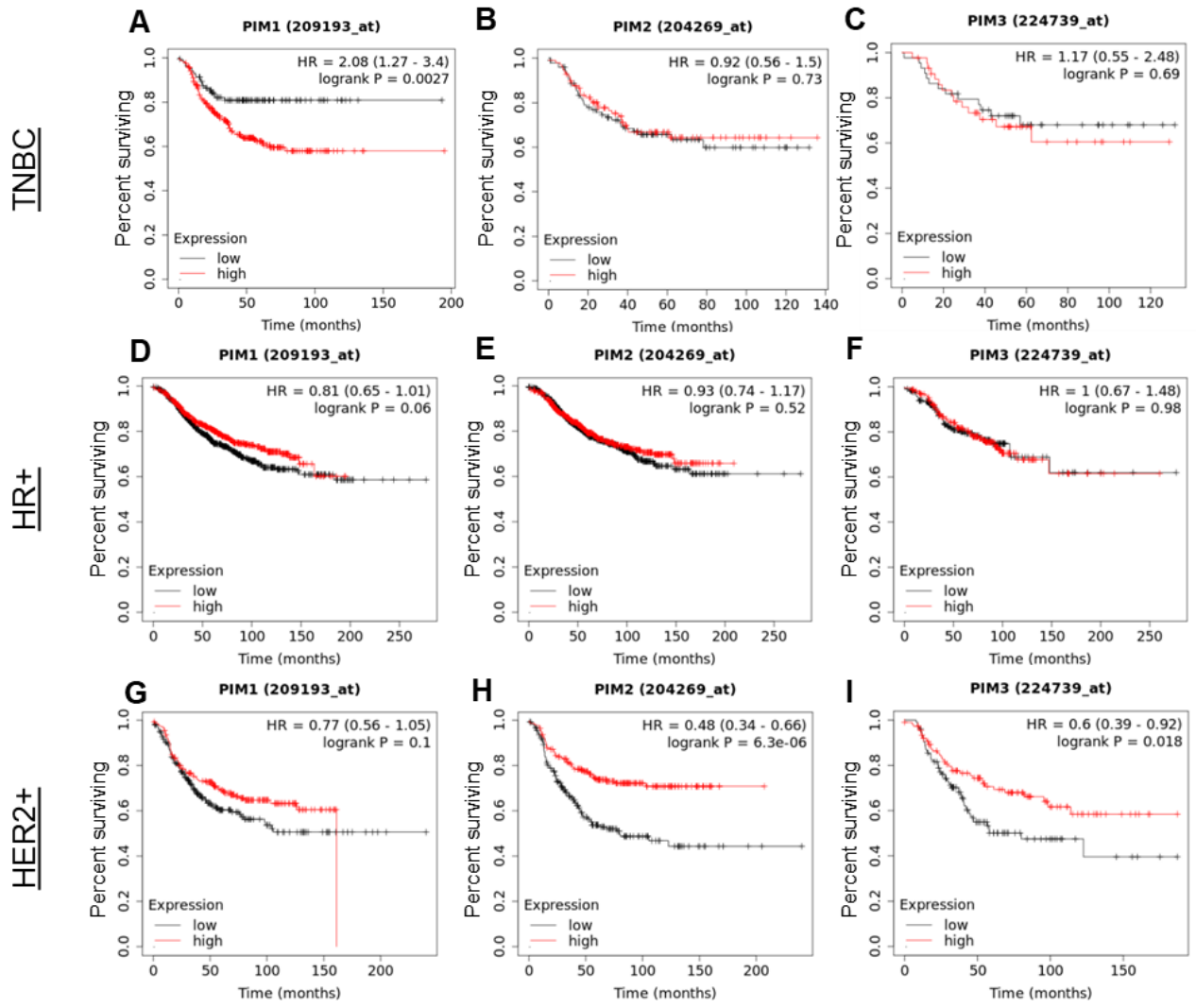

Figure 3. Kaplan-Meier survival analysis of breast cancer patients. We performed survival analysis on patient data from TCGA. Kaplan-Meier plots of A-C) TNBC patients, D-F) HR+ patients, and G-I) HER2+ patients. The high expressing group is shown in red, top 33% of patients were used for the high expressing group, the bottom 33% of patients were used for the low expressing group. (*P<0.05, **P<0.01, ***P<0.001).

Next, we evaluated PIM expression levels in breast cancer cell lines. We analyzed gene expression data from the cancer cell line encyclopedia data set. Breast cancer cell lines were grouped by receptor status, and PIM1, PIM2, and PIM3 expression was examined in these cells.

We found that PIM1 expression was significantly higher in TNBC cell lines when compared to HR+ or HER2+ cell lines (Fig. 4A). PIM2 expression was not significantly altered between the different groups (Fig. 4B), however, PIM3 expression was significantly lower in TNBC cell lines compared to HER2+ cell lines (Fig. 4C). These results indicate that the cell lines available show similar expression patterns to breast cancer patients. From this data we selected several cell lines for further experiments.

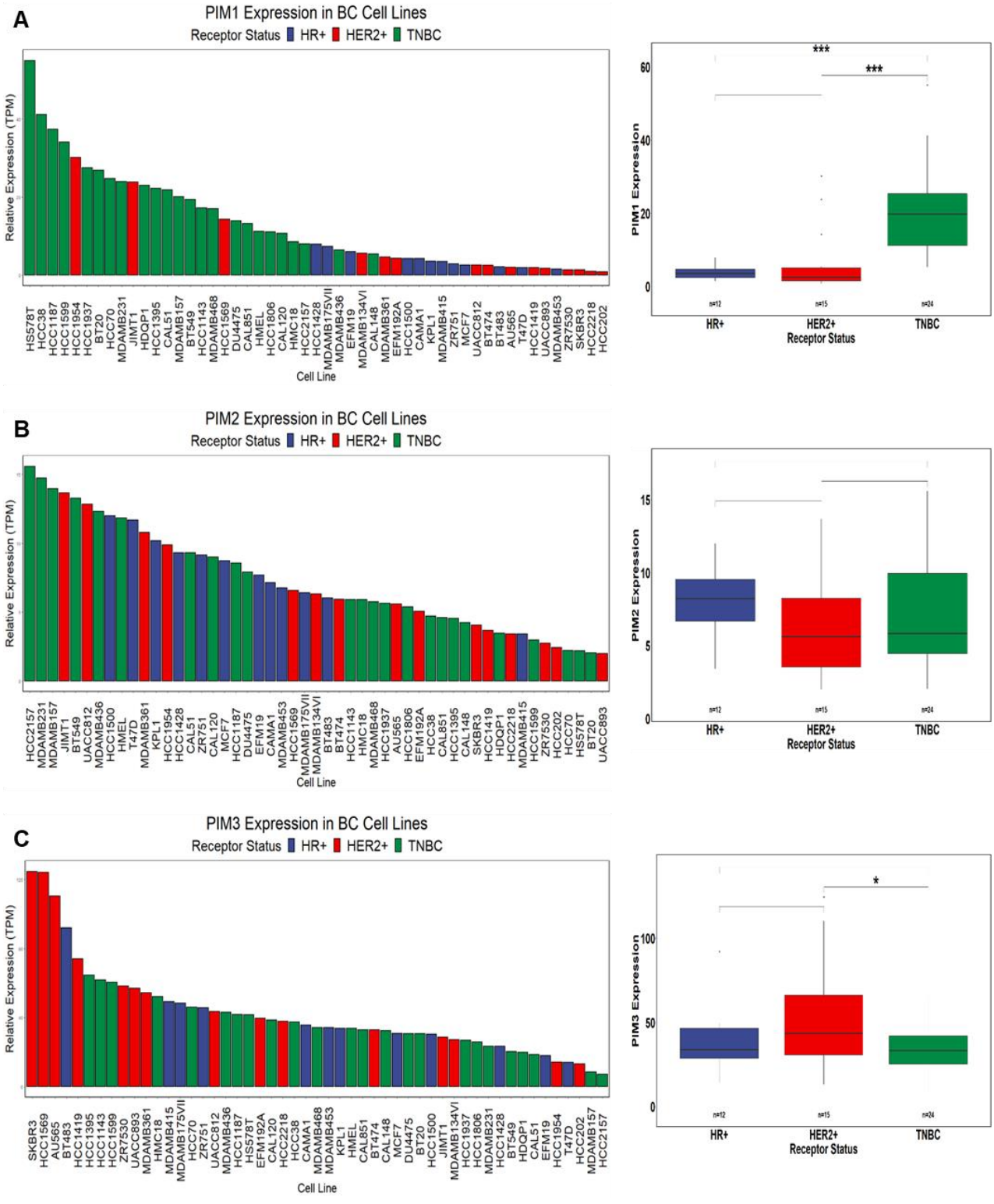

Figure 4. PIM kinase expression in breast cancer cell lines.

We performed RT-qPCR and immunoblot analysis to examine PIM1 and PIM2 expression in a panel of breast cancer cell lines. Similar to patient data, we found that PIM1 expression was increased at both the RNA and protein levels in breast cancer cell lines compared to control cell line HMEC (Fig. 5A). MDA-231, MDA-468, HS578T, BT-20, and MCF-7 cells had significantly higher PIM1 expression compared to control HMEC cells, with the highest expression in TNBC cell lines (Fig. 5A, C). PIM2 mRNA expression was also significantly higher in MDA-231 and MDA-468 cells compared to HMEC cells (Fig. 5B).

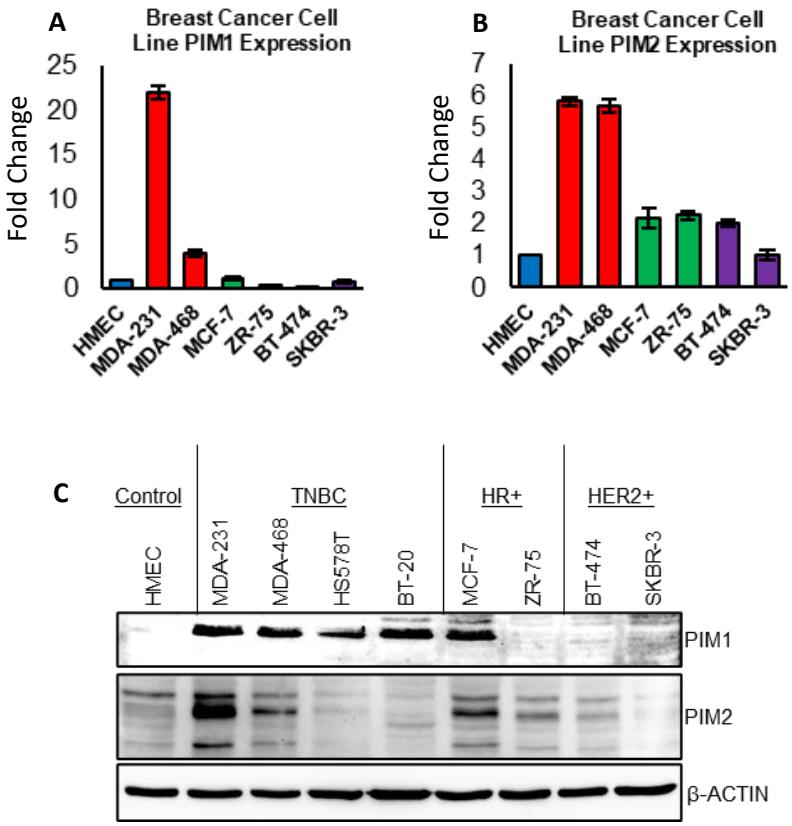

Figure 5: PIM expression in breast cancer cell lines. A) PIM1 and B) PIM2 mRNA expression was determined using RT-qPCR. GAPDH was used as an exogenous control. C) PIM1 and PIM2 expression was then examined by immunoblot. Beta-actin was used as a loading control. We found that TNBC cell lines had the highest expression of both PIM1 and PIM2.

PIM1 knockdown significantly reduces breast cancer cell growth

To study the role of PIM1 in breast cancer growth, we first knocked down PIM1 using two different shRNAs in a panel of breast cancer cell lines. Following PIM1 knockdown, cells were seeded and allowed to grow for 72 hours. PIM1 knockdown significantly reduced proliferation

of MDA-231, MDA-231-2B, MDA-468, BT-20, HCC-1806, HS578T, and MCF-7 cells (Figs. 6A-G). We also assessed the effects of PIM1 knockdown in non-tumorigenic MCF-10A breast epithelial cells and found that PIM1 knockdown modestly inhibited the MCF-10A cells (Fig 6 H), suggesting breast cancer cells are more dependent of PIM1.

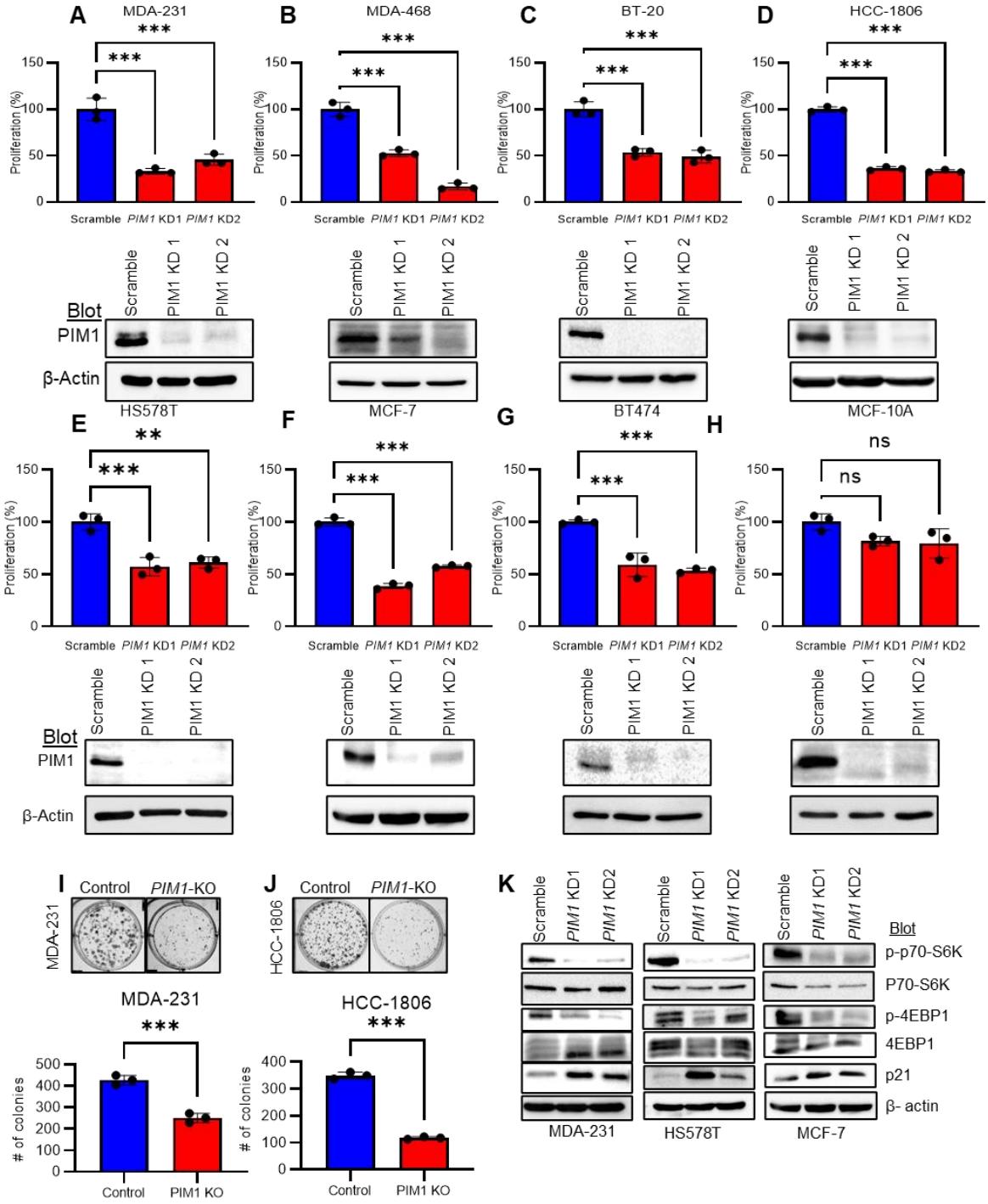

Figure 6. Knockdown of PIM1 significantly decreases breast cancer cell growth. A) MDA-231, B) MDA-468, C) BT-20, D) HCC-1806, E) HS578T, F) MCF-7, G) BT474 and H) MCF-10A cells were infected with two different PIM1 specific shRNAs. Cells were plated and allowed to grow for 72 hours. Viable cells were counted using trypan blue-exclusion and

quantified. Immunoblot analysis verifying knockdown of PIM1 are located below proliferation quantifications. Actin was used as a loading control. Colony formation assay showing PIM1 knockdown significantly impaired the colony forming ability of both I) MDA-231 and J) HCC-1806 cells. K) Immunoblot analysis showing a reduction in phosphorylation of p70-s6k and 4EBP1, and an increase in total p21 expression. (*P<0.05, **P<0.01, ***P<0.001)

We further examined the effects of PIM1 depletion on clonogenic outgrowth of breast cancer cells by performing colony-forming assays. MDA-231 and HCC-1806 cells were depleted of PIM1 using CRISPR/Cas9, and the cells were plated at a low density and allowed to grow for 7 days. We found that PIM1 deletion significantly reduced the colony forming ability of both MDA-231 (Fig 6I) and HCC-1806 cells (Fig 6J).

To get an insight into how PIM1 knockdown/depletion inhibits the growth of breast cancer cells, we performed immunoblot analysis. We observed that depletion of PIM1 reduced phosphorylation of p70S6K and 4EBP1 in MDA-231, HS578T, and MCF-7 cells (Fig. 6K). Additionally, we found that PIM1 knockdown increased the expression of cell cycle regulator p21 (Fig. 6K).

Interestingly, we found that PIM1 depletion resulted in increased expression of PIM2 (Fig. 7), suggesting the existence of a compensatory mechanism. This compensation effect was only seen in PIM1 depleted cells; PIM2 depletion, however, did not produce any changes in PIM1 expression.

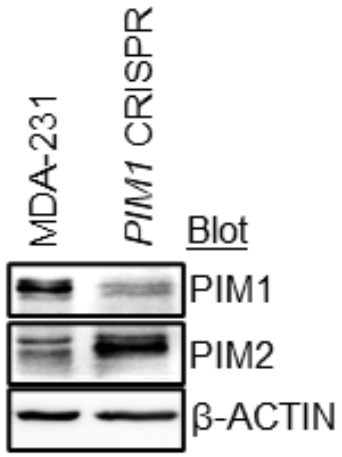

Figure 7. PIM2 expression is increased following PIM1 deletion. Immunoblot analysis of MDA-231 cells showed an increase in PIM2 expression following CRISPR mediated deletion of PIM1.

PIM2 knockdown reduces proliferation of breast cancer cells.

We found that PIM2 expression was also significantly altered in breast cancer patients. To evaluate the effect of PIM2 knockdown on the proliferation of breast cancer cells we performed similar experiments with PIM2 that we did with PIM1. We knocked down PIM2 using lentiviral shRNA in a panel of breast cancer cells and assessed the effect if PIM2 knockdown on cell growth. We found that knockdown of PIM2 only significantly reduced proliferation of MDA-468 and Hs578T cells (Fig 8). However, PIM2 knockdown did not show a significant reduction in proliferation of MDA-231 or MCF-10A cells (Fig. 8). Notably, PIM2 knockdown exhibited less inhibition of breast cancer cell proliferation compared to PIM1 knockdown (Fig. 7 and 8). This result suggests that PIM1 plays a greater role than PIM2 in breast cancer growth.

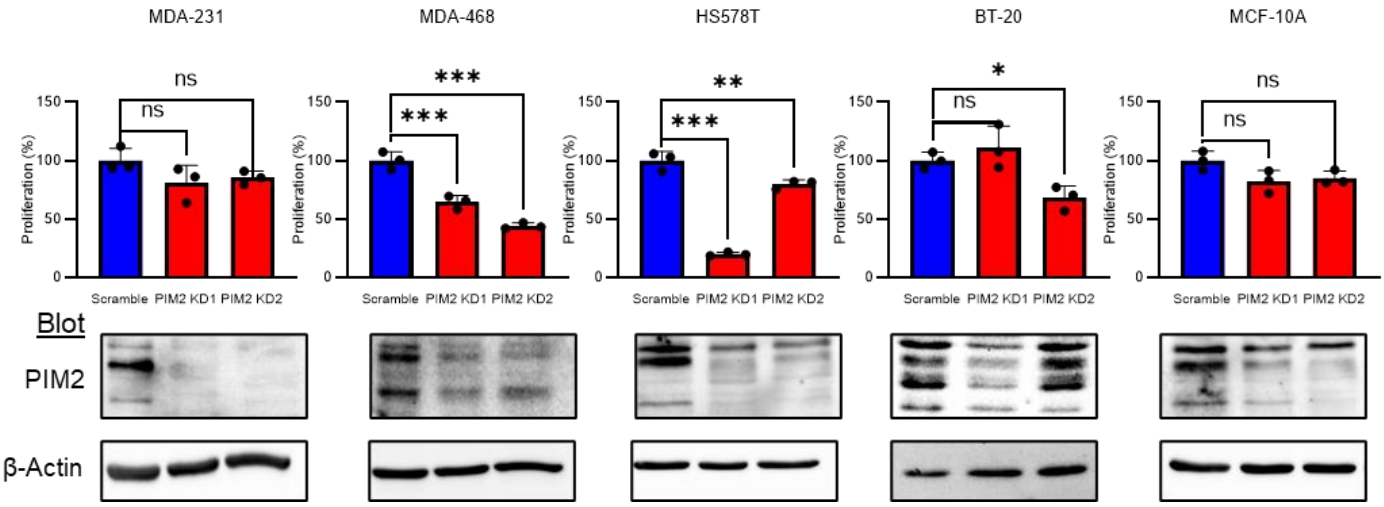

Figure 8: Knockdown of PIM2 significantly decreases breast cancer cells MDA-468 and HS578T growth. A) MDA-231, B) MDA-468, C) HS578T, D) BT-20, or E) MCF-10A cells were infected with two different PIM2 specific shRNAs. Cells were plated and allowed to grow for 72 hours. Viable cells were counted using trypan blue-exclusion and quantified. Immunoblot analysis verifying knockdown of PIM2. Beta-actin was used as a loading control.

Deletion of PIM1 significantly inhibited breast cancer cell migration, invasion, and anchorage-independent growth

Since we observed that PIM1 expression was significantly upregulated in metastatic breast cancer patients, we next examined the role of PIM1 in breast cancer cell migration and invasion. We first performed *in vitro* wound healing assays in MDA-231 (Fig 9A) and HCC-1806 (Fig 9B) cells. Following CRISPR/Cas9 mediated deletion of PIM1, cells were seeded and allowed to grow into a confluent layer. A scratch was then made using a pipette tip. The rate of scratch closing was then tracked for 18-36 hours by imaging the plate every 6 hours. PIM1 deletion significantly reduced cell migration/wound healing of both MDA-231 and HCC-1806 cell lines

(Figs. 9A-B). Next, we performed transwell migration (uncoated) and invasion (Matrigel coated) assays using highly invasive MDA-231 and HCC-1806 cells. Fourteen hours after seeding cells we stained the cells on the bottom of the transwell inserts using crystal violet. PIM1 depletion not only significantly reduced the number of migrating MDA-231 and HCC-1806 cells (Figs 9C, D), but also significantly reduced the number of invading cells through the Matrigel layer (Figs 9 E-F) suggesting PIM1 promotes breast cancer cell migration and invasion. To further examine the role of PIM1 in anchorage-independent growth (as a proxy measure of metastatic ability), we performed mammosphere assays using MDA-231 and HCC-1806 cells. The cells were seeded on ultra-low attachment plates and allowed to grow for 7-10 days, at which point they were imaged and quantified using Organoseg software (23). Mammosphere formation was significantly reduced upon PIM1 depletion in both MDA-231 and HCC-1806 cell lines (Figs. 9G-H), suggesting that PIM1 promotes anchorage independent growth and possibly metastatic ability in breast cancer cells.

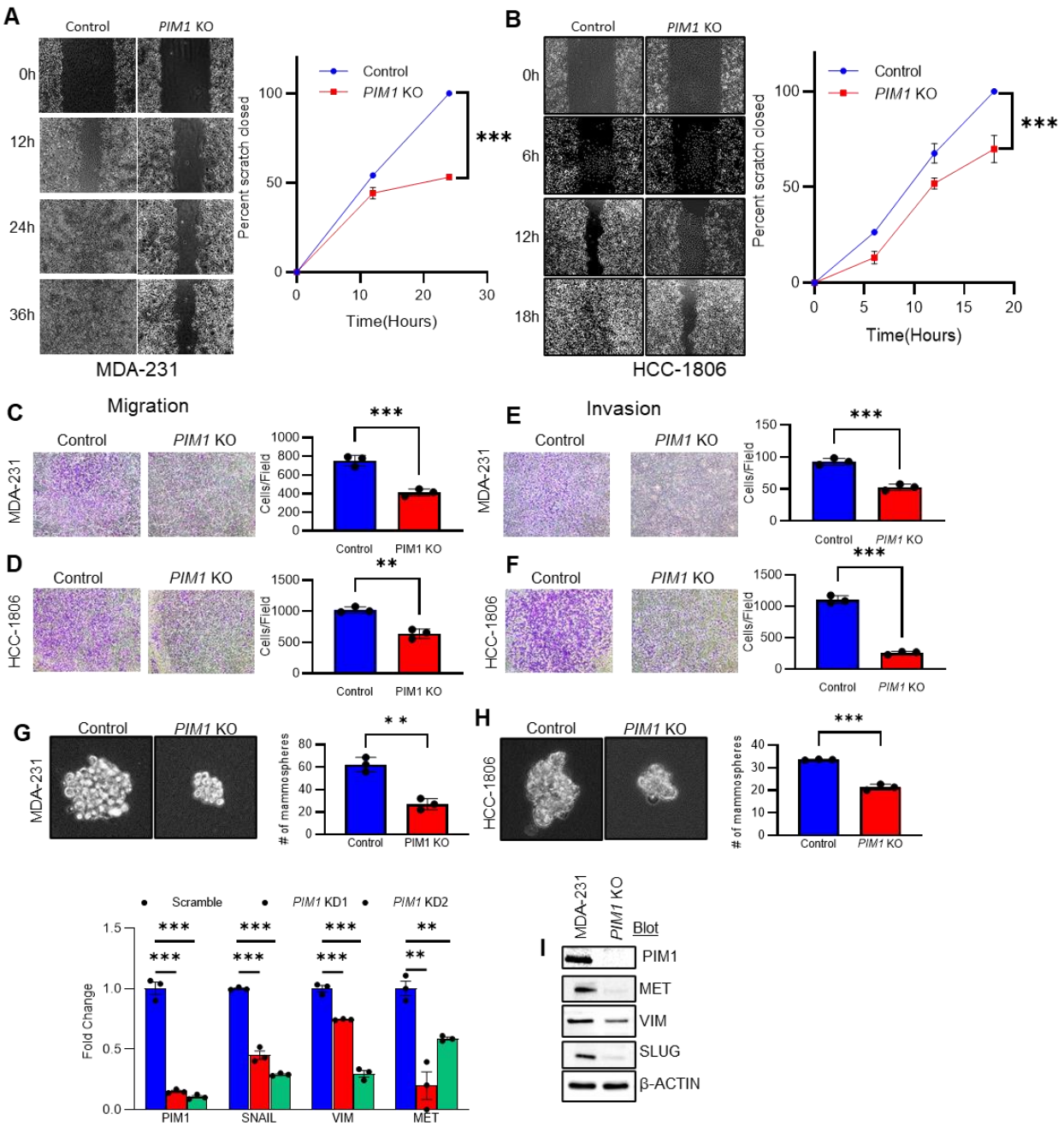

Figure 9. Knockdown of PIM1 significantly decreases breast cancer cell migration and invasion and anchorage independent growth. A) Wound-healing assay after CRISPR mediated deletion of PIM1 in A) MDA-231 and B) HCC-1806 cells showed significantly impaired cell migration. Plates were imaged every 12 hours for 36 hours. Quantification of wound closure was performed using ImageJ. Transwell assays were performed to evaluate the effects PIM1 deletion on the migration (C-D) and invasion (E-F) of MDA-231 and HCC-1806 cells. PIM1 deletion significantly reduced the migration and invasion of both cell lines. After cells were seeded on top of transwell inserts, they were fixed with cold methanol and stained

with crystal violet then counted. Mammosphere formation assays were then performed in G) MDA-231 and H) HCC-1806 cells to evaluate the effect of PIM1 deletion on anchorage-independent growth. PIM1 deletion significantly inhibited mammosphere formation and growth. I) Immunoblot analysis of EMT markers and pro-metastatic proteins following deletion of PIM1 showed a reduction in C-Met, vimentin and Slug. (* $p<0.05$; ** $p<0.005$; *** $p<0.001$).

In order to understand the mechanism of decreased cell migration and anchorage-independent growth in PIM1 depleted breast cancer cells, we performed immunoblot analysis following PIM1 deletion in MDA-231 cells. Immunoblot analysis revealed EMT markers Vimentin and SLUG, in PIM1-depleted MDA-231 cells (Fig. 9I). Additionally, deletion of PIM1 significantly reduced c-MET expression in MDA-231 cells (Fig. 9I). c-Met has been suggested to play an important role in breast cancer metastasis. Together, these results suggest that PIM1 promotes cell migration, invasion, anchorage independent growth, and EMT in breast cancer.

PIM kinase inhibition significantly reduces breast cancer cell growth

Next, we investigated the effects of PIM kinase inhibition on breast cancer cell growth. We used TP-3654, a novel second-generation PIM kinase inhibitor (39), which is more potent but less toxic than the first-generation PIM inhibitor SGI-1776 (40). TP-3654 also has a greater selectivity for PIM1 than PIM2 or PIM3 (39). We observed that treatment of TP-3654 significantly inhibited proliferation of MDA-231, MDA-468, 4T1, HCC-1806, HS578T, MCF-7, and BT474 breast cancer cells in a dose-dependent manner (Figs 10A-G). Interestingly, TNBC cell lines, which generally had higher PIM1 expression, were more sensitive to PIM kinase inhibition by TP-3654 treatment.

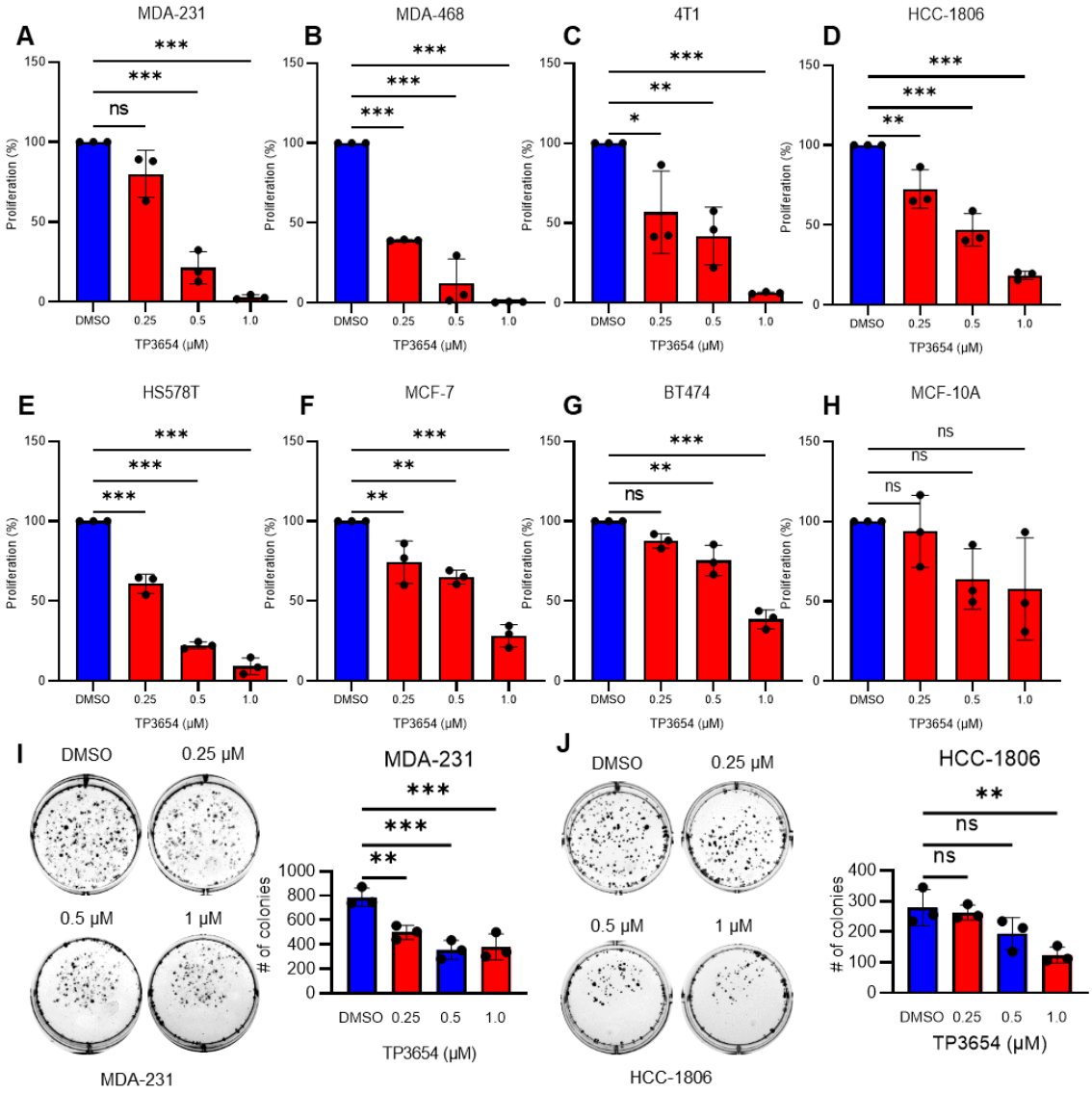

Figure 10. PIM1 inhibition with TP-3654 significantly reduces breast cancer cell growth and colony forming ability. (A-H) MDA-231(TNBC), MDA-468 (TNBC), 4T1 (Murine TNBC), BT-20 (TNBC), HD578T (TNBC), MCF7 (ER/PR+) and BT474 (HER2+), and MCF-10A (non-tumorigenic), cells were treated with varying concentrations of TP-3654 for 3 days and cell proliferation was assessed by viable cell counts. Note that treatment of TP-3654 (0.5-1 μM) significantly inhibited proliferation of MDA-231, MDA-468, 4T1, HCC-1806, HS578T, MCF-7, and BT474 cells. Non-tumorigenic MCF-10A cells were only modestly inhibited at higher concentrations. Clonogenic assays of I) MDA-231 and J) HCC-1806 cells treated with varying concentrations of TP3654 for 7 days. Assays were quantified, shown as mean +/- SD, n=3, with representative images are shown on the left. (* $p<0.05$; ** $p<0.005$; *** $p<0.001$).

We next investigated the effect of PIM kinase inhibition on clonogenic outgrowth of breast cancer cells. We plated MDA-231 and HCC-1806 cells at a low density (1,000 cells) and allowed them to grow for 7 days under varying concentrations of TP-36554. Treatment of TP-3654 significantly inhibited clonogenic outgrowth in both MDA-231 and HCC-1806 cells (Fig. 10 I-J).

We also tested the effects of TP-3654 on apoptosis of breast cancer cells using annexin V and PI staining followed by flow cytometry. We observed that TP-3654 treatment caused significant induction of apoptosis in 4T1, MDA-231, and HCC-1806 cells (Fig. 11).

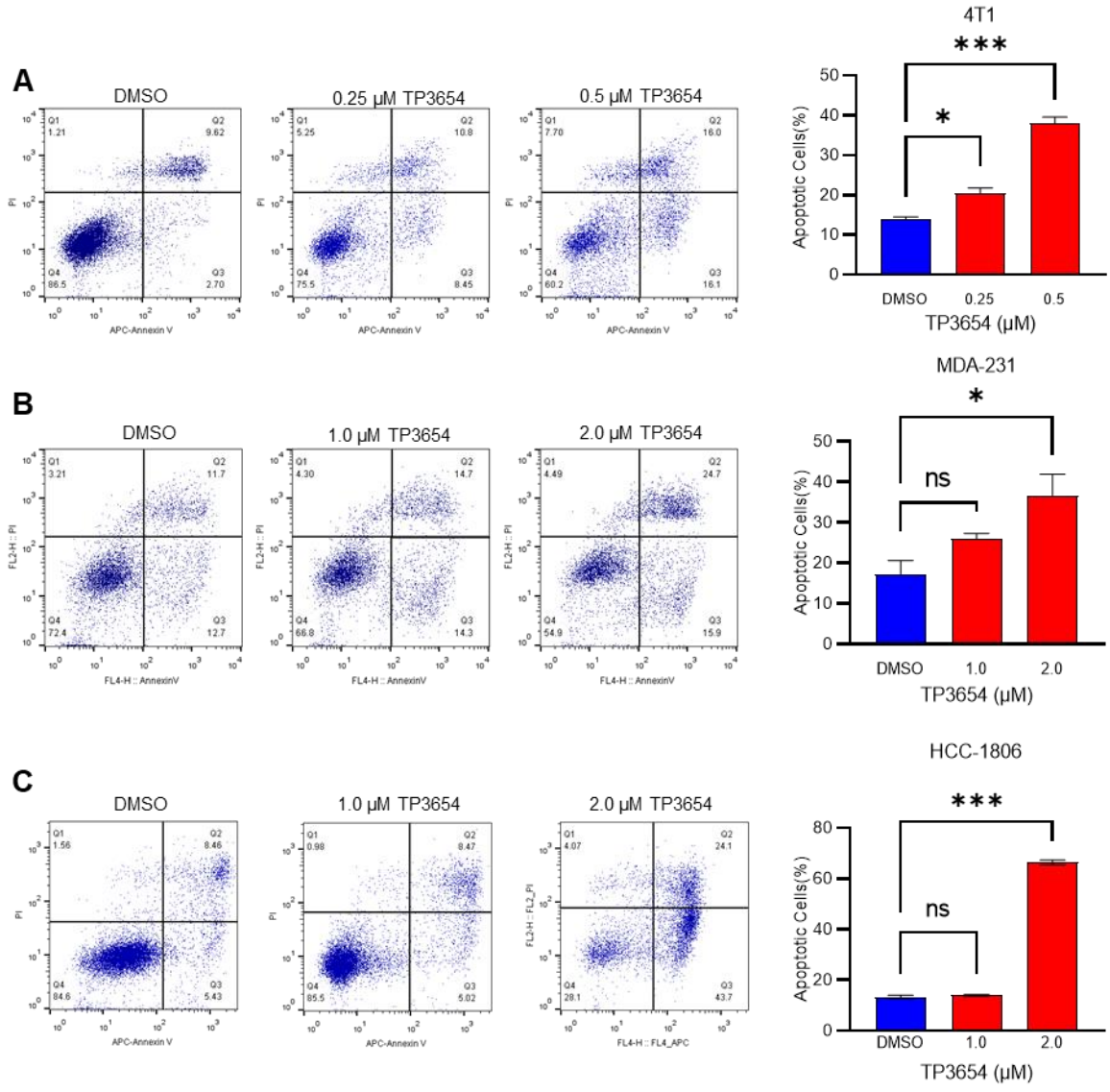

Figure 11. PIM kinase inhibition with TP3654 induces apoptosis in breast cancer cell lines. Apoptosis assays were performed in A) 4T1, B) MDA-231, and C) HCC-1806 cells. Cells were plated and treated with varying concentrations of TP3654 for 48 hours. Apoptosis was measured by PI/Annexin V staining. Values are reported as mean +/- SD, n=3. (*P<0.05, ***P<0.001)

Effects of PIM Kinase inhibition on cell cycle

We also assessed the effects of TP-3654 on cell cycle since inhibition of PIM kinase reduced breast cancer cell growth. Cell cycle analysis was performed in MDA-231 and HS578T TNBC cells by PI staining and flow cytometry following treatment of cells with TP-3654 for 48 hours. We found that treatment of MDA-231 and HS578T cells with TP-3654 resulted in a significantly higher percentage of cells in G0/G1 when compared to vehicle (DMSO) treatment (Fig. 12 A-B). We also found that HS578T cells had a significantly higher percentage of cells in G2 compared to vehicle treatment (Fig 12 B).

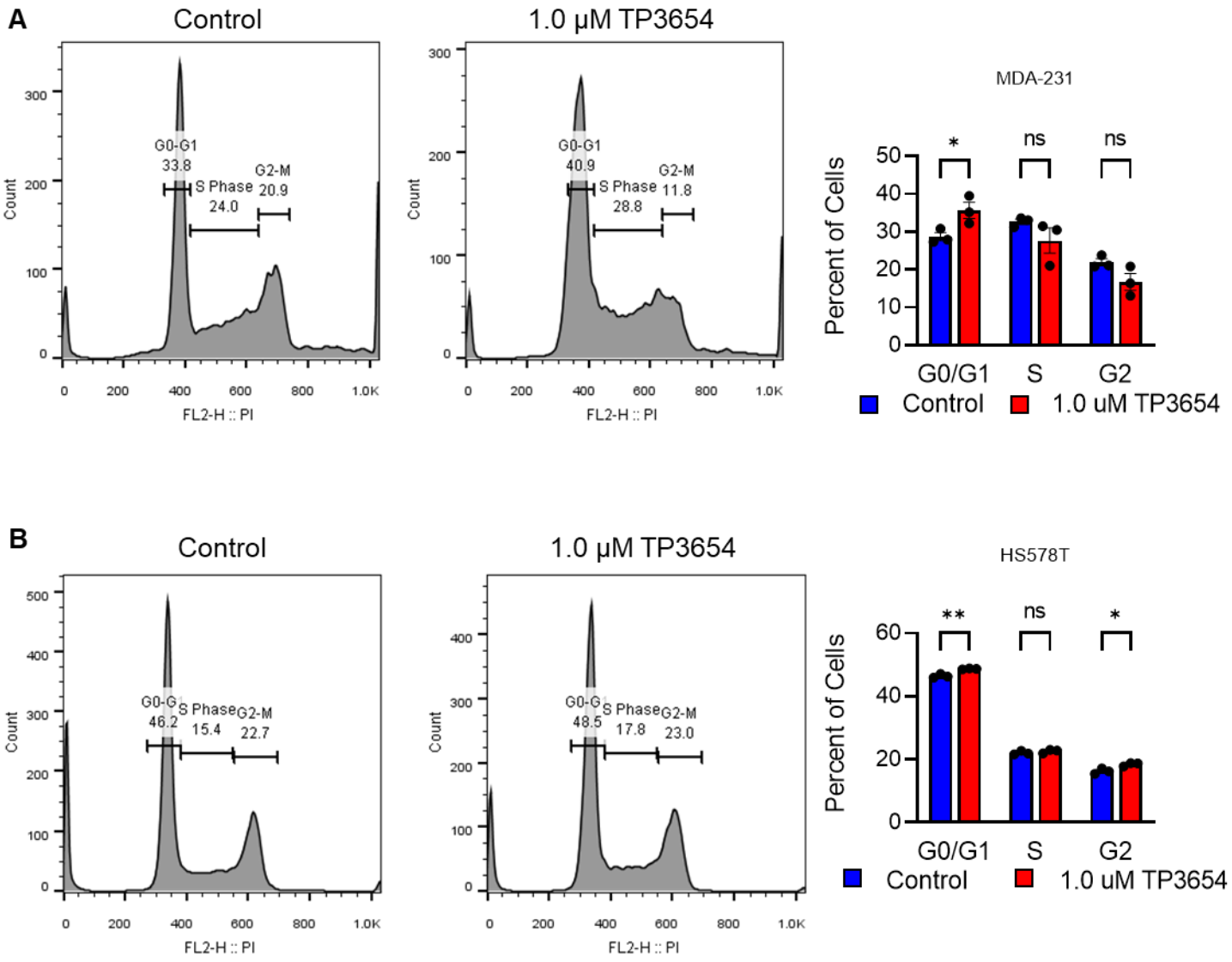

Figure 12. Effects of PIM kinase inhibition with TP3654 on cell cycle progression. PI staining was performed to evaluate cell cycle progression in A) MDA-231 and B) HS578T cells.

Inhibition of PIM kinase by TP-3654 significantly reduces breast cancer cell migration

We next investigated the effects of TP-3654 treatment on breast cancer cell migration. After seeding MDA-231 and HCC-1806 breast cancer cells and wound formation, cells were treated with vehicle (DMSO) or TP-3654 for 24 hours, and the cells were imaged every 12 hours. TP-3654 treatment significantly reduced cell migration/wound healing in both MDA-231 and HCC-1806 cells (Fig. 13 A-B).

We then examined the effect of PIM kinase inhibition by TP-3654 on mammosphere forming ability of breast cancer cells. Cells were plated in ultra-low attachment plates and allowed to grow for 7-10 days in the presence of vehicle (DMSO) or varying concentrations of TP3654. Similar to PIM1 depletion, inhibition of PIM kinase with TP-3654 significantly reduced mammosphere formation in both MDA-231 and HCC-1806 cells (Fig. 13 C-D).

To further understand the mechanism of inhibition of breast cancer cells by PIM kinase inhibitor TP-3654, we performed immunoblot analysis on MDA-231 cells after treatment with vehicle (DMSO) or TP-3654. We observed reduced phosphorylation of p70S6K and 4E-BP1, and increased expression of p21 in MDA-231 cells upon TP-3654 treatment (Fig. 13E). We also observed reduced expression of c-MET, Vimentin, and SLUG in MDA-231 cells upon TP-3654 treatment (Fig. 13 E), similar to that observed with PIM1 depletion. Therefore, it is plausible that PIM1 may contribute to breast cancer cell growth, migration, invasion, anchorage-independent growth and tumor sphere formation through modulation of these signaling molecules or pathways.

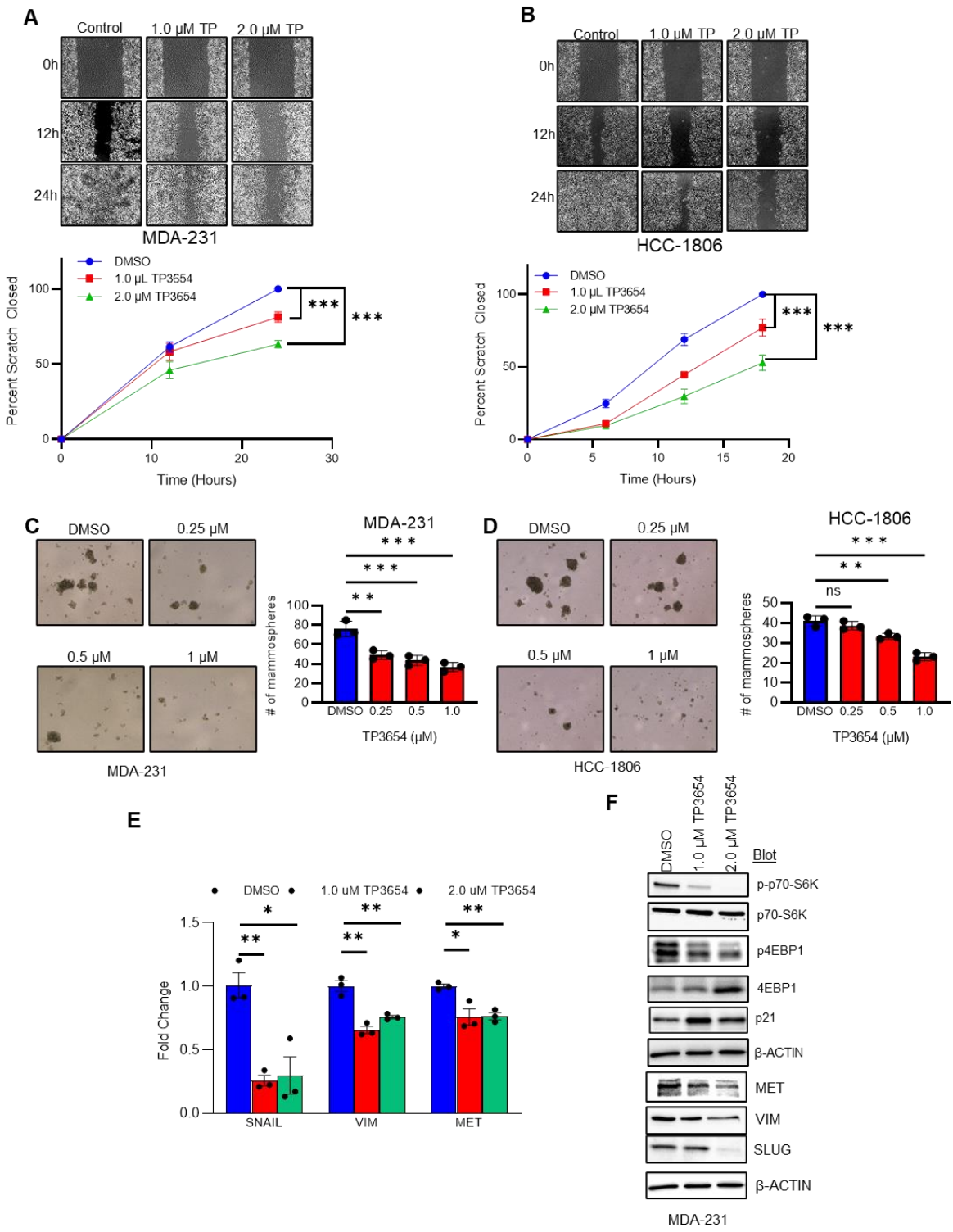

Figure 13. PIM1 inhibition with TP-3654 significantly reduces breast cancer cell migration and anchorage independent growth. Wound healing assays were performed in A) MDA-231 and B) HCC-1806 treated with varying concentrations of TP3654 to evaluate the effects of PIM kinase inhibition on the migration of breast cancer cells. Representative images are shown to the left. Wound healing assays were quantified using ImageJ, shown on the right. Mammosphere

assays were performed in C) MDA-231 and D) HCC-1806 cells treated with varying concentrations of TP3654 for 7 days to evaluate the effects of PIM kinase inhibition on anchorage independent growth. PIM kinase inhibition significantly inhibited mammosphere formation. Representative images and quantification (mean +/- SD, n=3) E) Immunoblot analysis showing a reduction in phosphorylation of p70-s6k and 4EBP1, and an increase in total p21 expression, and a decrease of EMT markers and pro-metastatic proteins C-Met, vimentin and Slug in MDA-231 cells treated with TP3654 for 48 hours. (* $p<0.05$; ** $p<0.005$; *** $p<0.001$).

PIM1/2 deletion decreases tumor growth and lung metastasis in MMTV-PyMT mouse breast cancer model

To examine the roles of PIM1 and PIM2 on breast tumor growth and metastasis *in vivo,* we crossed MMTV-PyMT mice with Pim1 or Pim2 knockout mice to generate Pim1 KO- MMTV-PyMT, Pim2 KO- MMTV-PyMT and Pim1/2 dKO- MMTV-PyMT mice. The MMTV-PyMT mouse model is an aggressive metastatic breast cancer model, with an intact immune system, in which mammary tumors develop at approximately 6 weeks of age, which readily metastasize to the lungs between 12 to 14 weeks of age (27). While MMTV-PyMT mice exhibited rapid increase in breast tumor growth, deletion of Pim1 significantly reduced tumor growth of MMTV-PyMT mice (Fig 14A). We also studied the effect of Pim2 deletion as well as Pim1/2 double deletion on breast tumor growth. We observed that Pim2 deletion also significantly reduced mammary tumor growth in MMTV-PyMT mice (Fig 14 A); However, Pim1 alone or Pim1/2 double deletion exhibited significantly greater reduction of breast tumor growth compared to Pim2 deletion alone in MMTV-PyMT mice (Fig 14A). H&E staining of the mammary gland tissues from Pim1 KO- MMTV-PyMT and Pim1/2 dKO- MMTV-PyMT mice also showed a significant decrease in tumor growth (Fig. 14B). Pim1/2 double knockout mice had much more adipose tissue present in addition to reduced tumor growth.

A major characteristic of MMTV-PyMT mice is that the tumors readily metastasize to the lungs (28). To evaluate the amount of lung metastases in these mice we sacrificed mice between 12 and 14 weeks of age and quantified the number of lung metastases per H&E-stained section. We found that deletion of Pim1 significantly reduced lung metastasis in MMTV-PyMT mice at 12 and 13 weeks of age (Fig. 14C). We also found that Pim2 deletion alone did not significantly alter lung metastasis, however Pim1/2 double deletion markedly inhibited lung metastasis in MMTV-PyMT mice at 13 weeks of age (Fig 14C). Overall, these results suggest that Pim1 plays an important role in breast cancer progression and metastasis.

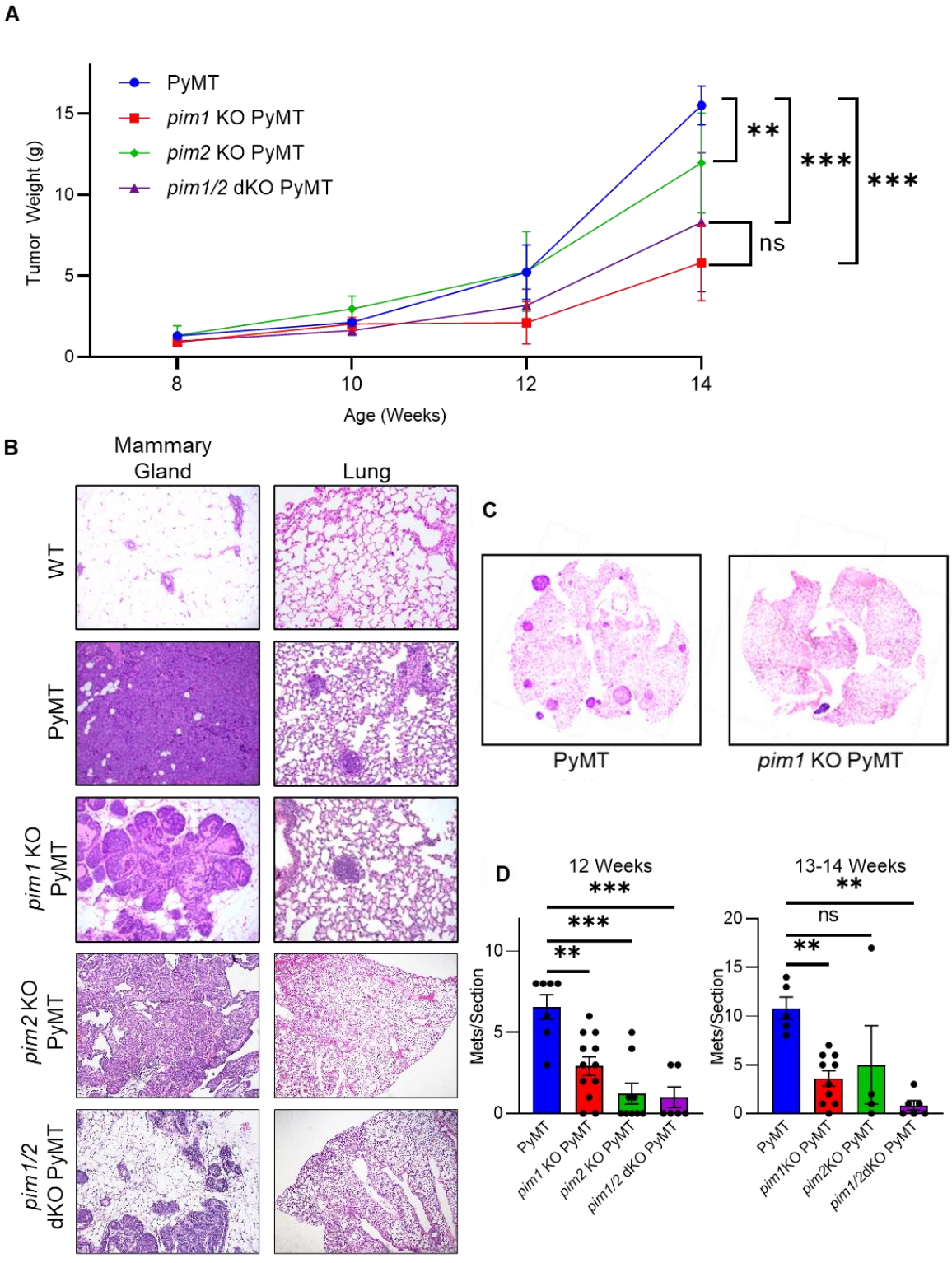

Figure 14. Deletion of PIM1 impairs tumor growth and metastasis in the MMTV-PyMT model of breast cancer. (A) Growth of PyMT and PIM1 KO PyMT tumors. Tumor growth was significantly decreased in PIM1 KO PyMT mice compared to PyMT control mice. (B) H&E

staining of mouse mammary glands and lungs at 12 weeks. (C) Representative Lung images of H&E-stained lung sections. (D) Quantification of lung metastasis. The number of lungs mets per section were counted. Pim1/2 KO PyMT mice had significantly reduced lung metastasis. Pim1 KO had significantly improved survival over PyMT mice. Significance was calculated using anova. (* p<0.05; ** p<0.005; *** p<0.001).

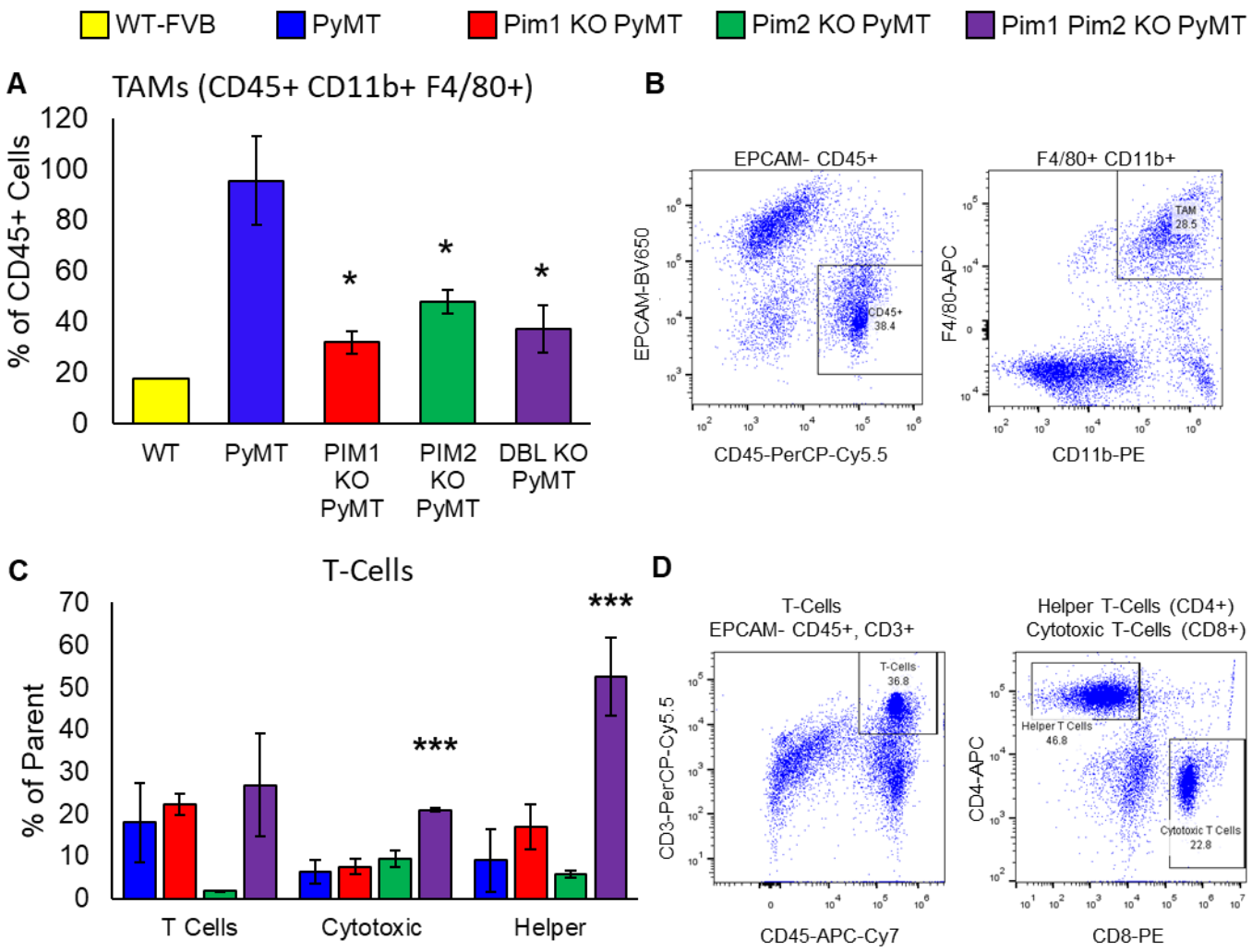

Figure 15. Effect of PIM1/2 deletion on immune invasion of MMTV-PyMT Tumors. A-B) Flow cytometry analysis of tumor immune cells reveled a significant decrease in tumor associated macrophages (TAMs) and a significant increase in both cytotoxic and helper T cells(C-D).

Tumor-associated macrophages (TAMs) are associated with poor prognosis in breast cancer due to their role in promoting immune suppression, inflammation, and metastasis (33-35). So, we assessed the effects of Pim1 deletion on TAMs in MMTV-PyMT mice. We found that Pim1 deletion significantly reduced the Tams in mammary tumors of MMTV-PyMT mice (Fig 15A). We also found that Pim2 deletion, and Pim1/2 double deletion also resulted in a significant decrease of TAMs in MMTV-PyMT mice (Fig 15A).

Conversely, T-cell infiltration of tumors is associated with a better prognosis in breast cancer, due to their ability to promote anti-tumor immune responses. We found that Pim1 and Pim2 deletion in MMTV-PyMT mice did not significantly alter T-cell infiltration of breast tumors. However, we did find that Pim1/2 double deletion resulted in a significant increase in the percentage of both CD8+ cytotoxic and CD4+ helper T cells in MMTV-PyMT mice.

Next, we examined the effects of Pim1 deletion on tumor cell signaling. Immunoblot analyses on mammary tumors showed reduced phosphorylation of p70S6K and 4E-BP1, and increased expression of p21 and p27 cell cycle regulators in Pim1 KO-MMTV-PyMT mice compared to MMTV-PyMT mice (Fig 16B). We also observed reduced expression of c-MET, Vimentin and Slug in mammary tumors of Pim1 KO-MMTV-PyMT mice compared to MMTV-PyMT mice (Fig. 16B). RT-qPCR analysis also showed significantly reduced mRNA expression of EMT markers, Snail, Vimentin and c-MET, in Pim1 KO-MMTV-PyMT mice compared to MMTV-PyMT mice (Fig. 16A). Taken together, these results suggest that PIM1 plays an important role in breast tumor growth and metastasis.

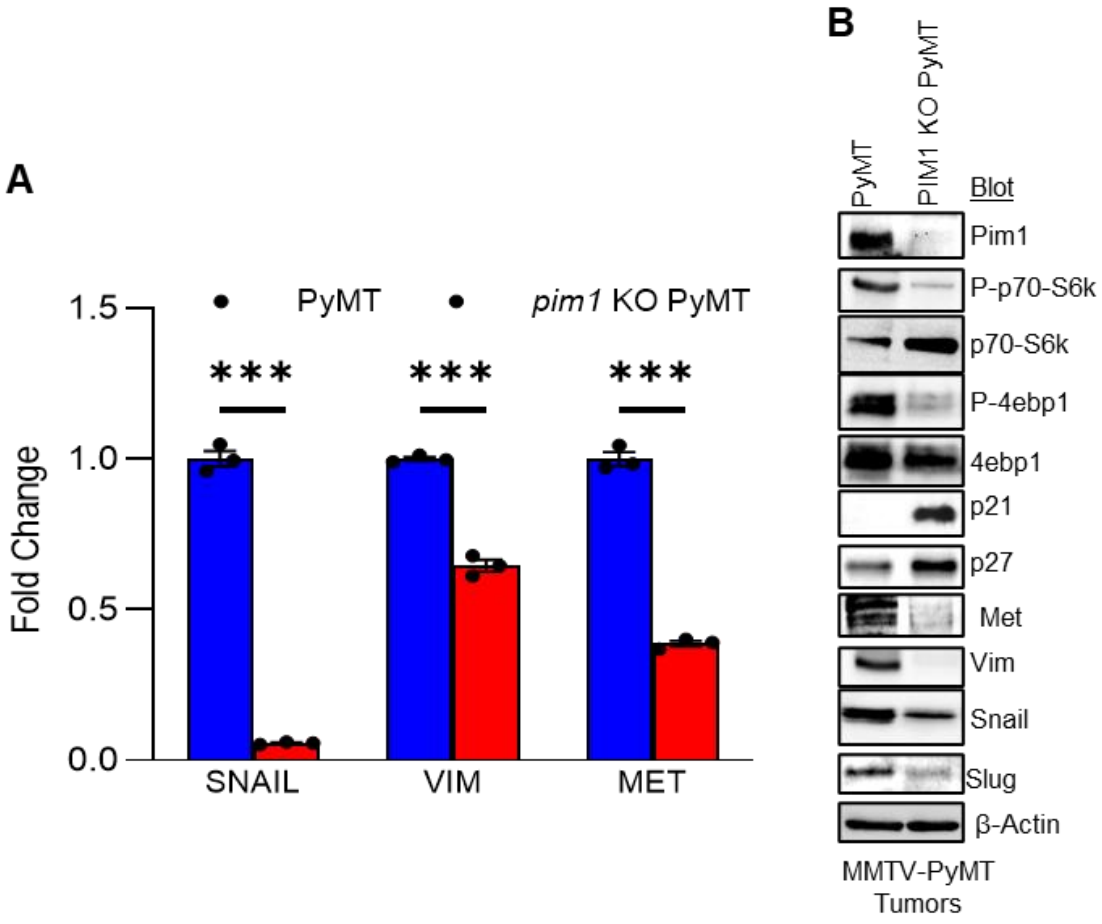

Figure 16. Effect of PIM1 KO on PyMT Tumor signaling. A) RT-qPCR analysis of PyMT tumors revealed PIM1 KO significantly decreased mRNA expression of SNAIL, Vimentin, and Met. B) Immunoblot analysis of PyMT tumors revealed decreased phosphorylation of p70s6K and 4EBP1. Total expression of p21 and p27 was increased. Total expression of Met, Vim, Snail, and Slug were decreased. Actin was used as a loading control.

PIM kinases have previously been shown to phosphorylate CXCR4, which stabilizes the protein in the membrane, increasing its overall expression and activity. CXCR4 has been suggested to promote metastasis. Since Pim1/2 deletion decreased lung metastasis in MMTV-PyMT mice, we also examined CXCR4 expression by immunoblotting. We found that PyMT mice had

significantly higher surface expression than both Pim1 KO PyMT mice and Pim2 KO PyMT mice. We also found that Pim1/Pim2 double knockout PyMT mice had greater reduction of CXCR4 expression (Fig. 17).

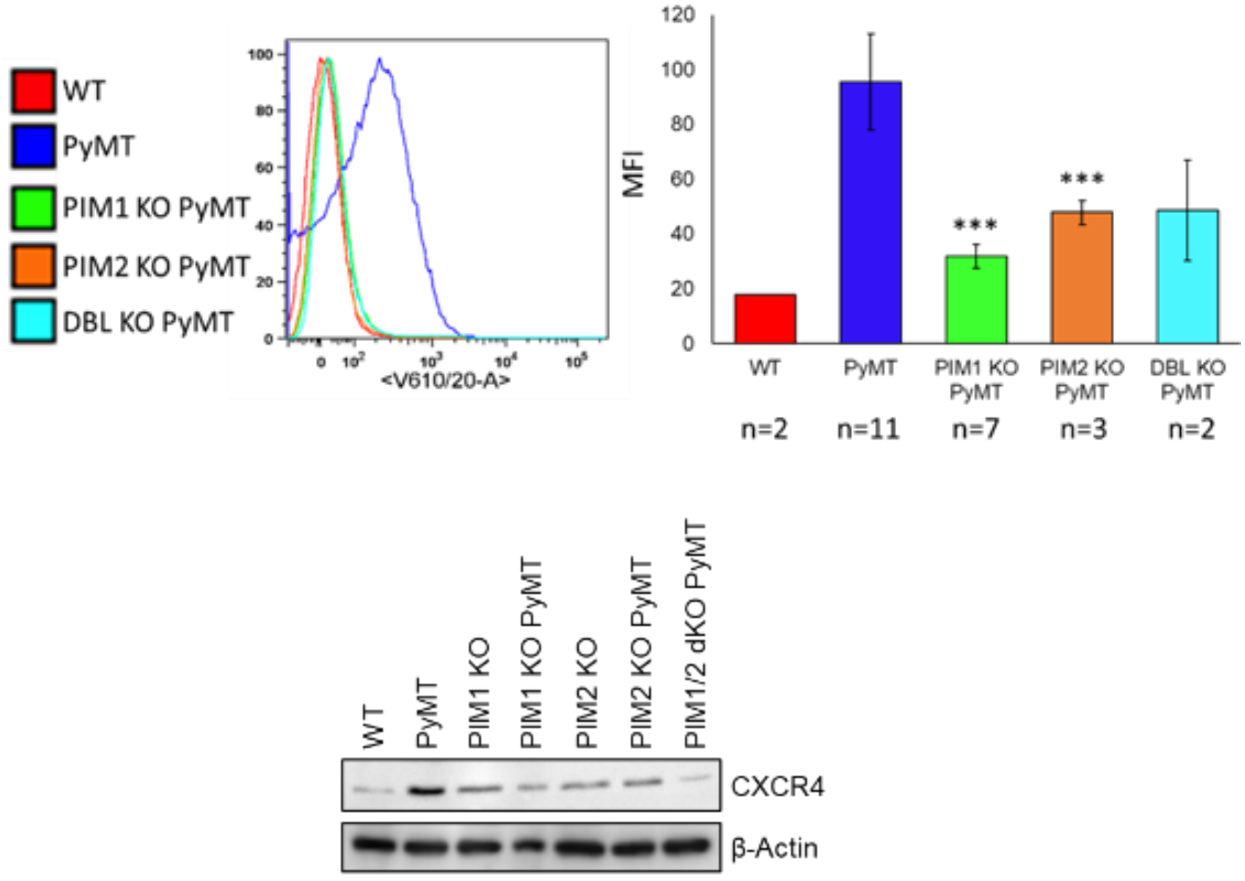

Figure 17. CXCR4 expression in MMTV-PyMT mouse mammary gland tumors. 12 week old PyMT mice were sacrificed and mouse mammary gland tumors were dissociated into single cell suspension and CXCR4 expression was evaluated by A) flow cytometry and B) western blot analysis.

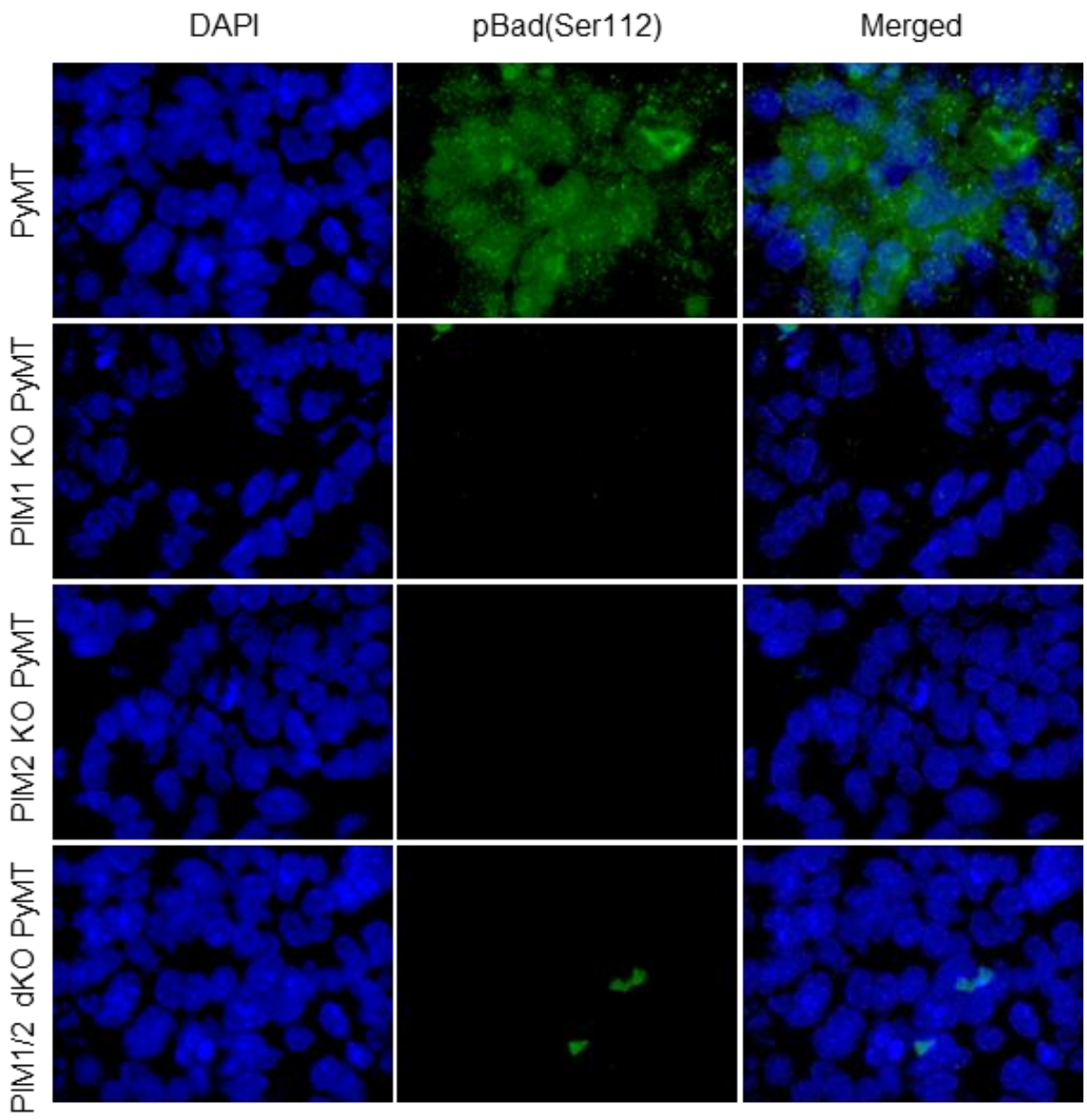

Figure 18. Effect of PIM1/PIM2 deletion BAD Phosphorylation at Ser112 in PyMT Mouse Tumors. 10-week-old MMTV-PyMT mouse tumor sections were stained using a primary antibody for pBAD (Ser112) and a goat-anti-rabbit secondary antibody conjugated to TRITC(Green). The nucleus was stained using DAPI (Blue). Images were obtained using an Olympus BX-51 epi-fluorescent microscope at 1000X magnification. Upon PIM1/2 deletion pBAD (Ser112) is dramatically decreased in PyMT tumors

We additionally examined the effects of PIM1/2 deletion on BAD phosphorylation by immunofluorescence. Tumors were cryosectioned and stained using a Ser112 phospho-BAD specific antibody and DAPI. We found that Pim1 deletion, Pim2 deletion, and Pim1/2 double deletion all decreased the levels of BAD phosphorylation in PyMT tumors. BAD is an important positive regulator of apoptosis, and a reduction in phosphorylation of Ser112 suggests Pim1/2 kinases play an role in preventing apoptosis of breast tumors.

Inhibition of PIM kinase by TP-3654 significantly reduces tumor growth and metastasis

To evaluate the effect of PIM kinase inhibition on breast cancer progression and metastasis we treated MMTV-PyMT mice with TP-3654. MMTV-PyMT mice at 6 weeks were treated with TP-3654 (200 mg/kg) daily via oral gavage for 6 weeks. Tumor size and mouse weight were monitored during the treatment. At 12 weeks of age the mice were euthanized, tumors and vital organs were then harvested to evaluate tumor progression and metastasis.

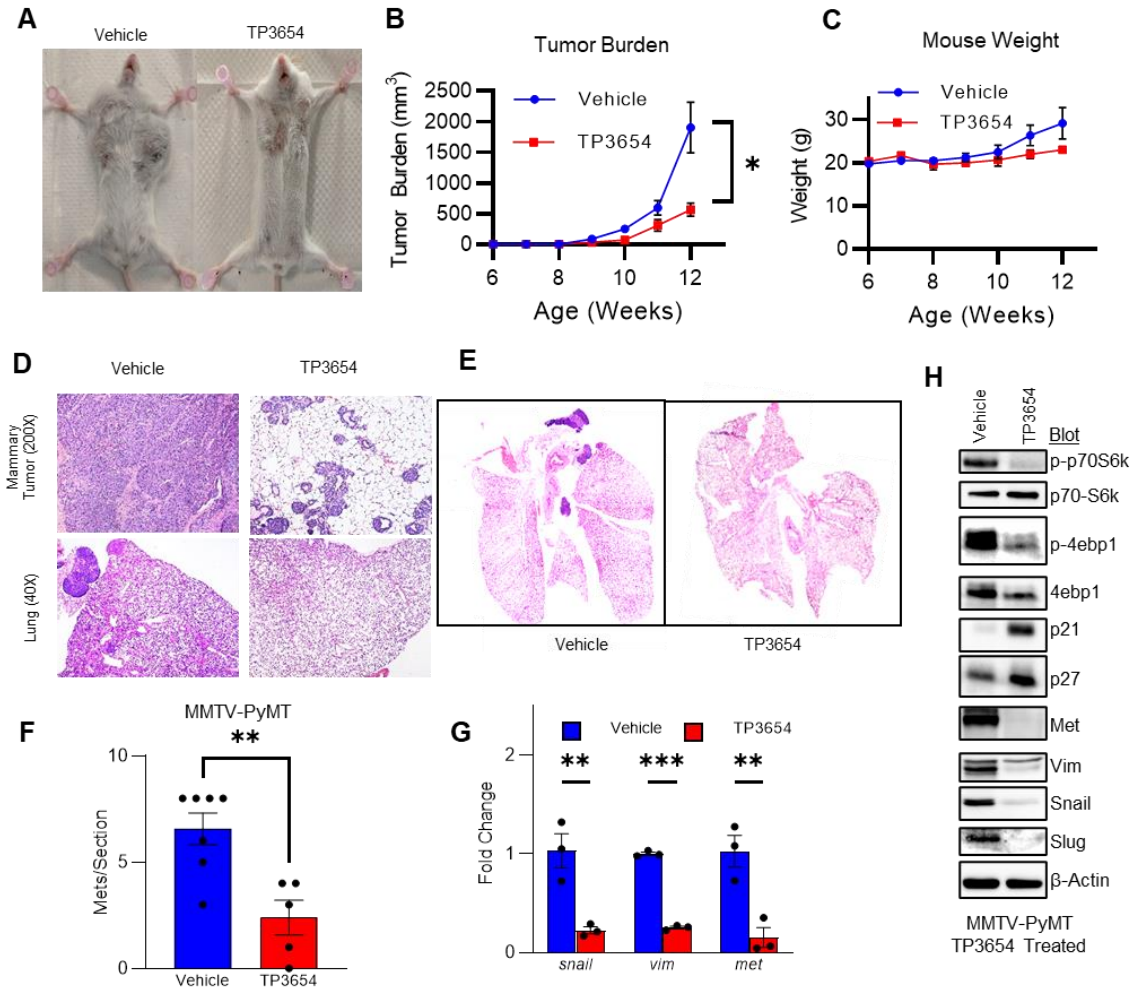

Figure 19. Inhibition of PIM1 with TP-3654 decreases tumor growth and lung metastasis of MMTV-PyMT mice. Mice were treated for 6 weeks with the PIM kinase inhibitor TP3654 (200mg/kg orally). A) Representative images of MMTV-PyMT and TP3654 treated mice at the end of treatment. B) Tumor growth of MMTV-PyMT and TP3654 treated mice (n=5 for each group). Tumor burden was calculated by measuring the length and width of the tumors each week C) Mouse weight over the course of treatment. D) H&E staining of tumor and lung sections. E) Representative images of mouse lungs following 6 weeks of treatment. F) Quantification of lung metastases. G) RT-qPCR analysis of tumors following treatment reveled SNAIL, VIM, and Met were significantly decreased. H) Immunoblot analysis of tumors following TP3654 treatment. Significance was calculated using Student's T-test. (* $p<0.05$; ** $p<0.005$; *** $p<0.001$).

We found that treatment of TP-3654 significantly inhibited tumor growth, without causing any significant decrease in the mouse weight (Fig 19A-C). Histological analysis of tumors also

showed that treatment of TP3654 significantly reduced tumor progression in MMTV-PyMT mice (Fig 19D). We also monitored the blood parameters of the mice and collected other vital organs and observed no significant side effects from the drug (data not shown). Treatment of TP-3654 significantly reduced mammary tumor growth and lung metastasis was markedly decreased in TP-3654 treated MMTV-PyMT mice (Fig. 19 E-F). RT-qPCR analysis showed significantly reduced mRNA expression of Snail, Vimentin and c-Met in TP-3654 treated MMTV-PyMT mice (Fig. 19G). We also performed immunoblot analysis on these mice and found a reduction in p70-S6K and 4EBP1 phosphorylation, increase in p21 expression, and a decrease in c-MET, SLUG, and Vimentin expression (Fig 19H) similar to that observed in Pim1 KO-MMTV-PyMT mice. Thus, the PIM kinase inhibitor TP-3654 reduces tumor growth and metastasis by inhibiting mTORC1 signaling, negatively regulating the cell cycle, and preventing EMT, a necessary step in breast cancer metastasis. Taken together, these results suggest that inhibition of PIM kinase by TP-3654 could be useful for the treatment of breast cancer.

Effects of combining TP3654 with Chemotherapies

We also evaluated if TP-3654 could be combined with currently available treatments to produce an even greater effect. TNBC patients receive a combination of a taxane, including paclitaxel or docetaxel, an anthracycline, such as doxorubicin, and a platinum agent, including cisplatin or carboplatin. We treated TNBC cell lines with TP-3654 in combination with these chemotherapy drugs.

First, we evaluated the single chemotherapy agents against TNBC cell growth. We tested cisplatin, doxorubicin, and docetaxel in MDA-231 cells. 100,000 cells were seeded in 6 well

plates in DMEM +2% FBS and allowed to grow in the presence of drug for 3 days. Viable cells were counted using trypan blue exclusion. We found that individual treatment resulted in a significant inhibition of growth, with IC50 values of 2.2 μM, 100.3 nM, and 1.9 nM for cisplatin, doxorubicin, and docetaxel respectively (Fig 20 A-C).

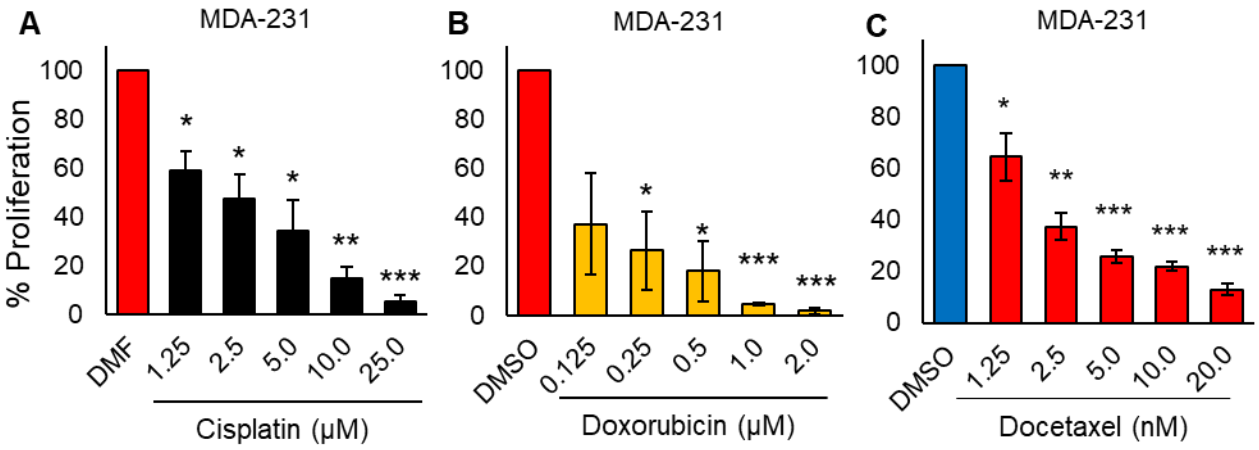

Figure 20. Effects of chemotherapy drugs in MDA-231 TNBC cells. Cells were plated and allowed to adhere overnight. The cells were then maintained in DMEM + 2%FBS and corresponding drug concentrations. Viable cells were harvested and counted by trypan blue exclusion. A) Cisplatin, B) Doxorubicin, and C) Docetaxel all significantly inhibited MDA-231 cell growth.

We then examined the effects of combined treatment of TP-3654 with these various chemotherapy drugs. We utilized TNBC cell lines MDA-231 and their highly metastatic subclone MDA-231-2B cells. Cells were treated with TP-3654 alone or in combination with doxorubicin or cisplatin for 72 hours and cell viability was measured using presto-blue HS.

We observed that treatment of TP-3654 alone significantly reduced proliferation of MDA-231 and MDA-231-2B cells in a dose-dependent manner (Fig. 21 A, B). Notably, highly metastatic MDA-231-2B cells were more pronouncedly inhibited by TP-3654 treatment than parent MDA-

231 cells with an IC50 value of 0.71 µM for MDA-231-2B cells and 1.3 µM for parent MDA-231 cells (Fig. 21A, B). Combined treatment of TP-3654 with doxorubicin or cisplatin resulted in significantly greater reduction of proliferation in MDA-231 and MDA-231-2B cells when compared to single drug treatment (Fig 21 C-F).

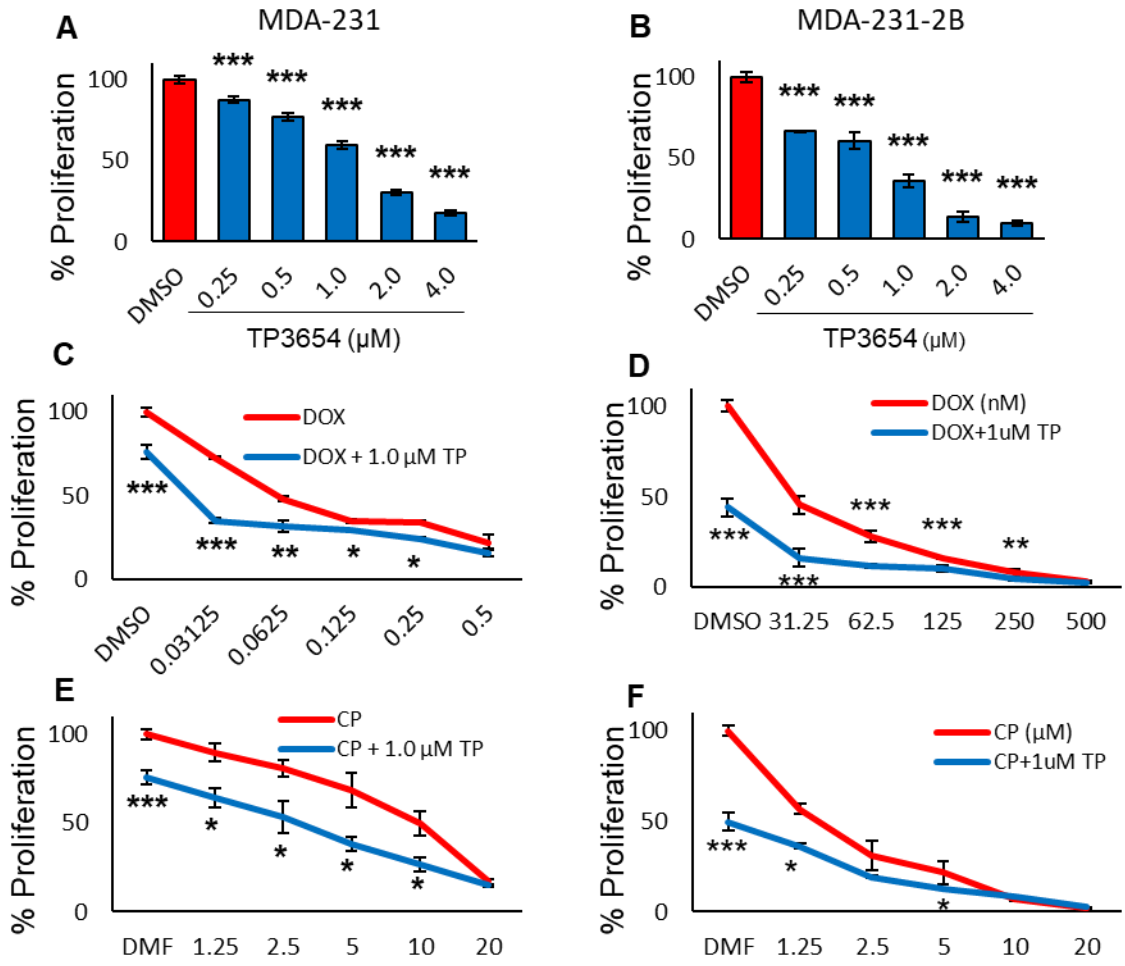

Figure 21. Combination treatment of TP3654 and chemotherapies in MDA-231 and MDA-231-2B cells. Cells were plated and allowed to adhere overnight. The cells were then maintained in DMEM + 2%FBS and corresponding drug concentrations. Viable cells were quantified using PrestoBlue-HS. TP3654 treatment in MDA-231 and MDA-231-2B cells resulted in significant decreases in proliferation. Combination treatment of 1.0 µM TP3654 and varying concentrations of C-D) Doxorubicin, and E-F) Cisplatin further significantly inhibited MDA-231 and MDA-231-2B cell growth when compared to single treatment alone.

Combination of TP3654 and doxorubicin or cisplatin resulted in further decreases in proliferation than single drug alone. This suggests that combination of these drugs may improve patient responses. To further evaluate these drug combinations, we then tested for synergy using COMPUSYN software, which utilizes the CI method for calculating synergy. If a CI is less than 1, the combination is considered synergistic, where if it is greater than 1 it is considered antagonistic. CI values equal to 1 indicate an additive effect.

We found that combination of TP3654 with both doxorubicin and cisplatin in MDA-231 cells produced a synergistic effect (Fig 22 A-B). We also found that in MDA-231-2B cells combination of TP3654 with doxorubicin produced a synergistic effect (Fig 22 C). Unexpectedly, we found that cisplatin and TP3654 combined treatment did not produce a synergistic effect, and instead it produced an antagonistic effect (Fig 22 D). These results suggest that TP3654 may work in combination with current therapies, however, further work must be done to further evaluate which drugs can be combined.

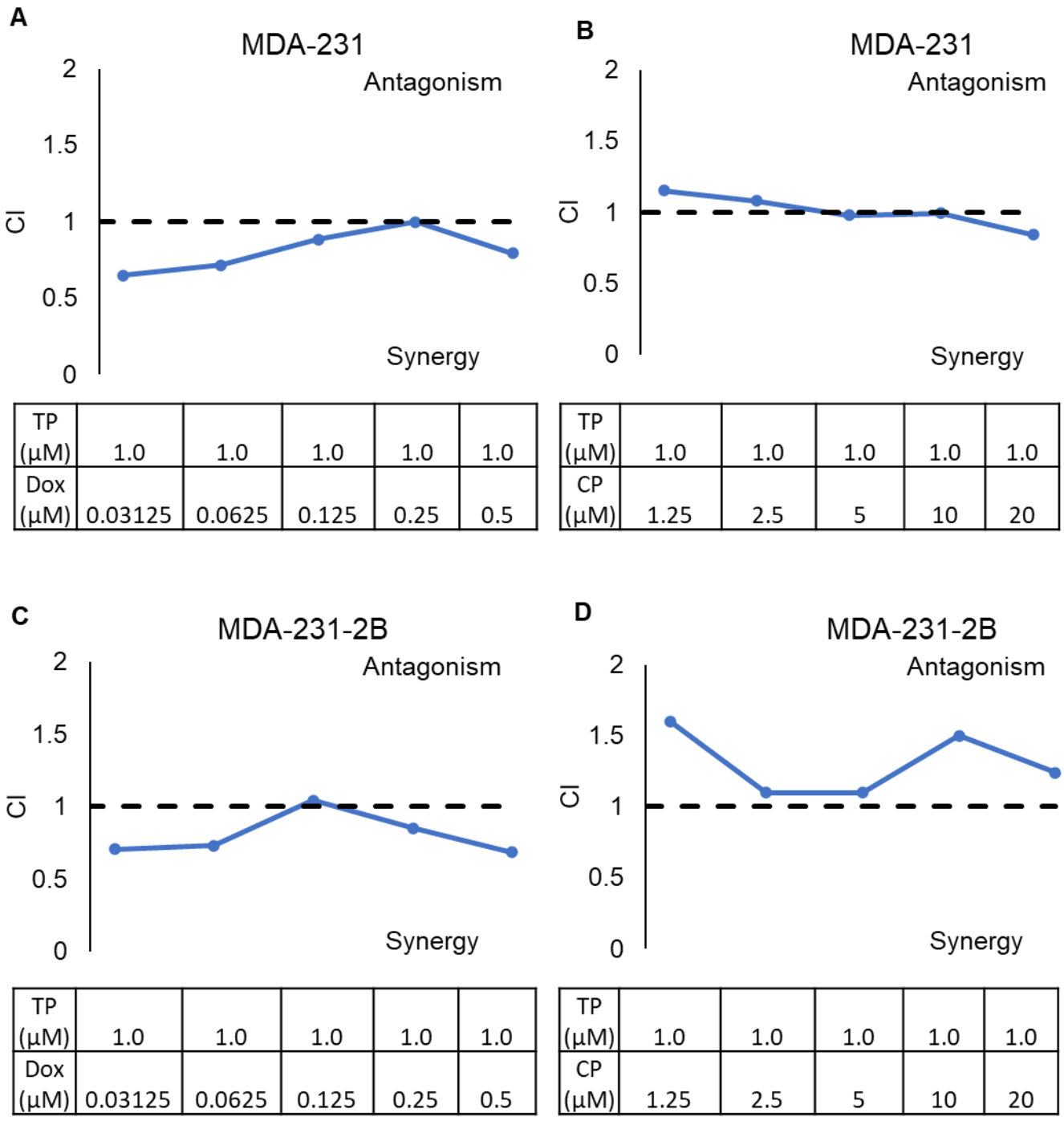

Figure 22. Formal synergy assay of TP3654 and doxorubicin/cisplatin combination. CI values were calculated from cell line proliferation data using CompuSyn software. MDA-231 cells treated with a combination of TP3654 and A) doxorubicin or B) cisplatin produced a synergistic effect. Combination of TP3654 with doxorubicin also produced a synergistic effect in MDA-231-2B cells (C), however combination with cisplatin resulted in an antagonistic effect (D).

In order to further study the in vivo effects of drug combination on breast tumor growth and metastasis we performed MDA-231-2B xenograft experiments. NSG mice were injected with $1x10^7$ cells, and tumors were allowed to reach 0.5 cm in width. After primary tumors were established in NSG mice, we treated these mice with placebo (vehicle), TP-3654, doxorubicin and TP-3654 plus doxorubicin for 5 weeks. TP-3654 was given at a dose of 200 mg/kg via oral gavage daily. Doxorubicin was administered at a dose of 2.5 mg/kg once per week via i.p. injection. Tumor growth and mice weight were monitored twice per week during the course of the treatment.

We found that treatment of TP-3654 or doxorubicin alone significantly reduced tumor growth in MDA-231-2B xenograft mice (Fig 23A). Combined treatment of TP-3654 plus doxorubicin resulted in greater reduction of tumor growth compared to vehicle treatment or doxorubicin treatment alone (Fig. 23A). The combination, however, was not significantly reduced when compared to TP-3654 treatment (Fig. 23A).

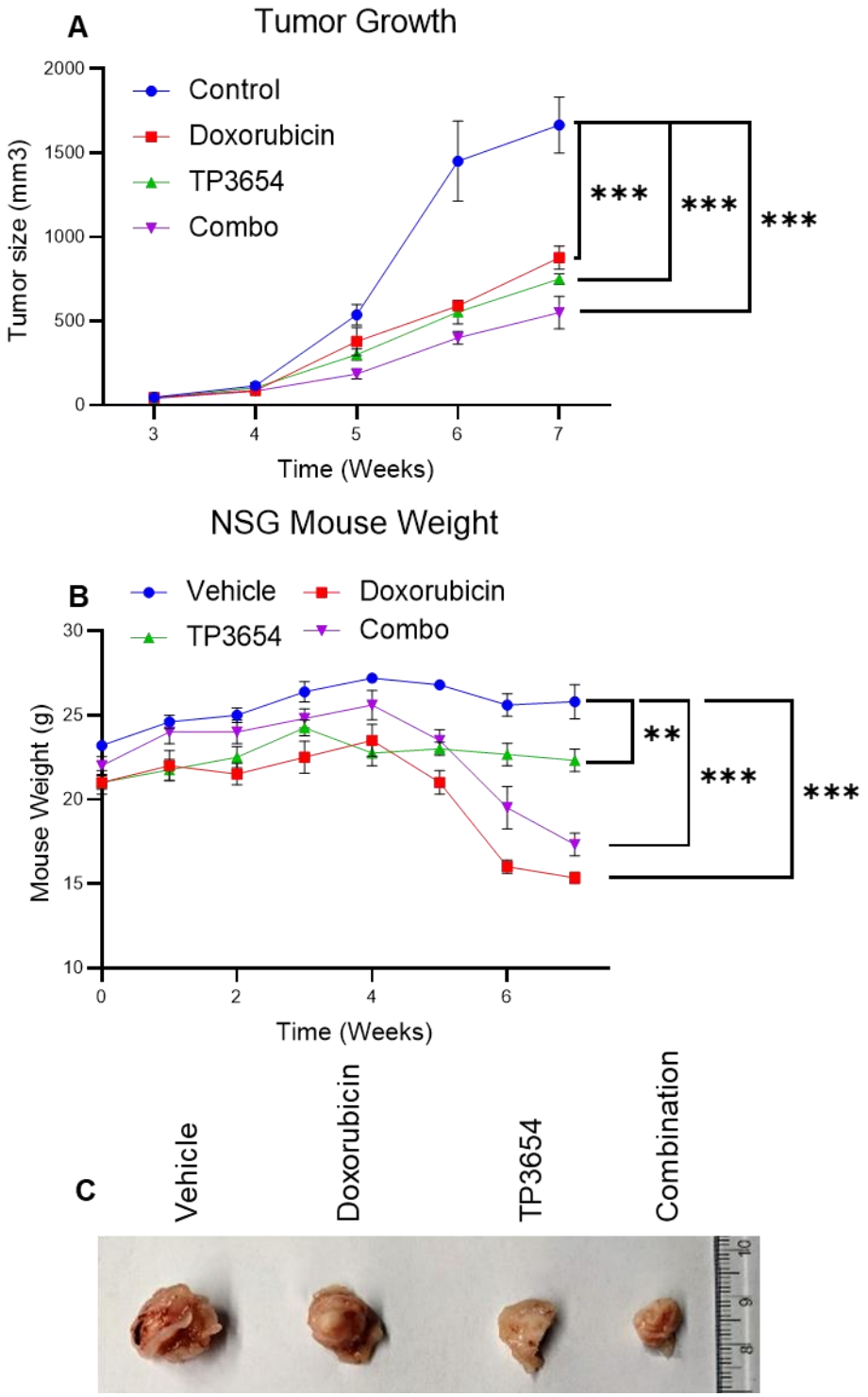

Figure 23. TP3654 and doxorubicin treatment in MDA-231-2B NSG mouse xenografts. NSG mice were injected with MDA-231-2B cells. Treatment started after tumors reached 0.5 cm in width. Treatment groups include vehicle, doxorubicin (2.5 mg/kg, weekly), TP3654 (200 mg/kg, daily), and combination. A) Tumor growth was monitored as well as B) mouse weight. C) Representative image of tumor size.

We also monitored the mouse weight during the course of the treatment as an indication of potential side effects. We found that mice treated with doxorubicin or combination lost a significant amount of weight (Fig 23B). TP3654 treated mice also exhibited significant weight loss, but it was to a much lesser extent than the mice receiving doxorubicin. These results indicate that doxorubicin has severe side effects, and a lower dosage may be needed in future experiments.

RNA-seq analysis revealed multiple pathways contributing to growth and metastasis

To gain further insights into the mechanisms by which PIM1 reduces breast cancer cell growth and metastasis, we performed RNA-seq analysis on MDA-231 cells that were PIM1 deleted or treated with TP-3654 for 48 hours. PCA analysis revealed separate clustering for each treatment group (Fig. 24 A). Volcano plots for each group were produced using DESeq2 (Fig 24 B-C). Gene set enrichment (GSEA) analysis revealed significant enrichment of genes related to translation elongation, mammary stemness, proliferation, metastasis, and EMT in control MDA-231 cells compared with PIM1 deleted or TP-3654 treated MDA-231 cells (Fig. 24 D-E).

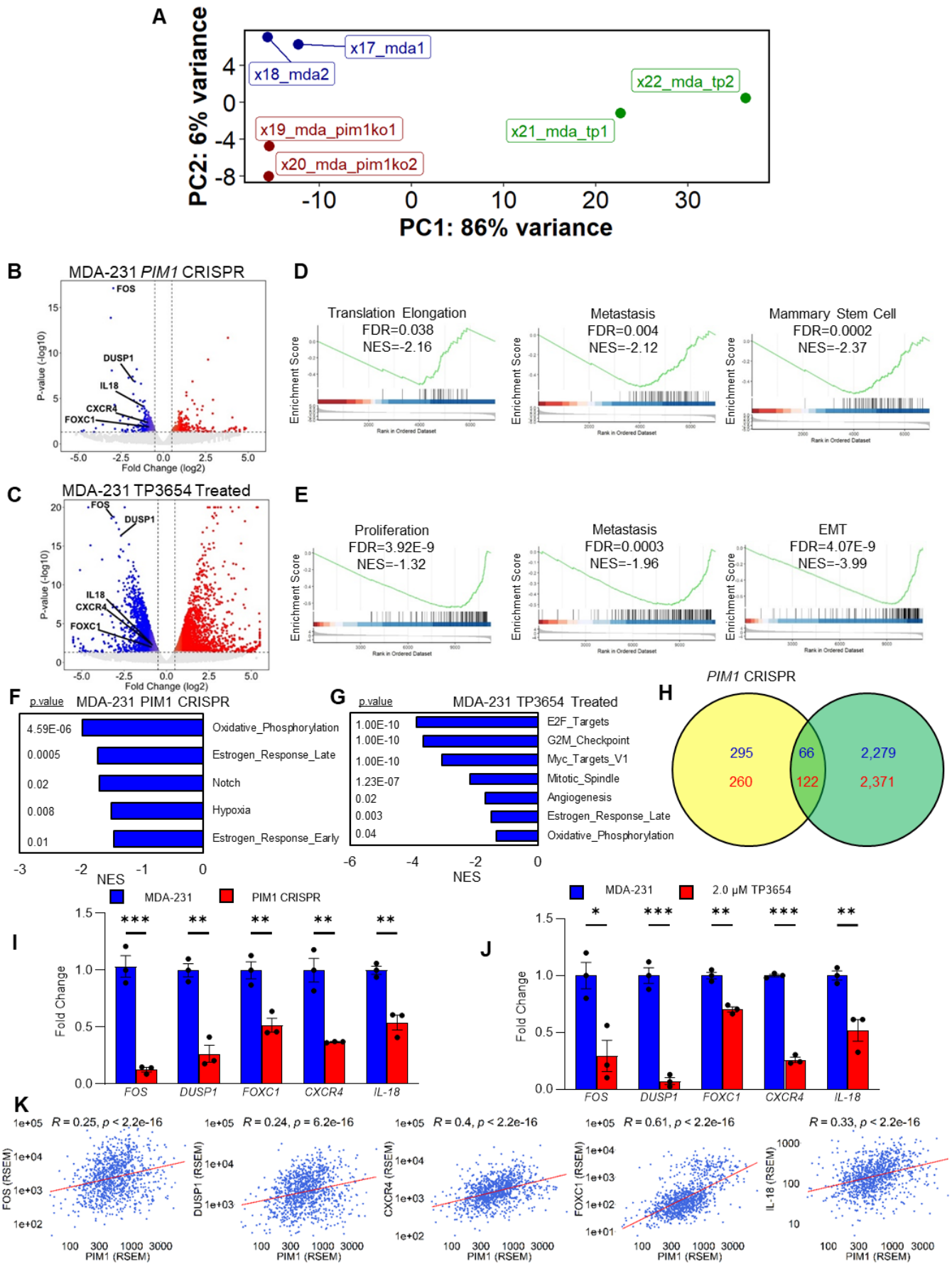
A
PC2: 6% variance
PC1: 86% variance
x17_mda1
x18_mda2
x22_mda_tp2
x21_mda_tp1
x19_mda_pim1ko1
x20_mda_pim1ko2
B
MDA-231 PIM1 CRISPR
P-value (-log10)
Fold Change (log2)
FOS
DUSP1
IL18
CXCR4
FOXC1
C
MDA-231 TP3654 Treated
D
Translation Elongation
FDR=0.038
NES=-2.16
Metastasis
FDR=0.004
NES=-2.12
Mammary Stem Cell
FDR=0.0002
NES=-2.37
Enrichment Score
E
Proliferation
FDR=3.92E-9
NES=-1.32
Metastasis
FDR=0.0003
NES=-1.96
EMT
FDR=4.07E-9
NES=-3.99
F
p.value
MDA-231 PIM1 CRISPR
4.59E-06 Oxidative_Phosphorylation
0.0005 Estrogen_Response_Late
0.02 Notch
0.008 Hypoxia
0.01 Estrogen_Response_Early
NES
G
p.value
MDA-231 TP3654 Treated
1.00E-10 E2F_Targets
1.00E-10 G2M_Checkpoint
1.00E-10 Myc_Targets_V1
1.23E-07 Mitotic_Spindle
0.02 Angiogenesis
0.003 Estrogen_Response_Late
0.04 Oxidative_Phosphorylation
NES
H
PIM1 CRISPR
295
260
66
122
2,279
2,371
I
MDA-231
PIM1 CRISPR
Fold Change
FOS
DUSP1
FOXC1
CXCR4
IL-18
J
MDA-231
2.0 μM TP3654
Fold Change
FOS
DUSP1
FOXC1
CXCR4
IL-18
K
R = 0.25, p < 2.2e-16
R = 0.24, p = 6.2e-16
R = 0.4, p < 2.2e-16
R = 0.61, p < 2.2e-16
R = 0.33, p < 2.2e-16
FOS (RSEM)
DUSP1 (RSEM)
CXCR4 (RSEM)
FOXC1 (RSEM)
IL-18 (RSEM)
PIM1 (RSEM)

Figure 24. RNA-seq analysis identified proliferation, EMT, and metastasis pathways were significantly altered by PIM1 deletion or inhibition with TP-3654 in MDA-231 cells. Volcano plots for genes differentially expressed in A) MDA-231 PIM1 KO cells and B) TP3654 treated MDA-231 cells. GSEA analysis showed a significant negative enrichment of genes related to proliferation, EMT, and metastasis in C) PIM1 KO MDA-231 cells and D) TP3654 treated MDA-231 cells. GSEA analysis also found several Hallmark pathways were enriched following E) PIM1 deletion or F) TP3654 treatment. G) Venn diagram showing the overlap of upregulated (red) and downregulated (blue) genes in PIM1 deleted and TP3654 treated MDA-231 cells. Further RT-qPCR validation confirmed H) PIM1 deletion or I) inhibition in MDA-231 cells significantly reduced expression of FOS, DUSP1, FOXC1, CXCR4 and IL-18. J) PIM1 correlation analysis of breast cancer patient expression data. Pearson correlations were performed and found FOS, DUSP1, CXCR4, FOXC1, and IL-18 were significantly positively correlated with PIM1. (*P<0.05, **P<0.01, ***P<0.001).

We also found several hallmark pathways were negatively regulated including oxidative phosphorylation, notch signaling, hypoxia, and estrogen response (Fig. 24 F-G). RNA-seq analysis also revealed 66 genes that were significantly reduced and 122 genes that were significantly increased in both PIM1 CRISPR and TP3654 treated cells (Fig. 24 H). We further validated FOS, DUSP1, FOXC1, CXCR4, IL18, and SMAD7 from these overlapping genes by RT-qPCR. Both PIM1 deletion and treatment with TP-3654 for 48 hours resulted in significant decreases in the mRNA expression of these selected targets (Fig. 24 I-J). These targets were chosen because previous studies have shown they are important for breast cancer progression and metastasis (36-47). Analysis of TCGA data also revealed that PIM1 expression was positively correlated with these targets (Fig. 24 K). We observed that PIM1 deletion or inhibition significantly reduced expression of FOS, DUSP1, FOXC1, CXCR4, and IL18 (Fig 24 I-J).

We also examined the effects of Pim1/2 deletion on the overall gene expression landscape. To do this, we purified RNA from 12-week-old PyMT mice and Pim1/2 double knockout mice and performed RNA-seq analysis.

PCA analysis revealed that the PyMT tumor samples clustered well together, and they were separated well from the Pim1/2 double knockout samples, although, the two double knockout samples did not cluster together (Fig 25A). Similar to MDA-231 PIM1 CRISPR and MDA-231 TP3654 treated samples, both Fos and DUSP1 expression was significantly reduced in Pim1/2 double knockout PyMT tumors, suggesting that PIM kinase signaling regulates expression of these targets.

Our analysis also found that S100a8 was significantly decreased in Pim1/2 double knockout PyMT mice. Both HR+ breast cancers and TNBCs have previously been found to have significantly higher expression of S100a8 and is highly correlated with significantly shorter overall survival. S100A8 is an inflammatory mediator and is associated with several different cancers. S100A8 promotes breast cancer progression by activating several different inflammatory signaling pathways including IL-6, TNF-α, TGF-β, NFKB, and MAPK.

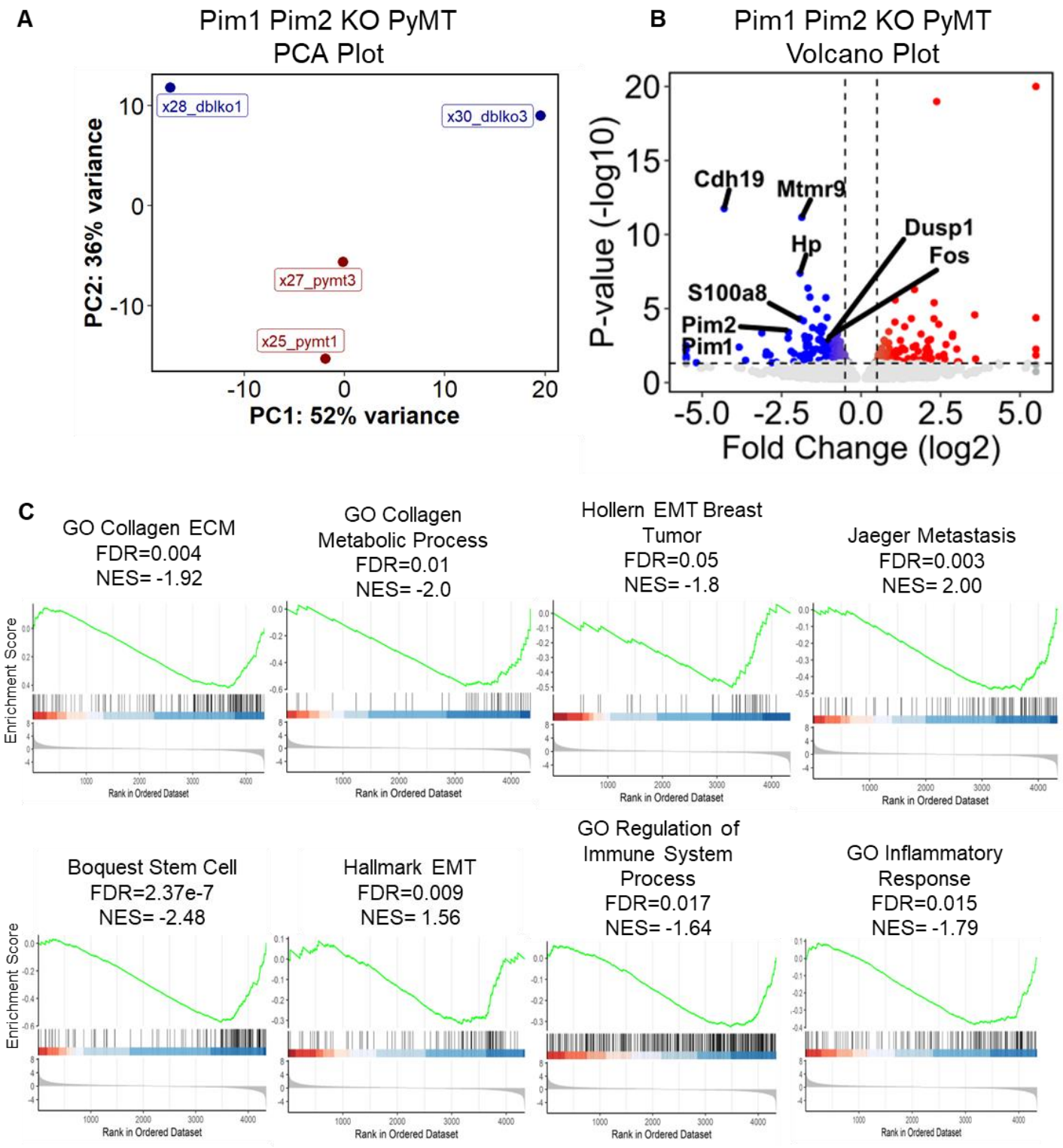

Figure 25. RNA-Seq Analysis of Pim1 Pim2 double knockout PyMT tumors. A) Principal Component Analysis of PyMT and Pim1 Pim2 KO PyMT samples. B) Volcano Plot of

significantly altered genes. C) Gene-set-Enrichment analysis (GSEA) of RNA-sequencing data with normalized enrichment score (NES) False discovery rate (FDR) are shown.

Additionally, we performed GSEA analysis to assess which pathways are being affected by Pim1 and Pim2 deletion in PyMT tumors (Fig 25C). Our analysis revealed that multiple pathways involving collagen and the extra cellular matrix were significantly enriched in PyMT tumors compared to Pim1/2 double knockout mice. Collagen and the extracellular matrix play an important role in breast cancer metastasis. We also found that pathways related to cell stemness, EMT, and metastasis were significantly enriched in PyMT tumors compared to Pim1/2 knockout PyMT mice (Fig. 25C). Finally, we also found that pathways related to inflammation and immune regulation were also affected by Pim1/2 knockout in PyMT tumors (Fig. 25C).

PIM1 deletion or inhibition significantly decreased breast cancer cell growth, invasion and metastasis. PIM1 deletion or inhibition in MMTV-PyMT mice significantly decreased tumor growth and metastasis in these mice. RNA-seq and immunoblot analysis revealed several EMT markers were also decreased with PIM1 deletion or inhibition. These results suggest that PIM1 plays an important role in breast cancer growth, EMT, and metastasis.

References

1. National Program of Cancer Registries and Surveillance, Epidemiology, and End Results Program SEER*Stat Database: NPCR and SEER Incidence – U.S. Cancer Statistics Public Use Research Database with Puerto Rico, 2021 submission (2005–2019), United States Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute. Released June 2022. Available at www.cdc.gov/cancer/uscs/public-use.

2. Zhuyan, J., Chen, M., Zhu, T., Bao, X., Zhen, T., Xing, K., Wang, Q., & Zhu, S. (2020). Critical steps to tumor metastasis: Alterations of tumor microenvironment and extracellular matrix in the formation of pre-metastatic and metastatic niche. In *Cell and Bioscience* (Vol. 10, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13578-020-00453-9

3. Massagué, J., & Obenauf, A. C. (2016). Metastatic colonization by circulating tumour cells. In *Nature* (Vol. 529, Issue 7586, pp. 298–306). Nature Publishing Group. https://doi.org/10.1038/nature17038

4. Puisieux, A., Brabletz, T., & Caramel, J. (2014). Oncogenic roles of EMT-inducing transcription factors. In *Nature Cell Biology* (Vol. 16, Issue 6, pp. 488–494). Nature Publishing Group. https://doi.org/10.1038/ncb2976

5. Kang, E., Seo, J., Yoon, H., & Cho, S. (2021). The post-translational regulation of epithelial–mesenchymal transition-inducing transcription factors in cancer metastasis. *International Journal of Molecular Sciences*, *22*(7). https://doi.org/10.3390/ijms22073591

6. Vuoriluoto, K., Haugen, H., Kiviluoto, S., Mpindi, J. P., Nevo, J., Gjerdrum, C., Tiron, C., Lorens, J. B., & Ivaska, J. (2011). Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. *Oncogene*, *30*(12), 1436–1448. https://doi.org/10.1038/onc.2010.509

7. Kessenbrock, K., Plaks, V., & Werb, Z. (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. In *Cell* (Vol. 141, Issue 1, pp. 52–67). Elsevier B.V. https://doi.org/10.1016/j.cell.2010.03.015

8. Cao, L., Wang, F., Li, S., Wang, X., Huang, D., & Jiang, R. (2019). PIM1 kinase promotes cell proliferation, metastasis and tumor growth of lung adenocarcinoma by potentiating the c-MET signaling pathway. *Cancer Letters*, *444*, 116–126. https://doi.org/10.1016/j.canlet.2018.12.015

9. Gao, X., Liu, X., Lu, Y., Wang, Y., Cao, W., Liu, X., Hu, H., & Wang, H. (2019). PIM1 is responsible for IL-6-induced breast cancer cell EMT and stemness via c-myc activation. *Breast Cancer*, *26*(5), 663–671. https://doi.org/10.1007/s12282-019-00966-3

10. Zhao, B., Liu, L., Mao, J., Zhang, Z., Wang, Q., & Li, Q. (2018). PIM1 mediates epithelial-mesenchymal transition by targeting Smads and c-Myc in the nucleus and potentiates clear-cell renal-cell carcinoma oncogenesis article. *Cell Death and Disease*, *9*(3). https://doi.org/10.1038/s41419-018-0348-9

11. Brault, L., Gasser, C., Bracher, F., Huber, K., Knapp, S., & Schwaller, J. (2010). Pim serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. In *Haematologica* (Vol. 95, Issue 6, pp. 1004–1015). https://doi.org/10.3324/haematol.2009.017079

12. Eichmann, A., Yuan, L., Breâ Ant, C., Alitalo, K., Ivi, P., & Koskinen, J. (2000). Developmental expression of Pim kinases suggests functions also outside of the hematopoietic system. In *Oncogene* (Vol. 19). www.nature.com/onc

13. Xie, Y., Xu, K., Linn, D. E., Yang, X., Guo, Z., Shimelis, H., Nakanishi, T., Ross, D. D., Chen, H., Fazli, L., Gleave, M. E., & Qiu, Y. (2008). The 44-kDa Pim-1 kinase phosphorylates BCRP/ABCG2 and thereby promotes its multimerization and drug-resistant activity in human prostate cancer cells. *Journal of Biological Chemistry*, *283*(6), 3349–3356. https://doi.org/10.1074/jbc.M707773200

14. Zemskova, M., Sahakian, E., Bashkirova, S., & Lilly, M. (2008). The PIM1 kinase is a critical component of a survival pathway activated by docetaxel and promotes survival of docetaxel-treated prostate cancer cells. *Journal of Biological Chemistry*, *283*(30), 20635–20644. https://doi.org/10.1074/jbc.M709479200

15. Cao, L., Wang, F., Li, S., Wang, X., Huang, D., & Jiang, R. (2019). PIM1 kinase promotes cell proliferation, metastasis and tumor growth of lung adenocarcinoma by potentiating the c-MET signaling pathway. *Cancer Letters*, *444*, 116–126. https://doi.org/10.1016/j.canlet.2018.12.015

16. Zhang, C., Qie, Y., Yang, T., Wang, L., Du, E., Liu, Y., Xu, Y., Qiao, B., & Zhang, Z. (2019). Kinase PIM1 promotes prostate cancer cell growth via c-Myc-RPS7-driven ribosomal stress. *Carcinogenesis*, *40*(1), 52–60. https://doi.org/10.1093/carcin/bgy126

17. Santio, N. M., Eerola, S. K., Paatero, I., Yli-Kauhaluoma, J., Anizon, F., Moreau, P., Tuomela, J., Härkönen, P., & Koskinen, P. J. (2015). Pim kinases promote migration and metastatic growth of prostate cancer xenografts. *PLoS ONE*, *10*(6). https://doi.org/10.1371/journal.pone.0130340

18. Jiang, R., Wang, X., Jin, Z., & Li, K. (2016). Association of nuclear PIM1 expression with lymph node metastasis and poor prognosis in patients with lung adenocarcinoma and squamous cell carcinoma. *Journal of Cancer*, *7*(3), 324–334. https://doi.org/10.7150/jca.13422

19. Oi-ning Leung, C., Chak-lui Wong, C., Ngo-yin Fan, D., Ka-lun Kai, A., Kwok-kwan Tung, E., Ming-jing Xu, I., Oi-lin Ng, I., & Cheuk-lam Lo, R. (2015). *PIM1 regulates glycolysis and promotes tumor progression in hepatocellular carcinoma*. www.impactjournals.com/oncotarget/

20. Santio, N. M., Vainio, V., Hoikkala, T., Mung, K. L., Lång, M., Vahakoski, R., Zdrojewska, J., Coffey, E. T., Kremneva, E., Rainio, E. M., & Koskinen, P. J. (2020). PIM1 accelerates prostate cancer cell motility by phosphorylating actin capping proteins. *Cell Communication and Signaling*, *18*(1). https://doi.org/10.1186/s12964-020-00618-6

21. Brasó-Maristany, F., Filosto, S., Catchpole, S., Marlow, R., Quist, J., Francesch-Domenech, E., Plumb, D. A., Zakka, L., Gazinska, P., Liccardi, G., Meier, P., Gris-Oliver, A., Cheang, M. C. U., Perdrix-Rosell, A., Shafat, M., Noël, E., Patel, N., McEachern, K., Scaltriti, M., … Tutt, A. N. (2016). PIM1 kinase regulates cell death, tumor growth and chemotherapy response in triple-negative breast cancer. *Nature Medicine*, *22*(11), 1303–1313. https://doi.org/10.1038/nm.4198

22. Horiuchi, D., Camarda, R., Zhou, A. Y., Yau, C., Momcilovic, O., Balakrishnan, S., Corella, A. N., Eyob, H., Kessenbrock, K., Lawson, D. A., Marsh, L. A., Anderton, B. N., Rohrberg, J., Kunder, R., Bazarov, A. v., Yaswen, P., McManus, M. T., Rugo, H. S., Werb, Z., & Goga, A. (2016). PIM1 kinase inhibition as a targeted therapy against triple-negative breast tumors with elevated MYC expression. *Nature Medicine*, *22*(11), 1321–1329. https://doi.org/10.1038/nm.4213

23. Borten, M. A., Bajikar, S. S., Sasaki, N., Clevers, H., & Janes, K. A. (2018). Automated brightfield morphometry of 3D organoid populations by OrganoSeg. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-017-18815-8

24. Xing, F., Liu, Y., Sharma, S., Wu, K., Chan, M. D., Lo, H. W., Carpenter, R. L., Metheny-Barlow, L. J., Zhou, X., Qasem, S. A., Pasche, B., & Watabe, K. (2016). Activation of the c-Met pathway mobilizes an inflammatory network in the brain microenvironment to promote brain metastasis of breast cancer. *Cancer Research*, *76*(17), 4970–4980. https://doi.org/10.1158/0008-5472.CAN-15-3541

25. Previdi, S., Abbadessa, G., Dalò, F., France, D. S., & Broggini, M. (2012). Breast cancer-derived bone metastasis can be effectively reduced through specific c-MET inhibitor tivantinib (ARQ 197) and shRNA c-MET knockdown. *Molecular Cancer Therapeutics*, *11*(1), 214–223. https://doi.org/10.1158/1535-7163.MCT-11-0277

26. Gautam, S. K., Kanchan, R. K., Siddiqui, J. A., Maurya, S. K., Rauth, S., Perumal, N., Atri, P., Venkata, R. C., Mallya, K., Mirza, S., Ponnusamy, M. P., Band, V., Mahapatra, S., Jain, M., Batra, S. K., & Nasser, M. W. (2020). Blocking c-met/erbb1 axis prevents brain metastasis in erbb2+ breast cancer. *Cancers*, *12*(10), 1–20. https://doi.org/10.3390/cancers12102838

27. Guy, C. T., Cardiff, R. D., & Muller`*, W. J. (1992). Induction of Mammary Tumors by Expression of Polyomavirus Middle T Oncogene: A Transgenic Mouse Model for Metastatic Disease. In *MOLECULAR AND CELLULAR BIOLOGY* (Vol. 12, Issue 3). https://journals.asm.org/journal/mcb

28. Franci, C., Zhou, J., Jiang, Z., Modrusan, Z., Good, Z., Jackson, E., & Kouros-Mehr, H. (2013). Biomarkers of Residual Disease, Disseminated Tumor Cells, and Metastases in the MMTV-PyMT Breast Cancer Model. *PLoS ONE*, *8*(3). https://doi.org/10.1371/journal.pone.0058183

29. Li, Z., Lin, F., Zhuo, C., Deng, G., Chen, Z., Yin, S., Gao, Z., Piccioni, M., Tsun, A., Cai, S., Zheng, S. G., Zhang, Y., & Li, B. (2014). PIM1 kinase phosphorylates the human transcription factor FOXP3 at serine 422 to negatively regulate its activity under inflammation. *Journal of Biological Chemistry*, *289*(39), 26872–26881. https://doi.org/10.1074/jbc.M114.586651

30. Liu, Z., Han, M., Ding, K., & Fu, R. (2020). The role of Pim kinase in immunomodulation. In *Am J Cancer Res* (Vol. 10, Issue 12). www.ajcr.us/ISSN:2156-6976/ajcr0118980

31. Narlik-Grassow, M., Blanco-Aparicio, C., Cecilia, Y., Perez, M., Muñoz-Galvan, S., Cañamero, M., & Carnero, A. (2013). Conditional Transgenic Expression of PIM1 Kinase in Prostate Induces Inflammation-Dependent Neoplasia. *PLoS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0060277

32. Volberding, P. J., Xin, G., Kasmani, M. Y., Khatun, A., Brown, A. K., Nguyen, C., Stancill, J. S., Martinez, E., Corbett, J. A., & Cui, W. (2021). Suppressive neutrophils require PIM1 for metabolic fitness and survival during chronic viral infection. *Cell Reports*, *35*(8). https://doi.org/10.1016/j.celrep.2021.109160

33. Zhang, M., Liu, Z. Z., Aoshima, K., Cai, W. L., Sun, H., Xu, T., Zhang, Y., An, Y., Chen, J. F., Hei Chan, L., Aoshima, A., Lang, S. M., Tang, Z., Che, X., Li, Y., Rutter, S. J., Bossuyt, V., Chen, X., Morrow, J. S., … Yan, Q. (2022). CECR2 drives breast cancer metastasis by promoting NF-κB signaling and macrophage-mediated immune suppression. In *Sci. Transl. Med* (Vol. 14). https://www.science.org

34. Zhao, X., Qu, J., Sun, Y., Wang, J., Liu, X., Wang, F., Zhang, H., Wang, W., Ma, X., Gao, X., & Zhang, S. (2017). Oncotarget 30576 www.impactjournals.com/oncotarget Prognostic significance of tumor-associated macrophages in breast cancer: a meta-analysis of the literature. In *Oncotarget* (Vol. 8, Issue 18). www.impactjournals.com/oncotarget/

35. Medrek, C., Pontén, F., Jirström, K., & Leandersson, K. (2012). The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer*, *12*. https://doi.org/10.1186/1471-2407-12-306

36. Muhammad, N., Bhattacharya, S., Steele, R., Phillips, N., & Ray, R. B. (2017). Involvement of c-Fos in the promotion of cancer stem-like cell properties in head and neck squamous cell carcinoma. *Clinical Cancer Research*, *23*(12), 3120–3128. https://doi.org/10.1158/1078-0432.CCR-16-2811

37. Weekes, D., Kashima, T. G., Zandueta, C., Perurena, N., Thomas, D. P., Sunters, A., Vuillier, C., Bozec, A., El-Emir, E., Miletich, I., Patinõ-Garcia, A., Lecanda, F., & Grigoriadis, A. E. (2016). Regulation of osteosarcoma cell lung metastasis by the c-Fos/AP-1 target FGFR1. *Oncogene*, *35*(22), 2852–2861. https://doi.org/10.1038/onc.2015.344

38. Saez, E., Rutberg, S. E., Mueller, E., Oppenheim, H., Smoluk, J., Yuspa, S. H., & Spiegelman, B. M. (1995). c-fos Is Required for Malignant Progression of Skin Tumors. In *Cell* (Vol. 82).

39. Foulks JM, Carpenter KJ, Luo B, et al. A small-molecule inhibitor of PIM kinases as a potential treatment for urothelial carcinomas. Neoplasia. 2014;16(5):403-412. doi:10.1016/j.neo.2014.05.004

40. Chen LS, Redkar S, Bearss D, Wierda WG, Gandhi V. Pim kinase inhibitor, SGI-1776, induces apoptosis in chronic lymphocytic leukemia cells. Blood. 2009;114(19):4150-4157. doi:10.1182/blood-2009-03-212852

Chapter 4

Discussion

Breast cancer remains one of the most frequently diagnosed cancer in women. Despite recent advances in imaging, early detection, and new treatments, approximately 42,000 women die each year in the United States (1). Breast cancers are a very heterogenous disease, with a wide array of mutations and genetic alterations driving the underlying disease progression. Several genes have been identified as frequently altered including overexpression of estrogen receptor, progesterone receptor, or HER2 (2). While inhibition of these receptors coupled with surgery and radiation has improved patient responses, there is still a great need to identify new targets in order to produce more effective treatments.

We found that PIM1 and PIM2 kinases are frequently amplified and overexpressed in breast cancers. We also found that TNBC patients had significantly higher expression of PIM1 and PIM2. PIM kinases have been linked to cell growth, survival, and drug-resistance in various hematologic and solid tumors. So, we assessed the role of PIM1/PIM2 in breast cancer growth, progression, and metastasis.

To evaluate our hypothesis, we started by knocking down PIM1 and PIM2 in a panel of breast cancer cell lines. We found that PIM1 knockdown significantly inhibited growth of the breast cancer cell lines MDA-231, MDA-468, BT-20, HCC-1806, HS578T, MCF-7, and BT474. MDA-231, MDA-468, BT-20, HCC-1806, and HS578T cells are all TNBC cell lines. Of the cell lines we tested, PIM1 knockdown was most effective in TNBC. This is possible due to the higher levels of PIM1 expression in TNBC cell lines. However, we did observe a significant decrease of proliferation by PIM1 depletion in HR+ cell line MCF-7 and HER2+ cell line

BT474. This suggests that PIM1 may also play a significant role in HR+ and HER2+ breast cancer, however due to a lack of directed therapies, we focused on TNBC.

We additionally knocked down PIM2 in a panel of breast cancer cell lines and found that PIM2 knockdown only significantly reduced proliferation of MDA-468 and HS578T cells. This could be due to redundancy in PIM signaling, and in most of the cell lines we examined, PIM1 expression is higher than PIM2. Because we observed greater effect on cells with PIM1 knockdown compared to PIM2 knockdown, our follow up experiments focused mostly on PIM1.

There are several different mechanisms that could explain why PIM1/2 are upregulated in breast cancers. PIM kinases are induced by several different cytokines (3). In breast cancer, several different cytokines have been fond to be misregulated in the local tumor environment including IL-1, IL-6, IL-11, and TGF-β (4). IL-6 has been shown to induce PIM1 expression in MCF-7 cells, so it is possible that this is one mechanism to explain the overexpression of PIM1 in breast cancers (5). We also found that PIM1 and PIM2 expression was significantly higher in TNBCs than the other receptor types. PIM expression can also be induced by aberrant JAK/STAT signaling (6). In TNBCs, amplifications of 9p24, the region containing JAK2 are common (7). Patients with this amplification were found to have significantly shorter survival than those without and it is possible that these genetic alterations can lead to the increased expression of PIM1/2 in TNBCs (7). PIM expression can also be induced under hypoxic conditions, which are commonly present in breast tumors due to their rapid growth (8).

Breast cancers are often inherently non-lethal, but associated mortalities with breast cancers occur when the primary tumor metastasizes to vital organs (9). So, we assessed the effects of PIM1 depletion on cell migration, invasion, and tumor sphere formation in TNBC cells. We showed that deletion of PIM1 diminishes MDA-231 and HCC-1806 cells' ability to migrate and

invade. We also showed that deletion of PIM1 significantly reduced anchorage independent growth and colony forming ability in these TNBC cells. These results indicate that PIM1 plays an important role in breast cancer metastasis.

One of the major questions we were investigating was what step of metastasis does PIM1 play an important role in. Metastasis requires cells to first locally invade into surround tissues. We found that several EMT markers were downregulated following PIM1 deletion or inhibition. We also found that in Matrigel invasion assays that MDA-231 cells were less invasive following PIM1 deletion. This suggests PIM1 plays an important role in the initial steps of metastasis.

During metastasis, after invading into local tissues, cancer cells then enter the bloodstream and must survive in circulation. EMT also plays a role in this process, and we evaluated cancer cell's ability to survive and proliferate in suspension, using tumorsphere formation assays, where cells were grown on ultra-low attachment plates. We found that deletion or inhibition of PIM1 significantly decreased tumorsphere formation ability. These results suggest that PIM1 may also promote anchorage independent growth in breast cancer, which is also contributing to the overall decrease in metastasis we saw in our MMTV-PyMT mouse experiments.

Data from our PIM1 knockdown/deletion studies prompted us to investigate the effects of PIM1 inhibition on breast cancer cells. We found that inhibition of PIM kinase by TP-3654 significantly reduced proliferation in several different breast cancer cell lines. Similar to our knockdown experiments TNBC cell lines tended to be the most sensitive to TP3654. However, we did also observe a significant reduction of proliferation in HR+ MCF-7 cells and HER2+ BT474 cells. Additionally, we showed that treatment of TP-3654 significantly reduced cell migration, anchorage independent growth, and colony forming ability.

We also provided some mechanistic insights into inhibition of breast cancer cells by PIM1 deletion or inhibition. We found that knockdown or inhibition of PIM1 resulted in a decrease in phosphorylation of p70S6K and 4E-BP1 and an increase in p21 expression. Phosphorylation of p70S6K and 4E-BP1 results in increased protein synthesis and cellular growth (10-11). p21 is a negative regulator of cell cycle, and its increase could also explain some of the decreased cell proliferation observed in breast cancer cells upon PIM1 deletion or inhibition (12).

PIM1 signaling overlaps significantly with PI3K/Akt/mTOR. The PI3K/Akt/mTOR pathway has been found to be altered in more than 70% of all breast tumors (53). This pathway has also been shown to have a significant role in breast cancer cell survival, proliferation, migration, and apoptosis (53). Because of these frequent alterations and their role in cancer, several clinical trials have focused on inhibitors of this pathway. Despite showing promise and being approved for treatment of HR+ breast cancers, a high number of adverse events during trials with everolimus have led limited success of targeting this pathway (54). It is important to note, while there is significant overlap between the pathways, it has also been reported that while treating cells with rapamycin, PIM kinase signaling was still able to activate downstream target 4EBP1, indicating an independent mechanism that is not dependent on mTOR (55). Additionally, it has also been reported that Akt and PIM kinases regulate eIF-4B through phosphorylation of separate sites; PIM1 phosphorylated eIF-4B at Ser406, where Akt phosphorylates eIF-4B at Ser422 (56). These independent mechanisms indicate that while there is significant overlap with Akt signaling, PIM signaling not only activates downstream mTOR signaling, but it does so independently of Akt. This may explain why targeting PIM kinases alone may be sufficient to affect these signaling pathways without targeting Akt. Further, the high number of adverse

events may from drugs targeting the PI3K/Akt/mTOR pathway may be circumvented by targeting PIM kinase to affect the same pathway. In addition, PIM kinase inhibition may also work synergistically with mTOR inhibitors such as everolimus.

EMT has been found to promote the metastatic propensity of several different cancers including breast cancer. Vimentin and SLUG have been implicated in cancer metastasis (13-14). We found that knockdown or inhibition of PIM1 resulted in decreased expression of EMT markers SLUG and Vimentin. Taken together, these results suggest that PIM1 plays an important role in promoting EMT and targeting PIM kinase may reduce breast cancer metastasis due to impaired migratory and invasive ability acquired through EMT.

We then went on to examine the in vivo effects of Pim1/2 deletion on tumor growth and metastasis using the MMTV-PyMT mouse model of breast cancer. We chose this model for multiple different reasons. This model most closely follows the normal progression of breast cancer development, where primary tumors first form, before becoming invasive and metastasizing to the lungs (15). In addition, the immune system is intact in this mouse model (15).

We found that deletion of Pim1 or Pim2 alone significantly decreased both tumor growth and metastasis in PyMT mice. Pim1 and Pim2 double deletion in MMTV-PyMT mice also resulted in a significant decrease in tumor growth that was significantly less than both control PyMT tumors and Pim2 KO PyMT tumors, however, was not significantly different from Pim1 deletion alone. We did, however, find that deletion of both Pim1 and Pim2 in PyMT mice resulted in the greatest decrease in metastasis to the lungs.

We also tested the in vivo efficacy of PIM kinase inhibitor TP-3654 in MMTV-PyMT mice. Similar to genetic deletion of Pim1in MMTV-PyMT mice, PIM kinase inhibition resulted in a significant decrease in tumor growth and lung metastasis. We also found that the mice tolerated the TP-3654 treatment very well as we did not see any significant changes in mouse weight throughout the course of the treatment, and endpoint analysis of organs showed no gross changes in organ function.

A major question is which step of metastasis does PIM1 play a role in. PIM1 knockdown resulted in decreases in EMT, which may explain some of the decreases in cellular migration and invasion, but we also saw anchorage independent growth, and colony formation ability impaired. This suggests that PIM1 may play an important role in several stages of metastasis, from the tumors initial invasive capabilities that are gained during EMT, to cell stemness that is needed to support anchorage independent growth and colony formation at distant sites. It has been reported that PIM1 promotes expression of c-Met through phosphorylation of eIF4B (16). We found that total c-Met expression was decreased with PIM1 deletion or inhibition but were unable to consistently show a decrease in eIF4B phosphorylation. This mechanism needs to be investigated further, but a decrease in c-Met also suggests a possible mechanism for the decrease in cell invasion/migration and metastasis.

We would also like to further evaluate how PIM1 is inhibiting EMT. Our study identified that we see a significant decrease in EMT marker expression upon knockdown or inhibition of PIM1, however we have not yet fully worked out how this is happening. We did find that PIM1 deletion or inhibition significantly reduced expression of c-Met both in vitro and in vivo. c-Met has been shown to promote EMT, however we expect there are additional signaling pathways related to EMT that are affected by PIM1/2. Previous studies have identified multiple different

pathways including TGF-β signaling, MAPK signaling, and NF-KB signaling contribute to EMT (17-20).

TGF-β activates phosphorylation of SMAD2 and SMAD3 (21). It has also been reported that PIM1 can phosphorylate these proteins (22). In addition, our RNA-seq analysis revealed a significant increase in SMAD7 expression. SMAD7 negatively regulates SMAD2/3 signaling, and an increase may result in impaired TGF-β/SMAD induced EMT (23). In preliminary experiments we have seen that SMAD2 phosphorylation is reduced with treatment of TP3654 (data not shown), but we were not able to show this result consistently with PIM1 or PIM2 knockdown.

Breast cancers are very heterogeneous diseases, and no single drug is sufficient to completely cure the disease. We saw the greatest effectiveness of PIM kinase inhibition with TP3654 in TNBC cells. TNBCs are treated with a combination of chemotherapy drugs including a taxane, an anthracycline, and a platinum salt. We showed that combined treatment of TP-3654 with doxorubicin or cisplatin resulted in greater inhibition of TNBC cells. Furthermore, combined treatment of TP-3654 and doxorubicin resulted in significantly greater reduction of tumor growth compared with vehicle or doxorubicin treatment in MDA-231-2B xenograft NSG mouse experiments. These results are still preliminary. We need to repeat these experiments to ensure reproducibility. In future experiments, we would also like to evaluate the combination of additional drugs used to treat breast cancer, including docetaxel and cisplatin.

Our studies also found that PIM1 and PIM2 play an important role in regulation of immune evasion of tumors. We found that deletion of Pim1 and deletion of both Pim1 and Pim2 resulted in a significant decrease in tumor associated macrophages (TAMs). Macrophages have been shown to release pro-inflammatory cytokines which promote EMT and metastasis in breast

cancer (24). In TNBC, TAMs are also a negative prognostic marker (25). We also found that PIM1 and PIM2 double knockout resulted in a significant increase in number of both CD4+ helper T cells and CD8+ cytotoxic t cells when compared to PyMT control tumors. T cell infiltration is associated with better patient survival and considered a positive prognostic marker (26-27). Our RNA-seq analysis found that PD-L1 was significantly decreased with PIM1 deletion or inhibition. PD-L1 promotes tumor immune evasion, and this decrease may explain why we see more T-cells in tumors where PIM1 or PIM2 is deleted. At this time, we do not know the exact mechanism of how PIM1/2 deletion leads to a decrease in PD-L1 but our RNA-seq analysis revealed some possible pathways. Overall, we see a decrease in multiple different EMT markers, and EMT has been found to play an important role in regulation of PD-L1 (44).

The PD-1/PDL1 interaction has been a major target identified in immunotherapy drugs (28). Pembrolizumab, an antibody that blocks PD-1 on lymphocytes has shown great promise as a new treatment option for cancer patients (29-31). It has shown great promise as a drug in melanoma, lung cancer, head and neck cancer, stomach and cervical cancers (29-30). Recent clinical trials (KEYNOTE-522) of pembrolizumab have examined it's potential as a neoadjuvant treatment for patients with stage III or higher TNBC. This study found a 7.5% improvement in pathologic complete response (pCR) in patients receiving pembrolizumab in combination to traditional chemotherapy treatment compared to those receiving chemotherapy alone. This clinical trial also reveled a significant improvement in patient event-free survival in patients receiving Keytruda. Despite these promising results, approximately 45% of TNBC patients in the study still did not respond to treatment with pembrolizumab. This suggests that additional drugs may be necessary to further improve patient responses. Because we see PD-L1 decreased by PIM1

deletion or inhibition, we hypothesize that combining TP-3654 with currently immunotherapy drugs targeting PD-1/PD-L1 may work synergistically together.

Our RNA-seq analysis revealed several pathways are altered by PIM1 deletion or inhibition. We observed significant enrichment of genes related to translation elongation, mammary stemness, proliferation, metastasis and EMT in control MDA-231 cells compared with PIM1 deleted or TP3654-treated MDA-231 cells. We observed that PIM1 deletion or inhibition significantly reduced expression of FOS, DUSP1, FOXC1, CXCR4, and IL18. RT-qPCR further validated the expression of these target genes are affected by PIM1 deletion or inhibition.

Our RNA-seq results revealed that c-FOS was significantly reduced in all 3 groups of samples we examined. c-FOS has previously been shown to induce PD-1 (32). Additionally, we found that IL-18 was significantly decreased with PIM1 deletion and PIM kinase inhibition. Tumor derived IL-18 has also been reported to induce PD-1 expression (33). In addition, our GSEA analysis revealed Notch signaling was significantly negatively enriched in PIM1 PIM2 double knockout tumors. Notch signaling has also been shown to increase PDL1 expression (34). Furthermore, in non-small cell lung cancer, notch mutations have been found to be a prognostic marker for response to immunotherapy, suggesting it plays an important role in patient response to immunotherapy (35). Combined treatment with TP-3654 and pembrolizumab should also be examined in future experiments.

PIM kinase deficient mice are viable, develop normally, and reproduce indicating that PIM kinase is an excellent drug target with minimal side effects (36). Several different PIM kinase inhibitors have been developed including SGI-1776, AZD-1208, and TP3654. In previous

clinical trials SGI-1776 failed due to cardiotoxicity and AZD-1208 also exhibited high rates of adverse events (37-38). TP-3654 is a novel second-generation PIM kinase inhibitor, which has already entered phase I/II clinical trials and shown great promise as a potential therapeutic for multiple different cancers including myelofibrosis and various solid tumors (NCT03715504, NCT04176198).

Future Directions

We have shown that PIM1 plays a key role in breast cancer growth, EMT, and metastasis. In future experiments we would like to further investigate the mechanisms by which PIM1 is contributing to these processes. We performed RNA-seq analysis on MDA-231 cells that had PIM1 deletion or were treated with the PIM inhibitor TP-3654. This analysis revealed several pathways related to breast cancer progression and metastasis that require additional validation. Our pathway analysis revealed significant enrichment in genes related to translation, proliferation, EMT, and metastasis. In future experiments we would like to further understand exactly how PIM1 is impacting these different targets, to do this we plan on first identifying which proteins are direct targets of PIM1 and which effects are downstream of those targets.

First, we would like to perform a non-biased screen of the phospho-proteome in MDA-231 cells. We plan on deleting PIM1 in these cells and comparing the protein phosphorylation to control cells. This will allow us to identify potential targets that are phosphorylated by PIM1. It is important to note these targets will then need to be narrowed down further to direct effects vs indirect effects of PIM1. A phospho-proteome experiment will however allow us to identify targets that are no longer phosphorylated when PIM1 is deleted, these targets can then be validated in future experiments.

To evaluate which proteins are direct targets of PIM1 we plan on overexpressing FLAG-tagged PIM1 in MDA-231 cells followed by immunoprecipitation experiments. We will use a flag specific antibody to pulldown PIM1, along with any interacting proteins. Several targets of PIM1 have already been identified in other settings, but our non-biased screen followed by immunoprecipitation will allow us to assess what additional targets may be having a significant

impact on our observed phenotype. This will also allow us to identify which interactions are directly the result of PIM1, rather than an indirect downstream effect.

Our results showed a reduction in phosphorylation of both p70-s6K and 4EBP1 when PIM1 was deleted or inhibited. These have previously been identified as targets of PIM1, and help explain some of the phenotype we observed, but further validation is needed to show that the changes in phosphorylation are what is driving our observed phenotype. To further evaluate these targets, we plan on deleting PIM1 and overexpressing these proteins. PIM1 mediated phosphorylation of 4EBP1 and p70-S6K may be due to downstream activation of mTORC1. PIM1 has been found to directly phosphorylate PRAS40 at Thr246 (45-46). PRAS40 is normally a negative regulator of mTORC1, and phosphorylation at Thr246 prevents this negative regulation, resulting in downstream phosphorylation of 4EBP1 and p70-S6K (45-46). It has also been shown that PIM1 overexpression in the presence of mTOR inhibitors is sufficient to increase the phosphorylation of 4EBP1, indicating a direct interaction exists (47). We plan on evaluating this interaction first with our immunoprecipitation experiments to show if a direct interaction exists, followed by downstream rescue experiments to determine its effects on our observed phenotype.

Phosphorylation of 4EBP1 at Thr37/46 prevents its association and subsequent inhibition of eIF4E (48). To study the effects of phosphorylation on cell growth, invasion, and EMT, we plan on deleting PIM1, while overexpressing 4EBP1 that has been mutated to prevent phosphorylation, or simply deleting the protein itself. It has previously been reported that cap-dependent translation is important for the regulation of EMT marker Snail as well as pro-metastatic c-Met (49). In cells that have both PIM1 and 4EPB1 deleted we will assess the effects on cell proliferation, invasion/migration, and EMT marker expression. We expect that deletion of PIM1 and 4EBP1 will result in little to no change in expression of downstream proteins c-Met

and Snail, suggesting that 4EBP1's negative regulation of cap-dependent translation is vital for the regulation of these proteins.

Conversely, several different activating mutations in p70-S6K have been identified, which can be overexpressed in rescue experiments to see if the changes in proliferation, migration/invasion, EMT, and metastasis are due to changes in the phosphorylation of these proteins. Thr412 mutations resulting in a single amino acid substitution to Glu in p70-s6K have been found to increase its activity 20-30-fold (50). So, to evaluate the role of p70-S6K, we plan on deleting PIM1 while overexpressing p70-S6K-T412G to see if activated p70-s6K is sufficient to rescue cells. We plan on first evaluating any effects on proliferation and migration/invasion in cells. We would additionally like to assess any changes in gene expression. We think that over-expression of p70-S6K-T412G will result in reactivation of protein synthesis, and we will not only see an effect on cell growth, but also on expression of EMT markers such as Snail/Slug. These experiments will allow us to further elucidate the mechanisms by which PIM1 is contributing to these processes.

Our RNA-Seq data revealed that FOS and DUSP1 were both significantly decreased with PIM1 deletion or inhibition. We started with these genes in common to validate by RT-qPCR. We selected these genes for their known roles in cancer, however we still need to determine how exactly PIM kinases are regulating these several different downstream targets. In future experiments, we plan on examining these targets further. Our current model suggests that deletion or inhibition of PIM1 results in decreased phosphorylation of 4EBP1, resulting in decreased cap-dependent translation, which ultimately results in a decrease of c-Met. C-Met is a receptor for hepatic growth factor (HGF) (51). HGF binding has been shown to activate the MAPK signaling cascade. This signaling has been shown to activate transcription of the c-Fos

gene as well as activate a negative feedback loop by increasing transcription of DUSP1. To evaluate if the changes we see in c-Fos and DUSP1 result from decreased expression of c-Met we first plan on knocking down c-Met in MDA-231 cells to see if this also results in decreased c-Fos and DUSP1 expression. In follow up experiments we also plan on deleting PIM1 while overexpressing c-Met to ensure the changes in FOS and DUSP1 are due to changes in c-Met expression, and not a different PIM1 dependent mechanism.

Several previous studies have found that PIM signaling from the different family members is redundant; however, we see very different results on tumor growth and metastasis in our PyMT mice when PIM1 or PIM2 is deleted. Additionally, we also expected that if this is true to see more of an effect on tumor growth and metastasis when we deleted both PIM1 and PIM2 in these mice. Instead, we found that there were no significant changes in tumor growth between Pim1 KO PyMT mice and Pim1 Pim2 KO PyMT mice. We also found that deletion of PIM1 in MDA-231 cells resulted in a compensatory effect, where PIM2 expression is upregulated, however despite this upregulation, we still see significant effects on cell proliferation, migration/invasion, and metastasis. We think that this may be because Pim1 is expressed at a higher level in PyMT tumors, so deletion of Pim1 has more of an effect, but this needs to be examined further. In future experiments we plan to further evaluate any differences between Pim1 and Pim2 by performing additional RNA-seq analysis on PyMT tumors. We plan to evaluate the effects of the single deletions on tumor signaling individually, as well as the double deletion together. This will allow us to assess if the same genes are being affected by deletion, or if there are different genes affected by Pim1 and Pim2. If PIM signaling is truly redundant, we expect that similar genes/pathways will be significantly altered by deletion of Pim1 or Pim2. If deletion of Pim1 or

Pim2 however results in several different genes and pathways being affected, this would suggest that different roles for Pim1 and Pim2 may exist that need to be evaluated further.

This project has focused on PIM1; We chose to focus on PIM1 and PIM2 initially because they were misregulated in breast cancer at higher rates than PIM3. While performing our initial knockdowns on a panel of breast cancer cells we found that PIM1 had a larger effect than PIM2, so we subsequent work focused on PIM1. PIM3 however, is highly expressed in the mammary tissue of both mice and humans, so we hypothesize that PIM3 may also play an important role in breast cancer growth and metastasis. In future experiments we would also like to delete PIM3 in a panel of breast cancer cell lines and perform similar experiments to evaluate the effects on cell growth, migration/invasion, and cell signaling. It has been reported that PIM signaling is largely redundant, so we expect that deletion of PIM3 may also produce similar effects. It may also be that PIM1 and PIM2 are induced in a cancer setting, and PIM3 is not, so deletion of PIM3 may also have less of an effect on these processes, but that must first be determined experimentally.

Another major question we would like to answer is what about breast cancer is leading to an increase in PIM1/2. We found that PIM1 and PIM2 were overexpressed or amplified in a large percentage of breast cancers, and that TNBCs had significantly higher PIM1 and PIM2 expression than the other subtypes of breast cancer. In the future, we would like to further analyze patient data to try and provide possible mechanisms of how PIM1 and PIM2 are being induced in breast cancer. To do this we plan on examining TCGA data to look for any possible mutations or other genetic changes that may explain why PIM kinases are being upregulated. There are several different ways PIM kinases are regulated, and we predict that it may not just be one thing. One possible explanation for TNBCs having higher PIM1/2 expression is that JAK2 amplifications are common in TNBCs, and the JAK/STAT pathway can induce PIM expression

(52). In future experiments we would like to evaluate the patient data, to provide possible explanations which could later be verified experimentally. For example, if we find that patients with amplification of PIM1 also have amplification of EGFR, we could overexpress EGFR in cells and see if it affects PIM expression. Additionally, we would also like to verify our results by performing immunohistochemistry staining of patient samples.

It is also worth noting that our PIM knockout mice are whole body knockouts, that are not inducible. In early studies we ensured that deletion of PIM1 or PIM2 did not significantly impact mammary gland development by performing whole mount analysis and found no significant impairments on mammary gland development (data not shown). This suggests that any changes we see in tumor growth and metastasis we see are not due to impaired mammary gland development in these mice. This, however, does not allow us to assess if the changes we see in tumor growth and metastasis are tumor intrinsic, or a result of additional changes outside the tumor itself. To assess if the effects we see resulting from PIM deletion are due to intrinsic changes in the tumor itself, we plan on performing allograft experiments where we transplant tumor cells from control or Pim1 deleted PyMT tumors into wild-type control mice. We plan on evaluating any changes in tumor growth as well as any changes in metastasis that may occur. In preliminary studies, we found that tumor growth was still significantly reduced with PIM1/2 deletion however, we did not see significant changes in metastasis. This indicates that the effects on tumor growth are intrinsic to the tumors themselves, however, metastasis may require PIM signaling outside of the tumor, but this needs to be evaluated further in future experiments.

One potential reason we see differences in our whole-body knockouts compared to transplanted tumors are in differences in immune cell function. Our data suggests that deletion of PIM1 has a significant impact on the immune milieu present in the tumor. We found that deletion of PIM1

resulted in a significant decrease in the number of tumor associated macrophages found in the tumor. TAMs are associated with a worse prognosis in breast cancer due to their release of pro-inflammatory cytokines which drive growth and metastasis. In future studies we would like to evaluate the effects of PIM1/2 deletion on macrophage growth and invasion. To do this, we plan on culturing macrophages from both control FVB mice as well as PIM1/2 deleted mice to first evaluate any changes in growth. After establishing the role PIM1/2 play in the growth of macrophages, we would also like to evaluate how deletion of PIM1/2 affects cell invasion.

We also found that PIM1 and PIM2 double knockout resulted in a significant increase in number of both CD4+ helper T cells and CD8+ cytotoxic t cells when compared to PyMT control tumors. T cell infiltration is associated with better patient survival and considered a positive prognostic marker (26-27). In future studies we would also like to further increase the number of animals for each these experiments. We also found no significant differences in the CD45+ CD3+ T-cells overall, so we would also like to examine Treg cells to see if any significant differences exist there. Treg cells play an important role in immune evasion in tumor progression, and because we see differences in CD4+ and CD8+ T cells in our PIM1/2 knockout mice we hypothesize there may also be differences in Treg cells as well.

We found significant differences between T cells and macrophages at the tumors after PIM1/2 knockout. We started looking at these cells because of previous reports suggesting that PIM1/2 play important roles in these cells. In future experiments we would like to examine any other changes that may be occurring in the regulation of any other immune cells such as natural killer cells or neutrophils. Our RNA-seq data showed that inflammation was significantly affected by PIM deletion, so we think these other cells may be affected as well. Along these lines we would also like to evaluate serum cytokine levels to see if PIM1/2 deletion affect these as well. We

have already collected serum from previously analyzed mice and plan to check cytokines such as TGF-β by ELISA.

Breast cancers are very heterogeneous diseases, and no single drug is sufficient to completely cure the disease. We saw the greatest effectiveness of PIM kinase inhibition with TP3654 in TNBC cells. TNBCs are treated with a combination of chemotherapy drugs including a taxane, an anthracycline, and a platinum salt. We showed that combined treatment of TP-3654 with doxorubicin or cisplatin resulted in greater inhibition of TNBC cells. Furthermore, combined treatment of TP-3654 and doxorubicin resulted in significantly greater reduction of tumor growth compared with vehicle or doxorubicin treatment in MDA-231-2B xenograft NSG mouse experiments. These results are still preliminary, and we need to repeat these experiments to ensure reproducibility. In future experiments, we would also like to evaluate the combination of additional drugs used to treat breast cancer, including docetaxel and cisplatin. In addition to evaluating the combination, we would also like to test if treatment with chemotherapy drugs induces PIM expression. DNA damage has been reported to induce PIM expression, and both cisplatin and doxorubicin cause DNA damage, so we hypothesize that treatment with these chemotherapies may induce PIM expression, so the combination may provide synergistic effects.

One of the issues we have had with combination treatments is trying to figure out the correct concentrations of each drug to use in order to achieve a synergistic effect. In future experiments, we would like to screen cells in a 96 well plate with varying concentrations of TP-3654 and the other drug. For TP-3654 we plan on screening 0.125 μM, 0.25 μM, 0.5 μM, 1.0 μM, and 2.0 μM. For the additional drug being screen we will set the concentrations used based on single treatments. Previously, we have been picking one concentration of TP-3654 and combining with multiple concentrations of additional drug. In future experiments, we plan on testing all of the

different concentrations for each drug in combination, using presto-blue HS to measure cell viability. This high throughput approach will allow us to test several different combinations at one time, without having to predict what concentration of drugs are needed. Based on this screen we can then perform additional drug treatments as needed.

In addition to chemotherapies in future experiments we would also like to evaluate the combination of directed therapies that are available for HR+ and HER2+ breast cancers. In preliminary experiments we have started testing tamoxifen in combination with TP-3654 in MCF-7 cells. We have seen at least an additive effect when combining TP-3654 with tamoxifen but would like to test additional concentrations of these drugs to further evaluate if the drugs can be combined to achieve a synergistic effect. If a synergistic effect is achieved, in future experiments we would also like to test the combination in animal models. The PyMT model of breast cancer has amplified estrogen receptor signaling, so we think this would be a good model to start evaluating this combination but would also like to test in NSG xenograft experiments as well.

In addition to tamoxifen, HR+ breast cancer patients can also receive a CDK4/6 inhibitor such as palbociclib. In preliminary experiments we have tested this combination in MDA-231 cells but would like to further evaluate the effect on HR+ breast cancer cell lines. Our studies have shown that p21/p27 expression is increased with TP-3654 treatment. Both p21 and p27 are potent cell cycle inhibitors, so we hypothesize that targeting the cell cycle at multiple points may produce a synergistic effect. We plan on testing this combination in MCF-7 cells to start, and also would like to evaluate the effects of combined treatments in animal models of HR+ breast cancer as well.

In addition to directed therapies for HR+ breast cancers, we would also like to assess the combination of TP-3654 with lapatinib. Our results showed that HER2+ breast cancer cells were less sensitive to PIM kinase inhibition, however we did see a significant reduction in cell growth. To further evaluate the potential of PIM kinase inhibition in HER2+ breast cancers we would also like to test the combination of lapatinib with TP-3654. We plan on first testing TP-3654 alone on a larger panel of HER2+ breast cancer cell lines, as well as the combination of TP-3654 and lapatinib in these cells. If the treatment proves effective in cells, we would also like to then test the combination in mouse models of breast cancer.

Our RNA-seq analysis revealed several pathways are altered by PIM1 deletion or inhibition. We observed significant enrichment of genes related to translation elongation, mammary stemness, proliferation, metastasis and EMT in control MDA-231 cells compared with PIM1 deleted or TP3654-treated MDA-231 cells. We observed that PIM1 deletion or inhibition significantly reduced expression of FOS, DUSP1, FOXC1, CXCR4, and IL18. RT-qPCR further validated the expression of these target genes are affected by PIM1 deletion or inhibition.

Our RNA-seq results revealed that c-FOS was significantly reduced in all 3 groups of samples we examined. c-FOS has previously been shown to induce PD-1 (32). Additionally, we found that IL-18 was significantly decreased with PIM1 deletion and PIM kinase inhibition. Tumor derived IL-18 has also been reported to induce PD-1 expression (33). In addition, our GSEA analysis revealed Notch signaling was significantly negatively enriched in PIM1 PIM2 double knockout tumors. Notch signaling has also been shown to increase PDL1 expression (34). Furthermore, in non-small cell lung cancer, notch mutations have been found to be a prognostic marker for response to immunotherapy, suggesting it plays an important role in patient response

to immunotherapy (35). Combined treatment with TP-3654 and pembrolizumab should also be examined in future experiments.

In addition to combining with current chemotherapy drugs, in future experiments we would also like to evaluate the combination of PIM kinase inhibition with radiation treatment. Radiation is commonly used to treat breast cancers of all receptor types prior to surgery to shrink tumors (39-40). Radiation causes damage to DNA, which could lead to an increase in PIM kinases (41). PIM1 has been identified as a biomarker for radiation response in other cancers including head and neck cancer and non-small cell lung cancer cells, and we hypothesize that PIM1/2 may also play an important role in radiosensitization of breast cancer cells (42-43). We first want to study if PIM kinases are induced in breast cancer cells in response to radiation. Next, we would like to pre-treat cells prior to irradiation with varying concentrations of TP-3654 to evaluate if the combination may be beneficial. We plan to use both cell lines and PyMT mice for these experiments.

Lastly, we would also like to test TP-3654 treatment in patient derived xenograft (PDX) models of breast cancer. We have tested TP-3654 in NSG xenograft experiments using MDA-231-2B cells and PyMT mice. MDA-231-2B xenograft experiments showed that TP-3654 was effective, but we would like to test TP-3654 in additional TNBC models. PyMT mice are not a true triple-negative model and there is no genetic TNBC model. PDX mice containing TNBC tumors are commercially available, and we would like to perform TP-3654 in these mice. This will allow us to further assess the effectiveness of TP-3654 in vivo, and in a patient derived TNBC setting. In future experiments we would like to treat mice with TP-3654 and assess the effect on tumor growth as well as metastasis. These experiments will provide additional evidence that TP-3654 may be a good candidate drug for clinical trials.

In conclusion, we demonstrate that PIM1 knockdown or inhibition significantly reduces both breast cancer cell growth, migration, invasion, and tumor sphere formation. Genetic deletion of Pim1 or pharmacologic inhibition resulted in significantly impaired breast tumor growth and lung metastasis. We also identified several downstream targets of PIM1 that are affected by PIM1 deletion or inhibition in breast cancer cell lines and MMTV-PyMT mice. In addition, we showed that PIM1 contributes to EMT and metastasis by regulating the expression of Vimentin, Slug and c-Met in breast cancer cells. Lastly, we show that combined treatment of TP-3654 and doxorubicin may result in greater inhibition of breast cancer cell growth. Taken together, these results suggest that PIM1, and to a lesser extent PIM2 play important roles in breast cancer growth and metastasis. Results from our study support the clinical investigation of PIM kinase inhibitor TP-3654 alone or in combination with chemotherapies in patients with TNBC.

References

1. National Program of Cancer Registries and Surveillance, Epidemiology, and End Results Program SEER*Stat Database: NPCR and SEER Incidence – U.S. Cancer Statistics Public Use Research Database with Puerto Rico, 2021 submission (2005–2019), United States Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute. Released June 2022. Available at www.cdc.gov/cancer/uscs/public-use

2. Dunnwald, L. K., Rossing, M. A., & Li, C. I. (2007). Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. *Breast cancer research*, *9*(1), 1-10.

3. Lilly, M., Holland, P., & Hendrickson, S. L. (1992). Sustained expression of the pim-1 kinase is specifically induced in myeloid cells by cytokines whose receptors are structurally related. *Oncogene*, *7*(4), 727-732.

4. Wang, L., Simons, D. L., Lu, X., Tu, T. Y., Avalos, C., Chang, A. Y., ... & Lee, P. P. (2020). Breast cancer induces systemic immune changes on cytokine signaling in peripheral blood monocytes and lymphocytes. *EBioMedicine*, *52*, 102631.

5. Gao, X., Liu, X., Lu, Y., Wang, Y., Cao, W., Liu, X., ... & Wang, H. (2019). PIM1 is responsible for IL-6-induced breast cancer cell EMT and stemness via c-myc activation. *Breast Cancer*, *26*(5), 663-671.

6. Čokić, V. P., Mitrović-Ajtić, O., Beleslin-Čokić, B. B., Marković, D., Buač, M., Diklić, M., ... & Raj, P. K. (2015). Proinflammatory cytokine IL-6 and JAK-STAT signaling pathway in myeloproliferative neoplasms. *Mediators of inflammation*, *2015*.

7. Balko, J. M., Schwarz, L. J., Luo, N., Estrada, M. V., Giltnane, J. M., Dávila-González, D., ... & Arteaga, C. L. (2016). Triple-negative breast cancers with amplification of JAK2 at the 9p24 locus demonstrate JAK2-specific dependence. *Science translational medicine*, *8*(334), 334ra53-334ra53.

8. Chen, J., Kobayashi, M., Darmanin, S., Qiao, Y., Gully, C., Zhao, R., ... & Lee, M. H. (2009). Hypoxia-mediated up-regulation of Pim-1 contributes to solid tumor formation. *The American journal of pathology*, *175*(1), 400-411.

9. Mettlin, C. (1999). Global breast cancer mortality statistics. *CA: a cancer journal for clinicians*, *49*(3), 138-144.

10. Zhang, F., Beharry, Z. M., Harris, T. E., Lilly, M. B., Smith, C. D., Mahajan, S., & Kraft, A. S. (2009). PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells. *Cancer biology & therapy*, *8*(9), 846-853.

11. Von Manteuffel, S. R., Gingras, A. C., Ming, X. F., Sonenberg, N., & Thomas, G. (1996). 4E-BP1 phosphorylation is mediated by the FRAP-p70s6k pathway and is independent of

mitogen-activated protein kinase. *Proceedings of the National Academy of Sciences*, *93*(9), 4076-4080.

12. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature*, *366*(6456), 701-704.

13. Satelli, A., & Li, S. (2011). Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cellular and molecular life sciences*, *68*(18), 3033-3046.

14. Ye, Y., Xiao, Y., Wang, W., Yearsley, K., Gao, J. X., Shetuni, B., & Barsky, S. H. (2010). ERα signaling through slug regulates E-cadherin and EMT. *Oncogene*, *29*(10), 1451-1462.

15. Attalla, S., Taifour, T., Bui, T., & Muller, W. (2021). Insights from transgenic mouse models of PyMT-induced breast cancer: recapitulating human breast cancer progression in vivo. *Oncogene*, *40*(3), 475-491.

16. Cao, L., Wang, F., Li, S., Wang, X., Huang, D., & Jiang, R. (2019). PIM1 kinase promotes cell proliferation, metastasis and tumor growth of lung adenocarcinoma by potentiating the c-MET signaling pathway. *Cancer letters*, *444*, 116-126.

17. Cho, E. S., Kang, H. E., Kim, N. H., & Yook, J. I. (2019). Therapeutic implications of cancer epithelial-mesenchymal transition (EMT). *Archives of pharmacal research*, *42*(1), 14-24.

18. Kotiyal, S., & Bhattacharya, S. (2014). Breast cancer stem cells, EMT and therapeutic targets. *Biochemical and biophysical research communications*, *453*(1), 112-116.

19. Antony, J., Thiery, J. P., & Huang, R. Y. J. (2019). Epithelial-to-mesenchymal transition: lessons from development, insights into cancer and the potential of EMT-subtype based therapeutic intervention. *Physical biology*, *16*(4), 041004.

20. Asgarova, A., Asgarov, K., Godet, Y., Peixoto, P., Nadaradjane, A., Boyer-Guittaut, M., ... & Hervouet, E. (2018). PD-L1 expression is regulated by both DNA methylation and NF-kB during EMT signaling in non-small cell lung carcinoma. *Oncoimmunology*, *7*(5), e1423170.

21. Weiss, A., & Attisano, L. (2013). The TGFbeta superfamily signaling pathway. *Wiley Interdisciplinary Reviews: Developmental Biology*, *2*(1), 47-63.

22. Zhao, B., Liu, L., Mao, J., Zhang, Z., Wang, Q., & Li, Q. (2018). PIM1 mediates epithelial-mesenchymal transition by targeting Smads and c-Myc in the nucleus and potentiates clear-cell renal-cell carcinoma oncogenesis. *Cell death & disease*, *9*(3), 1-14.

23. Yan, X., Liu, Z., & Chen, Y. (2009). Regulation of TGF-β signaling by Smad7. *Acta biochimica et biophysica Sinica*, *41*(4), 263-272.

24. Choi, J., Gyamfi, J., Jang, H., & Koo, J. S. (2018). The role of tumor-associated macrophage in breast cancer biology.

25. Yu, T., & Di, G. (2017). Role of tumor microenvironment in triple-negative breast cancer and its prognostic significance. *Chinese Journal of Cancer Research*, *29*(3), 237.

26. Oshi, M., Asaoka, M., Tokumaru, Y., Yan, L., Matsuyama, R., Ishikawa, T., ... & Takabe, K. (2020). CD8 T cell score as a prognostic biomarker for triple negative breast cancer. *International journal of molecular sciences*, *21*(18), 6968.

27. Matsumoto, H., Thike, A. A., Li, H., Yeong, J., Koo, S. L., Dent, R. A., ... & Iqbal, J. (2016). Increased CD4 and CD8-positive T cell infiltrate signifies good prognosis in a subset of triple-negative breast cancer. *Breast cancer research and treatment*, *156*(2), 237-247.

28. Meng, X., Huang, Z., Teng, F., Xing, L., & Yu, J. (2015). Predictive biomarkers in PD-1/PD-L1 checkpoint blockade immunotherapy. *Cancer treatment reviews*, *41*(10), 868-876.

29. Khoja, L., Butler, M. O., Kang, S. P., Ebbinghaus, S., & Joshua, A. M. (2015). Pembrolizumab. *Journal for immunotherapy of cancer*, *3*(1), 1-13.

30. Garon, E. B., Rizvi, N. A., Hui, R., Leighl, N., Balmanoukian, A. S., Eder, J. P., ... & Gandhi, L. (2015). Pembrolizumab for the treatment of non–small-cell lung cancer. *New England Journal of Medicine*, *372*(21), 2018-2028.

31. Schmid, P., Cortes, J., Pusztai, L., McArthur, H., Kümmel, S., Bergh, J., ... & O'Shaughnessy, J. (2020). Pembrolizumab for early triple-negative breast cancer. *New England Journal of Medicine*, *382*(9), 810-821.

32. Guo, J., Xue, Z., & Wang, L. (2022). Transcriptional regulation of the immune checkpoints PD-1 and CTLA-4. *Cellular & Molecular Immunology*, 1-2.

33. Park, I. H., Yang, H. N., Lee, K. J., Kim, T. S., Lee, E. S., Jung, S. Y., ... & Kong, S. Y. (2017). Tumor-derived IL-18 induces PD-1 expression on immunosuppressive NK cells in triple-negative breast cancer. *Oncotarget*, *8*(20), 32722.

34. Mansour, F. A., Al-Mazrou, A., Al-Mohanna, F., Al-Alwan, M., & Ghebeh, H. (2020). PD-L1 is overexpressed on breast cancer stem cells through notch3/mTOR axis. *Oncoimmunology*, *9*(1), 1729299.

35. Roper, N., Velez, M. J., Chiappori, A., Kim, Y. S., Wei, J. S., Sindiri, S., ... & Thomas, A. (2021). Notch signaling and efficacy of PD-1/PD-L1 blockade in relapsed small cell lung cancer. *Nature Communications*, *12*(1), 1-13.

36. Mikkers, H., Nawijn, M., Allen, J., Brouwers, C., Verhoeven, E., Jonkers, J., & Berns, A. (2004). Mice deficient for all PIM kinases display reduced body size and impaired responses to hematopoietic growth factors. *Molecular and cellular biology*, *24*(13), 6104-6115.

37. Batra, V., Maris, J. M., Kang, M. H., Reynolds, C. P., Houghton, P. J., Alexander, D., ... & Smith, M. A. (2012). Initial testing (stage 1) of SGI-1776, a PIM1 kinase inhibitor, by the pediatric preclinical testing program. *Pediatric blood & cancer*, *59*(4), 749-752.

38. Cortes, J., Tamura, K., DeAngelo, D. J., De Bono, J., Lorente, D., Minden, M., ... & Dean, E. (2018). Phase I studies of AZD1208, a proviral integration Moloney virus kinase inhibitor in solid and haematological cancers. *British journal of cancer*, *118*(11), 1425-1433.

39. Hortobagyi, G. N. (1998). Treatment of breast cancer. *New England Journal of Medicine*, *339*(14), 974-984.

40. Maughan, K. L., Lutterbie, M. A., & Ham, P. (2010). Treatment of breast cancer. *American family physician*, *81*(11), 1339-1346.

41. Baskar, R., Lee, K. A., Yeo, R., & Yeoh, K. W. (2012). Cancer and radiation therapy: current advances and future directions. *International journal of medical sciences*, *9*(3), 193.

42. Peltola, K., Hollmen, M., Maula, S. M., Rainio, E., Ristamäki, R., Luukkaa, M., ... & Jalkanen, S. (2009). Pim-1 kinase expression predicts radiation response in squamocellular carcinoma of head and neck and is under the control of epidermal growth factor receptor. *Neoplasia*, *11*(7), 629-IN1.

43. Kim, W., Youn, H., Seong, K. M., Yang, H. J., Yun, Y. J., Kwon, T., ... & Youn, B. (2011). PIM1-activated PRAS40 regulates radioresistance in non-small cell lung cancer cells through interplay with FOXO3a, 14-3-3 and protein phosphatases. *Radiation research*, *176*(5), 539-552.

44. Noman MZ, Janji B, Abdou A, et al. The immune checkpoint ligand PD-L1 is upregulated in EMT-activated human breast cancer cells by a mechanism involving ZEB-1 and miR-200. Oncoimmunology. 2017;6(1):e1263412. Published 2017 Jan 23. doi:10.1080/2162402X.2016.1263412

45. Subbannayya T, Leal-Rojas P, Zhavoronkov A, et al. PIM1 kinase promotes gallbladder cancer cell proliferation via inhibition of proline-rich Akt substrate of 40 kDa (PRAS40). J Cell Commun Signal. 2019;13(2):163-177. doi:10.1007/s12079-018-00503-5

46. Zhang F, Beharry ZM, Harris TE, et al. PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells. Cancer Biol Ther. 2009;8(9):846-853. doi:10.4161/cbt.8.9.8210

47. Hammerman PS, Fox CJ, Birnbaum MJ, Thompson CB. Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. Blood. 2005;105(11):4477-4483. doi:10.1182/blood-2004-09-3706

48. Müller D, Lasfargues C, El Khawand S, et al. 4E-BP restrains eIF4E phosphorylation. Translation (Austin). 2013;1(2):e25819. Published 2013 Jul 24. doi:10.4161/trla.25819

49. Li Y, Fan S, Koo J, et al. Elevated expression of eukaryotic translation initiation factor 4E is associated with proliferation, invasion and acquired resistance to erlotinib in lung cancer. Cancer Biol Ther. 2012;13(5):272-280. doi:10.4161/cbt.18923

50. Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J. Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. J Biol Chem. 1998;273(26):16621-16629. doi:10.1074/jbc.273.26.16621

51. Uchikawa E, Chen Z, Xiao GY, Zhang X, Bai XC. Structural basis of the activation of c-MET receptor. Nat Commun. 2021;12(1):4074. Published 2021 Jul 1. doi:10.1038/s41467-021-24367-3

52. Balko JM, Schwarz LJ, Luo N, et al. Triple-negative breast cancers with amplification of JAK2 at the 9p24 locus demonstrate JAK2-specific dependence [published correction appears in Sci Transl Med. 2019 Jan 23;11(476):]. Sci Transl Med. 2016;8(334):334ra53. doi:10.1126/scitranslmed.aad3001

53. Castaneda CA, Cortes-Funes H, Gomez HL, Ciruelos EM. The phosphatidyl inositol 3-kinase/AKT signaling pathway in breast cancer. Cancer Metastasis Rev. 2010;29(4):751-759. doi:10.1007/s10555-010-9261-0

54. Peterson, M.E. Management of adverse events in patients with hormone receptor-positive breast cancer treated with everolimus: observations from a phase III clinical trial. Support Care Cancer 21, 2341–2349 (2013). https://doi.org/10.1007/s00520-013-1826-3

55. Cen B, Xiong Y, Song JH, et al. The Pim-1 protein kinase is an important regulator of MET receptor tyrosine kinase levels and signaling. Mol Cell Biol. 2014;34(13):2517-2532. doi:10.1128/MCB.00147-14