The epigenetic regulation of p66ShcA and its role in promoting cellular plasticity and lung metastasis in breast cancer

By

Jesse Hudson

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Faculty of Medicine

Division of Experimental Medicine

Montreal, Quebec, Canada

Table of Contents

Abstract

Reactive oxygen species (ROS) are critical mediators of cell signalling and are implicated across different processes during tumorigenesis and progression. Depending on ROS levels, this can be beneficial or harmful to the tumor. p66ShcA is an adaptor protein that is involved in mediating an oxidative stress response by promoting the production of mitochondrial reactive oxygen species in response to stress stimuli. In cancer, p66ShcA has been shown to have both pro and anti-tumorigenic functions and is expressed variably. Our work has focused on characterizing whether p66ShcA is pro or anti-tumorigenic in breast cancer during tumor outgrowth and metastasis, if this depends on redox status, and whether this contributes to cellular plasticity. A key process that increases cellular plasticity and the malignant potential of breast tumors is the epithelial-to-mesenchymal-transition (EMT). To model this, we looked at the role of p66ShcA in ErbB2 positive luminal breast cancer versus aggressive triple-negative breast cancer (TNBC). We outline a novel role for p66ShcA in promoting cellular plasticity by inducing an EMT in HER2 positive luminal breast tumors through the Met RTK. p66ShcA-induced plasticity contributes to intratumoral heterogeneity, particularly in the luminal A subtype, where tumors are normally well-differentiated and express epithelial markers. We also identify p66ShcA as a biomarker of primary breast tumors possessing mesenchymal features, across molecular subtypes. Further studies revealed these effects appear to be independent of mitochondrial-p66ShcA.

Breast cancer is the most commonly diagnosed cancer in women and metastasis to distant organs is responsible for 90% of cancer-related deaths. The metastatic cascade involves a series of steps that contribute to successful colonization. While our understanding of the underlying molecular and cellular processes that contribute to metastatic disease has vastly improved, our ability to effectively treat patients has not. Hence, further studies in relevant pre-clinical models

are necessary to overcome current barriers in therapy. We discovered mitochondrial versus cytoplasmic pools of p66ShcA regulate different stages of the metastatic cascade in aggressive TNBC by employing expression vectors stably overexpressing wild-type p66ShcA or a nonphosphorylatable mutant (p66ShcAS36A) that cannot translocate into the mitochondria. Mitochondrial-p66ShcA is required for entry/survival in the circulation which leads to high levels of circulating tumor cells compared to p66ShcAS36A that is limited to the cytoplasm. In contrast, cytoplasmic p66ShcA was necessary for elevated migration from the primary site and increased focal adhesion turnover to facilitate colonization from the circulation. Therefore, in agreement with the literature, we show that ROS can be pro- or anti-tumorigenic both from the primary or metastatic site and depending on the molecular subtype. This work highlights p66ShcA's pleiotropic roles in breast cancer as a promiscuous molecule in tumorigenesis and metastasis. We identified high expression of p66ShcA in 1/3 of pre-existing parental TNBC clones, indicating high p66ShcA levels are enriched in TNBCs through metastatic *in vivo* selection and suggests that selection of p66ShcA as a metastasis progression gene. This evidence supports previous studies indicating metastases often resemble the primary tumor, that driver mutations are a rare event in breast cancer and metastatic progression genes often are already present in the primary tumor and are selected for through environmental factors such as stress and the microenvironment. Finally, p66ShcA has been shown to be epigenetically regulated through promoter methylation and hyperacetylation. We discovered that high p66ShcA expression correlates with the presence of active histone marks, including: H3K4Ac, H3K9Ac and H3K27Ac. Furthermore, the chromatin insulator, CTCF, binds to the p66ShcA promoter in breast cancer cells that express high levels of p66ShcA expression. These data suggest p66ShcA may be epigenetically regulated in breast cancer.

Résumé

Les espèces réactives de l'oxygène (ROS) sont des médiateurs critiques de la signalisation cellulaire et sont impliquées dans différents processus au cours de la tumorigenèse et de la progression. Selon les niveaux de ROS, cela peut être bénéfique ou néfaste pour la tumeur. p66ShcA est une protéine adaptatrice impliquée dans la médiation d'une réponse au stress oxydatif. Dans le cancer, il a été démontré que p66ShcA avait à la fois des fonctions pro et anti-tumorigènes et s'exprimait de manière variable. Notre travail a principalement consisté à déterminer si p66ShcA est un pro ou anti-tumorigène dans le cancer du sein au cours de la croissance tumorale et de la métastase, si cela dépend du statut redox et si cela contribue à la plasticité cellulaire. Un processus clé qui augmente la plasticité cellulaire et le potentiel malin des tumeurs du sein est la transition épithéliale-mésenchymateuse (EMT). Pour modéliser cela, nous avons examiné le rôle de p66ShcA dans le cancer du sein luminal positif ErbB2 par rapport au cancer du sein agressif triple négatif (TNBC). Nous décrivons un nouveau rôle pour p66ShcA dans la promotion de la plasticité cellulaire en induisant un EMT dans les tumeurs mammaires lumineuses HER2 positives par le biais de la RTK Met. La plasticité induite par p66ShcA contribue à l'hétérogénéité intratumorale, en particulier dans le sous-type luminal A, où les tumeurs sont normalement bien différenciées et expriment des marqueurs épithéliaux. Nous identifions également p66ShcA en tant que biomarqueur de tumeurs primitives du sein possédant des caractéristiques mésenchymateuses, sur différents sous-types moléculaires. D'autres études ont révélé que ces effets semblent être indépendants de p66ShcA mitochondrial. Le cancer du sein est le cancer le plus souvent diagnostiqué chez les femmes et les métastases à des organes distants sont responsables de 90% des décès liés au cancer. La cascade métastatique implique une série d'étapes qui contribuent au succès de la colonisation. Bien que notre compréhension des processus moléculaires et cellulaires

sous-jacents qui contribuent à la maladie métastatique se soit considérablement améliorée, notre capacité à traiter efficacement les patients ne s'est pas améliorée. Par conséquent, des études complémentaires sur des modèles précliniques pertinents sont nécessaires pour surmonter les obstacles actuels en matière de traitement. Nous avons découvert des pools mitochondriaux contre cytoplasmiques de p66ShcA régulant différents stades de la cascade métastatique dans une TNBC agressif. p66ShcA mitochondriale est nécessaire à l'entrée / à la survie dans la circulation, ce qui entraîne des taux élevés de cellules tumorales en circulation par rapport aux mutants VC et p66ShcAS36A limités au cytoplasme. Au contraire, p66ShcA cytoplasmique était nécessaire pour une migration élevée à partir du site primaire et une augmentation du renouvellement de l'adhésion focale afin de faciliter la colonisation par la circulation. Par conséquent, en accord avec la littérature, les ROS peuvent être pro- ou anti-tumorigènes à la fois du site primaire ou métastatique et en fonction du sous-type moléculaire. Ce travail met en évidence les rôles pléiotropes de p66ShcA dans le cancer du sein en tant que molécule promiscuité dans la tumorigenèse et les métastases. Nous avons identifié une expression élevée de p66ShcA dans 1/3 des clones TNBC parentaux préexistants, ce qui indique que des taux élevés de p66ShcA sont enrichis en TNBC par sélection métastatique in vivo et suggère que la sélection de p66ShcA en tant que gène de progression métastatique. Ces preuves corroborent les études antérieures indiquant que les métastases ressemblent souvent à la tumeur primitive, que les mutations du conducteur sont un événement rare dans le cancer du sein et que les gènes de progression métastatique sont déjà présents dans la tumeur primitive et sont sélectionnés en fonction de facteurs environnementaux tels que le stress et le microenvironnement. Enfin, il a été démontré que p66ShcA était régulé de manière épigénétique par le biais d'une méthylation et d'une hyperacétylation du promoteur. Nous avons découvert qu'une expression élevée de p66ShcA est corrélée à la présence de marques

d'histone actives, notamment : H3K4Ac, H3K9Ac et H3K27Ac. De plus, l'isolant de la chromatine, CTCF, se lie au promoteur p66ShcA dans les cellules du cancer du sein qui expriment des niveaux élevés d'expression de p66ShcA. Ces données suggèrent que p66ShcA pourrait être régulé épigénétiquement dans le cancer du sein.

Acknowledgements

First and foremost, I want to give a sincere thank you to my exceptional supervisor, Dr. Josie Ursini-Siegel, for supporting me as one of her first doctoral students for the past 7 years. None of this work would be possible without her guidance, leadership and encouragement. Josie demonstrates a level of enthusiasm and dedication to science that simply inspires and motivates. I feel very fortunate to have received a great deal of trust and independence to pursue novel projects and scientific avenues that involved a very enigmatic protein, all in an effort to enhance our understanding of breast tumorigenesis and progression. I have gained invaluable knowledge and experience throughout my graduate studies and it has been an absolute pleasure to learn and work alongside her and to have successfully persevered thanks to her guidance and patience.

Next, I would like to thank all of the granting agencies for supporting our research over the years. To the Canadian Institutes of Health Research, the Canadian Cancer Society and the Susan G. Komen foundation for providing our operating grants. To Le Fonds de recherche du Québec – Santé for supporting me with a prestigious doctoral studentship. I would also like to thank Experimental Medicine for their generosity in providing me with several travel awards and to CIHR for the ICS Travel Award to present my work at national and international conferences.

I would like to thank my astute research committee members Dr. Koren Mann, Dr. Michael Witcher, Dr. Rongtuan Lin, Dr. Teodoro and Dr. Lorraine Chalifour for their insightful scientific input over the years. A huge thank you goes to all of my extremely hardworking colleagues and collaborators for their invaluable research contributions that greatly enhanced the impact of our work. These studies would not have been possible without the support from all of the members of the Ursini-Siegel lab that have helped me a great deal both on the bench with experiments and advice and off of it by making the lab such a fun learning environment to be immersed in: Eduardo

Canedo, Kyle Lewis, Lauren Podmore, Laura Forrest, Matthew Siegel, Rachel Laselva, Ryuhjin Ahn, Stephanie Totten, Sophie Guerin, Valerie Sabourin and Young Im.

This thesis is dedicated to my loving family, friends and colleagues who have supported me throughout. It is with a heavy heart that I thank my father, mother, brother, cousins, aunts, uncles and grandparents for always supporting and encouraging me to pursue a life of continuous learning and growth. I appreciate Sarah, Helena, Sophie, Marek and Anne-Marie for welcoming me into their family and making life outside the lab just as enjoyable as it was in it. Their support will not be forgotten.

List of Abbreviations

4-HNE	4-hydroxynoneal
Akt	Protein kinase B
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BL1	Basal-like 1
BL2	Basal-like 2
BRCA	BReast CAncer
CD3	Cluster of differentiation 3
CH1	Collagen homology domain 1
CH2	Collagen homology domain 2
CK5/6	Cytokeratins 5/6
CK14	Cytokeratins 14
CTCF	CCCTC-binding factor is a transcription factor
NADH-Cyt B5	Cytochrome B5 reductase
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
DNMT1	DNA (cytosine-5)-methyltransferase 1
DNMT3b	DNA (cytosine-5)-methyltransferase 3b
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal-transition
ER	Estrogen receptor
ErbB2	Erythroblastic oncogene B
FAK	Focal adhesion kinase
FASL	Fas ligand
FGFR	Fibroblast growth factor receptor
GAB	Grb2-associated binding
GPX	Glutathione peroxidase
GATA3	GATA binding protein 3
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GRB2	GRB2 - Growth factor receptor-bound protein 2
HER1	The epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2 (neu in rodents)
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
HGF	Hepatocyte growth factor
HSP-90	Heat shock protein 90
IGF-1	Insulin-like growth factor 1
IM	Immunomodulatory
JNK1/2	C-Jun N-terminal kinases (JNKs)
Kit	Tyrosine-protein kinase Kit or CD117
LAR	Luminal androgen receptor
LCIS	Lobular carcinoma in situ
LDL	Low-density lipoproteins

M	Mesenchymal
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAP2K4	Mitogen-activated protein kinase kinase 4
MET	Mesenchymal-Epithelial-Transition (MET)
mTOR	Mammalian target of rapamycin
mtDNA	mutated mitochondrial DNA
MSL	Mesenchymal stem–like
MUC1	Sialomucin
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
nBSCs	Normal breast stem cells
NSCLC	Non-small-cell-lung-carcinoma
NTRK2	Neurotrophic receptor tyrosine kinase 2
PARP	Poly (ADP-ribose) polymerase
p38 MAPK	p38 mitogen-activated protein kinases
PDGFR	Platelet-derived growth factor receptors
PH domains	Pleckstrin homology domain (PH domain)
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic
PI(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate
PI4P	Phosphatidylinositol 4-phosphate
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PIN1	The prolyl isomerase PIN1
PKC	Protein kinase C
PPP2R2A	PPP2R2A protein phosphatase 2 regulatory subunit B alpha
PR	Progesterone receptors
Prx	Peroxiredoxin
PTP-1D	Protein-tyrosine phosphatase 1D
PTB	Phosphotyrosine-binding domain
PTB-PEST	Tyrosine-protein phosphatase non-receptor type 12
RAC1	Ras-related C3 botulinum toxin substrate 1
RANKL	Receptor activator of nuclear factor kappa-B ligand
RTK	Receptor tyrosine kinase
ROS	Reactive oxygen species
SFK	Src kinase family
SH2	Src Homology 2
SH3	Src Homology 3
ShcA	Src homology collagen A
SHIP2	The SH2 domain containing inositol 5-phosphatase 2 (SHIP2)
SHPS-1	Multifunctional transmembrane glycoprotein
SMA	Smooth muscle actin
SNTA1	Alpha-1-syntrophin
SOD	Superoxide dismutase
SOS	Son of sevenless
Src	Proto-oncogene tyrosine-protein kinase Src
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3

TEB	Terminal end buds
TGFß	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TNBC	Triple negative breast cancer
TRAIL	TNF-related apoptosis-inducing ligand
TrkA	Tropomyosin receptor kinase A (nerve growth factor receptor)
VEGF	Vascular endothelial growth factor
ZNF703	Zinc finger protein 703

Publications Arising from this Work Chapter

Chapter 2 was published as an original research article:

Jesse Hudson, Jacqueline R. Ha, Valerie Sabourin, Ryuhjin Ahn, Rachel La Selva, Julie Livingstone, Lauren Podmore, Jennifer Knight, Laura Forrest, Nicole Beauchemin, Michael Hallett, Morag Park, Josie Ursini-Siegel (2014). p66ShcA Promotes Breast Cancer Plasticity by Inducing an Epithelial-to-Mesenchymal Transition. *Molecular and Cellular Biology*, *34*(19), 3689–3701. https://doi.org/10.1128/MCB.00341-14

Chapter 3 contains material that was included in a published original research article:

Jesse Hudson*, Kyle Lewis*, Alexander Kiepas*, Julien Senécal, Matthew G. Annis, Jacqueline R. Ha, Valerie Sabourin, Sébastien Tabariès, Ryuhjin Ahn, Matthew Siegel, Rachel La Selva, Eduardo Cepeda, Peter Siegel, Josie Ursini-Siegel (2018). Mitochondrial and Cytoplasmic p66ShcA are Required for Different Stages of the Metastatic Cascade to Support Triple Negative Breast Cancer Lung Metastasis. Manuscript in preparation for submission to: *PNAS*.

Chapter 4 contains material that may be included in a future manuscript for publication as an original research article:

Jesse Hudson, Harvey Li, Valerie Sabourin, Tiejun Zhao, Michael Witcher, and Josie Ursini-Siegel. Epigenetic Regulation of p66ShcA in Breast Cancer. Manuscript in preparation.

The following publication included work performed by me during my PhD, however it is not presented in this thesis:

Young Kyuen Im, Ouafa Najyb, Simon-Pierre Gravel, Shawn McGuirk, Ryuhjin Ahn, Daina Z. Avizonis, Valérie Chénard, Valerie Sabourin, Jesse Hudson, Tony Pawson, Ivan Topisirovic, Michael Pollak, Julie St-Pierre, and Josie Ursini-Siegel (2018). Interplay between ShcA Signaling and PGC-1α Triggers Targetable Metabolic Vulnerabilities in Breast Cancer. *Cancer Research* (78) (17) 4826-4838; DOI: 10.1158/0008-5472.CAN-17-3696

Contributions of the Authors

I designed, executed and analyzed the majority of experiments under the guidance of my supervisor Dr. Ursini-Siegel. In addition to my work, several collaborations were established in order to complete the work presented in this thesis. The contributions provided by the authors are outlined below, for each chapter constituting this work. Finally, I wrote the dissertation with the guidance from my supervisor J.U.S.

In chapter 2, J.R.H. completed figures 4C-D, 6B, 6E-H and S5. V.S. completed figures 2A, 2C-D, 2F-G, 3D, 5A-C, S1D, S1F-G, S2 and S3. R.A. completed figures 1B and 6C-D. R.S. performed experiments studying p66ShcA-dependent signalling in response to oxidative stress during revisions. J.L. and M.H. completed RNA seq experiments for figures 7C-E and 8B-F. L.P. completed figures 2B, 2E, S1E and S6B-C. J.K. and M.P. provided the tumor lysates from transgenic mouse models of different breast cancer subtypes. L.F. completed figure 1A.

Note: Figures 1C-G, S1, S2 and S6 were used in L.P.'s M.Sc. thesis.

In chapter 3, K.L. and J.R.H. completed figure 2D-E, K. L. completed figure 5A, A.K. and J.S. completed figure 3A-C and figure 4A-E, M.G.A. completed figure 5B-C, V.S. completed tail vein IHC for 6A-C and S1D-G, S.T. and P.M.S. generated the in vivo selected metastatic variants, provided tumor lysates and taught our lab spontaneous metastasis assay, R.A. performed MFP injections for certain experiments, M.S. completed S2B, R.L and E.C. aided in experimental design and discussing results.

In chapter 4, H.L. completed figure 4B, V.S. completed figure 3A-B, J.Z. and M.W. provided cell lines, primers and guidance with CHIP assays and M.W. also generated figure 2A.

Original Contributions to Knowledge

1. We provide the first in vivo evidence of the role of p66ShcA in breast cancer. We showed that p66ShcA promotes an EMT in ErbB2 positive luminal breast cancer which enhances cellular plasticity and increases intratumoral heterogeneity. p66ShcA induces an EMT through activated Met signaling and is enriched in both the luminal A and claudin-low subtypes. We also discovered that p66ShcA acts as a biomarker of breast tumors possessing mesenchymal features regardless of molecular subtype.
2. p66ShcA can be pro or anti-tumorigenic during breast tumorigenesis depending on the context. In two ErbB2 positive luminal cell lines p66ShcA was sufficient to reduce tumor outgrowth by inhibiting cell proliferation. In contrast, in 4T1 parental TNBC tumors, p66ShcA was sufficient to elevate tumor outgrowth by reducing apoptosis.
3. Different pools of p66ShcA regulate early and late stages of the metastatic cascade during breast cancer metastasis to the lung. Mitochondrial-p66ShcA is important for intravasation and/or survival within the circulation. In contrast, cytoplasmic-p66ShcA controls migration from the primary site early on, enhances focal adhesion turnover to promote lung colonization and reactivate cell proliferation pathways during the late stages of the metastatic cascade. Hence, the role of ROS in promoting metastasis is context specific.
4. p66ShcA is epigenetically regulated in breast cancer. High p66ShcA expression correlates with active chromatin marks, binding of the chromatin boundary forming protein CTCF and inhibition of PARP activity reduces p66shcA expression. Furthermore, p66ShcA is transcriptionally regulated in lung metastatic variant breast cancer cells expressing high endogenous p66ShcA and these cells possess elevated levels of active chromatin within the p66ShcA promoter compared to parental cells.

Introduction – Rationale and objectives

p66ShcA is best characterized as a redox protein that regulates apoptosis in response to stress stimuli. p66ShcA expression is variable compared to the shorter ShcA isoforms, p46/52, adaptor proteins that mediate mitogenic signalling and are ubiquitously expressed (Giorgio et al. 2005a; G. Pelicci et al. 1992). Furthermore, the role of p66ShcA in breast cancer is inconsistent and poorly understood with some studies associating p66ShcA with favourable outcomes and others correlating p66ShcA with increased severity and recurrence (Frackelton et al. 2006; Grossman et al. 2007; Jackson et al. 2000). Indeed, reactive oxygen species (ROS) and various proteins can serve as promoters or suppressors of breast tumor progression and metastasis depending on the context and the molecular subtype (M. Feng et al. 2014; McLaughlin et al. 2013). Hence, my work has focused on understanding the role of p66ShcA in ErbB2 positive luminal versus basal breast cancer progression and lead to the first studies on the *in vivo* function of p66ShcA in these settings. In addition, an important aspect has been to delineate the mechanisms controlling p66ShcA expression in breast cancer with a focus on epigenetic control of the p66ShcA locus.

AIMS:

Aim #1: Characterizing the role of p66ShcA in ErbB2 positive luminal breast cancer as an inducer of cellular plasticity by promoting an EMT

Aim #2: Define the requirement and sufficiency of p66ShcA as a contextual regulator of breast cancer metastasis to the lung in aggressive TNBC

Aim #3: Delineate the epigenetic mechanisms controlling p66ShcA expression in breast cancer across molecular subtypes and in metastatic variants

Chapter 1 - Literature Review

General Cancer Overview and Hallmarks

Cancer is a remarkably heterogeneous disease that can occur in over 100 different forms and can arise from almost any tissue (Hanahan and Weinberg 2000). Moreover, unique features of the disease define virtually every individual cancer. This is due to the fact that tumors result from the sequential accumulation of mutations and are composed of complex tissues with multiple distinct cell types (Hanahan and Weinberg 2011). Despite this, certain characteristics, known as hallmarks are common to all cancers, (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) ability to avoid programmed cell death, (4) infinite replicative potential (5), stimulate blood vessel formation to supply nutrients to tumors and (6) they invade local tissue and spread to distant sites (Hanahan and Weinberg 2000). Furthermore, since the establishment of these initial hallmarks, new, emerging hallmarks have been defined involving deregulated cellular energetics, genomic instability, tumor-promoting inflammation and avoidance of immune destruction (Hanahan and Weinberg 2011). Malignant transformation of a cell occurs through the accumulation of genetic mutations within DNA, or through epigenetic modifications, and can occur years before clinical detection of the tumor. These changes provide growth and survival advantages and functionally contribute to intratumoral heterogeneity (Wahl and Spike 2017).

Genetic hits to two classes of master regulators, known as proto-oncogenes and tumor suppressors, often leads to the uncontrolled growth and spread of disease seen in human cancer (Visvader 2009). Proto-oncogenes act to accelerate excessive production or activation of growth stimuli either through enhanced growth factor production or through increased ligand-independent receptor activation. In contrast, tumor suppressor genes act to decelerate, signalling to the cell to reduce the activation of these stimulatory growth pathways and maintain homeostasis. Evasion or

reduction of these inhibitory signals promotes transformation and progression (Perou et al. 2000). In addition, these inhibitory cues can arise from neighboring cells within the stroma or from the tumor itself. In conclusion, the accumulation of genetic mutations and/or epigenetic modifications in key cell types and signalling molecules is required for successful transformation. Finally, crosstalk between the stroma and the tumor mass mediates critical interactions to promote tumor growth and progression.

Breast Cancer Epidemiology

Breast cancer is the most common cancer in Canadian women, making up over a 1/4 new cases with over 25,000 women being newly diagnosed each year (Smith et al. 2018). Of note, the incidence of breast cancer has remained stable for the past three years, with lifetime risk standing at 1/8 women. 1/2 of diagnoses occur in women aged between 50-69 with another 1/3 occurring in those aged 70 and above. Hence, breast cancer is rare in young women (aged below 50), with the largest group being 40-49. Currently, breast cancer is the second-leading cause of cancer-related death in Canadian women. Despite this, cancer mortality rates are at their lowest since 1950, largely due to the introduction of early screening and development of effective adjuvant treatments that target steroid receptors or RTK signalling.

Breast Cancer Prognosis

Approximately 7% of women with breast cancer are diagnosed before the age of 40 and survival rates for this group are poor (particularly in patients diagnosed with stage 3 and 4 breast cancer) when compared to those in older women. Multivariate analysis has shown younger age, BRCA1 and BRCA2 status to be independent predictors of adverse outcome, including metastasis (Anders et al. 2009; Elston and Ellis 1991; Fredholm et al. 2009). However, the incidence of breast

cancer is rising with age and the average diagnosis is now at age 61 and the majority of women who die from breast cancer are age 65 and older (Shachar, Hurria, and Muss 2016). Hence, there are at least two major age groups that are at increased risk of mortality.

Risk factors

Many of the established risk factors for breast cancer are linked to oestrogen levels, including: early menarche, late menopause, and obesity in postmenopausal women (Key, Verkasalo, and Banks 2001). Activation of sex steroids, including the nuclear estrogen (ER) and progesterone receptors (PR), combined with growth factors drive the development, growth and differentiation of breast epithelial tissue and are critical for breast cancer development and progression (B. Jones and Russo 1987). High estrogen levels allow the PR to be abundantly expressed together with alternative growth factor signalling. However, at low estrogen levels, the PR may be absent with an intact estrogen–ER pathway (Key, Verkasalo, and Banks 2001). ER positive cells secrete paracrine growth factors to ER negative epithelial cells that promote tumor cell proliferation (Clarke, Anderson, and Howell 2004).

Positive ER receptor status correlates with favorable prognostic features, including a lower rate of cell proliferation and histologic evidence of tumor differentiation. During the first several years after diagnosis, patients with ER positive tumors tend to have a lower recurrence rate; however, this is balanced by a higher recurrence rate in subsequent years (Bardou et al. 2003). In addition, when accurately measured, ER/PR status is an independent predictive factor for patients that would benefit from adjuvant endocrine therapy (Bardou et al. 2003). An early age at first birth and breastfeeding are components of childbearing that appear to provide a protective effect. Obesity, alcohol or tobacco use increases risk, whereas physical activity reduces it. Finally, hereditary genes significantly increase breast cancer risk, but represent a small number of cases

(Key, Verkasalo, and Banks 2001). Thus, a large proportion of environmental factors combined with a small number of genetic factors contribute to cancer development and progression.

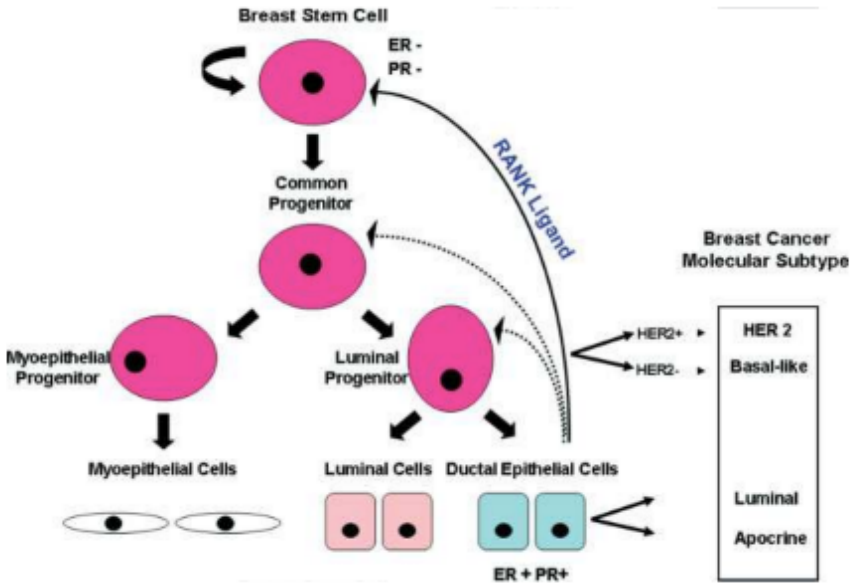
Structure, development and remodeling of the mammary gland

Most vertebrate organs develop during embryogenesis and the majority retain their basic structure throughout adulthood. Breast tissue, however, is unique in that it continually undergoes structural remodeling throughout the lifetime of reproductively active females. Breast cancer displays many of the characteristics seen during normal mammary gland development. In addition, several stromal factors that promote mammary development are also recruited during breast tumorigenesis. Crosstalk between the mammary epithelium and the mesenchyme leads to mammary bud formation at mid-gestation. The next phase occurs at puberty, due to the release of ovarian hormones, the distal ends of the mammary ducts swell into terminal end buds (TEBs), that consist of cuboidal epithelial cells. The TEBs are the invading fronts of the ducts that proliferate, extend into the fat pad, and branch by bifurcation until the ducts reach the limits of the fat pad. The major functional units of the mammary gland are the lobular structures comprising several small blind ended ductules situated at the end of the terminal ducts and known as terminal ductal lobular units (TDLUs). The entire ductal system is lined by a continuous layer of luminal epithelial cells surrounded by a layer of myoepithelial cells which, in turn, is surrounded by and in direct contact with a basement membrane. The TDLUs are then surrounded by delimiting fibroblasts and embedded in a specialized intralobular stroma. Each of these cell types can be differentiated in terms of unique patterns of antigen and cytokeratin expression. For example, only luminal epithelial cells express cytokeratins 8 and 18, the sialomucin MUC1 and low levels of cytokeratins 5 and 6, whereas myoepithelial cells express smooth muscle actin (SMA) and high levels of cytokeratins 5 and 6. Reproductive hormones induce the expansion and terminal differentiation of

the mammary epithelium into secretory, milk-producing, lobular cells. During pregnancy, the mammary epithelium invades from the nipple into the mammary fat pad and forms a small, branched ductal network. Once the pups no longer suckle on the mammary gland, the secretory epithelium of the mammary gland undergoes apoptosis and remodels back to its previous state.

Origins of Breast Cancer

Remodelling of the breast occurs during puberty and each pregnancy in response to the release of progesterone from stem cells. Normal breast stem cells are crucial to give rise to the various cell types required during these phases. Normal breast stem cells (nBSCs) are long-lived, capable of self-renewal activity and differentiate into a common progenitor that gives rise to luminal and myoepithelial progenitors that can differentiate into luminal/ductal epithelial or myoepithelial cells, respectively. Finally, the breast stem cell and breast progenitor pool is replenished during pregnancy and the reproductive cycle through the release of the RANKL ligand by ductal epithelial cells (Figure 1) (Frasor et al. 2003).



<u>Figure 1</u> - Hierarchy of normal breast epithelial development with possible links to the tumour-initiating cells of the different molecular cancer subtypes and to the role of steroid hormones in the control of the mammary stem and progenitor cells. Obtained from (Bombonati and Sgroi 2011).

Breast cancer derives from a luminal progenitor cell that gives rise to the human epidermal growth factor receptor 2 (HER2) positive and basal-like breast cancer subtypes (Molyneux et al. 2010; Shehata et al. 2012). In contrast, the more differentiated ductal epithelial cell likely gives rise to luminal breast cancers (Bombonati and Sgroi 2011). The BSC with intrinsic self-renewal potential differentiates into a common progenitor that gives rise to committed myoepithelial and luminal progenitors, which ultimately differentiate into myoepithelial, luminal and ductal epithelial cells. During puberty and pregnancy, the RANKL ligand is expressed by ductal epithelial cells in response to a surge in progesterone release to expand the stem cell population via paracrine signalling.

Breast Cancer Histology

The majority of the breast consists of fat (adipose tissue) that is embedded with a complex network of lobes/lobules that produce milk and ducts which transport it to the nipple. Mammary lobes/lobules and ducts are lined by a basal layer of myoepithelial cells that deposit fibronectin and collagen to maintain the integrity of the basement membrane and a surface layer of luminal epithelial cells that sit beneath these cells. Breast cancers are defined as carcinomas because they originate from these luminal epithelial cells within the duct or lobe (Molyneux et al. 2010; Shehata et al. 2012). Histopathological characterization of breast tumors can be broadly classified into in situ carcinoma and invasive (infiltrating) carcinoma (Figure 2). Invasive carcinoma makes up (70-80%) of breast tumors and includes seven subtypes: tubular, ductal lobular, invasive lobular, infiltrating ductal (well-differentiated), infiltrating ductal (poorly-differentiated), mucinous and medullary. Breast carcinoma in situ is further sub-classified as either ductal or lobular, where growth patterns and cytological features form the basis to distinguish between the two types. Ductal carcinoma in situ (DCIS) consists of a heterogeneous group of tumors and is far more common than lobular (LCIS) carcinoma in situ. DCIS has traditionally been further sub-classified based on architectural features, using histology, which gives rise to five well recognized subtypes: comedo, cribiform, micropapillary, papillary and solid. However, newer molecular markers have proven to have greater prognostic significance. In light of surgical advances leading to breast-conserving therapy, it has become necessary to more accurately stratify patients based on relative risk of recurrence or progression. These demands have led to the generation of several newer classification systems that incorporate molecular markers such as ER, PR, ErbB2 (Her2) and p53. (Malhotra et al. 2010)

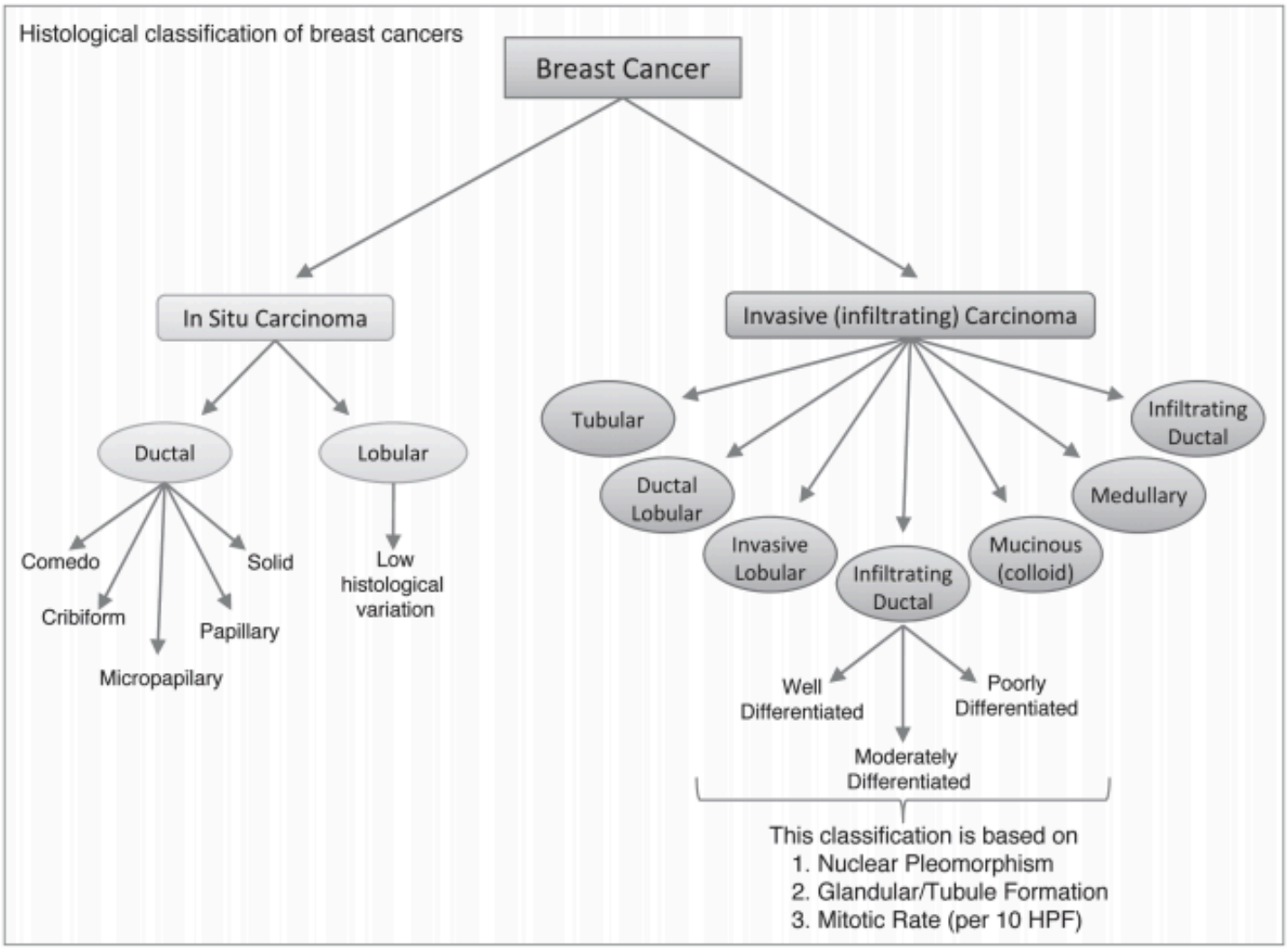


Figure 2 - Breast Cancer Histology. Obtained from (Malhotra et al. 2010)**.**

Histological classification of breast cancer subtypes based on architectural features and growth patterns that is currently used by clinicians and which categorizes the heterogeneity found in breast cancer. HPF: high power field.

Immunohistochemistry is also used to divide breast cancers based on receptor tyrosine kinase and hormone receptor expression within the tumor tissue and correlates well with gene expression profiling. The level of immunohistochemical staining for the estrogen receptor (ER), progesterone receptor (PR) and HER2 is calculated from patient samples and divided into 3 groups.

Those that are ER positive, PR positive and HER2 positive/negative are defined as luminal breast cancer and most closely resemble the luminal A and B subsets. ER negative, PR negative, but HER2 positive patient samples largely fall into the HER2 positive molecular subtype, making up 15-20% of breast tumors and are characterized by HER2 gene amplification, lack of ER expression and reduced survival (Slamon et al. 1987b). The lack of expression of all three receptors is classified as triple-negative breast cancer (TNBC), where the majority of breast tumors are invasive ductal carcinoma and associate with poor outcome. ~75% of triple negative breast cancers are classified as basal-like and the majority of these tumors possess mutations in p53 and are characterized by increased incidence of germline BRCA1 mutations (Koboldt et al. 2012).

Targeted Therapy

A range of therapeutic options exist for breast cancer patients. The current standard of care includes: surgery, radiation, hormone therapy, chemotherapy and targeted therapy. The estrogen receptor acts as a major driver of breast tumorigenesis and is expressed in the majority of breast cancers (75%) (Murphy and Dickler 2016). However, these cancers tend to be of lower grade with a high survival rate, ten years following therapy due to a high response rate to hormone therapy against the estrogen receptor (Tamoxifen). HER2 is the only other predictive marker that has been shown to be highly effective in treating breast tumors possessing amplified or overexpressed HER2 using anti-HER2 antibodies (Trastuzumab/Herceptin) (Goldhirsch et al. 2009). In addition, Lapatinib (a dual EGFR/HER2 tyrosine kinase inhibitor) is used in patients with HER2 positive metastatic breast cancer as the standard of care alongside conventional chemotherapy, like paclitaxel (Geyer et al. 2006; Masoud and Pagès 2017). Despite these significant benefits, however, resistance eventually develops in the majority of advanced cases (Geyer et al. 2006). A common resistance mechanism (to hormone or RTK inhibition) involves activation of

PI3K/Akt/mTOR signalling, as these pathways are engaged in the majority of breast cancers and particularly in ER positive tumors downstream of IGF-1 or in those expressing high levels of ErbB2 (Bahrami et al. 2018). One study indicates that PI3K pathway hyperactivation promotes estrogen-independent ER transcriptional activation (Miller, Balko, and Arteaga 2011). A second frequently observed mechanism involves Src activation and this has been suggested to be responsible for the resistance of HER2 positive breast cancer and to drive anti-estrogen tumor growth (Jin et al. 2017; Larsen et al. 2015). In contrast, systemic chemotherapy remains the standard of care in the TNBC subtype. Therefore, research aimed at identifying targetable molecular drivers in this class, as well as work aimed at overcoming therapeutic resistance and methods to effectively eradicate tumors at the secondary site are all critical research priorities.

Molecular Subtypes

As outlined above, treatment options have certainly improved through the development of therapies targeting the ER or HER2, but they are not effective against all subtypes. Breast cancer is a heterogeneous disease that can be divided into at least 6 intrinsic molecular subtypes based on gene expression profiling that correlate with patient outcome (Dai et al. 2015; Sorlie et al. 2001). These include: Luminal A, luminal B, HER2, basal-like, claudin-low and normal-like subtypes (Perou et al. 2000; Prat and Perou 2011). The advantages of this intrinsic classification system lies in the fact that the differences seen in gene expression between tumors also reflects the fundamental differences at the molecular level, as these molecular subtypes persist even in independent data sets (Sorlie et al. 2003).

Breast tumors that are ER positive based on molecular profiling mostly fall into the luminal A (good outcome) or luminal B (intermediate prognosis) subtypes and constitute approximately 60% of breast cancer patients. Luminal A tumors are characterized by mutations in GATA3,

PIK3CA and MAP3K1 (Koboldt et al. 2012). However, a significant portion of Luminal B tumors express HER2 in addition to possessing a high number of DNA copy number changes and a higher proliferative index (Koboldt et al. 2012). The remaining subtypes are ER negative and are associated with poor prognosis, resistance to chemotherapy and a higher proliferative index (Ki67) (Balko et al. 2012). The human epidermal growth factor receptor 2 (HER2) subtype is defined by the absence of ER expression, amplification and overexpression of HER2, as well as 9 other genes within an amplicon contained on chromosome 17q21. Basal-like breast cancer is characterized by the absence or low levels of ER, very low levels of HER2, in addition to the expression of genes characteristic of myoepithelial and basal epithelial cells within a normal mammary duct (Abd El-Rehim et al. 2004; Finak et al. 2006).

Gene expression profiles of morphologically normal epithelial and stromal tissue revealed standard clinical characteristics, but did cluster ER/PR/HER2 negative breast cancers with basal-like subtype expression profiles with poor prognosis (Finak et al. 2006). The "claudin-low" subtype is defined by low expression of adherens and tight junctional proteins, lack of cell polarity and was discovered in 5-10% of breast cancer patients (Prat 2011). These "claudin-low" tumors are enriched in the basal subtype, correlate with poor prognosis and are characterized by both elevated stem cell properties and induction of an epithelial-mesenchymal-transition (EMT) (Taube et al. 2010)**.** The final breast cancer subtype is characterized by the expression of genes most closely resembling the normal breast epithelium, known as normal-like (Prat et al. 2010). Gene expression profiling can also be used to predict those at an elevated risk of recurrence that would benefit from adjuvant therapy, as genetic profiling outperforms even the best histological prognostic factors such as lymph node status (van 't Veer et al. 2002). More recently, a PAM50

assay was developed based on the expression of 50 genes designed to classify single samples into each of the five intrinsic subtypes (Bernard et al. 2009).

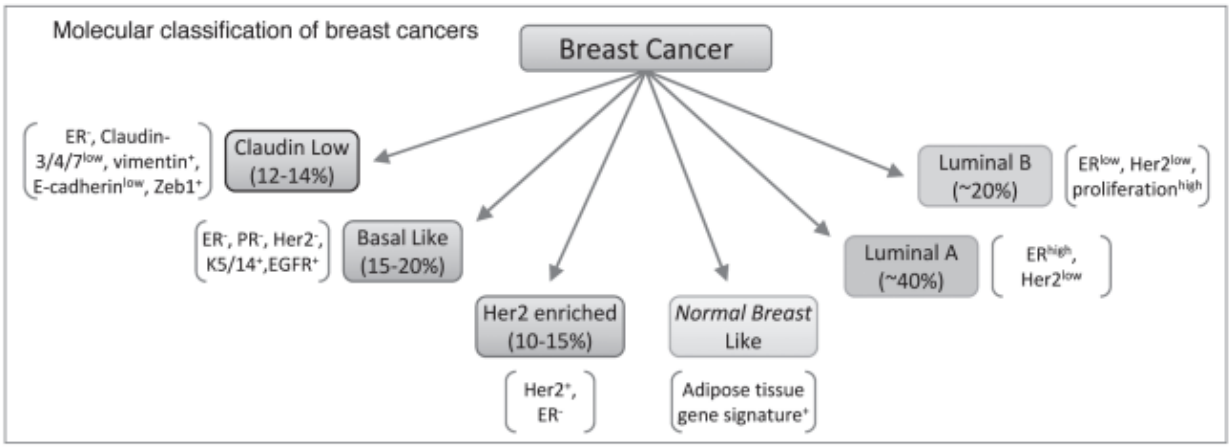


Figure 3 - Molecular subtypes of breast cancer and their characteristics. Obtained from (Malhotra et al. 2010)**.**

Classification of the intrinsic molecular subtypes of breast cancer identified by microarray analysis of gene expression data from patient tumor specimens.

More recent studies have further classified breast cancers into various clusters or subtypes based on gene expression profiling. TNBC is a heterogeneous group of tumours and further classification is crucial to tailor patient treatment. Cluster analysis has identified 6 TNBC subtypes displaying unique expression profiles, including: 2 basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem–like (MSL), and a luminal androgen receptor (LAR) subtype (Lehmann et al. 2011). Mesenchymal-like TNBC subtypes were found to be sensitive to BCR/ABL/Src inhibition (Dasatinib) and mTOR blockade (NVP-BEZ235). In contrast, LAR tumors are effectively inhibited by HSP-90 inhibition (17-DMAG).

Hence, identifying predicted “driver” signaling pathways is a worthy approach to identify pharmacologically relevant targets.

10 clusters were identified that correlated to a degree with hormone and growth factor receptor expression (Dawson et al. 2013). Integrative cluster 1 is characterized by high proliferating ER positive tumours/luminal B tumors and high genomic instability. Integrative cluster 2 is comprised of ER positive tumours of both luminal A and luminal B subtypes and remarkably is associated with the worst prognosis of all ER positive tumours with a 10-year disease-specific survival rate of only 50%. This cluster shows a characteristic ‘firestorm’ pattern due to the amplification of 11q13/14 and associated genomic instability. Integrative cluster 3 is composed primarily of luminal A cases with a good prognosis and is enriched for invasive lobular and tubular carcinomas. Clinically, this is significant, as patients from this cluster could potentially be spared systemic chemotherapy. Integrative cluster 4 is a unique cluster incorporating tumors from a mixture of subtypes including ER positive/negative and TNBC. These tumors possess low levels of genomic instability. Integrative cluster 5 encompasses the ErbB2 amplified ER positive/negative cancers and this group demonstrated the worst disease-specific survival at 10 years of around 45% (Before the widespread use of Herceptin). Integrative cluster 6 represents a distinct subgroup of ER positive tumours, comprising both luminal A and luminal B cases that have amplified the 8p12 amplicon that contains ZNF703, which is associated with invasiveness and increased stem-cell features. Clinically, this cluster shows an intermediate prognosis and a 10-year disease-specific survival of around 60%. Integrative cluster 7 is comprised predominately of ER positive luminal A tumours with a good prognosis and the highest levels of MAP3K1 and CTCF mutations. Furthermore, tumours within integrative cluster 8 demonstrate high levels of PIK3CA, GATA3 and MAP2K4 mutations. Integrative cluster 9 is another mostly Luminal B

cluster group that shows an intermediate prognosis with a high proportion of deletions of PPP2R2A, a phosphatase that functions as a tumor suppressor. Finally, integrative cluster 10 incorporates mostly triple negative tumours from the core basal-like intrinsic subtype with the highest rates of TP53 mutations despite displaying only intermediate levels of genomic instability and are enriched in DNA damage repair and apoptosis genes. These tumors often present in young women and have the worst survival rates for the first five years, after which prognosis is good. Incorporating molecular information from genomic, transcriptomic and proteomic profiling along with tumour-specific information determined by histopathology will allow for improved subtype-specific diagnostic, prognostic and predictive tests.

Tumor morphology

Morphological tumour differentiation (graded on three features: degree of tubule formation, variation in shape and size of nuclei and mitotic rate, with a score of 1-3 for each feature that is summed to determine the overall grade of 1, 2 or 3) provides a good prediction of prognosis in breast cancer. In the Nottingham/Tenovus study, patients with well differentiated tumours have a significantly better survival rate than those with poorly differentiated tumours (Elston 1984). It was also discovered that tumor grade forms an important prognostic index together with tumour size and lymph node stage and that these factors should be used to stratify patients for appropriate therapy (Elston 1984). Tumor grade; HER2, ER, and PR status; and multigene panel (such as Oncotype DX) status were recently incorporated into the ACJC guidelines (Giuliano et al. 2017).

Stages of breast cancer

Staging describes or classifies a cancer based on the amount of cancer present in the body in addition to where it was when first diagnosed. The most common staging system for breast

cancer is the anatomic TNM system. It provides quantitative classification categories for the primary tumor (T), regional lymph nodes (N), and distant metastases (M), which are combined to determine an overall stage group. Historically, the TNM anatomic stage groups have been associated with outcome measures, including overall survival (OS) and disease-free survival (Giuliano et al. 2017). Numerically, non-invasive or in situ breast cancer is defined as stage 0, when the tumor remains in the duct or lobule. Stages 1A, 1B and 2A defines early stage breast cancer, tumors smaller than 5cm or those that have not spread to more than 3 lymph nodes. Locally advanced breast cancer (includes inflammatory breast cancer) defines tumors larger than 5cm that have started to spread to adjacent tissues or to more than 3 lymph nodes and it includes the stages 2B, 3A, 3B and 3C. Metastatic breast cancer refers to stage 4 tumors that have disseminated to other organs (Giuliano et al. 2017).

RTK Signalling

Fifty-eight receptor tyrosine kinases (RTKs) are encoded within the human genome, belonging to 20 subfamilies as defined by genetic phylogeny (Lemmon and Schlessinger 2010). Some are expressed ubiquitously, such as IGF-IR due to its anti-apoptosis function, while others vary like EGFR (Bhargava et al. 2005). The flow of molecular information through normal and oncogenic signaling pathways frequently depends on protein phosphorylation, mediated by specific kinases (Tony Pawson and Kofler 2009). Receptor tyrosine kinases (RTKs) are a subclass of cell-surface growth-factor receptors with an intrinsic, ligand-controlled tyrosine-kinase activity (Gschwind, Fischer, and Ullrich 2004). Ligand-mediated activation of RTKs results in hetero- or homo-dimerization of tyrosine kinases followed by trans-phosphorylation of both the receptor catalytic domain and noncatalytic regions of the cytoplasmic domain, including: HER2, FGFR, TrkA, insulin and KIT (Lemmon and Schlessinger 2010; Tony Pawson 2002). Catalytic domain

phosphorylation can lead to activation and potentiation (autophosphorylation) of kinase activity in most RTKs (EGFR and Ret are exceptions) (Lemmon and Schlessinger 2010).

Noncatalytic domain phosphorylation creates docking sites for downstream cytoplasmic targets, such as SH2 and PTB (phosphotyrosine binding) domains, which bind to specific receptor phosphotyrosine residues. In addition, SH3 domains recognize protein-rich motifs. Domains that recognize phosphoserine and phosphothreonine sequences have also been identified, indicating that phosphorylation of serine and threonine also plays a role in mediating protein-protein interactions. Other domains show specificity for non-protein molecules. PH domains recognize specific membrane-associated phosphoinositides, allowing juxtaposition of a signalling protein alongside both the receptor and additional downstream intracellular targets. Downstream signalling pathways are constructed in a modular fashion. The arrangement and re-arrangement of various combinations of modular domains in different signaling proteins (combinatorial use) has allowed for the creation of complex signaling networks and pathways. In addition to performing catalytic functions, signaling proteins serve as scaffolds for the assembly of multiprotein signaling complexes, as adaptors, as transcription factors and as signal pathway regulators (Tony Pawson 2002).

RTKs as Molecular Drivers of Breast Cancer

The epidermal growth factor RTK family consists of four members: EGFR (ErbB1, HER1), ErbB2 (HER2, neu in rodents), ErbB3 (HER3) and ErbB4 (HER4) (Wieduwilt and Moasser 2008). These structurally related receptors are single chain transmembrane glycoproteins consisting of an extracellular ligand-binding ectodomain, a transmembrane domain, a short juxtamembrane section, a tyrosine kinase domain and a tyrosine-containing C-terminal tail. ErbB2 is frequently amplified in breast cancer (Slamon et al. 1987a, 1989). ErbB3 is the preferred binding

partner for HER2/ErbB2 and is frequently overexpressed in HER2 positive tumors (Siegel et al. 1999; Wallasch et al. 1995). ErbB3 strongly activates downstream PI3K signalling, despite possessing weak intrinsic kinase activity, by heterodimerizing with other ErbB family members (Balko et al. 2012). Intriguingly, ErbB3 levels are highest in the luminal mammary epithelium and loss of ErbB3 in epithelial cells leads to the expression of a mammary basal stem cell signature (Balko et al. 2012).

In contrast, basal/triple-negative breast cancers (TNBCs) variably express both luminal and basal markers, including: cytokeratins CK5/6, CK14 and are characterized by heterogeneous expression of a number of RTKs capable of propagating tumor proliferation and survival, including: Met, EGFR, Focal adhesion kinase (FAK), Lyn and Src (Abd El-Rehim et al. 2004; Hochgräfe et al. 2010; Taylor-Papadimitriou et al. 1989). The lack of a singular driver in basal breast cancer has hindered efforts to develop a targeted therapy. For example, while overexpression of Met alone is sufficient to recapitulate the disease in a subset of animal models based on gene expression profiling, a significant number of tumors stratify to the luminal subtype (Ponzo et al. 2009). Furthermore, a clinical trial using Tivantinib to effectively block Met signalling was only effective in 5% of TNBC patients for a duration of 6 months, demonstrating the breadth and redundancy of the RTK kinome in basal breast cancer (Tolaney et al. 2015). In breast cancer cells, several clusters of RTKs from the same class occurred and correlated with resistance, including: an EGFR/FGFR1/c-Met class, an IGF-1R/NTRK2 class and a PDGFRb class. Intriguingly, abundance of an RTK or ligand of one class generally did not affect sensitivity to a drug targeting an RTK of a different class. Hence, classifying RTKs by their networks and then targeting multiple receptors within a class may reduce or prevent the onset of resistance (Wagner, Wolf-yadlin, and Macbeath 2013).

ShcA adaptor proteins

Src homology and collagen domain (Shc) adaptor proteins are best characterized as mediators of growth factor signalling, especially in the MAPK and PI3K/Akt cascades downstream of RTKs during development/tumorigenesis and are highly conserved across species (Luschnig et al. 2000; Josie Ursini-Siegel et al. 2008). The ShcA adaptor protein family consists of four genes: ShcA, ShcB, ShcC and ShcD (N. Jones et al. 2007; G Pelicci et al. 1996; Giuliana Pelicci et al. 1992). While ShcA is ubiquitously expressed in mammalian cells, ShcB and ShcC are largely limited to neuronal cells, while ShcD is expressed both in brain and skeletal muscle (N. Jones et al. 2007). Indeed, loss of ShcA expression is embryonic lethal at day 11.5 playing a key role in RTK signalling during heart development and vasculogenesis (Lai and Pawson 2000).

Focusing on the ShcA allele, it encodes three proteins that originate through alternative promoter usage (p66) or alternate translation initiation (p46, p52) (Giuliana Pelicci et al. 1992; Ventura 2002). ShcA adaptor proteins possess two phospho-tyrosine binding motifs; an amino terminal PTB domain that functions to bind a number of RTKs at the plasma membrane, including EGFR, HER2 and integrins, a carboxy terminal SH2 domain capable of binding Src, FAK, Lyn and cytoplasmic proteins. ShcA also possesses a proline-rich CH1 domain which contains key tyrosine phosphorylation sites that transduce mitogenic signalling (Y239, Y240 and Y317) (Peter van der Geer et al. 1995; Migliaccio et al. 1997; Giuliana Pelicci et al. 1992; Ravichandran et al. 1997; Josie Ursini-Siegel and Muller 2008a). p66ShcA, the longest isoform, is unique in that it primarily mediates an oxidative stress response and is only expressed in certain cell types. For clarity, the focus of this review will first characterize p46/p52 related functions before distinguishing aspects specific to p66ShcA.

PTB domain of ShcA

The PTB domain of ShcA is essential for binding of phospholipids, membrane localization and association with membrane-bound RTKs. Indeed, 5% of endogenous Shc proteins are localized to the plasma membrane even in unstimulated cells (Ravichandran et al. 1997). The PTB domain is capable of binding to activated receptors, through arginine 175 (R175), by recognizing phospho-tyrosine motifs possessing the NPXpY target sequence (P van der Geer et al. 1996; M. M. Zhou et al. 1995). Consistent with these findings, ShcA is able to bind PI(4,5)P2, PI4P and PI(3,4,5)P3 at the cell membrane via the PTB domain (Rameh et al. 1997; M. M. Zhou et al. 1995).

CH1 domain of ShcA

In response to ligand binding and RTK activation, such as EGFR, ShcA is phosphorylated at Y239/240 or Y317 within the CH1 domain. Grb2 is recruited to p-Tyr motifs within the CH1 domain, where it can then recruit the GTP exchange factor, SOS, to activate RAS/MAPK signalling. In contrast, when GAB is recruited to Grb2 bound to ShcA, this activates the PI3K/Akt cascade (Wills and Jones 2012). A fraction of ShcA proteins remain phosphorylated even at low levels of EGF stimulation and EGFR receptor density, indicating the importance of these adaptor proteins in mediating basal levels of tyrosine dependent cell signalling through different protein-protein interactions (Soler et al. 1994). While EGF stimulation leads to phosphorylation of both the p46 and p52 isoforms of ShcA, insulin stimulation preferentially phosphorylates p52 ShcA (Okada, Yamauchi, and Pessin 1995). ShcA also organizes cytoskeletal rearrangement in response to integrin signaling (Lai and Pawson 2000). Serine/Threonine phosphorylation also plays an important role in regulating ShcA signalling, phosphorylation of serine29 on ShcA promotes binding of the negative regulator PTP-PEST to dampen downstream signalling (Faisal et al. 2002).

SH2 domain of ShcA

SH2 domains recognize short motifs containing phospho-tyrosine residues followed by three-five COOH terminal residues, such as those on activated receptor tyrosine kinases (T. Pawson 1997). In response to ligand binding and tyrosine phosphorylation of the Met receptor by HGF, ShcA binds to Met through its SH2 domain. HGF stimulation leads to RAS pathway activation through Grb2. In this context, Shc overexpression leads to enhanced cell growth and migration upon HGF stimulation, outlining a role for Shc downstream of HGF in both the mitogenic and motogenic response (Giuliana Pelicci et al. 1995). The SH2 domain of ShcA is also key in recruitment and binding to CD3 of the T cell receptor and the platelet derived growth factor receptor (PDGFR) (Gelderloos et al. 1998; Ravichandran et al. 1993). ShcA proteins mediate cell survival and cell cycle progression in response to adhesion molecules, including binding classes of integrins through the PTB and the CH1 domain. In response to mitogens, recruitment and binding of ShcA to integrins is sufficient to promote MAPK pathway activation, transcription through the FOS response element and G1 transit. In the absence of Shc, mitogen stimulation and adhesion led to cell cycle arrest and apoptosis (Wary et al. 1996).

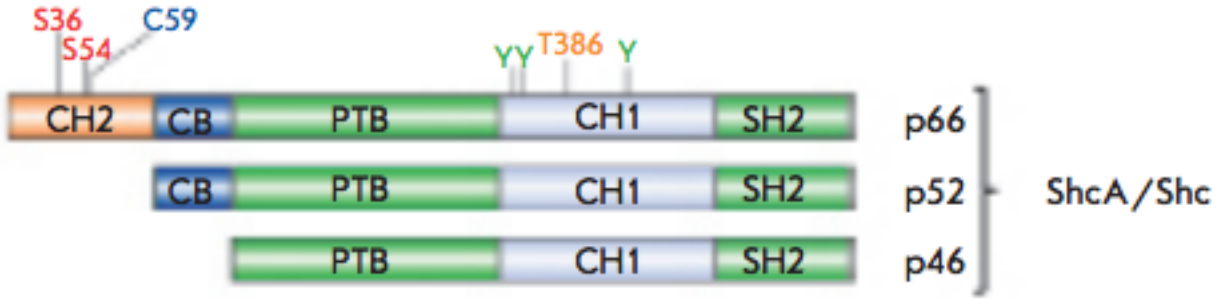


Figure 4 - ShcA Structure and Domains.

ShcA isoforms possesses 3-4 domains, including: CH2 domain at the N-terminus with a phosphorylatable serine (S36) residue (p66ShcA isoform specific), PTB domain, CH1 domain

with 3 phosphorylatable tyrosines (Y239/240 and Y317) and a C-terminal SH2 domain. Figure obtained from (Galimov 2010).

Functional role of ShcA Signaling in Breast Cancer

The ShcA adaptor protein relays extracellular signals from receptor tyrosine kinases (RTK), inducing cell proliferation, survival, invasion, angiogenesis and immune suppression during mammary tumorigenesis (Ahn et al. 2017; Im et al. 2015; Saucier et al. 2004; Josie Ursini-Siegel et al. 2010; Josie Ursini-Siegel and Muller 2008a). In fact, ShcA signalling is so crucial downstream of the ErbB2 receptor, that ablation of ShcA virtually prevents transformation and metastasis in transgenic mouse models of ErbB2-driven breast cancer (Josie Ursini-Siegel et al. 2008; Webster et al. 1998). Y239/240 and Y313, within the CH1 domain, were shown to have important and non-redundant roles in tumor initiation and progression, primarily in controlling cell survival (Y313) or regulating angiogenesis (Y239/240) in luminal breast cancer models at steady state levels in the tumor (Josie Ursini-Siegel et al. 2008). Indeed, ShcA was found to interact with Grb2 and Src in a distinct and major signalling subnetwork that defined the human interactome of breast cancer patients and predicted patient outcome (Taylor et al. 2009). Hence, cell signalling downstream of ShcA acts as a molecular driver in breast cancer.

PTB domain of ShcA in breast cancer

The ShcA PTB domain has been shown to be crucial for mediating ErbB2-dependent tumor initiation (Webster et al. 1998). Intriguingly, breast cancer cells deficient in phosphotyrosine-dependent RTK signaling (PTB-R175Q mutants) exhibit delayed tumor initiation, but increased tumor outgrowth in vivo (Ahn et al. 2013). These effects were found to be largely dependent on increased integrin signaling and fibronectin production, leading to elevated VEGF production and

increased angiogenesis. Collectively, these results suggest that the strength of PTB-dependent ShcA signaling must be tightly regulated to promote tumorigenesis. Indeed, phosphatases have been shown to act as negative regulators SHIP2 and PTP-PEST act as negative regulators of ShcA and others by dephosphorylating phospho-tyrosine dependent signalling and leading to signal termination. Indeed, the PTB domain has been shown to be controlled by negative regulators involved in signal termination both through the phospho-tyrosine binding pocket (SHIP2 and PTP-PEST) and through phospho-tyrosine independent mechanisms (PTPepsilon, ERK) (Davidson and Veillette 2001; Faisal et al. 2002)(Kraut-Cohen, Muller, and Elson 2008).

CH1 domain of ShcA in breast cancer

P-Tyr signalling downstream of ShcA is important in a number of cellular processes. The CH1 domain regulates mitogenic and apoptotic RTK cues in cancer through phosphorylation of Y239/240 and Y317 to promote cell survival through recruitment of a Grb2/GAB complex that activates PI3K/Akt signalling or a Grb2/SOS avenue to potentiate MAPK signalling and to promote cell proliferation (Josie Ursini-Siegel et al. 2008). In addition, ShcA has been shown to mediate resistance to chemotherapy in HER2 positive luminal breast cancer through the P-Tyr motifs (Lucs, Muller, and Muthuswamy 2010). ShcA potentiates immune suppression through simultaneous inhibition of STAT1 anti-tumor immunity and augmentation of STAT3 immunosuppressive signaling via P-Tyr motifs within the CH1 domain (Ahn et al. 2017). Intriguingly, ShcA is required for enhanced motility and invasion through the ErbB2 receptor in response to TGF-ß stimulation and this mechanism necessitates the 3 tyrosine phosphorylation sites within the CH1 domain of ShcA to alter the cytoskeletal architecture through Rac1 (Northey et al. 2008).

SH2 domain of ShcA in breast cancer

Using ErbB2-driven transgenic mouse models of breast cancer, the SH2 domain was shown to control breast tumor outgrowth, survival and the development of lung metastases via enhanced 14-3-3/PI3K/Akt signalling (J. Ursini-Siegel et al. 2012). PTB-independent ShcA pools require a functional SH2 domain, but not the tyrosine phosphorylation sites to promote mammary tumorigenesis to activate multiple Src family kinases (SFK), including Src and Fyn, in ErbB2-positive breast cancers (Ha et al. 2018). Intriguingly, SFK inhibition overcomes ErbB2-dependent and independent tumor progression that requires ShcA signalling and provides a potential therapeutic strategy to overcome responsiveness to RTK inhibitors (Ha et al. 2018).

p66ShcA signalling

The longest isoform, p66ShcA, is characterized by a unique CH2 domain and cytochrome c-binding domain located at the N-terminal region that are associated with the induction of oxidative stress (Migliaccio et al. 1999). Within the CH2 domain are two serine residues S36 and S54, that are important for mitochondrial localization and protein stability, respectively (Mains, Sulston, and Wood 2006). Under stress conditions, p66ShcA is phosphorylated on S36 by PKC or JNK1/2 within the CH2 domain, which allows prolyl isomerase 1 (PIN1) to bind and induce a conformational change that results in p66ShcA dimerization and its mitochondrial import (Clark et al. 2007; Khalid et al. 2016; Migliaccio et al. 1999). p66ShcA undergoes a second conformational change within the mitochondria, in response to oxidizing conditions and disulphide bond formation at Cys59, which allows it to catalyze the transfer of electrons from cytochrome c to molecular oxygen (Gertz et al. 2008). This leads to the production of H_2O_2 which opens the permeability transition pore, stimulates organelle swelling and cytochrome c release. Once in the cytosol, cytochrome c can activate caspases and promote activation of the apoptosome (Giorgio et

al. 2005b). Indeed, p66ShcA-deficient mice display increased resistance to oxidative stress and a 30% increase in lifespan (Migliaccio et al. 1999). However, some clinical cancer studies associate elevated p66ShcA with good prognosis (Davol et al. 2003; Frackelton et al. 2006), while others link high p66ShcA levels with increased severity and recurrence of breast, colon and prostate cancer (Grossman et al. 2007; Jackson et al. 2000; M.-S. Lee et al. 2004). Consequently, the role of p66ShcA during breast cancer progression is conflicting and poorly understood.

Anti-Tumorigenic properties of p66ShcA

p66ShcA signalling in normal cells

p66ShcA is tyrosine phosphorylated upon EGF stimulation and binds to activated EGFR and Grb2, but does not induce transformation of fibroblasts unlike p46 and p52 (Migliaccio et al. 1997). This is due to the fact that p66ShcA can recruit a Grb2/SOS complex, however the proline-rich CH2 domain of p66ShcA displaces SOS and prevents downstream signalling. Phosphorylation of S36 on p66ShcA played a negative role in H_2O_2-induced ERK activation and survival by reducing the expression of and formation of protein complexes with the p46 and p52 ShcA isoforms that reduced P-Tyr-dependent signalling (Mains, Sulston, and Wood 1990). Indeed, p66ShcA functions to negatively regulate the formation of a signaling complex (SHPS-1/SHP-2/Src/p52shc) that is required for p52Shc activation in response to IGF-I, which leads to the attenuation of cell proliferation and migration through MAPK signalling in untransformed cells (Xi, Shen, and Clemmons 2008). A fraction of p66ShcA is localized to the mitochondrial intermembrane space (Giorgio et al. 2005a). p66ShcA has even been shown to control the oxidative stress response in early mammalian embryo development (Betts, Bain, and Madan 2014). In macrophages, where NADPH-oxidase serves as the major source of ROS, knockout of the p66shcA gene leads to a 40% decrease in ROS formation (Tomilov et al. 2010). Residues Q132–

Q133 and E132–E133, within the CH2 domain, were shown to be essential for the transfer of electrons between cytochrome c and complex 3 (G. Pelicci et al. 1992). Notably, overexpression of p66ShcA in non-transformed cells induces anoikis through the PTB domain to control focal adhesion formation (FAK) (Zhenyi; Ma et al. 2007).

p66ShcA signalling in cancer

In lung cancer, epigenetic silencing of p66ShcA lead to inhibition of anoikis and promoted metastatic behaviour (X. Li et al. 2014). p66ShcA is also downregulated in highly aggressive lung carcinoma cell lines (Zhenyi; Ma et al. 2007). Furthermore, loss of p66ShcA leads to unrestrained hyperactivation of Ras thereby promoting RhoA activation, inhibiting Rac1 activity and inhibiting apoptosis upon detachment from the ECM, an effect that at least partially depends on S54 and promotes lung metastasis (Z. Ma et al. 2010; Mains, Sulston, and Wood 2006). Supporting these findings, p66ShcA gene transcription is positively regulated by Nrf2 binding to the p66ShcA promoter, but only occurs in cells lacking methylation at a consensus binding site. High NRF2 and low p66ShcA correlate with increasing tumor grade and aggressiveness in lung cancer patients, suggesting a link between chromatin state, ROS formation and antioxidant levels in lung cancer progression (W. Du et al. 2013). Collectively, p66ShcA appears to possess anti-tumorigenic properties by influencing cell proliferation, regulating apoptosis to control cell survival and inducing anoikis to restrain metastatic progression. Rac1 was shown to govern p66ShcA protein stability in a p38 MAPK dependent manner and through phosphorylation of S54 within the CH2 domain to induce ROS formation (Mains, Sulston, and Wood 2006).

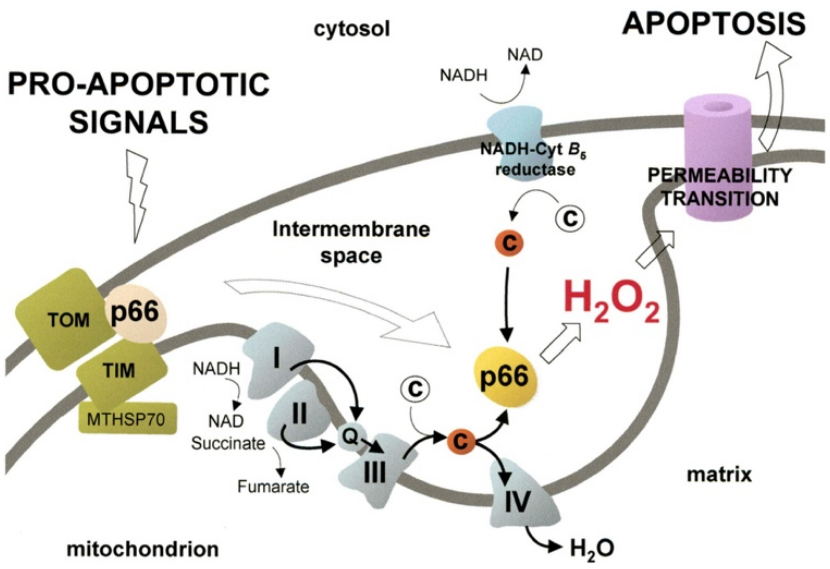


Figure 5 - Model of p66ShcA mediated response to oxidative stress. Obtained from (Giorgio et al. 2005b).

Proapoptotic signals induce release of p66Shc from a putative inhibitory complex and lead to its mitochondrial translocation. Active p66ShcA then oxidizes reduced cyt c and catalyzes the reduction of O_2 to H_2O_2. Opening of the permeability transition pore by H_2O_2 then leads to swelling and apoptosis. NADH-Cyt B_5 reductase is indicated as additional putative source of reduced cyt c (Bernardi and Azzone 1981).

Pro-Tumorigenic properties of p66ShcA in cancer

Some tumor cells are dependent on ROS for tumorigenicity and elevated ROS can promote tumor aggressiveness (Weinberg et al. 2010). In this context, p66ShcA is upregulated by steroid hormones in hormone-sensitive cancers and functions in a ROS-dependent fashion to promote tumor growth and carcinogenesis (M.-S. Lee et al. 2004; Muniyan et al. 2015). In ovarian cancer cells, whether induced or overexpressed, p66ShcA promotes ROS production, MAPK signalling and cell proliferation (Muniyan et al., 2015). p66ShcA forms a trimeric complex with alpha-1-syntrophin and Grb2, which can trigger cell proliferation and migration in breast cancer cell lines

(Bhat et al., 2014). Hence, clearly establish a causal relationship between p66Shc protein levels and cell proliferation. In other cancer types, including breast tumors p66ShcA appears to be associated with heightened metastatic potential through increased invasiveness and motility, rather than through MAPK activation (Arany et al. 2008; Jackson et al. 2000). In breast cancer p66ShcA also displaced Sos1 protein from Grb2 when SNTA1 was overexpressed with p66 to increase ROS production and migratory potential (Bhat et al. 2014). ROS is also a well-characterized inducer of an EMT, which is associated with increased invasiveness and poor prognosis (M. a Cichon and Radisky 2014; Hur et al. 2013). p66ShcA was shown to play a role in VEGF signalling and angiogenesis in endothelial cells (Oshikawa et al. 2012). Collectively these data suggest a role for p66ShcA in tumor cell proliferation, angiogenesis or metastasis depending on the molecular and cellular context.

Apoptosis

Transformed cells also develop mechanisms to circumvent programmed cell death or apoptosis. The apoptotic machinery is activated through distinct pathways: the extrinsic pathway and the intrinsic pathway, which consists of both upstream and downstream effectors (Hanahan & Weinberg). Extrinsic cell death occurs through death receptor activation on the cell surface, by ligands such as the TNF family, FASL and TRAIL. Ligand activation leads to the stimulation of proteases known as caspases (Adams and Cory 2007). This mechanism occurs in response to external physiological stress cues on the cell (UV, radiation, chemotherapy) and leads to the activation of several executioner caspases, promotes cell disassembly and cell consumption (Hanahan and Weinberg 2011). In contrast, the more ancient and evolutionary conserved intrinsic cell death pathway occurs through the activation of various intracellular stress cues, including oxidative stress, and in response to cytochrome c release within the mitochondria (Adams and

Cory 2007). This pathway is also primarily regulated by the Bcl-2 family of apoptotic proteins (Adams and Cory 2007). These pathways are largely independent from one another and programmed cell death largely functions as a natural barrier to cancer development (Hanahan and Weinberg 2011).

Reactive Oxygen Species

Reactive oxygen species (ROS) are radicals, ions or molecules that possess an unpaired electron in their outermost shell, making them highly reactive (Liou and Storz 2010). There exists two kinds of ROS: free oxygen radical (superoxide anion (O_2^-), hydroxyl radical (OH^-) and non-free radical (H_2O_2) (Liou and Storz 2010). ROS are generated as normal by products of mitochondrial oxidative phosphorylation to produce ATP for energy (Ray, Huang, and Tsuji 2012; Huiqin Zhong and Yin 2015). The very nature of the alternating one-electron oxidation-reduction reaction at a time predispose each electron carrier, within the electron transport chain, to side reactions with molecular oxygen. This "leaky" transfer of electrons results in mitochondrial O_2^- generation from approximately 1–2% of the total daily oxygen consumed (Cadenas, Davies, and Adenas 2000). Cancer cells rely on the "Warburg Effect", an increased emphasis on aerobic glycolysis to fuel rapid growth and expansion whereby much of the carbon from glucose is spared for biosynthesis, instead of diverting into the citric acid cycle, resulting in increased lactate production. In order to maintain a rapid growth rate, glucose transporters are significantly upregulated by cancer cells, including GLUT 1 and 3 compared to normal cells (DeBerardinis et al. 2007). The generation of superoxide anion occurs at the cytosolic side of the inner mitochondrial membrane (Han, Williams, and Cadenas 2001).

Normally, the electron transport chain (ETC) transfers electrons from NADH to generate water molecules within the mitochondrial matrix at complex one and coenzyme Q (CoQ) facilitates

electron transfer to produce H_20 and (O_2^-), but this also leads to (O_2^-) release (Finkel 2011). O_2^- is then converted to H_2O_2 predominantly by superoxide dismutases (SOD) (Huiqin Zhong and Yin 2015). At complex 3, Coenzyme Q (CoQ) facilitates electron transfer and water and (O_2^-) are released into both the inner mitochondrial space and mitochondrial matrix, while cytochrome c and p66ShcA regulate H_20_2 production in the inner mitochondrial space (Giorgio et al. 2005b; Muller, Liu, and Remmen 2004). NADPH oxidase is the main source of O_2^- within the cytosol and was even found to have transforming properties (Suh et al. 1999). Low levels of lipid oxidation radically increases the passive permeability of lipid bilayers (Runas and Malmstadt 2015).

ROS Production and RTK Signalling

Growth factors and cytokines, elevated metabolic activity, mitochondrial dysfunction (ETC leakage), peroxisome activity, oncogene activity, increased cellular receptor signalling, production by immune cells and oxidases, cyclooxygenases and lipoxygenases can all lead to ROS induction (Liou and Storz 2010; Morgan and Liu 2011). EGF and PDGF signalling lead to the production of H_20_2 (Furui 1995; Rhee et al. 1997), while FGF and TNFα have also been shown to induce ROS formation (Lo and Cruz 1995). Critical cysteine thiol groups of target proteins exist as a thiolate anion (S) and are readily oxidized by H_20_2 (Wood, Poole, and Karplus 2003). Hence, RTKs signalling can stimulate ROS formation and alter downstream protein signalling to modulate various processes including cell proliferation (S. R. Lee et al. 2002).

Antioxidant Defence System

Elevated levels of reactive oxygen species are seen in cancer however, a concomitant increase in antioxidant expression occurs to cope with elevated oxidative stress, suggesting a delicate balance between tumor promoting and tumor suppressive ROS (Szatrowski & Nathan, 1991). Cellular

redox status is maintained through both enzymatic and non-enzymatic antioxidant systems. Enzymatic systems include catalase, superoxide dismutase (SOD), glutathione peroxidase (Gpx) and peroxiredoxin (Prdx). SOD converts 0_2^- to H_20_2, while catalase catalyzes the formation of H_20 from H_20_2. Gpx converts H_20_2 to H_20. In the process, glutathione (GSH) is converted to its oxidized form, glutathione disulfide (GSSG) (Galadari et al. 2017). Glutathione (GSH) is the major non-enzymatic anti-oxidant and is predominantly found in its reduced form and is distributed within the cytosol, nucleus, endoplasmic reticulum and mitochondria (Muller, Liu, and Remmen 2004). Other non-enzymatic sources include: vitamins A (retinoic acid), C (ascorbic acid), E (alpha-tocopherol), folic acid, calcium, and multivitamins, which have been shown to reduce the risk of cancer (Shannon 1997).

Oxidative Damage and Cancer

Moderate ROS levels can promote cell survival and proliferation by activating signalling pathways that can contribute to tumor growth in stressful tumor microenvironments. However, failure of ROS scavenging mechanisms, or antioxidant scarcity can result in severe damage of biomolecules, even triggering cell death through excessive ROS accumulation (Bansal and Simon 2018). Hence, cancer cells must fine tune antioxidant levels to balance ROS and survive. Direct or indirect-ROS-mediated damage can occur within nucleic acid, proteins and lipids and has been implicated in carcinogenesis as well as diabetes, aging and neurodegeneration (Ray, Huang, and Tsuji 2012). Indeed, DNA damage repair and metabolism are determinants of species longevity (Siming Ma et al. 2016). One of the main targets of ROS in cancer is the membrane lipid bilayer and one of the main products of lipid peroxidation is 4-hydroxynoneal (4-HNE) formation (Huiqin Zhong and Yin 2015). However, there is increasing evidence that ROS does not contribute to disease-state solely through damage to macromolecules. For example, ROS was shown to

contribute to metastasis through gene activation (K. Ishikawa et al. 2008). Moreover, cancer cells expressing mutated mitochondrial DNA (mtDNA) exhibited higher levels of ROS production compared to cells without mutated mtDNA and elevated ROS lead to higher metastatic potential in breast cancer cells expressing mutated mtDNA (K. Ishikawa et al. 2008).

Anoikis - Resisting Cell Death

Anoikis is a programmed cell death that is activated upon cell detachment from extracellular matrix. Resisting anoikis is a critical mechanism in promoting adherent-independent cell growth and attachment to an inappropriate matrix, which aids in the colonization of distant organs to promote metastasis (Paoli, Giannoni, and Chiarugi 2013). Anchorage-independent growth and the epithelial–mesenchymal transition are two features associated with anoikis resistance and are vital steps during cancer progression (Frisch and Francis 1994; Kumar et al. 2011). Integrin signalling through $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_1\beta_1$ integrins are all able to activate an anti-anoikis pathway by enhancing anchorage independent growth (Brassard et al. 1999; Wary et al. 1996).

The initiation and execution of anoikis is mediated by different pathways, all of which terminally converge into the activation of caspases and effectors to promote cell death (Paoli, Giannoni, and Chiarugi 2013). Overall, the anoikis program is executed through the interplay of the intrinsic and extrinsic pathways. The perturbation of the mitochondria (the intrinsic pathway) or the triggering of cell surface death receptors (the extrinsic pathway). BCL-2 and their associated family members fine tune the regulation of these apoptotic programs by providing pro-survival (antiapoptotic) or death cues (Meredith, Fazeli, and Schwartz 1993).

Detached or migrating cancer cells can adopt different strategies to compensate the loss of integrin signals and overcome anoikis. PI3K/Akt signalling is one of the most commonly activated pathways as it provides the majority of survival cues for a cell via integrins and growth factors

(Bacus et al. 2002; Garcia Pedrero et al. 2005; Kumar et al. 2011; Liu et al. 1998; Tanno, Mitsuuchi, and Altomare 2001). Overexpression of ErbB2 also rescues ATP deficiency during cell detachment from the ECM and promotes cell survival through PI3K activation (Schafer et al. 2009). Elevated levels of ROS occur during cell detachment from the ECM and antioxidants, including superoxide dismutase and catalase, have been shown to increase cell viability during anoikis (Davison et al. 2013). Multicellular aggregation during cell detachment has also been shown to allow tumour cells to evade anoikis in an ErbB2 and E-cadherin dependent fashion (Rayavarapu et al. 2015).

EMT

During embryogenesis and development cellular migration of different cell types to distant sites allows for the formation of new organs. The epithelial-to-mesenchymal transition (EMT) is a remodelling process that is tightly controlled by a number of master transcription factors and facilitates the acquisition of migratory characteristics by reducing cell contacts and remodelling the actin cytoskeleton (Grille et al. 2003). Upon successful migration, cells must differentiate to generate additional epithelia and this is accomplished through a reverse EMT known as the Mesenchymal–Epithelial-Transition (MET). There is also a need for the creation of supportive mesenchymal cells within epithelial tissues during development and an EMT induces the expression of mesenchymal markers vimentin and N-cadherin, while simultaneously reducing epithelial marker expression, including: E-cadherin, claudins 3,4 and 7 (Herranz et al. 2008; K. Li et al. 2009; Seftor et al. 2006). Normally specialized functions are only acquired through cellular differentiation. Thus, reversible increases in cellular plasticity, through an EMT, allow committed cells to acquire new functions in response to changing microenvironments (Grille et al. 2003; Huber et al. 2004). Among the growth factors known to induce EMT are transforming growth

factor β (TGFβ), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF), members of the epidermal growth factor (EGF) family and insulin-like growth factor (IGF) (J. Kim et al. 2016; Lorenzatti et al. 2011; Savagner, Yamada, and Thiery 1997; Zavadil et al. 2004).

During cancer development, tumor cells hi-jack these developmental processes to promote tumor initiation, growth and progression. EMT induction has been shown to increase the number of CTCs, measured by vimentin expression of cells isolated from the blood (Satelli et al. 2017). Indeed, the presence of circulating tumor cells, prior to treatment, predicts metastasis and poor survival in breast cancer patients (Cristofanilli and Budd 2004). Evidence exists that cancer cells are only fully permissive to induction of an EMT once specific oncogenic initiating events have occurred. For example, transgenic mice deficient in p53-/- only display characteristics of an EMT after activation of constitutive Met signaling (J. F. Knight et al. 2013).

Metastatic Cascade

There are several steps involved for successful metastasis to a distant secondary site, termed the metastatic cascade (Chambers, Groom, and MacDonald 2002). Cancer cells must first invade locally and gain access to the vasculature. Then they must survive in the circulation and extravasate at secondary sites in order to form micrometastases. Only once overt macroscopic metastases have formed, by reactivating survival and proliferation pathways, is the cascade complete.

Migration and local invasion

There are several modes of migration undertaken by cancer cells to invade locally and enter the circulation successfully and broadly can be divided into individual or collective invasion. Cell migration involves the polarization of cells toward a leading edge at the invasive front and a

lagging edge at the rear (Richardson and Lehmann 2010). Protusion and adhesion then occurs at the leading edge while retraction happens at the rear. For collective invasion, mechanisms include: multicellular streaming, collective cell invasion and tumor budding. Multicellular streaming is dependent on tensile forces at the leading front to create microtracks that cells follow by deforming and re-aligning the ECM into forms conducive for migration (Gjorevski et al. 2015). Hegerfeldt (2002) showed that collective cell invasion was dependent on β1 integrin expression and clustering at the leading edge in melanoma and collective invasion has also been shown to be important in breast cancer (Patsialou et al. 2013). Finally, tumor budding involves the tumor mass extending several finger-like multicellular projections at the invasive front, taking on both spindle-like and rounded morphology and has been seen in breast, lung, colorectal and pancreatic cancer (Bronsert et al. 2014). There is also plasticity that exists with collective cell invasion converting to single cell modes, such as through induction of a full or partial EMT (Van Zijl, Krupitza, and Mikulits 2011).

The family of matrix metalloproteinases play a crucial role in degrading the extracellular matrix and includes collagenases, gelatinases, stromelysins and membrane type MMPs (Itoh et al. 1999). These proteins play important roles during development, involution, tissue repair and display altered expression in cancer (Itoh et al. 1999; H. Zheng et al. 2006). Interactions with the extracellular matrix (ECM) have been shown to control lung metastatic potential as Maric et al. (2015) showed that $\alpha_5\beta_1$ integrin expression can be regulated by GNMB, through FAK and Src signaling, to increase adhesion and promote metastasis in HER2+ luminal breast cancer. Moreover, expression of MMP-2 and MMP-9 (Gelatinase B) have been shown to be important for growth, invasion, and metastasis in several cancers (Itoh et al. 1999; H. Zheng et al. 2006).

Angiogenesis in Metastasis

By promoting angiogenesis, tumor cells are able to acquire sufficient nutrients to fuel their metabolic requirements and expand in size. An additional benefit to the tumor is that the newly formed vessels can provide an additional escape route to secondary sites. Indeed, GFP-tagged metastatic cells were found to protrude toward blood vessels and migrate with a greater number of host cells than non-metastatic cells (Wyckoff et al. 2000). Moreover, microvessel density correlates with increasing grade and severity in breast cancer patients with invasive disease (Weidner et al. 1991) Tumor cells sometimes gain access to the blood through the lymphatic system and the angiogenic factor VEGF-D was shown to promote metastasis in this fashion (Stacker et al. 2001).

Chemokines in metastasis

Various factors secreted by tumor cells and the surrounding stroma also modulate the tumor microenvironment to promote metastasis. Chemokines are a superfamily of small cytokine-like proteins that can drive the recruitment of cell types that promote tumor development and metastasis, some of which are pro-inflammatory (Müller et al. 2001). Indeed, altered expression of alpha (CXC) and beta (CC) inflammatory chemokines regulates breast cancer cell migration (Youngs et al. 1997). In addition to promoting directional migration, chemokines can induce focal adhesion and cytoskeletal rearrangements in tumor cells helping tumor cells to reach distant organs (Müller et al. 2001). In fact, the chemokine receptors CXCR4 CCR7 and their respective ligands CXCL12/SDF-1alpha and CCL21 were shown to regulate breast cancer metastasis by promoting actin polymerization and pseudopod formation and were found at the highest levels in organs that are first destinations from the breast (lymph nodes, lung, live and bone) (Müller et al. 2001). In addition to promoting directional migration, chemokines can induce focal adhesion and

cytoskeletal rearrangements in tumor cells helping tumor cells to reach distant organs (Müller et al. 2001).

CTCs and survival in the circulation

Once in the circulation, tumor cells must evade apoptosis and reach the secondary site. One mechanism involves the upregulation of periostin, which promotes cell survival by Akt under stressful conditions, including: serum deprivation, hypoxia and anoikis and increased the formation of micrometastases by prostate cancer cells in mice (Bao et al. 2004). Circulating tumor cells (CTCs) were shown to upregulate genes involved in evading immune surveillance and a subset possessed mutations in p53 and/or RAS that were not seen at the primary site (Steinert et al. 2014). These results indicate avoiding immune detection and promoting survival cues are likely key factors for navigating the circulatory system in vivo. Entry into the circulation can occur very early on as CTC's can be identified from the blood while only benign lesions are present in patient biopsies from the primary site (Franken et al. 2012).

Extravasation

Another important component in extravasation is platelet activation at the secondary site. Reduction of the number of circulating platelet cells significantly reduced the lung metastatic potential in mice (Gasic, Gasic, and Stewart 1968). Platelets appear to contribute to metastases by their adhesive interaction with tumor cells via the adhesive proteins fibronectin and von Willebrand factor in pulmonary metastasis of melanoma cells (Karpatkin et al. 1988). The ability of tumor cells to generate 12(S)-HETE is positively correlated to their metastatic potential (Honn et al. 1994). Enhanced tumor cell adhesion was blocked by treating endothelial cells with antibodies against the alpha v beta 3 complex (Gasic, Gasic, and Stewart 1968).

Colonization

In order to successfully colonize secondary organs, tumor cells must extravasate from the circulation and adhesion plays a prominent role. Several processes are activated during cell detachment in addition to anoikis, including survival proteins and remodelling of the actin-cytoskeleton (Buchheit, Weigel, and Schafer 2014). Focal adhesion formation promotes cell survival and proliferation through activation of signalling cascades like MAPK, PI3K or stress kinase JNK and this is dependent on the type of integrin implicated like ahpha$_5$,beta$_1$ (Buchheit, Weigel, and Schafer 2014). Paxillin acts to modulate the formation of focal adhesions and stress fibers by binding alpha-integrins which allows for the binding and activation of FAK (Buchheit, Weigel, and Schafer 2014). The loss of E-cadherin during induction of an EMT is a common method for resisting anoikis (Kumar et al. 2011). Furthermore, primary tumor-derived exosome release from tumor cells facilitates cellular adhesion in recipient CTCs and promotes metastasis (Fu et al. 2018). Indeed, exosome cargo was shown to contain molecules that enhanced SMAD3 signalling through increased production of ROS (Fu et al. 2018). Recently, strategies aimed at preventing metastasis through adhesion blocked have been tested for effectiveness as anti-metastatic therapy. In ER negative/CD44 positive breast cancer, E-selectin preferentially promoted shear-resistant adhesion and transendothelial migration and a single intravenous injection of an E-selectin targeted aptamer (ESTA) reduced metastasis to a baseline level in both syngeneic and xenogeneic forced breast cancer metastasis models without relocating the site of metastasis (Kang et al. 2015). Indeed, inhibition of cellular adhesion by CTCs at the secondary site using dual-antibody coated nanomaterial significantly inhibited metastasis of colon cancer cells (Margueron et al. 2009).

The Tumor Microenvironment and the Pre-metastatic niche

Stephen Paget proposed in 1892 that particular cancer cells (the "seed") have a propensity to target specific organs at distant sites ("soil") and that this is dependent on crosstalk between the tumor and the microenvironment of secondary organs (Fidler 2003). The main barrier to treatment of patients with metastatic disease is due to the heterogeneity that exists between primary and secondary tumors that leads to resistance to treatment. The microenvironment plays a large role in shaping the response to systemic therapies. Indeed, leukocyte complexity has been shown to predict breast cancer survival and regulate the response to chemotherapy (DeNardo et al. 2011). The composition of stromal and immune cells within the local tumor microenvironment dictates whether a permissive or suppressive metastatic niche develops. Inflammatory conditions can be driven by oncogenes and are often responsible for activating key inflammatory transcription factors such as NF-KB, STAT-3 and HIF1-alpha. Activation of these master regulators promotes chemokine and cytokine release from tumours to modulate and recruit inflammatory and stromal cells within the tumor microenvironment (Mantovani et al. 2008). Recently, it was shown that lung and liver metastases from the breast are enriched in immune infiltrates, including: T cells, neutrophils and myeloid-derived cells (Tabariès et al. 2015). Organotropism was shown to be dependent on metabolic reprogramming, where 4T1 liver cells (that also possess high levels of immune infiltrates) display reduced mitochondrial metabolism compared to lung metastatic variants that are highly reliant on oxidative phosphorylation (Dupuy et al. 2015). Tumor cells have been shown to secrete microRNAs in order to reduce glucose uptake in neighboring stromal cells at the metastatic niche and thereby prime the microenvironment (increase glucose availability for the tumor cells) for tumor colonization (Fong et al. 2015). Tumors can also secrete exosomes to promote crosstalk at the primary site in order to promote metastasis (Luga et al. 2012).

ROS, antioxidants and metastasis

Research shows ROS levels are altered during metastasis and that ROS is produced from both cancer cells and the surrounding inflammatory stroma (Babior, Kipnes, and Cumvu 1973; Szatrowski and Nathan 1991). ROS are mainly derived from oxygen consuming reactions within peroxisomes, the endoplasmic reticulum and the mitochondria. While it is known that aerobic glycolysis influences metastatic progression, more recently, a pro-metastatic role for the mitochondria was established through ETC inhibition (Pelicano, Carney, and Huang 2004). Consequently, antioxidants to reduce oxidative stress levels have been proposed as a potential therapeutic strategy. Indeed, Piskounova et al. (2015) showed that metastasizing melanoma cells increase their antioxidant defenses upon loss of contact with the extracellular matrix of the basement membrane. Moreover, Le Gal et al. (2015) found that antioxidant supplementation promoted proliferation, survival and metastatic spread by increasing the ratio between reduced (GSH) and oxidized (GSSG) GSH/GSSG, suggesting combined therapies inhibiting both the antioxidant response and promoting oxidative stress to be the most effective. However, these findings are in contrast to several studies indicating inhibition of mitochondrial-derived ROS as an effective means of reducing metastasis (Goh et al. 2011; Porporato et al. 2014). One potential explanation for this discrepancy is that antioxidant supplementation must effectively target mitochondrial-derived ROS to be an effective therapeutic strategy. Furthermore, clinical trials using various antioxidant compounds have failed to consistently show clinical efficacy. Hence, further studies are required to overcome the complexity in identifying the ideal context for using anti-oxidant supplementation as a beneficial treatment. One potential mechanism is that intermediate levels of ROS fuel pro-metastatic signalling, but high levels are required to induce sufficient cellular damage to activate cell death pathways. Indeed, chemotherapy has been shown

to lead to high levels of free radicals and promotes DNA damage (Pelicano, Carney, and Huang 2004). An increased reliance on oxidative stress signalling suggests tumor cells must upregulate their antioxidant capacity to maintain oxidative stress levels at a tolerable level. In terms of receptor status and organ specific metastasis, one study found bone metastases to correlate with ER positivity and high ROS levels while the presence of lung metastases correlated with ER negativity and high levels of antioxidant expression (H. M. Kim, Jung, and Koo 2014). Stromal GST expression was also found to be higher in liver and bone metastases while Catalase was lower in bone metastases (H. M. Kim, Jung, and Koo 2014).

Epigenetics and Cancer

Traditionally cancer was thought to be a genetic disease. The discovery that chromatin modifications, methylation of DNA and RNA-dependent regulation often precede the accumulation of genetic abnormalities and nuclear reprogramming of tumor cells has furthered our understanding of the onset and development of carcinogenesis and the molecular pathways that regulate it (Kelly and Issa 2016). The field of epigenetics was coined to bridge developmental biology and genetics; to explain the phenomenology of undifferentiated embryos developing into adult organisms. Hence, *"epigenetics"* is broadly defined as the "unfolding of the genetic program for development" (Holliday 2006). The best characterized epigenetic marker is DNA methylation; when a methyl group is covalently added to a cytosine residue that precedes guanine by a DNA methylase at the 5' regulatory end of genes. The consequences of DNA methylation in cancer were found to include hypomethylated or hypermethylated regions of the genome (Feinberg and Vogelstein 1983; Sakai et al. 1991). These hypo and hypermethylated regions occur in CpG islands and lead to the activation of oncogenes and inactivation of tumor suppressors respectively to promote the development of cancer (Flavahan et al. 2016; Sakai et al. 1991). DNA

hypomethylation is commonly seen in cancer and recently has become a therapeutic target of interest given its influence on tumor progression and metastasis (Stefanska et al. 2014).

It is now known that alterations to histone structure regulate DNA hypermethylation and these interactions occur in complicated chromatin networks (Flavahan et al. 2016). Lysine acetylation is associated with transcriptional activation (Hebbes, Thorne, and Crane-Robinson 1988). In contrast, lysine methylation promotes transcriptional activation or repression depending upon which residue is modified and the degree of methylation (Liang et al. 2004). Common histone modifications that occur in cancer include: H3K4me3, H3K9Ac and H3K27Ac near the transcription start site that correlate with low levels of DNA methylation and open, actively transcribed chromatin (Liang et al. 2004; Sharma, Kelly, and Jones 2009). In contrast, H3K9me3 and H3K27me3 are repressive chromatin marks and the two main mechanisms associated with gene silencing in mammalian cells (Hon et al. 2012; Margueron et al. 2009). The polycomb repressive complex consisting of PRC1 and PRC2 control methylation of H3K27 (Lund, Lohuizen, and M 2004). G9a has been shown to regulate methylation of H3K9 and regulates p16 gene silencing along with DNA methylation (Kondo et al. 2007). In contrast, HAT's p300 and CBP maintain active chromatin (Lund, Lohuizen, and M 2004). Finally, during replication most histones are assembled into nucleosomes to package genomic DNA. However, at particular regions of chromosomes several variant histones are deposited independently of replication.

The zinc finger CCCTC-binding factor (CTCF) functions as an epigenetic regulator of gene transcription by preventing the spread of repressive heterochromatin at promoter elements (Witcher and Emerson 2009a). Basal breast tumors exhibit genomic instability, which activates poly(ADP-ribose) polymerase (PARP). In turn, PARP catalyzes the post-translational addition of

poly-ADP ribose (PAR) units onto proteins involved in DNA damage repair, which increases their function (Smith D.C, Simon M., Alldredge A.L. 1992). PARP-dependent parylation of CTCF is required for CTCF to bind DNA (Witcher and Emerson 2009a). Loss of CTCF at a domain boundary permits a constitutive enhancer to interact aberrantly and activate PDGFRA expression and promotes tumor cell proliferation (Fang et al. 2011a). This work demonstrates the link between tumor progression and chromatin boundaries. Collectively, epigenetic regulation of gene expression has been shown to correlate with aggressiveness and cellular plasticity in various cancers, including breast tumors.

Epigenetic Regulation of p66ShcA

While the p46 and p52 isoforms are expressed ubiquitously in breast cancer cell lines and primary tumors, p66 levels are variable (Stevenson and Frackelton 1998). This is in agreement with published work indicating p66ShcA is regulated at the epigenetic level through promoter methylation and deacetylation (Ventura 2002). Intriguingly, endothelial cell exposure to LDL was shown to induce p66 expression through a mechanism dependent on the DNA methyltransferases DNMT1 and DNMT3b (Y.-R. Kim et al. 2012). Indeed, homocysteine was shown to epigenetically regulate p66 expression by controlling methylation of CpG dinucleotides 6 and 7 (CpG6,7) within the p66 promoter (C. S. Kim et al. 2011). Sirtuin1, a class 3 histone deacetylase, has also been shown to epigenetically regulate the p66 promoter through modifications on histone 3 (S. Zhou et al. 2011). In lung cancer, ChIP analyses for histone marks in the $p66^{Shc}$ promoter region indicated epigenetic silencing of p66 as revealed through decreased association of activating histones (H3K9Ac, H3K4me2, and H3K4me3) and enrichment of repressing histones (H3K9me2) in SCLC cells. Moreover, occupancy of this region was controlled by the lymphocyte lineage-restricted transcription factor, Aiolos (X. Li et al. 2014). Our work focused on identifying mechanisms

regulating p66ShcA expression in breast cancer cell lines and in vivo selected metastatic variants. Functionally, we tested whether p66ShcA is pro or anti-tumorigenic in breast cancer depending on the redox status.

References for Introduction

Abd El-Rehim, D. M., Pinder, S. E., Paish, C. E., Bell, J., Balmey, R. W., Robertson, J. F. R., … Ellis, I. O. (2004). Expression of luminal and basal cytokeratins in human breast carcinoma. *Journal of Pathology*, *203*(2), 661–671. https://doi.org/10.1002/path.1559

Adams, J. M., & Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*, *26*(9), 1324–1337. https://doi.org/10.1038/sj.onc.1210220

Ahn, R., Sabourin, V., Bolt, A. M., Hébert, S., Totten, S., De Jay, N., … Ursini-Siegel, J. (2017). The Shc1 adaptor simultaneously balances Stat1 and Stat3 activity to promote breast cancer immune suppression. *Nature Communications*, *8*. https://doi.org/10.1038/ncomms14638

Ahn, R., Sabourin, V., Ha, J. R., Cory, S., Maric, G., Im, Y. K., … Ursini-Siegel, J. (2013). The ShcA PTB domain functions as a biological sensor of phosphotyrosine signaling during breast cancer progression. *Cancer Research*, *73*(14), 4521–4532. https://doi.org/10.1158/0008-5472.CAN-12-4178

Anders, C. K., Johnson, R., Litton, J., Phillips, M., & Bleyer, A. (2009). Breast Cancer Before Age 40 Years. *Seminars in Oncology*, *36*(3), 237–249. https://doi.org/10.1053/j.seminoncol.2009.03.001

Arany, I., Faisal, A., Nagamine, Y., & Safirstein, R. L. (2008). p66shc inhibits pro-survival epidermal growth factor receptor/ERK signaling during severe oxidative stress in mouse renal proximal tubule cells. *Journal of Biological Chemistry*, *283*(10), 6110–6117. https://doi.org/10.1074/jbc.M708799200

Babior, B. M., Kipnes, R. S., & Cumvu, J. T. (1973). CONCISE Biological Defense Mechanisms, *52*(March), 741–744. https://doi.org/10.1172/JCI107236.00.02

Bacus, S. S., Altomare, D. A., Lyass, L., Chin, D. M., Farrell, M. P., Gudkov, A., & Testa, J. R. (2002). AKT2 is frequently upregulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. *Oncogene*, *21*(22), 3532–3540. https://doi.org/10.1038/sj.onc.1205438

Bahrami, A., Khazaei, M., Shahidsales, S., Hassanian, S. M., Hasanzadeh, M., Maftouh, M., … Avan, A. (2018). The Therapeutic Potential of PI3K/Akt/mTOR Inhibitors in Breast Cancer: Rational and Progress. *Journal of Cellular Biochemistry*, *119*(1), 213–222. https://doi.org/10.1002/jcb.26136

Balko, J. M., Cook, R. S., Vaught, D. B., Kuba, M. G., Miller, T. W., Bhola, N. E., … Arteaga, C. L. (2012). Profiling of residual breast cancers after neoadjuvant chemotherapy identifies DUSP4 deficiency as a mechanism of drug resistance. *Nature Medicine*, *18*(7), 1052–1059. https://doi.org/10.1038/nm.2795

Bansal, A., & Simon, M. C. (2018). Glutathione metabolism in cancer progression and treatment resistance. *The Journal of Cell Biology*, *217*(7), jcb.201804161. https://doi.org/10.1083/jcb.201804161

Bao, S., Ouyang, G., Bai, X., Huang, Z., Ma, C., Liu, M., … Wang, X. F. (2004). Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway. *Cancer Cell*, *5*(4), 329–339. https://doi.org/10.1016/S1535-6108(04)00081-9

Bardou, V. J., Arpino, G., Elledge, R. M., Osborne, C. K., & Clark, G. M. (2003). Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *Journal of Clinical Oncology*, *21*(10), 1973–1979. https://doi.org/10.1200/JCO.2003.09.099

Bernard, P. S., Parker, J. S., Mullins, M., Cheung, M. C. U., Leung, S., Voduc, D., … Perou, C. M. (2009). Supervised risk predictor of breast cancer based on intrinsic subtypes. *Journal of Clinical Oncology*, *27*(8), 1160–1167. https://doi.org/10.1200/JCO.2008.18.1370

Bernardi, P., & Azzone, G. F. (1981). Cytochrome c as an electron shuttle between the outer and inner mitochondrial membranes. *Journal of Biological Chemistry*, *256*(14), 7187–7192.

Betts, D. H., Bain, N. T., & Madan, P. (2014). The p66Shc adaptor protein controls oxidative stress response in early bovine embryos. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0086978

Bhargava, R., Gerald, W. L., Li, A. R., Pan, Q., Lal, P., Ladanyi, M., & Chen, B. (2005). EGFR gene amplification in breast cancer: Correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. *Modern Pathology*, *18*(8), 1027–1033. https://doi.org/10.1038/modpathol.3800438

Bhat, H. F., Baba, R. A., Adams, M. E., & Khanday, F. A. (2014). Role of SNTA1 in Rac1 activation, modulation of ROS generation, and migratory potential of human breast cancer cells. *British Journal of Cancer*, *110*(3), 706–714. https://doi.org/10.1038/bjc.2013.723

Bombonati, A., & Sgroi, D. C. (2011). The molecular pathology of breast cancer progression. *The Journal of Pathology*, *223*(November 2010), 307–317. https://doi.org/10.1002/path.2808

Brassard, D. L., Maxwell, E., Malkowski, M., Nagabhushan, T. L., Kumar, C. C., & Armstrong, L. (1999). Integrin alpha(v)beta(3)-mediated activation of apoptosis. *Experimental Cell Research*, *251*(1), 33–45. https://doi.org/10.1006/excr.1999.4559

Bronsert, P., Enderle-Ammour, K., Bader, M., Timme, S., Kuehs, M., Csanadi, A., … Wellner, U. F. (2014). Cancer cell invasion and EMT marker expression: a three-dimensional study of the human cancer-host interface. *The Journal of Pathology*, *234*(3), 410–422. https://doi.org/10.1002/path.4416

Buchheit, C. L., Weigel, K. J., & Schafer, Z. T. (2014). Cancer cell survival during detachment from the ECM: Multiple barriers to tumour progression. *Nature Reviews Cancer*, *14*(9), 632–641. https://doi.org/10.1038/nrc3789

Cadenas, E., Davies, K. J. A., & Adenas, E. N. C. (2000). MITOCHONDRIAL FREE RADICAL GENERATION, OXIDATIVE STRESS, AND AGING. *Free Radical Biology & Medicine*, *29*(00), 201–383.

Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer*, *2*, 563. Retrieved from http://dx.doi.org/10.1038/nrc865

Cichon, M. a, & Radisky, D. C. (2014). ROS-induced epithelial-mesenchymal transition in mammary epithelial cells is mediated by NF-kB-dependent activation of Snail. *Oncotarget*, *5*(9), 2827–2838. https://doi.org/10.18632/oncotarget.1940

Clark, E., Pyle, G. F., Zhou, N., Kawaoka, Y., Webster, R., Fanning, T. G., … Taubenberger, J. K. (2007). Protein Kinase C b and Prolyl Isomerase, *315*(February), 659–663.

Clarke, R. B., Anderson, E., & Howell, A. (2004). Steroid receptors in human breast cancer. *Trends in Endocrinology and Metabolism*, *15*(7), 316–323. https://doi.org/10.1016/j.tem.2004.07.004

Cristofanilli, M., & Budd, G. (2004). Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *England Journal of Medicine*, *351*(8), 781–791. https://doi.org/10.1056/NEJMoa040766

Dai, X., Li, T., Bai, Z., Yang, Y., Liu, X., Zhan, J., & Shi, B. (2015). Breast cancer intrinsic subtype classification, clinical use and future trends. *American Journal of Cancer Research*, *5*(10), 2929–2943. https://doi.org/www.ajcr.us/ISSN:2156-6976/ajcr0014158

Davidson, D., & Veillette, Â. (2001). PTP-PEST , a scaffold protein tyrosine phosphatase , negatively regulates lymphocyte activation by targeting a unique set of substrates, *20*(13).

Davison, C. A., Durbin, S. M., Thau, M. R., Zellmer, V. R., Chapman, S. E., Diener, J., … Schafer, Z. T. (2013). Antioxidant enzymes mediate survival of breast cancer cells deprived of extracellular matrix. *Cancer Research*, *73*(12), 3704–3715. https://doi.org/10.1158/0008-5472.CAN-12-2482

Davol, P. A., Bagdasaryan, R., Elfenbein, G. J., Maizel, A. L., & Frackelton, A. R. (2003). Shc proteins are strong, independent prognostic markers for both node-negative and node-positive primary breast cancer. *Cancer Research*, *63*(20), 6772–6783. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14583473

Dawson, S. J., Rueda, O. M., Aparicio, S., & Caldas, C. (2013). A new genome-driven integrated classification of breast cancer and its implications. *EMBO Journal*, *32*(5), 617–628. https://doi.org/10.1038/emboj.2013.19

DeBerardinis, R. J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S., & Thompson, C. B. (2007). Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proceedings of the National Academy of Sciences*, *104*(49), 19345–19350. https://doi.org/10.1073/pnas.0709747104

DeNardo, D. G., Brennan, D. J., Rexhepaj, E., Ruffell, B., Shiao, S. L., Madden, S. F., … Coussens, L. M. (2011). Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discovery*, *1*(1), 54–67. https://doi.org/10.1158/2159-8274.CD-10-0028

Du, W., Jiang, Y., Zheng, Z., Zhang, Z., Chen, N., Ma, Z., … Liu, Z. (2013). Feedback loop between p66Shcand Nrf2 promotes lung cancer progression. *Cancer Letters*, *337*(1), 58–65. https://doi.org/10.1016/j.canlet.2013.05.016

Dupuy, F., Tabariès, S., Andrzejewski, S., Dong, Z., Blagih, J., Annis, M. G., … Siegel, P. M. (2015). PDK1-dependent metabolic reprogramming dictates metastatic potential in breast cancer. *Cell Metabolism*, *22*(4), 577–589. https://doi.org/10.1016/j.cmet.2015.08.007

Elston and Ellis. (1991). pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*, *19*(5), 151–153. https://doi.org/10.1111

Elston, C. W. (1984). the Assessment of Histological Differentiation in Breast Cancer. *Australian and New Zealand Journal of Surgery*, *54*(1), 11–15. https://doi.org/10.1111/j.1445-2197.1984.tb06677.x

Faisal, A., el-Shemerly, M., Hess, D., & Nagamine, Y. (2002). Serine/threonine phosphorylation of ShcA. Regulation of protein-tyrosine phosphatase-pest binding and involvement in insulin signaling. *The Journal of Biological Chemistry*, *277*(33), 30144–30152. https://doi.org/10.1074/jbc.M203229200

Fang, F., Turcan, S., Rimner, A., Kaufman, A., Giri, D., Morris, L. G. T., … Chan, T. A. (2011). Breast cancer methylomes establish an epigenomic foundation for metastasis. *Science Translational Medicine*, *3*(75). https://doi.org/10.1126/scitranslmed.3001875

Feinberg, A. P., & Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, *301*(5895), 89–92.

https://doi.org/10.1038/301089a0
Feng, M., Bao, Y., Li, Z., Li, J., Gong, M., Lam, S., … Yu, Q. (2014). RASAL2 activates RAC1 to promote triple-negative breast cancer progression. *Journal of Clinical Investigation*, *124*(12), 5291–5304. https://doi.org/10.1172/JCI76711
Fidler, I. J. (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited, *3*(June), 1–6.
Finak, G., Sadekova, S., Pepin, F., Hallett, M., Meterissian, S., Halwani, F., … Park, M. (2006). Gene expression signatures of morphologically normal breast tissue identify basal-like tumors. *Breast Cancer Research*, *8*(5), 1–18. https://doi.org/10.1186/bcr1608
Finkel, T. (2011). Signal transduction by reactive oxygen species. *Journal of Cell Biology*, *194*(1), 7–15. https://doi.org/10.1083/jcb.201102095
Flavahan, W. A., Drier, Y., Liau, B. B., Gillespie, S. M., Venteicher, A. S., Stemmer-Rachamimov, A. O., … Bernstein, B. E. (2016). Insulator dysfunction and oncogene activation in IDH mutant gliomas. *Nature*, *529*(7584), 110–114. https://doi.org/10.1038/nature16490
Fong, M. Y., Zhou, W., Liu, L., Alontaga, A. Y., Chandra, M., Ashby, J., … Wang, S. E. (2015). Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. *Nature Cell Biology*, *17*(2), 183–194. https://doi.org/10.1038/ncb3094
Frackelton, A. R., Lu, L., Davol, P. A., Bagdasaryan, R., Hafer, L. J., & Sgroi, D. C. (2006). p66 Shc and tyrosine-phosphorylated Shc in primary breast tumors identify patients likely to relapse despite tamoxifen therapy. *Breast Cancer Research*, *8*(6), 1–8. https://doi.org/10.1186/bcr1631
Franken, B., de Groot, M. R., Mastboom, W. J. B., Vermes, I., van der Palen, J., Tibbe, A. G. J., & Terstappen, L. W. M. M. (2012). Circulating tumor cells, disease recurrence and survival in newly diagnosed breast cancer. *Breast Cancer Research*, *14*(5). https://doi.org/10.1186/bcr3333
Frasor, J., Danes, J. M., Komm, B., Chang, K. C. N., Richard Lyttle, C., & Katzenellenbogen, B. S. (2003). Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: Insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology*, *144*(10), 4562–4574. https://doi.org/10.1210/en.2003-0567
Fredholm, H., Eaker, S., Frisell, J., Holmberg, L., Fredriksson, I., & Lindman, H. (2009). Breast cancer in young women: Poor survival despite intensive treatment. *PLoS ONE*, *4*(11), 1–9. https://doi.org/10.1371/journal.pone.0007695
Frisch, S., & Francis, H. (1994). Disruption of epithelial cell matrix interactions induces apoptosis. *J. Cell Biol.*, *124*(4), 619–626. Retrieved from internal-pdf:/Anoikis (Frisch, 1994).pdf
Fu, Q., Zhang, Q., Lou, Y., Yang, J., Nie, G., Chen, Q., … Liang, T. (2018). Primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer. *Oncogene*, 1–14. https://doi.org/10.1038/s41388-018-0391-0
Furui, J. (1995). Requirement fir Generation of H202 for Platelet-Derived Growth Factor Signal Transduction.
Galadari, S., Rahman, A., Pallichankandy, S., & Thayyullathil, F. (2017). Reactive oxygen species and cancer paradox: To promote or to suppress? *Free Radical Biology and Medicine*, *104*(December 2016), 144–164. https://doi.org/10.1016/j.freeradbiomed.2017.01.004

Galimov, E. R. (2010). The Role of p66shc in Oxidative Stress and Apoptosis. *Acta Naturae*, *2*(4), 44–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22649663%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3347587

Garcia Pedrero, J. M., Garcia Carracedo, D., Muñoz Pinto, C., Herrero Zapatero, A., Rodrigo, J. P., Suarez Nieto, C., & Gonzalez, M. V. (2005). Frequent genetic and biochemical alterations of the PI3K/AKT/PTEN pathway in head and neck squamous cell carcinoma. *International Journal of Cancer*, *114*(2), 242–248. https://doi.org/10.1002/ijc.20711

Gasic, G. J., Gasic, T. B., & Stewart, C. C. (1968). Antimetastatic effects associated with platelet reduction. *Proceedings of the National Academy of Sciences*, *61*(1), 46–52. https://doi.org/10.1073/pnas.61.1.46

Gelderloos, J. A., Rosenkranz, S., Bazenet, C., & Kazlauskas, A. (1998). A Role for Src in Signal Relay by the Platelet-derived Growth Factor α Receptor. *Journal of Biological Chemistry* , *273*(10), 5908–5915. https://doi.org/10.1074/jbc.273.10.5908

Gertz, M., Fischer, F., Wolters, D., & Steegborn, C. (2008). Activation of the lifespan regulator p66Shc through reversible disulfide bond formation. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(15), 5705–5709. https://doi.org/10.1073/pnas.0800691105

Geyer, C. E., Forster, J., Lindquist, D., Chan, S., Romieu, C. G., Pienkowski, T., … Cameron, D. (2006). Lapatinib plus Capecitabine for HER2-Positive Advanced Breast Cancer. *New England Journal of Medicine*, *355*(26), 2733–2743. https://doi.org/10.1056/NEJMoa064320

Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi, C., … Pelicci, P. G. (2005a). Electron Transfer between Cytochrome c and $p66^{Shc}$ Generates Reactive Oxygen Species that Trigger Mitochondrial Apoptosis. *Cell*, *122*(2), 221–233. https://doi.org/10.1016/j.cell.2005.05.011

Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi, C., … Pelicci, P. G. (2005b). Electron transfer between cytochrome c and p66Shcgenerates reactive oxygen species that trigger mitochondrial apoptosis. *Cell*, *122*(2), 221–233. https://doi.org/10.1016/j.cell.2005.05.011

Giuliano, A. E., Connolly, J. L., Edge, S. B., & Mittendorf, E. A. (2017). Breast Cancer — Major Changes in the American Joint Committee on Cancer Eighth Edition Cancer Staging Manual. *A Cancer Journals for Clinicians*, *67*(0), 290–303. https://doi.org/10.3322/caac.21393.

Gjorevski, N., Piotrowski, A. S., Varner, V. D., & Nelson, C. M. (2015). Dynamic tensile forces drive collective cell migration through three-dimensional extracellular matrices. *Scientific Reports*, *5*(February), 11458. https://doi.org/10.1038/srep11458

Goh, J., Enns, L., Fatemie, S., Hopkins, H., Morton, J., Pettan-Brewer, C., & Ladiges, W. (2011). Mitochondrial targeted catalase suppresses invasive breast cancer in mice. *BMC Cancer*, *11*. https://doi.org/10.1186/1471-2407-11-191

Goldhirsch, A., Ingle, J. N., Gelber, R. D., Coates, A. S., Thürlimann, B., & Senn, H. J. (2009). Thresholds for therapies: Highlights of the St Gallen international expert consensus on the primary therapy of early breast cancer 2009. *Annals of Oncology*, *20*(8), 1319–1329. https://doi.org/10.1093/annonc/mdp322

Grille, S. J., Bellacosa, A., Upson, J., Klein-szanto, A. J., Roy, F. Van, Lee-kwon, W., … Larue, L. (2003). The Protein Kinase Akt Induces Epithelial Mesenchymal Transition and Promotes Enhanced Motility and Invasiveness of Squamous Cell Carcinoma Lines The

Protein Kinase Akt Induces Epithelial Mesenchymal Transition and Promotes Enhanced Motility and Invasiven. *Cancer Research*, *63*, 2172–2178. https://doi.org/10.1016/0092-8674(91)90143-m

Grossman, S. R., Lyle, S., Resnick, M. B., Sabo, E., Lis, R. T., Rosinha, E., … Hafer, L. J. (2007). p66 Shc tumor levels show a strong prognostic correlation with disease outcome in stage IIA colon cancer. *Clinical Cancer Research*, *13*(19), 5798–5804. https://doi.org/10.1158/1078-0432.CCR-07-0073

Gschwind, A., Fischer, O. M., & Ullrich, A. (2004). The discovery of receptor tyrosine kinases: Targets for cancer therapy. *Nature Reviews Cancer*, *4*(5), 361–370. https://doi.org/10.1038/nrc1360

Ha, J. R., Ahn, R., Smith, H. W., Sabourin, V., Hébert, S., Cepeda Cañedo, E., … Ursini-Siegel, J. (2018). Integration of distinct ShcA signaling complexes promotes breast tumor growth and tyrosine kinase inhibitor resistance. *Molecular Cancer Research : MCR*, *16*(May), 894–908. https://doi.org/10.1158/1541-7786.MCR-17-0623

Han, D., Williams, E., & Cadenas, E. (2001). Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. *Biochemical Journal*, *353*(2), 411. https://doi.org/10.1042/0264-6021:3530411

Hanahan, D., & Weinberg, R. A. (2000). The Hallmarks of Cancer, *100*, 57–70. https://doi.org/10.1007/s00262-010-0968-0

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hebbes, T. R., Thorne, A. W., & Crane-Robinson, C. (1988). A direct link between core histone acetylation and transcriptionally active chromatin. *The EMBO Journal*, *7*(5), 1395–1402. https://doi.org/10.1007/BF00777468

Herranz, N., Pasini, D., Diaz, V. M., Franci, C., Gutierrez, A., Dave, N., … Peiro, S. (2008). Polycomb Complex 2 Is Required for E-cadherin Repression by the Snail1 Transcription Factor. *Molecular and Cellular Biology*, *28*(15), 4772–4781. https://doi.org/10.1128/MCB.00323-08

Hochgräfe, F., Zhang, L., O'Toole, S. A., Browne, B. C., Pinese, M., Cubas, A. P., … Daly, R. J. (2010). Tyrosine phosphorylation profiling reveals the signaling network characteristics of basal breast cancer cells. *Cancer Research*, *70*(22), 9391–9401. https://doi.org/10.1158/0008-5472.CAN-10-0911

Holliday, R. (2006). Epigenetics: A historical overview. *Epigenetics*, *1*(2), 76–80. https://doi.org/10.4161/epi.1.2.2762

Hon, G. C., Hawkins, R. D., Caballero, O. L., Lo, C., Lister, R., Pelizzola, M., … Ren, B. (2012). Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer, 246–258. https://doi.org/10.1101/gr.125872.111

Honn, K. V., Tang, D. G., Gao, X., Butovich, I. A., Liu, B., Timar, J., & Hagmann, W. (1994). 12-Lipoxygenases and 12(S)-HETE: role in cancer metastasis. *Cancer and Metastasis Reviews*, *13*(3–4), 365–396. https://doi.org/10.1007/BF00666105

Huber, M. a, Azoitei, N., Baumann, B., Grünert, S., Sommer, A., Pehamberger, H., … Wirth, T. (2004). NF- κ B is essential for epithelial- mesenchymal transition and metastasis in a model of breast cancer progression. *The Journal of Clinical Investigation*, *114*(4), 569–581. https://doi.org/10.1172/JCI200421358.The

Hur, K., Toiyama, Y., Takahashi, M., Balaguer, F., Nagasaka, T., Koike, J., … Goel, A. (2013). MicroRNA-200c modulates epithelial-tomesenchymal transition (EMT) in human

colorectal cancer metastasis. *Gut*, *62*(9), 1315–1326. https://doi.org/10.1136/gutjnl-2011-301846

Im, Y. K., La Selva, R., Gandin, V., Ha, J. R., Sabourin, V., Sonenberg, N., … Ursini-Siegel, J. (2015). The ShcA adaptor activates AKT signaling to potentiate breast tumor angiogenesis by stimulating VEGF mRNA translation in a 4E-BP-dependent manner. *Oncogene*, *34*(13), 1729–1735. https://doi.org/10.1038/onc.2014.110

Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., … Hayashi, J.-I. (2008). ROS-Generating Mitochondrial DNA Mutations Can Regulate Tumor Cell Metastasis. *Science*, *320*(5876), 661–664. https://doi.org/10.1126/science.1156906

Itoh, T., Tanioka, M., Matsuda, H., Nishimoto, H., Yoshioka, T., Suzuki, R., & Uchira, M. (1999). Experimental metastasis is suppressed in MMP-9-deficient mice. *Clinical and Experimental Metastasis*, *17*(2), 177–181. https://doi.org/10.1023/A:1006603723759

Jackson, J. G., Yoneda, T., Clark, G. M., Yee, D., Jackson, J. G., Yoneda, T., … Yee, D. (2000). Elevated Levels of p66 Shc Are Found in Breast Cancer Cell Lines and Primary Tumors with High Metastatic Potential Elevated Levels of p66 Shc Are Found in Breast Cancer Cell Lines and Primary Tumors with High Metastatic Potential 1, *6*(March), 1135–1139.

Jin, M. H., Nam, A.-R., Park, J. E., Bang, J.-H., Bang, Y.-J., & Oh, D.-Y. (2017). Resistance Mechanism against Trastuzumab in HER2-Positive Cancer Cells and Its Negation by Src Inhibition. *Molecular Cancer Therapeutics*, *16*(6), 1145–1154. https://doi.org/10.1158/1535-7163.MCT-16-0669

Jones, B., & Russo, J. (1987). Influence of steroid hormones on the growth fraction of human breast carcinomas. *American Journal of Clinical Pathology*, *88*(2), 132–138.

Jones, N., Hardy, W. R., Friese, M. B., Jorgensen, C., Smith, M. J., Woody, N. M., … Pawson, T. (2007). Analysis of a Shc Family Adaptor Protein, ShcD/Shc4, That Associates with Muscle-Specific Kinase. *Molecular and Cellular Biology*, *27*(13), 4759–4773. https://doi.org/10.1128/MCB.00184-07

Kang, S. A., Hasan, N., Mann, A. P., Zheng, W., Zhao, L., Morris, L., … Tanaka, T. (2015). Blocking the adhesion cascade at the premetastatic niche for prevention of breast cancer metastasis. *Molecular Therapy*, *23*(6), 1044–1054. https://doi.org/10.1038/mt.2015.45

Karpatkin, S., Pearlstein, E., Ambrogio, C., & Coller, B. S. (1988). Role of adhesive proteins in platelet tumor interaction in vitro and metastasis formation in vivo. *Journal of Clinical Investigation*, *81*(4), 1012–1019. https://doi.org/10.1172/JCI113411

Kelly, A. D., & Issa, J.-P. J. (2016). Epigenetics and Cancer. *Epigenetics, Energy Balance, and Cancer*, *44106*, 1–28. https://doi.org/10.1152/japplphysiol.00066.2010.

Key, T. J., Verkasalo, P. K., & Banks, E. (2001). Epidemiology of breast cancer. *The Lancet Oncology*, *2*(3), 133–140. https://doi.org/10.1016/S1470-2045(00)00254-0

Khalid, S., Drasche, A., Thurner, M., Hermann, M., Ashraf, M. I., Fresser, F., … Troppmair, J. (2016). CJun N-terminal kinase (JNK) phosphorylation of serine 36 is critical for p66Shc activation. *Scientific Reports*, *6*(October 2015), 1–13. https://doi.org/10.1038/srep20930

Kim, C. S., Kim, Y. R., Naqvi, A., Kumar, S., Hoffman, T. A., Jung, S. B., … Irani, K. (2011). Homocysteine promotes human endothelial cell dysfunction via site-specific epigenetic regulation of p66shc. *Cardiovascular Research*, *92*(3), 466–475. https://doi.org/10.1093/cvr/cvr250

Kim, H. M., Jung, W. H., & Koo, J. S. (2014). Expression of reactive oxygen species-related proteins in metastatic breast cancer is dependent on the metastatic site. *International Journal of Clinical and Experimental Pathology*, *7*(12), 8802–8812.

Kim, J., Kong, J., Chang, H., Kim, H., Kim, A., Kim, J., … Kim, A. (2016). EGF induces epithelial-mesenchymal transition through phospho-Smad2/3-Snail signaling pathway in breast cancer cells. *Oncotarget*, *7*(51), 85021–85032. https://doi.org/10.18632/oncotarget.13116

Kim, Y.-R., Kim, C.-S., Naqvi, A., Kumar, A., Kumar, S., Hoffman, T. A., & Irani, K. (2012). Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction. *AJP: Heart and Circulatory Physiology*, *303*(2), H189–H196. https://doi.org/10.1152/ajpheart.01218.2011

Knight, J. F., Lesurf, R., Zhao, H., Pinnaduwage, D., Davis, R. R., Saleh, S. M. I., … Park, M. (2013). Met synergizes with p53 loss to induce mammary tumors that possess features of claudin-low breast cancer. *Proceedings of the National Academy of Sciences*, *110*(14), E1301–E1310. https://doi.org/10.1073/pnas.1210353110

Koboldt, D. C., Fulton, R. S., McLellan, M. D., Schmidt, H., Kalicki-Veizer, J., McMichael, J. F., … Palchik, J. D. (2012). Comprehensive molecular portraits of human breast tumours. *Nature*, *490*(7418), 61–70. https://doi.org/10.1038/nature11412

Kondo, Y., Shen, L., Suzuki, S., Kurokawa, T., Masuko, K., Tanaka, Y., … Sekido, Y. (2007). Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. *Hepatology Research*, *37*(11), 974–983. https://doi.org/10.1111/j.1872-034X.2007.00141.x

Kraut-Cohen, J., Muller, W. J., & Elson, A. (2008). Protein-tyrosine phosphatase ε regulates Shc signaling in a kinase-specific manner: Increasing coherence in tyrosine phosphatase signaling. *Journal of Biological Chemistry*, *283*(8), 4612–4621. https://doi.org/10.1074/jbc.M708822200

Kumar, S., Park, S. H., Cieply, B., Schupp, J., Killiam, E., Zhang, F., … Frisch, S. M. (2011). A Pathway for the Control of Anoikis Sensitivity by E-Cadherin and Epithelial-to-Mesenchymal Transition. *Molecular and Cellular Biology*, *31*(19), 4036–4051. https://doi.org/10.1128/MCB.01342-10

Lai, K. M. V., & Pawson, T. (2000). The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo. *Genes and Development*, *14*(9), 1132–1145. https://doi.org/PMC316585

Larsen, S. L., Laenkholm, A. V., Duun-Henriksen, A. K., Bak, M., Lykkesfeldt, A. E., & Kirkegaard, T. (2015). Src drives growth of antiestrogen resistant breast cancer cell lines and is a marker for reduced benefit of tamoxifen treatment. *PLoS ONE*, *10*(2), 1–19. https://doi.org/10.1371/journal.pone.0118346

Lee, M.-S., Igawa, T., Chen, S.-J., Van Bemmel, D., Lin, J. S., Lin, F.-F., … Lin, M.-F. (2004). p66Shc protein is upregulated by steroid hormones in hormone-sensitive cancer cells and in primary prostate carcinomas. *International Journal of Cancer. Journal International Du Cancer*, *108*(5), 672–678. https://doi.org/10.1002/ijc.11621

Lee, S. R., Yang, K. S., Kwon, J., Lee, C., Jeong, W., & Rhee, S. G. (2002). Reversible inactivation of the tumor suppressor PTEN by H2O2. *Journal of Biological Chemistry*, *277*(23), 20336–20342. https://doi.org/10.1074/jbc.M111899200

Lehmann, B. D. B., Bauer, J. a J., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., … Li, Y. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *Journal of Clinical Investigation*, *121*(7), 2750–2767. https://doi.org/10.1172/JCI45014DS1

Lemmon, M. A., & Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. *Cell*,

141(7), 1117–1134. https://doi.org/10.1016/j.cell.2010.06.011
Li, K., He, W., Lin, N., Wang, X., & Fan, Q. X. (2009). N-cadherin knock-down decreases invasiveness of esophageal squamous cell carcinoma in vitro. *World Journal of Gastroenterology*, *15*(6), 697–704. https://doi.org/10.3748/wjg.15.697
Li, X., Xu, Z., Du, W., Zhang, Z., Wei, Y., Wang, H., … Liu, Z. (2014). Aiolos promotes anchorage independence by silencing p66Shctranscription in cancer cells. *Cancer Cell*, *25*(5), 575–589. https://doi.org/10.1016/j.ccr.2014.03.020
Liang, G., Lin, J. C. Y., Wei, V., Yoo, C., Cheng, J. C., Nguyen, C. T., … Jones, P. A. (2004). Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. *Proceedings of the National Academy of Sciences*, *101*(19), 7357–7362. https://doi.org/10.1073/pnas.0401866101
Liou, G.-Y., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radical Research*, *44*(5), 479–496. https://doi.org/10.3109/10715761003667554
Liu, a X., Testa, J. R., Hamilton, T. C., Jove, R., Nicosia, S. V, & Cheng, J. Q. (1998). AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. *Cancer Research*, *58*(14), 2973–2977.
Lo, Y. Y., & Cruz, T. F. (1995). Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *The Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.270.20.11727
Lorenzatti, G., Huang, W., Pal, A., Cabanillas, A. M., & Kleer, C. G. (2011). CCN6 (WISP3) decreases ZEB1-mediated EMT and invasion by attenuation of IGF-1 receptor signaling in breast cancer. *Journal of Cell Science*, *124*(10), 1752–1758. https://doi.org/10.1242/jcs.084194
Lucs, A. V., Muller, W. J., & Muthuswamy, S. K. (2010). Shc is required for ErbB2-induced inhibition of apoptosis but is dispensable for cell proliferation and disruption of cell polarity. *Oncogene*, *29*(2), 174–187. https://doi.org/10.1038/onc.2009.312
Luga, V., Zhang, L., Viloria-Petit, A. M., Ogunjimi, A. A., Inanlou, M. R., Chiu, E., … Wrana, J. L. (2012). Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell*, *151*(7), 1542–1556. https://doi.org/10.1016/j.cell.2012.11.024
Lund, a H., Lohuizen, V., & M. (2004). b. Epigenetics and cancer. *Genes & Dev*, *18*(May 2016), 2315–2335. https://doi.org/10.1101/gad.1232504.Per
Luschnig, S., Krauss, J., Bohmann, K., Desjeux, I., & Nüsslein-Volhard, C. (2000). The Drosophila SHC adaptor protein is required for signaling by a subset of receptor tyrosine kinases. *Molecular Cell*, *5*(2), 231–241. https://doi.org/10.1016/S1097-2765(00)80419-0
Ma, S., Upneja, A., Galecki, A., Tsai, Y. M., Burant, C. F., Raskind, S., … Gladyshev, V. N. (2016). Cell culture-based profiling across mammals reveals DNA repair and metabolism as determinants of species longevity. *ELife*, *5*(NOVEMBER2016), 1–25. https://doi.org/10.7554/eLife.19130
Ma, Z., Liu, Z., Wu, R. F., & Terada, L. S. (2010). P66Shc restrains Ras hyperactivation and suppresses metastatic behavior. *Oncogene*, *29*(41), 5559–5567. https://doi.org/10.1038/onc.2010.326
Ma, Z., Myers, D. P. ., Ru, F. W., Nwariaku, F. E. ., & Terada, L. S. (2007). p66Shcmediates anoikis through RhoA. *Journal of Cell Biology*, *179*(1), 23–31. https://doi.org/10.1083/jcb.200706097

Mains, P. E., Sulston, I. A., & Wood, W. B. (1990). Dominant maternal-effect mutations causing embryonic lethality in Caenorhabditis elegans. *Genetics*, *125*(2), 351–369. https://doi.org/10.1091/mbc.E05

Mains, P. E., Sulston, I. A., & Wood, W. B. (2006). Rac1 Leads to Phosphorylation-dependent Increase in Stability of the p66shc Adaptor Protein: Role in Rac1-induced Oxidative Stress. *Genetics*, *17*, 122–129. https://doi.org/10.1091/mbc.E05

Malhotra, G. K., Zhao, X., Band, H., & Band, V. (2010). Histological, molecular and functional subtypes of breast cancers. *Cancer Biology and Therapy*, *10*(10), 955–960. https://doi.org/10.4161/cbt.10.10.13879

Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature*, *454*(7203), 436–444. https://doi.org/10.1038/nature07205

Margueron, R., Justin, N., Ohno, K., Sharpe, M. L., Son, J., Drury, W. J., … Gamblin, S. J. (2009). Role of the polycomb protein EED in the propagation of repressive histone marks. *Nature*, *461*(7265), 762–767. https://doi.org/10.1038/nature08398

Masoud, V., & Pagès, G. (2017). Targeted therapies in breast cancer: New challenges to fight against resistance. *World Journal of Clinical Oncology*, *8*(2), 120. https://doi.org/10.5306/wjco.v8.i2.120

McLaughlin, S. K., Olsen, S. N., Dake, B., De Raedt, T., Lim, E., Bronson, R. T., … Cichowski, K. (2013). The RasGAP Gene, RASAL2, Is a Tumor and Metastasis Suppressor. *Cancer Cell*, *24*(3), 365–378. https://doi.org/10.1016/j.ccr.2013.08.004

Meredith, J. E., Fazeli, B., & Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. *Molecular Biology of the Cell*, *4*(9), 953–961. https://doi.org/10.1091/mbc.4.9.953

Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., … Pelicci, P. G. (1999). The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature*, *402*(6759), 309–313. https://doi.org/10.1038/46311

Migliaccio, E., Mele, S., Salcini, A. E., Pelicci, G., Lai, K. V., Superti-furga, G., … Pelicci, P. G. (1997). Opposite effects of the p52 shc / p46 shc and p66 shc splicing isoforms on the EGF receptor – MAP kinase – fos signalling pathway. *The EMBO Journal*, *16*(4), 706–716.

Miller, T. W., Balko, J. M., & Arteaga, C. L. (2011). Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. *Journal of Clinical Oncology*, *29*(33), 4452–4461. https://doi.org/10.1200/JCO.2010.34.4879

Molyneux, G., Geyer, F. C., Magnay, F. A., McCarthy, A., Kendrick, H., Natrajan, R., … Smalley, M. J. (2010). BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. *Cell Stem Cell*, *7*(3), 403–417. https://doi.org/10.1016/j.stem.2010.07.010

Morgan, M. J., & Liu, Z. (2011). Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Research*, *21*(1), 103–115. https://doi.org/10.1038/cr.2010.178

Müller, a, Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., … Zlotnik, a. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, *410*(6824), 50–56. https://doi.org/10.1038/35065016

Muller, F. L., Liu, Y., & Remmen, H. Van. (2004). Metabolism and Bioenergetics : Complex III Releases Superoxide to Both Sides of the Inner Mitochondrial Membrane Complex III Releases Superoxide to Both Sides of the Inner Mitochondrial Membrane *. *Journal of Biological Chemistry*, *279*(210), 49064–49073. https://doi.org/10.1074/jbc.M407715200

Muniyan, S., Chou, Y. W., Tsai, T. J., Thomes, P., Veeramani, S., Benigno, B. B., … Lin, M. F. (2015). p66Shc longevity protein regulates the proliferation of human ovarian cancer cells.

Molecular Carcinogenesis, *54*(8), 618–631. https://doi.org/10.1002/mc.22129
Murphy, C. G., & Dickler, M. N. (2016). Endocrine resistance in hormone-responsive breast cancer: Mechanisms and therapeutic strategies. *Endocrine-Related Cancer*, *23*(8), R337–R352. https://doi.org/10.1530/ERC-16-0121
Northey, J. J., Chmielecki, J., Ngan, E., Russo, C., Annis, M. G., Muller, W. J., & Siegel, P. M. (2008). Signaling through ShcA Is Required for Transforming Growth Factor - and Neu/ErbB-2-Induced Breast Cancer Cell Motility and Invasion. *Molecular and Cellular Biology*, *28*(10), 3162–3176. https://doi.org/10.1128/MCB.01734-07
Okada, S., Yamauchi, K., & Pessin, J. E. (1995). Shc isoform-specific tyrosine phosphorylation by the insulin and epidermal growth factor receptors. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.270.35.20737
Oshikawa, J., Kim, S.-J., Furuta, E., Caliceti, C., Chen, G.-F., McKinney, R. D., … Ushio-Fukai, M. (2012). Novel role of p66Shc in ROS-dependent VEGF signaling and angiogenesis in endothelial cells. *American Journal of Physiology-Heart and Circulatory Physiology*, *302*(3), H724–H732. https://doi.org/10.1152/ajpheart.00739.2011
Paoli, P., Giannoni, E., & Chiarugi, P. (2013). Anoikis molecular pathways and its role in cancer progression. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1833*(12), 3481–3498. https://doi.org/10.1016/j.bbamcr.2013.06.026
Patsialou, A., Bravo-Cordero, J. J., Wang, Y., Entenberg, D., Liu, H., Clarke, M., & Condeelis, J. S. (2013). Intravital multiphoton imaging reveals multicellular streaming as a crucial component of in vivo cell migration in human breast tumors. *IntraVital*, *2*(2), e25294. https://doi.org/10.4161/intv.25294
Pawson, T. (1997). Signaling Through Scaffold, Anchoring, and Adaptor Proteins. *Science*, *278*(5346), 2075–2080. https://doi.org/10.1126/science.278.5346.2075
Pawson, T. (2002). Regulation and targets of receptor tyrosine kinases. *European Journal of Cancer*, *38*, S3–S10. https://doi.org/10.1016/S0959-8049(02)80597-4
Pawson, T., & Kofler, M. (2009). Kinome signaling through regulated protein-protein interactions in normal and cancer cells. *Current Opinion in Cell Biology*, *21*(2), 147–153. https://doi.org/10.1016/j.ceb.2009.02.005
Pelicano, H., Carney, D., & Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates*, *7*(2), 97–110. https://doi.org/10.1016/j.drup.2004.01.004
Pelicci, G., Dente, L., De Giuseppe, A., Verducci-Galletti, B., Giuli, S., Mele, S., … Pelicci, P. G. (1996). A family of Shc related proteins with conserved PTB, CH1 and SH2 regions. *Oncogene*, *13*(3), 633–641.
Pelicci, G., Giordano, S., Zhen, Z., Salcini, A. E., Lanfrancone, L., Bardelli, A., … Pelicci, P. (1995). *The motogenic and mitogenic responses to HGF are amplified by the SHC adaptor protein*. *Oncogene* (Vol. 10).
Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., … Giuseppe Pelicci, P. (1992). A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*, *70*(1), 93–104. https://doi.org/https://doi.org/10.1016/0092-8674(92)90536-L
Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., … Pelicci, P. (1992). A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*, *70*, 93–104.
Perou, C. M. C. M., Sørile, T., Eisen, M. B. M. B., van de Rijn, M., Jeffrey, S. S. S., Ress, C. A.

C. A., … Botstein, D. (2000). Molecular portraits of human breast tumours. *Nature*, *406*(6797), 747–752. https://doi.org/10.1038/35021093

Ponzo, M. G., Lesurf, R., Petkiewicz, S., O'Malley, F. P., Pinnaduwage, D., Andrulis, I. L., … Park, M. (2009). Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(31), 12903–12908. https://doi.org/10.1073/pnas.0810402106

Porporato, P. E., Payen, V. L., Pérez-Escuredo, J., De Saedeleer, C. J., Danhier, P., Copetti, T., … Sonveaux, P. (2014). A mitochondrial switch promotes tumor metastasis. *Cell Reports*, *8*(3), 754–766. https://doi.org/10.1016/j.celrep.2014.06.043

Prat, A. (2011). Molecular Characterization of Basal-LikeandNon-Basal-Like Triple-Negative Breast Cancer. *New York*, 36–45. https://doi.org/10.1634/theoncolo-gist.2011-0022

Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., … Perou, C. M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Research*, *12*(5). https://doi.org/10.1186/bcr2635

Prat, A., & Perou, C. M. (2011). Deconstructing the molecular portraits of breast cancer. *Molecular Oncology*, *5*(1), 5–23. https://doi.org/10.1016/j.molonc.2010.11.003

Rameh, L. E., Arvidsson, A., Iii, K. L. C., Anthony, D., Rathbun, G., Crompton, A., … Burakoff, S. J. (1997). CELL BIOLOGY AND METABOLISM : A Comparative Analysis of the Phosphoinositide Binding Specificity of Pleckstrin Homology Domains A Comparative Analysis of the Phosphoinositide Binding Specificity of Pleckstrin Homology Domains *, *272*(35), 22059–22066.

Ravichandran, K. S., Lee, K. K., Songyang, Z., Cantley, L. C., Burn, P., & Burakoff, S. J. (1993). Interaction of Shc with the zeta chain of the T cell receptor upon T cell activation. *Science*, *262*(5135), 902 LP-905. Retrieved from http://science.sciencemag.org/content/262/5135/902.abstract

Ravichandran, K. S., Zhou, M. M., Pratt, J. C., Harlan, J. E., Walk, S. F., Fesik, S. W., & Burakoff, S. J. (1997). Evidence for a requirement for both phospholipid and phosphotyrosine binding via the Shc phosphotyrosine-binding domain in vivo. *Mol Cell Biol*, *17*(9), 5540–5549. https://doi.org/10.1128/MCB.17.9.5540

Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling*, *24*(5), 981–990. https://doi.org/10.1016/j.cellsig.2012.01.008

Rayavarapu, R. R., Heiden, B., Pagani, N., Shaw, M. M., Shuff, S., Zhang, S., & Schafer, Z. T. (2015). The role of multicellular aggregation in the survival of ErbB2-positive breast cancer cells during extracellular matrix detachment. *Journal of Biological Chemistry*, *290*(14), 8722–8733. https://doi.org/10.1074/jbc.M114.612754

Rhee, S. G., Kang, S. W., Tekle, E., Baines, I. C., Seo, M. S., Chock, P. B., & Bae, Y. S. (1997). Epidermal Growth Factor (EGF)-induced Generation of Hydrogen Peroxide. *Journal of Biological Chemistry*, *272*(1), 217–221. https://doi.org/10.1074/jbc.272.1.217

Richardson, B. E., & Lehmann, R. (2010). Mechanisms guiding primordial germ cell migration: Strategies from different organisms. *Nature Reviews Molecular Cell Biology*, *11*(1), 37–49. https://doi.org/10.1038/nrm2815

Runas, K. A., & Malmstadt, N. (2015). Low levels of lipid oxidation radically increase the passive permeability of lipid bilayers. *Soft Matter*, *11*(3), 499–505. https://doi.org/10.1039/c4sm01478b

Sakai, T., Toguchida, J., Ohtani, N., Yandell, D. W., Rapaport, J. M., & Dryja, T. P. (1991). Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *American Journal of Human Genetics*, *48*(5), 880–888. https://doi.org/10.1007/BF02890404

Satelli, A., Batth, I., Brownlee, Z., Mitra, A., Zhou, S., Noh, H., … Li, S. (2017). EMT circulating tumor cells detected by cell-surface vimentin are associated with prostate cancer progression. *Oncotarget*, *8*(30), 49329–49337. https://doi.org/10.18632/oncotarget.17632

Saucier, C., Khoury, H., Lai, K.-M. V., Peschard, P., Dankort, D., Naujokas, M. A., … Park, M. (2004). The Shc adaptor protein is critical for VEGF induction by Met/HGF and ErbB2 receptors and for early onset of tumor angiogenesis. *Proceedings of the National Academy of Sciences*, *101*(8), 2345–2350. https://doi.org/10.1073/pnas.0308065101

Savagner, P., Yamada, K. M., & Thiery, J. P. (1997). The Zinc-Finger Protein Slug Causes DesmosomeDissociation, an Initial and Necessary Step forGrowth Factor–induced Epithelial–Mesenchymal Transition, *137*(6), 1–17. https://doi.org/10.1083/jcb.137.6.1403

Schafer, Z. T., Grassian, A. R., Song, L., Jiang, Z., Gerhart-Hines, Z., Irie, H. Y., … Brugge, J. S. (2009). Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature*, *461*(7260), 109–113. https://doi.org/10.1038/nature08268

Seftor, E. A., Meltzer, P. S., Kirschmann, D. A., Margaryan, N. V., Seftor, R. E. B., & Hendrix, M. J. C. (2006). The epigenetic reprogramming of poorly aggressive melanoma cells by a metastatic microenvironment. *Journal of Cellular and Molecular Medicine*, *10*(1), 174–196. https://doi.org/10.1111/j.1582-4934.2006.tb00299.x

Shachar, S. S., Hurria, A., & Muss, H. B. (2016). Breast Cancer in Women Older Than 80 Years. *Journal of Oncology Practice*, *12*(2), 123–132. https://doi.org/10.1200/JOP.2015.010207

Shannon, S. (1997). Relationship between Vitamin and Calcium Colon Cancer ' Supplement Use and, *6*(October), 769–774.

Sharma, S., Kelly, T. K., & Jones, P. A. (2009). Epigenetics in cancer. *Carcinogenesis*, *31*(1), 27–36. https://doi.org/10.1093/carcin/bgp220

Shehata, M., Teschendorff, A., Sharp, G., Novcic, N., Russell, I. A., Avril, S., … Watson, C. J. (2012). Phenotypic and functional characterisation of the luminal cell hierarchy of the mammary gland. *Breast Cancer Research*, *14*(5), 1–19. https://doi.org/10.1186/bcr3334

Siegel, P. M., Ryan, E. D., Cardiff, R. D., & Muller, W. J. (1999). Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *The EMBO Journal*, *18*(8), 2149–2164. https://doi.org/10.1093/emboj/18.8.2149

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., & Mcguire, W. L. (1987a). Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., & Mcguire, W. L. (1987b). Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2lneu Oncogene. *Science*, *235*(4785), 177–182.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keth, D. E., … Press, M. F. (1989). Studies of the HER-2/neu Proto-oncogene in Human Breast and Ovarian Cancer.

Smith D.C, Simon M., Alldredge A.L., A. F. (1992). © 19 9 2 Nature Publishing Group. *Letters to Nature*, *359*, 139–141.

Smith, L., Bryan, S., De, P., Rahal, R., Shaw, A., Turner, D., … Dixon, M. (2018). *Canadian Cancer Statistics 2018*. https://doi.org/2018

Soler, C., Alvarez, C. V, Beguinot, L., & Carpenter, G. (1994). Potent SHC tyrosine

phosphorylation by epidermal growth factor at low receptor density or in the absence of receptor autophosphorylation sites. *Oncogene*, *9*(8), 2207–2215.
Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., … Borresen-Dale, A.-L. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences*, *98*(19), 10869–10874. https://doi.org/10.1073/pnas.191367098
Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., … Botstein, D. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences*, *100*(14), 8418–8423. https://doi.org/10.1073/pnas.0932692100
Stacker, S. A., Caesar, C., Baldwin, M. E., Thornton, G. E., Williams, R. A., Prevo, R., … Achen, M. G. (2001). VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nature Medicine*, *7*(2), 186–191. https://doi.org/10.1038/84635
Stefanska, B., Cheishvili, D., Suderman, M., Arakelian, A., Huang, J., Hallett, M., … Szyf, M. (2014). Genome-wide study of hypomethylated and induced genes in patients with liver cancer unravels novel anticancer targets. *Clinical Cancer Research*, *20*(12), 3118–3132. https://doi.org/10.1158/1078-0432.CCR-13-0283
Steinert, G., Schölch, S., Niemietz, T., Iwata, N., García, S. A., Behrens, B., … Koch, M. (2014). Immune escape and survival mechanisms in circulating tumor cells of colorectal cancer. *Cancer Research*, *74*(6), 1694–1704. https://doi.org/10.1158/0008-5472.CAN-13-1885
Stevenson, L. E., & Frackelton, A. R. (1998). Constitutively tyrosine phosphorylated p52 Shc in breast cancer cells: correlation with ErbB2 and p66 Shc expression. *Breast Cancer Research and Treatment*, *49*(2), 119–128. https://doi.org/10.1023/A:1006007227747
Suh, Y. A., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., … Lambeth, J. D. (1999). Cell transformation by the superoxide-generating oxidase Mox1. *Nature*, *401*(6748), 79–82. https://doi.org/10.1038/43459
Szatrowski, T. P., & Nathan, C. F. (1991). Production of Large Amounts of Hydrogen Peroxide by Human Tumor Cells. *Cancer Research*, *51*(3), 794 LP-798. Retrieved from http://cancerres.aacrjournals.org/content/51/3/794.abstract
Tabariès, S., Ouellet, V., Hsu, B. E., Annis, M. G., Rose, A. A. N., Meunier, L., … Siegel, P. M. (2015). Granulocytic immune infiltrates are essential for the efficient formation of breast cancer liver metastases. *Breast Cancer Research*, *17*(1), 1–18. https://doi.org/10.1186/s13058-015-0558-3
Tanno, S., Mitsuuchi, Y., & Altomare, D. (2001). AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells. *Cancer Research*, 589–593. Retrieved from http://cancerres.aacrjournals.org/content/61/2/589.short
Taube, J. H., Herschkowitz, J. I., Komurov, K., Zhou, A. Y., Gupta, S., Yang, J., … Mani, S. A. (2010). Erratum: Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudinlow and metaplastic breast cancer subtypes (Proceedings of the National Academy of Sciences of the United States of America (2010) 107:35 (1. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(44), 19132. https://doi.org/10.1073/pnas.1015095107
Taylor-Papadimitriou, J., Stampfer, M., Bartek, J., Lewis, a, Boshell, M., Lane, E. B., & Leigh, I. M. (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium. *Journal of*

Cell Science, *94 (Pt 3)*, 403–413. https://doi.org/12629510
Taylor, I. W., Linding, R., Warde-farley, D., Liu, Y., Pesquita, C., Faria, D., … Wrana, J. L. (2009). Dynamic modularity in protein interaction networks predicts breast cancer outcome, *27*(2), 199–204. https://doi.org/10.1038/nbt.1522
Tolaney, S. M., Tan, S., Guo, H., Barry, W., Van Allen, E., Wagle, N., … Krop, I. E. (2015). Phase II study of tivantinib (ARQ 197) in patients with metastatic triple-negative breast cancer. *Investigational New Drugs*, *33*(5), 1108–1114. https://doi.org/10.1007/s10637-015-0269-8
Tomilov, A. A., Bicocca, V., Schoenfeld, R. A., Giorgio, M., Migliaccio, E., Ramsey, J. J., … Cortopassi, G. A. (2010). Decreased superoxide production in macrophages of long-lived p66Shc knock-out mice. *Journal of Biological Chemistry*, *285*(2), 1153–1165. https://doi.org/10.1074/jbc.M109.017491
Ursini-Siegel, J., Cory, S., Zuo, D., Hardy, W. R., Rexhepaj, E., Lam, S., … Muller, W. J. (2010). Receptor tyrosine kinase signaling favors a protumorigenic state in breast cancer cells by inhibiting the adaptive immune response. *Cancer Research*, *70*(20), 7776–7787. https://doi.org/10.1158/0008-5472.CAN-10-2229
Ursini-Siegel, J., Hardy, W. R., Zheng, Y., Ling, C., Zuo, D., Zhang, C., … Muller, W. J. (2012). The ShcA SH2 domain engages a 14-3-3/PI3′K signaling complex and promotes breast cancer cell survival. *Oncogene*, *31*(48), 5038–5044. https://doi.org/10.1038/onc.2012.4
Ursini-Siegel, J., Hardy, W. R., Zuo, D., Lam, S. H. L., Sanguin-Gendreau, V., Cardiff, R. D., … Muller, W. J. (2008). ShcA signalling is essential for tumour progression in mouse models of human breast cancer. *The EMBO Journal*, *27*(6), 910–920. https://doi.org/10.1038/emboj.2008.22
Ursini-Siegel, J., & Muller, W. J. (2008). The ShcA adaptor protein is a critical regulator of breast cancer progression. *Cell Cycle*, *7*(13), 1936–1943. https://doi.org/10.4161/cc.7.13.6205
van ’t Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A. M., Mao, M., … Friend, S. H. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, *415*(6871), 530–536. https://doi.org/10.1038/415530a
van der Geer, P., Wiley, S., Gish, G. D., Lai, V. K., Stephens, R., White, M. F., … Pawson, T. (1996). Identification of residues that control specific binding of the Shc phosphotyrosine-binding domain to phosphotyrosine sites. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(3), 963–968. https://doi.org/10.1073/pnas.93.3.963
van der Geer, P., Wiley, S., Ka-Man Lai, V., Olivier, J. P., Gish, G. D., Stephens, R., … Pawson, T. (1995). A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides. *Current Biology*, *5*(4), 404–412. https://doi.org/https://doi.org/10.1016/S0960-9822(95)00081-9
Van Zijl, F., Krupitza, G., & Mikulits, W. (2011). Initial steps of metastasis: Cell invasion and endothelial transmigration. *Mutation Research - Reviews in Mutation Research*, *728*(1–2), 23–34. https://doi.org/10.1016/j.mrrev.2011.05.002
Ventura, A. (2002). The p66Shc Longevity Gene Is Silenced through Epigenetic Modifications of an Alternative Promoter. *Journal of Biological Chemistry*, *277*(25), 22370–22376. https://doi.org/10.1074/jbc.M200280200
Visvader, J. E. (2009). Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes and Development*, *23*(22), 2563–2577. https://doi.org/10.1101/gad.1849509

Wagner, J. P., Wolf-yadlin, A., & Macbeath, G. (2013). Receptor Tyrosine Kinases Fall into Distinct Classes Based on Their Inferred Signaling Networks RESULTS, *6*(284).

Wahl, G. M., & Spike, B. T. (2017). Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity. *Npj Breast Cancer*, *3*(1), 1–12. https://doi.org/10.1038/s41523-017-0012-z

Wallasch, C., Weiss, F. U., Niederfellner, G., Jallal, B., Issing, W., & Ullrich, A. (1995). Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *The EMBO Journal*, *14*(17), 4267–4275. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7556068%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC394510

Wary, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., & Giancotti, F. G. (1996). The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell*, *87*(4), 733–743. https://doi.org/10.1016/S0092-8674(00)81392-6

Webster, M. a, Hutchinson, J. N., Rauh, M. J., Muthuswamy, S. K., Anton, M., Tortorice, C. G., … Muller, W. J. (1998). Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. *Molecular and Cellular Biology*, *18*(4), 2344–2359. https://doi.org/10.1128/MCB.18.4.2344

Weidner, N., Semple, J. P., Welch, W. R., & Folkman, J. (1991). Tumor Angiogenesis and Metastasis — Correlation in Invasive Breast Carcinoma. *New England Journal of Medicine*, *324*(1), 1–8. https://doi.org/10.1056/NEJM199101033240101

Weinberg, F., Hamanaka, R., Wheaton, W. W., Weinberg, S., Joseph, J., Lopez, M., … Chandel, N. S. (2010). Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proceedings of the National Academy of Sciences*, *107*(19), 8788–8793. https://doi.org/10.1073/pnas.1003428107

Wieduwilt, M. J., & Moasser, M. M. (2008). The epidermal growth factor receptor family: Biology driving targeted therapeutics. *Cellular and Molecular Life Sciences*, *65*(10), 1566–1584. https://doi.org/10.1007/s00018-008-7440-8

Wills, M. K. B., & Jones, N. (2012). Teaching an old dogma new tricks: twenty years of Shc adaptor signalling. *Biochemical Journal*, *447*(1), 1–16. https://doi.org/10.1042/BJ20120769

Witcher, M., & Emerson, B. M. (2009). Epigenetic Silencing of the p16INK4a Tumor Suppressor Is Associated with Loss of CTCF Binding and a Chromatin Boundary. *Molecular Cell*, *34*(3), 271–284. https://doi.org/10.1016/j.molcel.2009.04.001

Wood, Z. A., Poole, L. B., & Karplus, P. A. (2003). Peroxiredoxin Evolution and the Signaling. *Science*, *300*(April), 6506–6553. https://doi.org/10.1126/science.1080405

Wyckoff, J. B., Jones, J. G., Condeelis, J. S., & Segall, J. E. (2000). A Critical Step in Metastasis : In Vivo Analysis of Intravasation at the Primary Tumor A Critical Step in Metastasis : In Vivo Analysis of Intravasation at the, 2504–2511.

Xi, G., Shen, X., & Clemmons, D. R. (2008). p66shc Negatively Regulates Insulin-Like Growth Factor I Signal Transduction via Inhibition of p52shc Binding to Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase Substrate-1 Leading to Impaired Growth Factor Receptor-Bound Protein-2 Membrane . *Molecular Endocrinology*, *22*(9), 2162–2175. https://doi.org/10.1210/me.2008-0079

Youngs, S. J., Ali, S. A., Taub, D. D., & Rees, R. C. (1997). Chemokines induce migrational responses in human breast carcinoma cell lines. *International Journal of Cancer*, *71*(2), 257–266. https://doi.org/10.1002/(SICI)1097-0215(19970410)71:2<257::AID-IJC22>3.0.CO;2-D

Zavadil, J., Cermak, L., Soto-Nieves, N., & Böttinger, E. P. (2004). Integration of TGF-β/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *EMBO Journal*, *23*(5), 1155–1165. https://doi.org/10.1038/sj.emboj.7600069

Zheng, H., Takahashi, H., Murai, Y., Cui, Z., Nomoto, K., Niwa, H., … Takano, Y. (2006). Expressions of MMP-2, MMP-9 and VEGF are closely linked to growth, invasion, metastasis and angiogenesis of gastric carcinoma. *Anticancer Research*, *26*(5 A), 3579–3583.

Zhong, H., & Yin, H. (2015). Role of lipid peroxidation derived 4-hydroxynonenal (4-HNE) in cancer: Focusing on mitochondria. *Redox Biology*, *4*, 193–199. https://doi.org/10.1016/j.redox.2014.12.011

Zhou, M. M., Ravichandran, K. S., Olejniczak, E. T., Petros, A. M., Meadows, R. P., Sattler, M., … Fesik, S. W. (1995). Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature*, *378*(6557), 584–592. https://doi.org/10.1038/378584a0

Zhou, S., Chen, H. Z., Wan, Y. Z., Zhang, Q. J., Wei, Y. S., Huang, S., … Liang, C. C. (2011). Repression of P66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction. *Circulation Research*, *109*(6), 639–648. https://doi.org/10.1161/CIRCRESAHA.111.243592

Chapter 2 - p66ShcA promotes breast cancer plasticity by inducing an epithelial-to-mesenchymal transition

Jesse Hudson,[a] Jacqueline R. Ha,[a] Valerie Sabourin,[a] Ryuhjin Ahn,[a] Rachel La Selva,[a] Julie Livingstone,[b] Lauren Podmore,[a] Jennifer Knight,[b] Laura Forrest,[a,b] Nicole Beauchemin,[b,c,d] Michael Hallett,[b,d] Morag Park,[b,c,d] Josie Ursini-Siegel[a,c]

Lady Davis Institute for Medical Research, Montreal, Quebec, Canada[a];

Goodman Cancer Research Centre, Montreal, Quebec, Canada[b] ;

Department of Oncology, McGill University, Montreal, Quebec, Canada[c];

Department of Biochemistry, McGill University, Montreal, Quebec, Canada[d]

2.1 Preface

This chapter explores the concept of tumor plasticity or reversible programming of cell state through induction of EMT in breast cancer. Breast cancers are a heterogenous mixture of genetically distinct tumor cells and adjacent stromal cells that make up the tumor microenvironment. Combined, tumor heterogeneity and tumor plasticity influence progression and therapeutic responsiveness. This study outlines p66ShcA dependent mechanisms that contribute to these aspects both within and across molecular subtypes of breast cancer. In addition, prior to this study, the role of p66ShcA in cancer was conflicting with some studies linking p66ShcA to pro-tumorigenic functions and reduced survival, while others suggesting p66ShcA was anti-tumorigenic and correlated with improved patient outcome. Moreover, p66ShcA to date, has been best characterized as a redox protein that contributes to aging, cardiovascular disease, cancer and other chronic diseases. Notably, we were the first to examine the *in vivo* role of p66ShcA in breast cancer. This work outlines p66ShcA as a novel inducer of an EMT through Met receptor tyrosine kinase signalling, a promoter of cell plasticity in ErbB2+ luminal breast cancer, a biomarker of mesenchymal tumors across molecular subtypes and a suppressor of primary tumor growth all of which correlate with serine36 phosphorylation and elevated ROS formation *in vitro* and *in vivo*.

2.2 Abstract

Breast cancers are stratified into distinct subtypes, which influence therapeutic responsiveness and patient outcome. Patients with luminal breast cancers are often associated with a better prognosis relative to that with other subtypes. However, subsets of patients with luminal disease remain at increased risk of cancer-related death. A critical process that increases the malignant potential of breast cancers is the epithelial-to-mesenchymal transition (EMT). The p66ShcA adaptor protein stimulates the formation of reactive oxygen species in response to stress stimuli. In this paper, we report a novel role for p66ShcA in inducing an EMT in HER2+ luminal breast cancers. p66ShcA increases the migratory properties of breast cancer cells and enhances signaling downstream of the Met receptor tyrosine kinase in these tumors. Moreover, Met activation is required for a p66ShcA-induced EMT in luminal breast cancer cells. Finally, elevated p66ShcA levels are associated with the acquisition of an EMT in primary breast cancers spanning all molecular subtypes, including luminal tumors. This is of high clinical relevance, as the luminal and HER2 subtypes together comprise 80% of all newly diagnosed breast cancers. This study identifies p66ShcA as one of the first prognostic biomarkers for the identification of more aggressive tumors with mesenchymal properties, regardless of molecular subtype.

2.3 Introduction

Breast cancer is classified into distinct molecular subtypes, which include basal and claudin-low (both typically ER-PR-HER2-), luminal A/B (ER+), and HER2+ cancers (1, 2). Luminal A tumors are generally associated with a more favorable outcome, while luminal B, HER2+, basal, and claudin-low tumors predict a worse prognosis. Breast cancers are often classified based on expression of markers that define distinct cell types within a mammary duct. Luminal epithelial cells express cytokeratin 8/18 (CK8/18), along with adherens (E-cadherin) and tight junctional (ZO-1 and claudin) proteins. Myoepithelial cells, which are cytokeratin 14 and smooth muscle actin (SMA) positive, provide structural support to the luminal epithelial layer. Luminal and HER2+ breast cancers retain CK8/18 and E-cadherin expression (3), while basal breast cancers often coexpress CK14 and SMA (4). Molecular profiling studies have shown that triple-negative breast cancers stratify into basal and claudin-low subtypes. This is also reflected in cultivated breast cancer cell lines, which cluster into basal A (basal) and basal B (claudin-low) subgroups by gene expression profiling (5). Basal A tumors coexpress luminal (CK8/18) and myoepithelial (CK14 and SMA) markers. Basal B tumors, also referred to as claudin-low, uniformly lack luminal epithelial markers but express mesenchymal markers that are indicative of an epithelial-to-mesenchymal transition (EMT) (6, 7).

During an EMT, epithelial cells acquire mesenchymal properties, including loss of cell polarity and cell-cell contacts, which augment their migratory properties (8). The EMT process is governed by a network of transcription factors, including Snail1/2, Zeb1/2, and Twist1/2, which coordinately repress E-cadherin and increase the expression of mesenchymal markers to impart a more spindle-like and migratory phenotype. Elevated levels of these transcription factors are associated with increased recurrence and poor disease-free and overall survival in breast cancer

patients (8). Claudin-low tumors, representing 5 to 8% of all breast cancers, have undergone a full EMT (6, 7). However, several studies have unequivocally demonstrated that some breast cancers express EMT-like genes (vimentin, N-cadherin, Snai1/2, Zeb1/2, and Twist1/2 genes) irrespective of E-cadherin levels (9–11). This suggests that many breast cancers can acquire mesenchymal characteristics without the obligate loss of epithelial features. This partial transdifferentiation of breast tumors within other molecular subtypes increases their plasticity by promoting many prometastatic properties that are associated with an EMT (8).

The ShcA gene encodes three proteins that are produced through differential promoter usage (p66) or alternate translation initiation (p46 and p52) (12, 13). While the p46/52ShcA isoforms are ubiquitously expressed, p66ShcA levels are highly variable in cancer cells (14). The p46/52ShcA isoforms transduce mitogenic signals by recruiting Grb2/SOS and Grb2/Gab complexes to activate the extracellular signal-regulated kinase (ERK) and AKT pathways, respectively (15, 16). Paradoxically, while p66ShcA is tyrosine phosphorylated and binds Grb2, it neither has transforming properties nor activates ERK (12). p66ShcA contains a unique N-terminal domain, including a serine residue (S36) that is phosphorylated in response to stress stimuli (17, 18). Binding of Pin1 to pSer36 of p66ShcA induces its translocation into the inner mitochondrial matrix (19, 20), where p66ShcA promotes the formation of reactive oxygen species (ROS) (21). While it is well established that the p46/52ShcA isoforms are critical for breast cancer progression (22–25), the biological significance of p66ShcA during this process is poorly understood. We provide the first experimental evidence that p66ShcA is a major driver of breast cancer plasticity, both in vitro and in vivo, by inducing an epithelial-to-mesenchymal transition.

2.4 Material and Methods

Cell lines. MDA-MB-231 and BT474 cells were obtained from the ATCC. MDA-MB-231 cells were cultured in 10% fetal bovine serum (FBS)–Dulbecco modified Eagle medium (DMEM), while BT474 cells were cultured in 10% FBS–RPMI medium. The NIC tumor cell line was established from a mouse mammary tumor virus (MMTV)/Neu-internal ribosome entry site (IRES)-Cre (NIC) transgenic mouse mammary tumor (25) and maintained in 5% FBS–DMEM supplemented with mammary epithelial growth supplement (Invitrogen). NIC cells were cultured in 10% FBS–DMEM for 3 to 4 days prior to experimental analysis. A C-terminal, FLAG-tagged mouse p66ShcA cDNA was subcloned into the XhoI/EcoRI sites of pMSCV-puro (Clontech) or the NotI/EcoRI sites of pQXCIB-blast (Addgene) and used for the generation of stable cell lines. MDA-MB-231 and NIC cells were transfected with the pMSCV-puro vector and maintained in 2 ug/ml of puromycin, while the BT474 cells were transfected with the pQXCIB/p66ShcA vector and maintained in 10 ug/ml of blasticidin. Parental BT474 cells were employed as the negative control.

Small interfering RNA (siRNA) studies. Cells were transfected with a pool of three DICER substrate duplex siRNAs targeting mouse Met or with a universal scrambled control (Origene) as described previously (22). Cells were also cultured in the presence of 1uM crizotinib (LC Laboratories) or an equivalent volume of dimethyl sulfoxide (DMSO) for 4 days prior to cell lysis. The medium was replenished, and fresh inhibitor was added on the second day.

In vivo studies. Tumor cells (1 x 10^6) were injected into the fourth mammary gland of 6- to 8-week-old female SCID-beige mice (Taconic). Following the first palpation, tumor volumes were measured on a biweekly basis, as described previously (25). Animal studies were approved by the

Animal Resources Centre at McGill University and complied with guidelines set by the Canadian Council of Animal Care. Immunoblotting, ELISA, and qRT-PCR.

Immunoprecipitation experiments. 200ug quantities of cytoplasmic extracts were immunoprecipitated with FLAG-specific antibodies (Sigma).

Immunoblotting. Immunoblots were performed as described previously (25) using the antibodies listed in Table S1 in the supplemental material.

ELISA. Hepatocyte growth factor (HGF) enzyme-linked immunosorbent assay (ELISA) (R&D) was performed on 50ug of whole-cell lysate.

RT-qPCR. For the reverse transcription-quantitative PCR (RT-qPCR) studies, total RNA was isolated using RNeasy midi-kits (Qiagen) and cDNA was generated using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using the QuantiTect SYBR green RT-PCR kit (Qiagen) with primers listed in Table S2 in the supplemental material. CSF1, CCL2, CCL5, MMP9, and tumor necrosis factor alpha (TNF-alpha) mRNA levels were measured using TaqMan probes (Invitrogen). Semiquantitative analysis of immunoblots was conducted using ImageJ software.

Immunohistochemistry. Immunohistochemical and immunohistofluorescent staining of paraffin- and OCT-embedded sections was performed as described previously (25). The antibodies are listed in Table S1 in the supplemental material. Quantification of stained sections was performed using Aperio Imagescope software. For the immunohistofluorescent images, the percent green and percent red pixels were quantified using ImageJ software. For ex vivo dihydroethidium (DHE) staining, OCT-embedded sections were incubated with 2uM DHE (Invitrogen) and quantified by ImageJ software. Boyden chamber assays and scratch assays.

Cell migration and invasion assays. Boyden chamber assays were performed as described previously (26) using the following numbers of cells: NIC cells, 200,000; BT474 cells, 125,000; and MDA-MB-231 cells, 100,000. For the scratch assays, cells were allowed to reach a monolayer prior to initiation of the experiment. Wound closure was monitored for specific specific time periods, and images were captured using a bright-field microscope.

Bioinformatics. Samples from the McGill Genome Quebec data set (n = 84) with matching RT-qPCR-derived p66ShcA expression values were used. Samples were hybridized on GeneChip Human Gene 1.0 ST arrays (Affymetrix) and normalized in R using the bioconductor RMA (27) and hugene10stv1cdf annotation packages. All patients were assigned a genomic subtype based on genes in the PAM50 centroids (28). We also employed publicly available level 1 Illumina HiSeq RNA sequencing data from breast invasive carcinoma (TCGA) (29). Relative p66ShcA levels were measured based on the read depth in the p66ShcA specific region (chr1, 154942676 to 154943043). Rsamtools was used to index downloaded bam files, extract reads, and calculate read depth coverage (http://bioconductor.org/packages/release/bioc/html/Rsamtools.html). Tumors were annotated by their PAM50 subtype. Expression of EMT genes was investigated in level 3 TCGA microarray data where matching transcriptome sequencing (RNA-seq) data were available (n = 660).

Statistical analysis. All statistical analysis was performed using a two-tailed Student t test with the exception the data shown in Fig. 7F and G, which were evaluated using single-factor analysis of variance (ANOVA), and Fig. 8A and G, which were determined using Fisher's exact probability test (2 X 2 contingency).

2.5 Results

p66ShcA is enriched in claudin-low breast cancer cell lines

We sought to interrogate how p66ShcA expression is regulated in hu- man breast cancer cell lines that resemble the luminal or basal subtypes (5). While p46/52ShcA is ubiquitously expressed, p66ShcA is absent or weakly expressed in all luminal breast cancer cell lines examined (Fig. 1A). Indeed, p66ShcA is also weakly ex- pressed in normal mouse mammary gland tissue (Fig. 1B). In contrast, p66ShcA expression is aberrantly elevated in many hu- man basal breast cancer cell lines and is particularly enriched within the claudin-low subtype (basal A, 2/5; basal B, 4/5) (Fig. 1A). We also examined the relationship between p66ShcA levels and expression of the ErbB2, epidermal growth factor receptor (EGFR), and MET receptor tyrosine kinases in this panel of hu- man breast cancer cell lines. We show that p66ShcA tends to be enriched in MET-positive cell lines and is excluded from ErbB2-expressing cell lines, with a few exceptions for each receptor tyrosine kinase. In contrast, we did not observe any association be- tween p66ShcA and EGFR levels in any of the breast cancer cell lines that we studied (Fig. 1A). We extended our observations and show that p66ShcA mRNA levels are also exceedingly low in luminal cell lines and highly enriched in p66ShcA-positive basal breast cancer cell lines (Fig. 1C). Using a publicly available data set (5), we show that p66ShcA protein levels are elevated in a larger panel of basal breast cancer cell lines in which the highest p66ShcA levels are observed in the claudin-low subgroup (Fig. 1D).

p66ShcA inhibits the growth of ErbB2+ luminal breast cancers in vivo

In general, p66ShcA is restricted from luminal breast cancer cell lines and enriched in those resembling the claudin-low subtype. Moreover, p66ShcA and ErbB2 levels are inversely

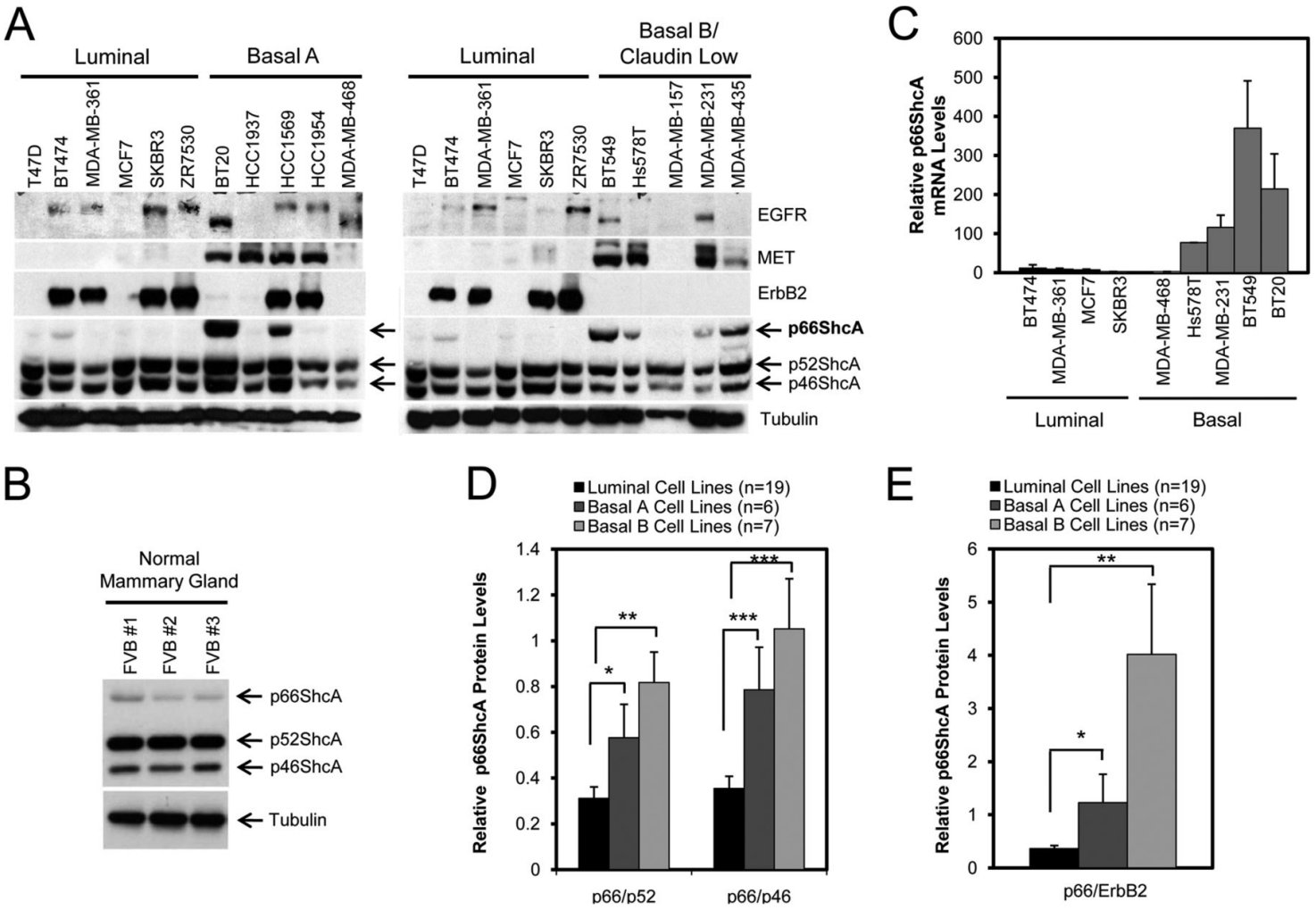


Figure 1: p66ShcA is enriched in basal breast cancer cell lines. (A) Immunoblot analysis of whole cell lysates using ShcA, MET, EGFR, ErbB2 and Tubulin specific antibodies. Cells are classified as luminal or basal (a/b) as described (5). **(B)** Immunoblot analysis of whole cell lysates generated from mammary glands of three FVB female mice using ShcA and Tubulin-specific antibodies. **(C)** Quantification of p66ShcA mRNA levels in the indicated cell lines by RT-qPCR analysis. The data is normalized to GAPDH levels and is representative of three replicates. **(D)** Semi-quantitative assessment of p66ShcA protein levels in breast cancer cells lines comprising the luminal, basal A and B subtypes. The data is obtained from densitometric analysis of published immunoblots (5) and is represented as the p66/p46 and 555 p66/52 ratio in luminal (n=19), basal a (n=6) and basal b (n=7) cell lines ± SEM (*p=0.036, 556 **p=0.034, ***p=0.002). **(E)** Semi-quantitative assessment of the p66/ErbB2 ratio ± SEM 557 (*p=0.001; **p<0.001).

correlated in several, but not all, of the luminal breast cancer cell lines examined (Fig. 1A and E). Therefore, we sought to define whether p66ShcA functionally alters ErbB2-driven mammary tumorigenesis. We ectopically expressed p66ShcA in a cell line derived from ErbB2- driven mammary tumors (NIC) (25), which retains a luminal phenotype (Fig. 2A) and expresses low endogenous p66ShcA levels (Fig. 2B). Unsupervised hierarchical clustering analysis previously revealed that MMTV/ErbB2 mouse mammary tumors most closely resemble human luminal breast cancer (30).

We also overexpressed p66ShcA in MDA-MB-231 breast cancer cells, which are claudin low and express moderate p66ShcA levels (Fig. 1A; see also Fig. S1A in the supplemental material). We confirmed that p66ShcA is overexpressed in NIC and MDA-MB-231 cells (Fig. 2B; see also Fig. S1B in the supplemental material) to levels that correspond to those observed in many basal breast cancer cell lines (Fig. 1A and D). Overexpression of p66ShcA moderately decreased the growth of NIC luminal tumors (Fig. 2C) but did not affect the growth of MDA-MB-231 claudin-low tumors in vivo (see Fig. S1C in the supplemental material). Despite this fact, both NIC/p66ShcA and MDA-MB-231/p66ShcA tumors displayed elevated ROS production relative to that of vector controls (VCs). These results suggest that claudin-low tumors, but not luminal tumors, can adapt to elevated ROS levels induced by p66ShcA (Fig. 2D; see also Fig. S1D). Consistently, NIC/p66ShcA tumors, but not MDA-MB-231/p66ShcA tumors, display increased p38 mitogen-activated protein kinase (MAPK) activation (Fig. 2E; see also Fig. S1E).

Moreover, the reduced growth potential of NIC/p66ShcA tumors is associated with impaired cell proliferation (Fig. 2F), while the proliferative indices of control and p66ShcA-overexpressing MDA-MB-231 tumors were comparable (see Fig. S1F). Interestingly, however, we also observe increased microvessel density in NIC/p66ShcA tumors but not in MDA-MB-231

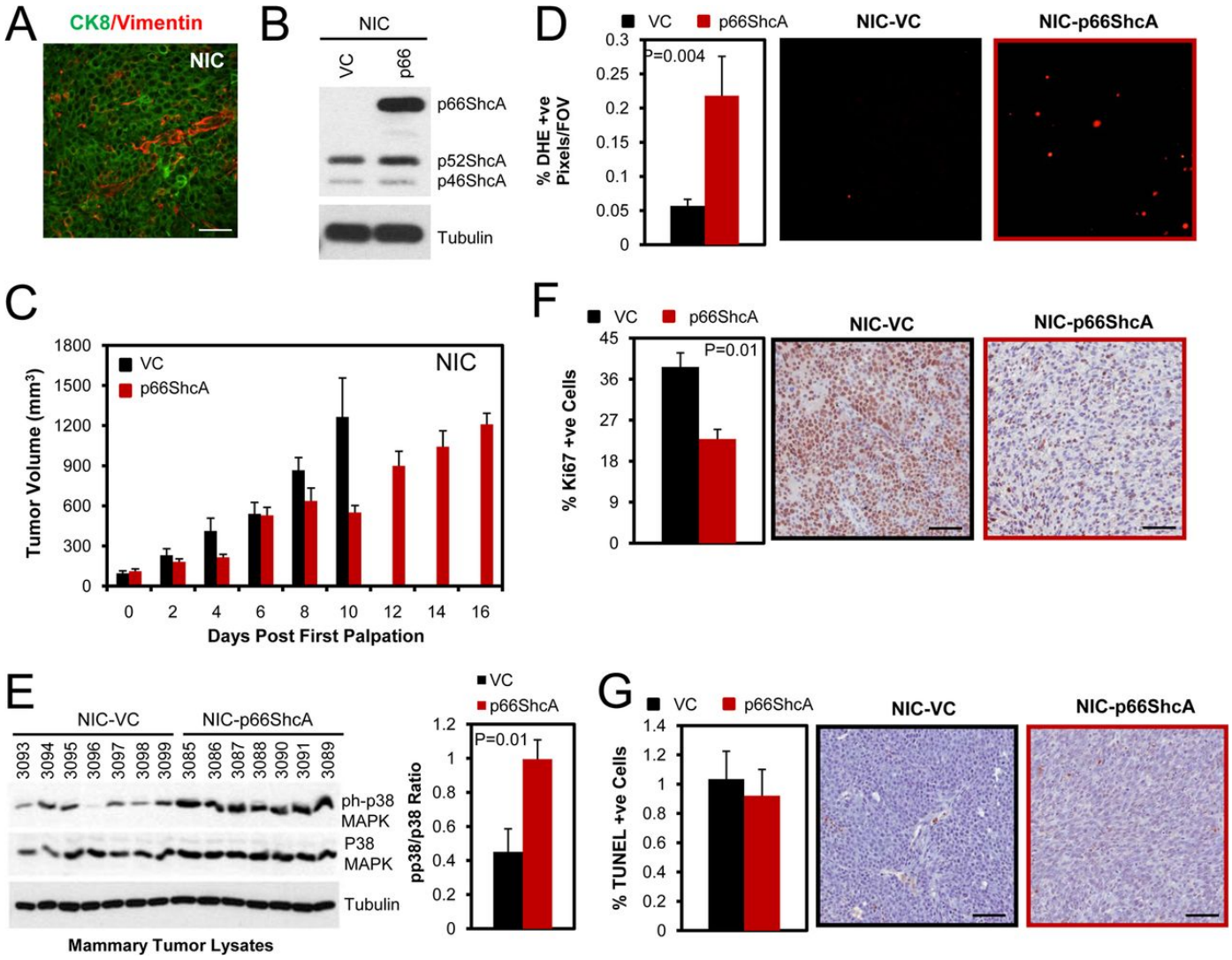
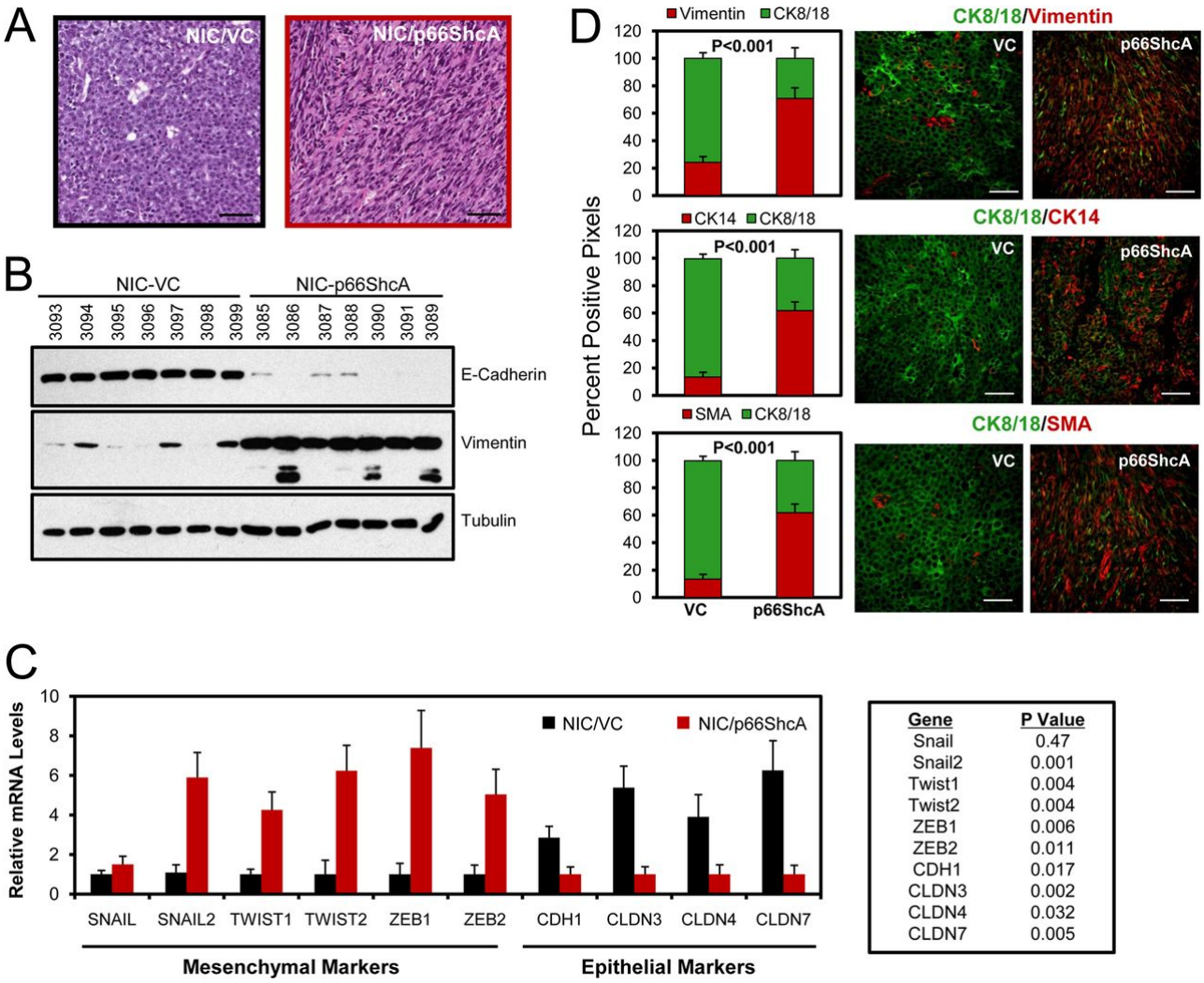


Figure 2: p66ShcA reduces the growth of ErbB2-positive luminal mammary tumors.
(A) Immunohistofluorescent staining of ErbB2-driven (NIC) tumors using Cytokeratin 8/18 (CK8) and Vimentin-specific antibodies. **(B)** Immunoblot of vector control (VC) and p66ShcA overexpressing NIC cell lysates using ShcA and Tubulin specific antibodies. **(C)** Mammary fat pad injection of NIC-VC and NIC-p66ShcA cells (1 x 10^6). The data is recorded as tumor volume (mm3) ± SEM and is representative of 7 mice each. **(D)** Percentage of dihydroethidium (DHE) positive cells present in cryosections from NIC-VC and NIC-p66ShcA mammary tumors. The data is representative of 40-46 fields (20X) and 6 tumors per cell line and is shown as % DHE positive cells/field of view ± SEM. **(E)** Mammary tumors were probed with phospho-p38MAPK, p38MAPK and Tubulin-specific antibodies. **(F)** Ki67 immunohistochemical staining of paraffin-embedded sections from NIC-VC and NIC-p66ShcA mammary tumors. The data is representative of 7 tumors each and is depicted as % Ki67 positive cells ± SEM. **(G)** TUNEL staining of paraffin-embedded sections from vector control and p66ShcA-expressing tumors. For each section, a minimum of 20,000 nuclei were counted using Image Scope software. The data is presented as the percentage TUNEL positive cells ± SEM (n=7 tumors each).

tumors relative to that in vector controls (see Fig. S2). Finally, p66ShcA overexpression does not significantly alter the apoptotic potential of NIC or MDA-MB-231tumors relative to that of their vector controls (Fig. 2G; see also Fig. S1G). These observations suggest that p66ShcA impairs the in vivo growth potential of ErbB2-driven luminal breast cancers but does not appreciably alter the growth of claudin-low tumors.

p66ShcA induces an EMT in ErbB2+ luminal breast cancers

Given that claudin-low tumors already possess mesenchymal features and endogenously express p66ShcA, we examined whether p66ShcA overexpression increases the mesenchymal properties of luminal breast tumors. Indeed, NIC/p66ShcA mammary tumors acquired a spindle-like morphology (Fig. 3A) coincident with exceedingly low E-cadherin and high vimentin levels relative to those in vector controls (Fig. 3B). Moreover, NIC/p66ShcA tumors significantly upregulate EMT-promoting transcription factors (Slug, Twist1/2, and Zeb1/2) and display reduced expression of genes encoding adherens and tight junction proteins (E-cadherin and claudin-3 [Cldn3], Cldn4, and Cldn7) (Fig. 3C). These data were confirmed by immunofluorescent staining, which revealed NIC/VC tumors uniformly retain expression of luminal epithelial markers (CK8/18) and display residual SMA and vimentin staining that is reflective of fibroblast/myofibroblast infiltration. While small subsets of NIC/p66ShcA tumor cells retain CK8/18 positivity, a majority of them have lost their luminal characteristics and acquire expression of basal/mesenchymal markers (SMA, vimentin, and CK14) (Fig. 3D). These data suggest that p66ShcA induces an EMT in ErbB2-driven luminal breast cancers.



Gene	P Value
Snail	0.47
Snail2	0.001
Twist1	0.004
Twist2	0.004
ZEB1	0.006
ZEB2	0.011
CDH1	0.017
CLDN3	0.002
CLDN4	0.032
CLDN7	0.005

Figure 3. p66ShcA induces an EMT in luminal mammary tumors.
(A) Hematoxylin and eosin (H&E)-stained sections from NIC/VC and NIC/p66ShcA mammary tumors. Scale bars=40 um. **(B)** Immunoblot analysis of NIC/VC and NIC/p66ShcA tumor lysates using E-cadherin-, vimentin-, and tubulin-specific antibodies. Lower-molecular-weight species in the vimentin blot represent proteolytic fragments. **(C)** RT-qPCR analysis of RNA isolated from NIC/VC or NIC/p66ShcA mammary tumors using primers specific for mesenchymal (Snail1/2, Twist1/2, and Zeb1/2) and epithelial (E-cadherin/Cdh1 and Cldn3/Cldn4/ Cldn7) markers. The data are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels ± SEM (n=7 tumors each). **(D)** Paraffin-embedded sections from NIC/VC and NIC/p66ShcA mammary tumors were subjected to immunohistofluorescent staining with CK8/18-specific antibodies (green) and costained with vimentin-, CK14-, or SMA-specific antibodies (red). The data are representative of results for seven tumors each. The following numbers of 20X fields were quantified: for VC, 141 (CK8/vimentin), 140 (CK8/SMA), and 116 (CK8/CK14), and for p66ShcA, 151 (CK8/vimentin) and 141 (CK8/SMA and CK8/CK14). The data are shown as mean percent positive staining per field ± SEM. Scale bars=40μm

We next examined whether p66ShcA functions in a cell-autonomous fashion to promote an EMT using two ErbB2-positive luminal breast cancer cell lines (NIC and BT474). BT474 cells express low levels of endogenous p66ShcA (Fig. 1A). Both NIC and BT474 cells are E-cadherin positive, with little vimentin expression in vitro (Fig. 4A). However, p66ShcA overexpression is sufficient to substantially increase vimentin levels in both NIC and BT474 cells (Fig. 4A), which is associated with elevated p38MAPK activation (Fig. 4B), similar to what we observed in ErbB2-positive mammary tumors (Fig. 2E). We next assessed the phosphorylation status of p66ShcA in NIC and BT474 overexpressers to evaluate whether serine or tyrosine phosphorylation of this adaptor protein is associated with its ability to endow breast tumor cells with mesenchymal features. We demonstrate a significant, albeit variable, increase in p66ShcA Ser36 phosphorylation in NIC and BT474 cells (Fig. 4C). In contrast, p66ShcA is only weakly phosphorylated on key tyrosine residues within the CH1 domain in NIC and BT474 cells relative to levels that are achieved with the p46/52ShcA isoforms (Fig. 4D). These data suggest that the S36 phosphorylation site may contribute to a p66ShcA-induced EMT. To extend these observations, we evaluated E-cadherin levels in control and p66ShcA expression luminal breast cancer cell lines in vitro. Interestingly, E-cadherin levels do not vary appreciably between control and p66ShcA-expressing cells (Fig. 4A), and the majority of NIC/p66ShcA and BT474/p66ShcA cells retain both E-cadherin and vimentin expression in the same cell (see Fig. S3 in the supplemental material). This contrasts with results of our in vivo studies, which demonstrate that CK8/18 and vimentin are reciprocally regulated in NIC/p66ShcA luminal breast tumors (Fig. 3).

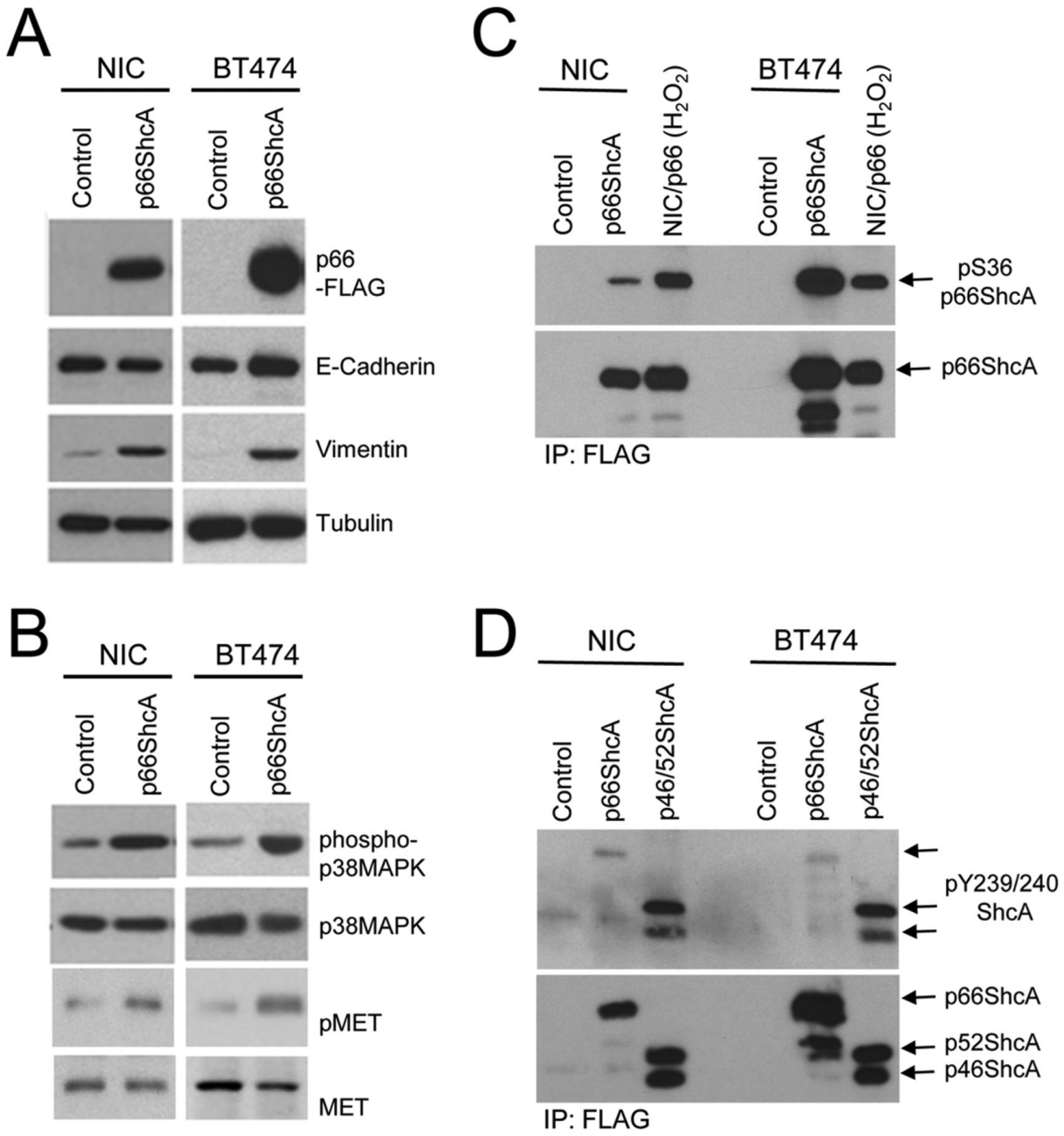


Figure 4. p66ShcA is S36 phosphorylated and increases vimentin expression in ErbB2-driven luminal breast cancer cell lines. (A) Total cell lysates were generated from control and p66ShcA-expressing NIC and BT474 cells and subsequently probed with FLAG-, E-cadherin-, vimentin-, and tubulin-specific antibodies. **(B)** Total cell lysates were generated from control and p66ShcA-expressing NIC and BT474 cells and subsequently probed pp38 MAPK-, p38 MAPK-, pMET-, and MET-specific antibodies. **(C)** FLAG immunoprecipitates from control and p66ShcA overexpressing NIC and BT474 cells probed with pS36-p66ShcA- and ShcA-specific antibodies. The positive control represents the NIC/FLAG-p66ShcA overexpressing cell line stimulated with 1mM H2O2 for 1h prior to cell lysis. **(D)** FLAG immunoprecipitates from control and p66ShcA-overexpressing NIC and BT474 cells probed with pY239/240-ShcA- and ShcA-specific antibodies. The positive control represents a breast cancer cell line stably overexpressing a FLAG-tagged p46/42ShcA construct.

To interrogate the reproducibility of these findings in an independent model of ErbB2-driven breast cancer, we injected parental and p66ShcA-overexpressing BT474 cells into the mammary fat pads of immunodeficient mice. We also observed a significant growth inhibition in BT474/p66ShcA tumors (see Fig. S4A), similar to what we observed with NIC cells (Fig. 2C). How- ever, p66ShcA significantly increased vimentin expression in BT474 tumors in vivo without a corresponding reduction in E- cadherin levels (see Fig. S4B and C). Taken together, these data suggest that p66ShcA primarily functions to increase breast cancer plasticity by stimulating the expression of mesenchymal genes in luminal breast cancer cells. However, p66ShcA expression is not necessarily sufficient to correspondingly inhibit the expression of luminal markers, such as E-cadherin. Thus, we conclude that p66ShcA primarily induces a partial EMT in luminal breast cancers and that its ability to induce a full EMT in vivo requires integration of additional signaling pathways derived from the mammary tumor itself or from cells within the stromal microenvironment.

It is well established that an EMT increases the migratory properties of cancer cells. We next employed both Boyden chamber and scratch assays to examine whether a p66ShcA-induced EMT in ErbB2+ luminal breast cancers renders them more motile in vitro (8). We show that NIC/p66ShcA and BT474/p66ShcA cells displayed a 2.5-fold increase in their migratory properties relative to those of their respective controls (Fig. 5A and B). Moreover, NIC/p66ShcA cells displayed a comparable increase in their invasive properties (Fig. 5A). The inability of p66ShcA to increase the invasive properties of BT474 cells (Fig. 5B) may reflect differences in expression of matrix-degrading proteases between NIC and BT474 cells. Despite this fact, we did observe a robust increase in p66ShcA-induced cell migration in both cell types.

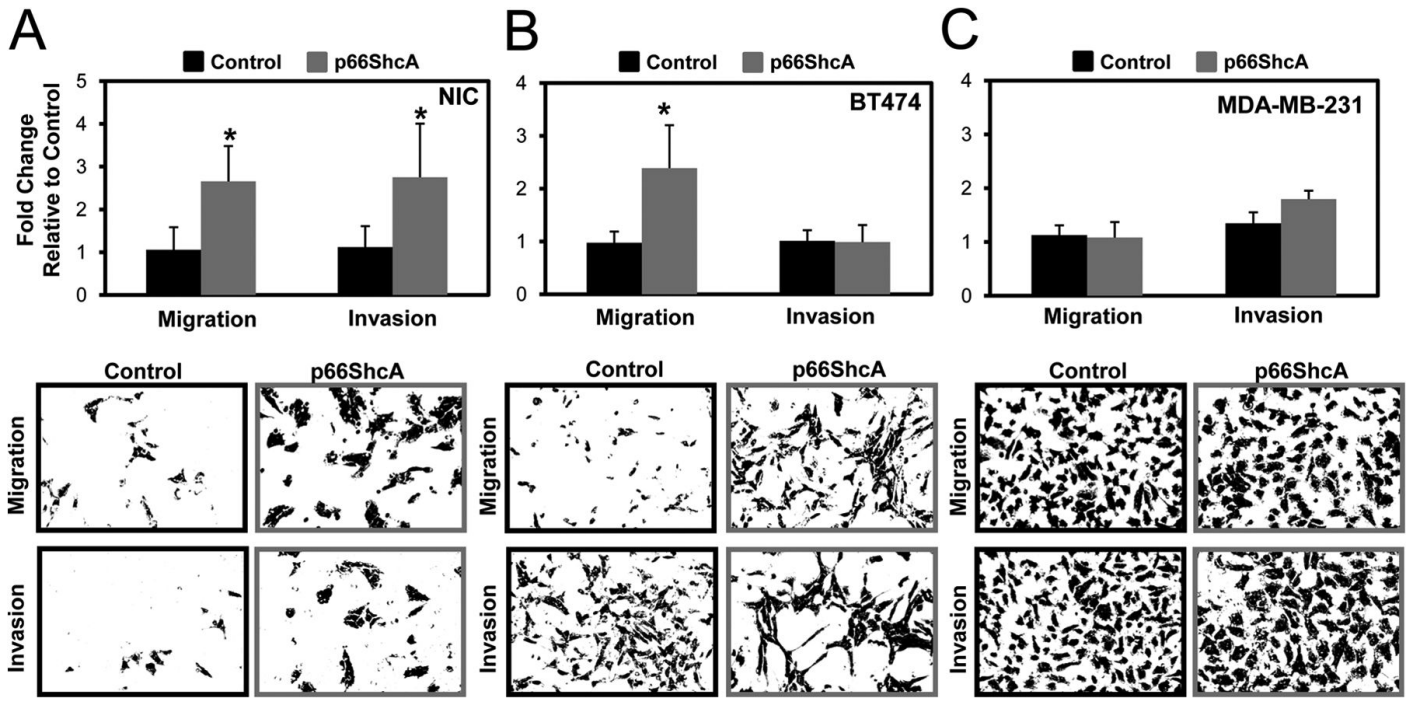


Figure 5. p66ShcA increases the migratory property of luminal breast cancer cells.
(A-C) Control and p66ShcA-expressing NIC **(A)**, BT474 **(B)**, and MDA-MB-231 **(C)** cells were screened by Boyden chamber assays to assess cell migration and invasion. The data are representative of results for 8 inserts from two independent experiments (NIC migration, $P<0.001$; NIC invasion, $P=0.004$; and BT474 migration, $P<0.001$). For the MDA-MB-231 cells, the data represents the averages for 6 inserts from one experiment.

We further validated these observations using an in vitro scratch assay. Despite the fact that p66ShcA significantly inhibited the growth potential of NIC cells in vitro, it stimulated a 2-fold increase in cell migration in a scratch assay (see Fig. S5A to C in the supplemental material). By the same token, p66ShcA did not significantly impact the proliferative ability of BT474 cells but accelerated wound closure (>4-fold) in a scratch assay (see Fig. S5C to E). In contrast, p66ShcA overexpression had no appreciable effect on the migratory or invasive characteristics of MDA-MB-231 cells, which are claudin low (Fig. 5C). These data demonstrate that p66ShcA augments the migratory properties of luminal breast cancers but may not be required in breast cancer cells that have already undergone a stable EMT. Interestingly, these observations contrast recent studies which showed a role for p66ShcA in impairing leukocyte migration and chemotaxis by inhibiting actin polymerization and its subsequent disassembly, coincident with reduced Vav phosphorylation (31, 32). This suggests either that p66ShcA exerts differential effects on the actin cytoskeleton in epithelial cells or that the ability of p66ShcA to increase the migratory properties of breast cancer cells is secondary to its ability to induce an EMT.

p66ShcA relies on increased Met signaling to induce an EMT in ErbB2+ luminal breast cancers.

Oxidative stress induces an EMT (33), and p66ShcA stimulates ROS production in response to stress stimuli (21). Indeed, p66ShcA overexpression increases ROS production (Fig. 2D) and the acquisition of an EMT phenotype in luminal breast tumors (Fig. 3). One mechanism by which ROS stimulates an EMT is via activation of the NF-KB pathway (34). Thus, we examined whether p66ShcA overexpression was associated with elevated NF-KB signaling in luminal breast cancer cells. Under steady-state conditions, phospho-NF-KB levels are comparable in control and

p66ShcA-expressing NIC and BT474 cells (see Fig. S6A) in the supplemental material. However, p66ShcA could potentiate NF-KB signaling in mammary tumors in vivo, given their exposure to a plethora of stress stimuli. Indeed, NIC/p66ShcA mammary tumors significantly and uniformly increase NF-KB signaling (see Fig. S6B), coincident with elevated expression of NF-KB target genes that confer a tumorigenic and angiogenic phenotype (see Fig. S6C). In contrast, NF-KB signaling is not appreciably altered in control and p66ShcA-expressing BT474 tumors (see Fig. S3C). These observations demonstrate that p66ShcA expression is not necessarily sufficient to increase NF-KB signaling in mammary tumors in vivo but rather may rely on collaborative stromally derived signals to activate this pathway.

The transforming growth factor ß (TGF-ß) pathway is another key promoter of an EMT in ErbB2-driven breast cancer cells (26, 35). However, the degree of activation of canonical TGF-ß/SMAD signaling in NIC/VC mammary tumors is comparable to that in NIC/p66ShcA mammary tumors (Fig. 6A and B). Another critical inducer of an EMT is the Met receptor tyrosine kinase (36). Transgenic mouse models have demonstrated that Met overexpression in the mammary epithelium synergizes with p53 deficiency to induce the formation of mammary tumors resembling the claudin-low subtype (37).

We show that p66ShcA and Met are coordinately regulated in several, but not all, human claudin-low breast cancer cell lines examined (Fig. 1A) and that both NIC/p66ShcA and BT474/p66ShcA breast cancer cells activate Met in vitro (Fig. 4B). Moreover, both NIC/p66ShcA and BT474/p66ShcA tumors with mesenchymal properties demonstrate increased Met activation (Fig. 6A and B; see also Fig. S4B in the supplemental material), which coincides with elevated HGF (the Met ligand) production from p66ShcA-overexpressing mammary tumors in vivo (Fig. 6C). Thus, p66ShcA overexpression increases Met signaling in luminal breast cancer cells, both in vitro

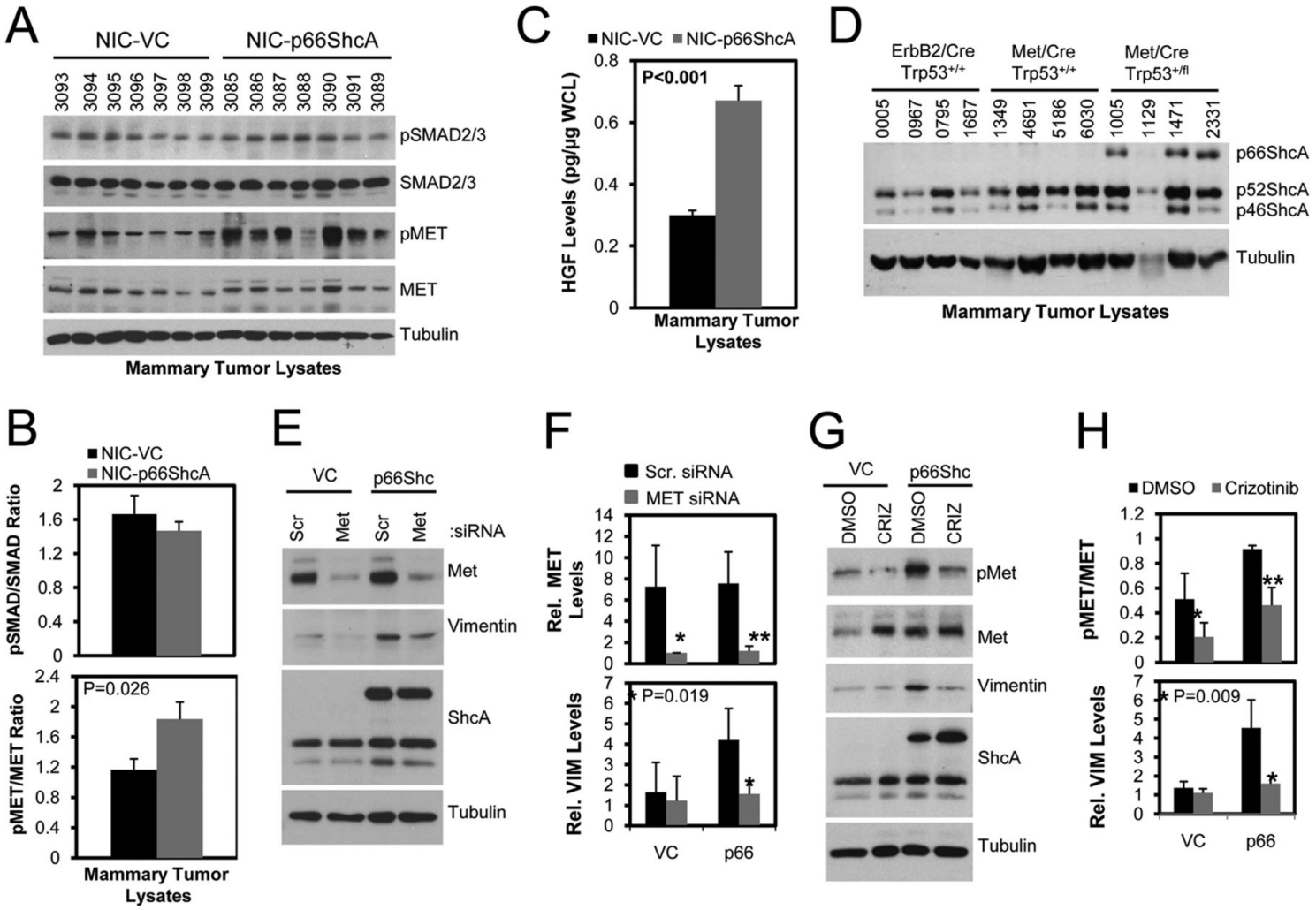


Figure 6. p66ShcA activated Met signaling increases vimentin expression in luminal breast cancer cells. (A) Immunoblot analysis of NIC/VC and NIC/p66ShcA tumor lysates using pSMAD2/3-, SMAD2/3-, pMET, MET-, and tubulin-specific antibodies. **(B)** Quantification of the relative ratios of pSMAD2/3 to SMAD2/3 and pMET to MET in the mammary tumor lysates shown in panel A. **(C)** HGF ELISA of NIC/VC and NIC/p66ShcA tumor lysates (n=7 tumors each). **(D)** Immunoblot analysis of tumor lysates from MMTV/NIC, MMTV/Met;Cre;$p53^{+/+}$, and MMTV/Met;Cre;$p53^{fl/+}$ transgenic mice using ShcA- and tubulin- specific antibodies. **(E)** Immunoblot analysis of whole-cell lysates from NIC/VC and NIC/p66ShcA breast cancer cells transfected with scrambled or Met-specific siRNAs using pMET-, E-cadherin-, vimentin-, and tubulin-specific antibodies. **(F)** Quantification of relative MET (*, P = 0.018; **, P == 0.005) and vimentin levels (normalized to tubulin levels) in NIC/VC and NIC/p66ShcA cells transfected with scrambled and Met-specific siRNAs as outlined for panel E. The data are representative of results from four independent experiments. **(G)** Immunoblot analysis of whole-cell lysates from NIC-p66ShcA breast cancer cells treated with 1µM crizotinib or DMSO control over a 4-day period. **(H)** Quantification of the pMET/MET ratio (*, P = 0.04; **, P = 0.008) and relative vimentin levels (normalized to tubulin levels) in NIC/VC and NIC/p66ShcA cells treated with DMSO or crizotinib as outlined for panel G. The data are representative of results from four independent experiments.

and in vivo. Finally, we examined whether p66ShcA expression is differentially regulated in transgenic mouse models of breast cancer. These include ErbB2-driven (NIC) tumors, resembling the luminal sub- type (25), and Met/p53$^{+/+}$ tumors, which give rise to tumors that coexpress luminal and basal markers but do not acquire a mesenchymal phenotype (38). In contrast, Met-driven, p53-deficient tumors lose their epithelial characteristics and undergo a stable EMT (37). We demonstrate that p66ShcA is absent from NIC and Met/p53$^{+/+}$ tumors but is upregulated in Met-driven models of claudin-low breast cancer (Fig. 6D). Combined, these data suggest that p66ShcA and Met are coordinately regulated in mammary tumors with mesenchymal properties.

Finally, we evaluated whether reduced Met expression or signaling impacted a p66ShcA-induced EMT. Using Met-specific siRNAs, we were able to achieve a >4-fold reduction in Met levels in control and p66ShcA-expressing NIC cells. While vimentin levels were low and unaltered in NIC/VC control cells, reduced Met expression resulted in a 3-fold decrease in vimentin expression in NIC/ p66ShcA cells (Fig. 6E and F). To substantiate these findings, we also employed crizotinib, a pharmacological Met inhibitor, and show a 2-fold reduction in Met phosphorylation in both control and p66ShcA-expressing NIC cells. Again, crizotinib had no effect on baseline vimentin levels in NIC cells but significantly attenuated (3.5-fold) vimentin expression in NIC/p66ShcA expressors (Fig. 6G and H). Taken together, these data suggest that p66ShcAacooperates with Met signaling to support a mesenchamal phenotype in breast cancer cells

Elevated p66ShcA levels are associated with an EMT phenotype in primary human breast cancers.

The relationship between p66ShcA and an EMT cannot be interrogated by microarray data given that the ShcA probes recognize all three isoforms. To circumvent this issue, we took a two-pronged approach. First, we quantified p66ShcA mRNA levels from 84 primary breast tumors (Genome Quebec) by RT-qPCR using primers specific to the CH2 domain (Fig. 7A) and generated matching gene expression profiling data for each tumor. We also determined relative p66ShcA levels in a publicly available RNA-seq data set (n = 660; TCGA) (29), including luminal A/B, HER2+, and basal breast cancers. In both data sets, tumors were rank ordered based on p66ShcA levels and stratified into two groups: p66ShcA-low (bottom 50%) and p66ShcA-high (top 50%) (Fig. 7B). We show that increasing p66ShcA levels are associated with increased Met and HGF levels, elevated expression of mesenchymal markers (vimentin, Snai1/2, Zeb1/2, and Twist1/2), and a reduction of several claudins (Cldn3, Cldn4, and Cldn7) across all breast cancer subtypes (Fig. 7C and D). Interestingly, utilization of a ShcA probe spanning all three isoforms cannot stratify breast tumors with increased mesenchymal features (Fig. 7E), suggesting that p66ShcA is uniquely associated with the acquisition of EMT characteristics. Similar to observations made in our preclinical models, we show that a corresponding increase in p66ShcA levels in primary breast tumors is significantly associated with a robust increase in vimentin expression and has no appreciable effect on E-cadherin levels (Fig. 7F). This is associated with a particularly robust increase in Snail2, Zeb1, and Zeb2 mRNA levels in p66ShcA-high mammary tumors (Fig. 7G).

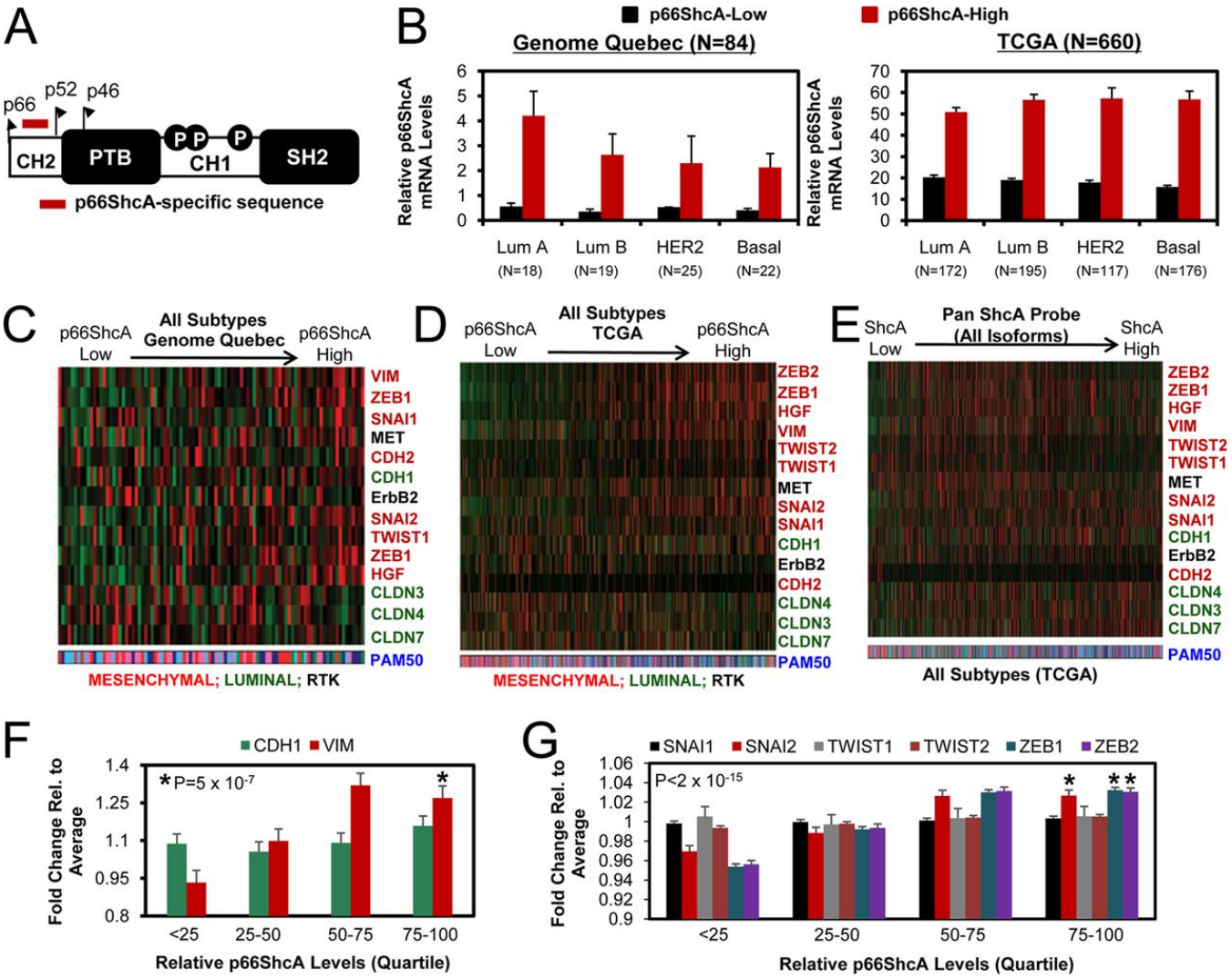


Figure 7. p66ShcA levels stratify primary breast tumors with an EMT phenotype irrespective of molecular subtype. (A) Region within the CH2 domain used to interrogate p66ShcA expression levels. **(B)** Relative p66ShcAmRNA levels were determined from 84 primary breast cancers by RT-qPCR (Genome Quebec). We also screened p66ShcAmRNAlevels in 660 primary breast cancers by RNA-seq (TCGA). Tumors were stratified based on relative p66ShcA expression levels (low, bottom 50%; high, top 50%). **(C and D)** Stratification of breast tumors with the Genome Quebec **(C)** and TCGA **(D)** data sets based on increasing p66ShcA expression levels. A similar analysis was performed across all subtypes within the Genome Quebec and TCGA data sets. **(E)** Stratification of breast tumors within the TCGA data set based on increasing ShcA levels using a probe that spans all three isoforms. A heat map depicting the relative expression levels of luminal (green) and mesenchymal (red) genes is shown. **(F)** Relative E-cadherin (CDH1) and vimentin (VIM) expression levels in breast cancer patients from the TCGA data set (n = 660). For each patient, fold change gene expression values were calculated by first normalizing expression levels within a tumor to the average expression value across all tumors (n = 660). Normalized expression values were then log2 transformed, and tumors were segregated into quartiles based on relative p66ShcA levels over the entire cohort. **(G)** Relative SNAI1, SNAI2, TWIST1, TWIST2, ZEB1, and ZEB2 gene expression levels in breast tumors from the TCGA data.

We next interrogated whether p66ShcA expression is specific to particular breast cancer subtypes. Surprisingly, p66ShcA is expressed in all breast cancer subtypes, including luminal A tumors (Fig. 8A), which seemingly contradicts our observation in breast cancer cell lines (Fig. 1A). However, upon closer inspection, increased p66ShcA levels specifically within luminal A breast cancers are associated with increased Met signaling (HGF and Met) and elevated expression of mesenchymal markers (vimentin, Snai1/2, Zeb1/2, and Twist1/2) concomitant with reduced claudin levels (Cldn3, Cldn4, and Cldn7) (Fig. 8B). Indeed, high p66ShcA levels correlate with an EMT across all molecular subtypes, including luminal B, HER2, and basal breast tumors (Fig. 8C to E). Taken together, these observations suggest that p66ShcA is a universal driver of breast cancer cell plasticity by promoting the acquisition of mesenchymal features in all breast cancers, including the luminal subtypes. Given this observation, we next examined whether increasing p66ShcA levels were associated with the acquisition of a claudin-low signature, which is indicative of a full and stable EMT (6). Breast tumors expressing elevated p66ShcA levels are enriched in a subset of genes that identify the claudin-low subtype (Fig. 8F). However, p66ShcA-high luminal A/B and HER2tumors are not defined as claudin low (Fig. 8G). In contrast, p66ShcA-high basal breast tumors are significantly enriched in the claudin-low subtype (Fig. 8G), which is consistent with our observations in basal breast cancer cell lines (Fig. 1) and in transgenic mouse models of claudin-low breast cancer (Fig. 6D). This suggests that within the basal subtype, p66ShcA overexpression may promote the development of claudin-low tumors. Combined, our data demonstrate that p66ShcA may serve as a clinical biomarker for breast cancer plasticity, through the induction of an EMT, irrespective of molecular subtype. The functional implication of p66ShcA overexpression in luminal and basal breast tumors warrants future investigation.

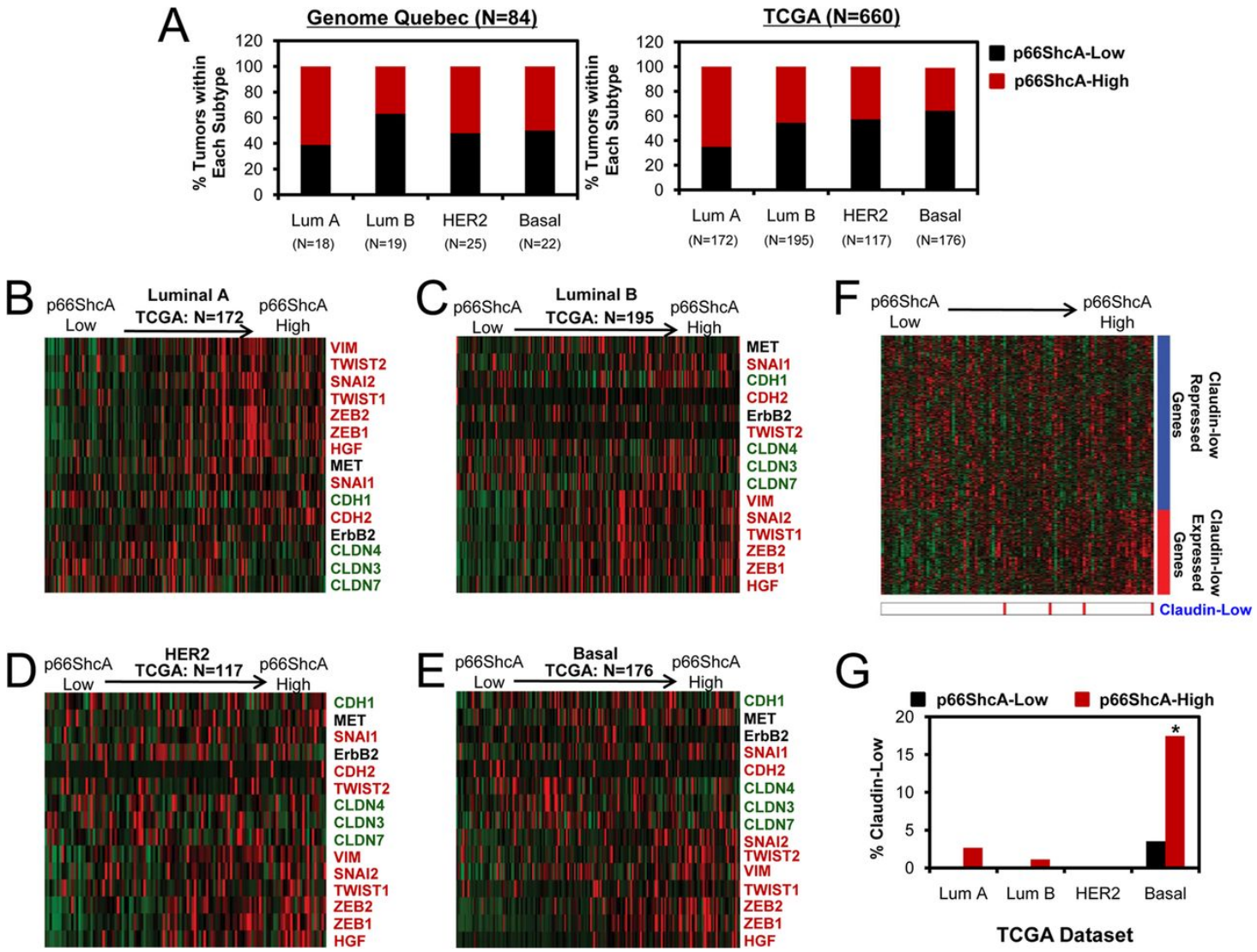


Figure 8. p66ShcA is enriched in the claudin-low subtype of basal breast cancer.
(A) The percentage of p66ShcA-low and p66ShcA-high tumors within each molecular subtype is shown for the Genome Quebec and TCGA data sets. **(B to E)** Stratification of luminal A **(B)**, luminal B **(C)**, HER2 **(D)**, and basal **(E)** tumors within the TCGA data set based on increasing p66ShcA levels. Heat maps depicting the relative expression levels of luminal (green) and mesenchymal (red) genes are shown. **(F)** Tumors were ordered by increasing p66ShcA levels, and expression levels of genes within a claudin-low signature were determined (Genome Quebec). **(G)** For the TCGA data set, the relative percentage of p66ShcA-low and p66ShcA-high tumors that are defined as claudin low by gene expression profiling is shown. For the TCGA data set in panel A, the P values for the association between high p66ShcA levels (top 50%) between breast cancer subtypes was determined using Fisher's exact probability test and are as follows: luminal A versus luminal B, P=0.0002; luminal A versus HER2, P=0.00019; luminal A versus basal, P = 7.3 X 10^{-8}; luminal B versus HER2, P = 0.64; luminal B versus basal, P = 0.07; and HER2 versus basal, P = 0.27. *, P = 0.004 (p66ShcALow versus p66ShcAHigh within the basal subtype.

2.6 Discussion

Breast cancers are stratified into distinct subtypes, which influences patient outcome. Despite this fact, a significant degree of heterogeneity exists *within* breast cancer subtypes, which impacts therapeutic responsiveness. Our data reinforces the concept of such plasticity, even within breast cancer subtypes. Indeed, we show that a significant number of primary breast tumors which acquire mesenchymal features neither display reduced E-cadherin levels nor are defined as claudin-low. Several studies support the notion that breast cancers can acquire mesenchymal genes without the obligate loss of epithelial features (9-11). By combining orthotopic studies with established breast cancer cell lines and primary breast tumor material, we demonstrate a causal role for the p66ShcA adaptor in inducing an EMT, in part, through activation of the Met receptor tyrosine kinase (Fig. 9).

This is clinically relevant as tumors with luminal features, comprise up to 80% of all newly diagnosed breast cancers. Indeed, p66ShcA expression is elevated in breast tumors with mesenchymal features, including the luminal A subtype, which is typically associated with a more epithelial-like state and better patient outcome. This is consistent with retrospective studies, which showed that a significant number of luminal A breast cancer patients are at elevated risk of cancer-related death more than 10 years following diagnosis despite adjuvant endocrine therapy and chemotherapy (39). It is possible that a subset of tumor cells, with increased plasticity, confer therapeutic resistance and relapse in these women with luminal A breast cancer. The importance of p66ShcA in promoting therapeutic resistance and/or relapse in luminal tumors requires additional experimentation.

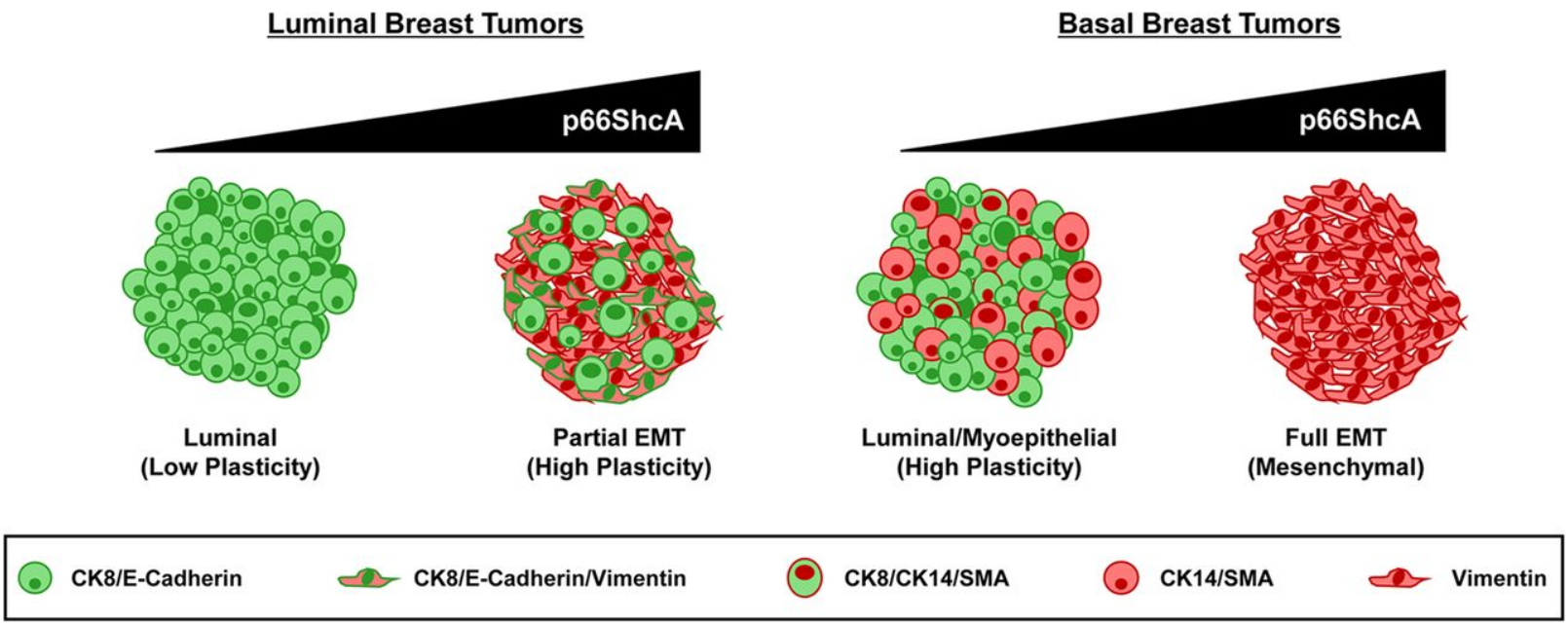


Figure 9. Proposed model for the role of p66ShcA in promoting plasticity among the various breast cancer subtypes. Within the luminal subtype, breast tumors uniformly express cytokeratin 8/18, along with adherens (E-cadherin) and tight junctional (ZO-1 and claudin) proteins. However, a subset of these tumors also express EMT-like genes (vimentin, N-cadherin, Snai1/2, Zeb1/2, and Twist1/2) without the obligate loss of E-cadherin expression. We propose that elevated p66ShcA expression in luminal breast cancer cells endows them with such a partial EMT phenotype to increase their plasticity, leading to enhanced cell migration and invasion. In contrast, within the basal subtype, basal-like tumors coexpress luminal (CK8/18) and myoepithelial (CK14 and SMA) markers, while claudin- low tumors uniformly lack luminal epithelial markers and stably express mesenchymal genes indicative of a complete EMT. We propose that elevated p66ShcA overexpression in the basal subtype further drives an EMT to promote the development of claudin-low breast tumors.

Paradoxically, we also demonstrate that p66ShcA is enriched in the majority of basal breast cancer cell lines, particularly within the claudin-low subtype, and is expressed at exceedingly low levels or is absent from luminal breast cancer cell lines. This seemingly contradicts our observations in primary breast tumors, which show elevated p66ShcA expression across all subtypes, including luminal breast cancers. However, our observation that p66ShcA can stratify mammary tumors with mesenchymal features, irrespective of breast cancer subtype, reconciles these observations and reinforces the notion that a significant degree of heterogeneity exists within breast tumors in vivo, even within a specific subtype. Interestingly, while elevated p66ShcA levels induce the acquisition of mesenchymal features in all breast tumors, they are significantly enriched only in claudin-low tumors within the basal subtype. This suggests that p66ShcA increases plasticity of luminal breast tumors, by favoring coexpression of luminal and mesenchymal genes. Ironically, within the basal subtype, p66ShcA may limit plasticity by pushing these already more plastic tumors toward a full EMT and favor the emergence of claudin-low breast tumors (Fig. 9). The importance of p66ShcA in the emergence and tumorigenic potential of claudin-low breast tumors requires further experimentation. Finally, this study suggests that although established breast cancer cell lines retain many luminal or basal features that are observed in primary tumors at the molecular level, they do not capture the inherent heterogeneity that is found in primary clinical material. Thus, they are a useful surrogate for, but not an exact representation of, what is observed in primary breast cancers.

Numerous studies highlight a critical role for the ShcA isoforms in breast cancer. Increased ShcA signaling predicts lymph node positivity, high grade, and relapse in breast cancer patients (40). High ShcA levels are enriched in HER2+ and basal tumors and are associated with reduced survival of breast cancer patients (23). In transgenic mouse models, deletion of all three ShcA

isoforms in the mammary epithelium virtually ablates ErbB2-driven tumor induction (23, 25). Using xenograft approaches, an important role for p46/52ShcA during mammary tumor growth, angiogenesis, and metastasis was shown (22, 24, 25). Our in vivo studies provide the first experimental evidence that p66ShcA contributes to tumor heterogeneity in luminal breast cancers by inducing an EMT. However, we also show a modest p66ShcA-induced impairment of luminal tumor growth. These dichotomous roles for p66ShcA in breast cancers are in agreement with contradictory studies that interrogated the relationship between p66ShcA and disease outcome. While some studies suggest that high p66ShcA levels are associated with good outcome in breast cancers (40), others correlate high p66ShcA levels with increased lymph node positivity, grade, and recurrence in breast, prostate, and colon cancer patients (41–43). We suggest that the association between high p66ShcA levels and good outcome is reflective of the fact that p66ShcA is enriched in luminal breast cancers. In these studies, the predictive power of p66ShcA as a good prognosis biomarker is significantly increased when stratifying for tumors with reduced ShcA tyrosine phosphorylation (40). Thus, it is possible that the combination of p66ShcAHigh and ShcA-pTyrLow as stratification tools selects for luminal breast tumors with reduced receptor tyrosine kinase signaling. In contrast, an association between p66ShcA levels and poor outcome (41–43) may be reflective of increased tumor heterogeneity and is consistent with our observation that p66ShcA increases the migratory properties of luminal breast cancers.

Consistent with these clinical correlates, numerous studies have suggested both pro- and antitumorigenic properties for p66ShcA signaling. In response to stress stimuli, p66ShcA catalyzes electron transport from cytochrome c and promotes the formation of reactive oxygen species (ROS) (21). Extremely high ROS levels disrupt the mitochondrial membrane potential, leading to opening of the permeability transition pore, cytochrome c release, and apoptosis (18). We

hypothesize that reduced p66ShcA-induced tumor cell proliferation is likely reflective of increased oxidative stress, which is consistent with the observation that p66ShcA is Ser36 phosphorylated in the luminal breast cancer cell lines examined in this study. However, most solid tumors exhibit moderately elevated ROS levels, which facilitate neoplastic growth by stimulating an inflammatory response (44). By inducing the NF-KB pathway, ROS increases tumor cell proliferation and survival (45) and stimulates the recruitment of stromal cells that facilitate tumor growth and metastasis (46). Our observations suggest that p66ShcA signaling increases Met and NF-KB signaling in breast tumors, coincident with a robust increase in tumor angiogenesis. Indeed, both NF-KB and Met transduce proangiogenic stimuli in cancer cells (47, 48). Thus, this study suggests that p66ShcA functions in a cell-autonomous manner and may also collaborate with the microenvironment to establish an EMT in vivo. Moreover, p66ShcA expression is sufficient to increase Met signaling in vitro, which, in turn, pro- motes the acquisition of mesenchymal markers in breast cancer cells. Interestingly, while the Met receptor tyrosine kinase is a clinically relevant biomarker of poor outcome basal breast cancers (38), a subset of luminal breast tumors examined in this work coordinately display increased Met, HGF, and p66ShcA expression, coincident with the attainment of an EMT phenotype. The observation that Met-driven, claudin-low transgenic mammary tumors further increase endogenous p66ShcA expression rein- forces the hypothesis that a Met/p66ShcA-driven signaling axis is central for the ability of a mammary tumor to undergo an epithelial-to-mesenchymal transition.

In contrast, p66ShcA overexpression only activates NF-KB signaling in mammary tumors in vivo. Moreover, it is intriguing that we only observe elevated NF-KB activation, in addition to in- creased expression of protumorigenic NF-KB target genes, in p66ShcA-expressing mammary tumors that have undergone a full EMT. In contrast, BT474/p66ShcA tumors enhance vimentin

expression but retain their luminal characteristics. Interestingly, the degree of NF-KB signaling in control BT474 tumors is comparable to that in p66ShcA-expressing BT474 tumors. Given that NF-KB is a known inducer of an EMT (34), this raises the intriguing possibility that tumor microenvironmental cues that increase stress signaling in vivo, including tumor hypoxia and inflammation, collaborate with p66ShcA to engage the NF-KB pathway and fully drive the EMT process. This study has important clinical implications, as it demonstrates that p66ShcA functions as a molecular driver of an EMT, irrespective of breast cancer subtype. Thus, it may assist in the development of broadly applicable targeted therapies that span all molecular subtypes.

2.7 References

1. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. 2000. Molecular portraits of human breast tumours. Nature 406:747–752. http://dx.doi.org/10.1038/35021093.
2. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R. 2002. A gene expression signature as a predictor of survival in breast cancer. N. Engl. J. Med. 347:1999–2009. http://dx.doi.org/10.1056/NEJMoa021967.
3. Visvader JE. 2009. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes Dev. 23:2563–2577. http://dx.doi.org/10 .1101/gad.1849509.
4. Gusterson BA, Ross DT, Heath VJ, Stein T. 2005. Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. Breast Cancer Res. 7:143–148. http://dx.doi.org/10.1186 /bcr1041.
5. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW. 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10:515–527. http://dx.doi.org/10.1016/j.ccr.2006.10.008.
6. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X, Perou CM. 2010. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 12:R68. http://dx.doi.org/10.1186/bcr2635.
7. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, Hartwell K, Onder TT, Gupta PB, Evans KW, Hollier BG, Ram PT, Lander ES, Rosen JM, Weinberg RA, Mani SA. 2010. Core epithelial-to-mesenchymal transition interactome gene expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc. Natl. Acad. Sci. U. S. A. 107:15449–15454. http://dx.doi.org/10.1073/pnas .1004900107.

8. Drasin DJ, Robin TP, Ford HL. 2011. Breast cancer epithelial-to-mesenchymal transition: examining the functional consequences of plas- ticity. Breast Cancer Res. 13:226. http://dx.doi.org/10.1186/bcr3037.
9. Côme C, Magnino F, Bibeau F, De Santa Barbara P, Becker KF, Theillet C, Savagner P. 2006. Snail and slug play distinct roles during breast carcinoma progression. Clin. Cancer Res. 12:5395–5402. http://dx.doi.org /10.1158/1078-0432.CCR-06-0478.
10. Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, Notorfrancesco KL, Cardiff RD, Chodosh LA. 2005. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell 8:197– 209. http://dx.doi.org/10.1016/j.ccr.2005.07.009.
11. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117:927–939. http://dx.doi.org/10.1016/j.cell.2004.06.006.
12. Migliaccio E, Mele S, Salcini AE, Pelicci G, Lai KM, Superti-Furga G, Pawson T, Di Fiore PP, Lanfrancone L, Pelicci PG. 1997. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J. 16:706–716. http: //dx.doi.org/10.1093/emboj/16.4.706.
13. Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, Nicoletti I, Pawson T, Pelicci PG. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell 70:93–104. http://dx.doi.org/10.1016/0092-8674(92)90536-L.
14. Ventura A, Luzi L, Pacini S, Baldari CT, Pelicci PG. 2002. The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. J. Biol. Chem. 277:22370–22376. http://dx.doi.org/10.1074 /jbc.M200280200.
15. Gu H, Maeda H, Moon JJ, Lord JD, Yoakim M, Nelson BH, Neel BG. 2000. New role for Shc in activation of the phosphatidylinositol 3-kinase/ Akt pathway. Mol. Cell. Biol. 20:7109–7120. http://dx.doi.org/10.1128 /MCB.20.19.7109-7120.2000.
16. Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D. 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 363:83–85. http://dx.doi.org/10.1038 /363083a0.
17. Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG. 1999. The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 402:309–313. http://dx.doi.org/10.1038/46311.
18. Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, Martin-Padura I, Pelliccia G, Trinei M, Bono M, Puri C, Tacchetti C, Ferrini M, Mannucci R, Nicoletti I, Lanfrancone L, Giorgio M, Pelicci PG. 2004. The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. J. Biol. Chem. 279:25689–25695. http://dx.doi .org/10.1074/jbc.M401844200.
19. Gertz M, Fischer F, Wolters D, Steegborn C. 2008. Activation of the lifespan regulator p66Shc through reversible disulfide bond formation. Proc. Natl. Acad. Sci. U. S. A. 105:5705–5709. http://dx.doi.org/10.1073 /pnas.0800691105.
20. Pinton P, Rimessi A, Marchi S, Orsini F, Migliaccio E, Giorgio M, Contursi C, Minucci S, Mantovani F, Wieckowski MR, Del Sal G, Pelicci PG, Rizzuto R. 2007. Protein kinase C beta and prolyl isomerase 1 regulate mitochondrial effects of the life-span determinant p66Shc. Science 315:659–663. http://dx.doi.org/10.1126/science.1135380.
21. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M, Pinton P, Rizzuto R, Bernardi P, Paolucci F, Pelicci PG. 2005. Electron

transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 122:221–233. http://dx.doi.org/10.1016/j .cell.2005.05.011.
22. Ahn R, Sabourin V, Ha JR, Cory S, Maric G, Im YK, Hardy WR, Zhao H, Park M, Hallett M, Siegel PM, Pawson T, Ursini-Siegel J. 2013. The ShcA PTB domain functions as a biological sensor of phosphotyrosine signaling during breast cancer progression. Cancer Res. 73:4521–4532. http://dx.doi.org/10.1158/0008-5472.CAN-12-4178.
23. Ursini-Siegel J, Cory S, Zuo D, Hardy WR, Rexhepaj E, Lam S, Schade B, Jirstrom K, Bjur E, Piccirillo CA, Denardo D, Coussens LM, Brennan DJ, Gallagher WM, Park M, Pawson T, Hallett M, Muller WJ. 2010. Receptor tyrosine kinase signaling favors a protumorigenic state in breast cancer cells by inhibiting the adaptive immune response. Cancer Res. 70: 7776–7787. http://dx.doi.org/10.1158/0008-5472.CAN-10-2229.
24. Ursini-Siegel J, Hardy WR, Zheng Y, Ling C, Zuo D, Zhang C, Podmore L, Pawson T, Muller WJ. 2012. The ShcA SH2 domain engages a 14-3-3/PI3K signaling complex and promotes breast cancer cell survival. Oncogene 31:5038–5044. http://dx.doi.org/10.1038/onc.2012.4.
25. Ursini-Siegel J, Hardy WR, Zuo D, Lam SH, Sanguin-Gendreau V, Cardiff RD, Pawson T, Muller WJ. 2008. ShcA signalling is essential for tumour progression in mouse models of human breast cancer. EMBO J. 27:910–920. http://dx.doi.org/10.1038/emboj.2008.22.
26. Northey JJ, Chmielecki J, Ngan E, Russo C, Annis MG, Muller WJ, Siegel PM. 2008. Signaling through ShcA is required for transforming growth factor beta- and Neu/ErbB-2-induced breast cancer cell motility and invasion. Mol. Cell. Biol. 28:3162–3176. http://dx.doi.org/10.1128 /MCB.01734-07.
27. Gautier L, Cope L, Bolstad BM, Irizarry RA. 2004. Affyanalysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20:307–315. http://dx.doi.org/10.1093/bioinformatics/btg405.
28. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, Davies S, Fauron C, He X, Hu Z, Quackenbush JF, Stijleman IJ, Palazzo J, Marron JS, Nobel AB, Mardis E, Nielsen TO, Ellis MJ, Perou CM, Bernard PS. 2009. Supervised risk predictor of breast cancer based on intrinsic subtypes. J. Clin. Oncol. 27:1160–1167. http://dx.doi.org/10 .1200/JCO.2008.18.1370.
29. The Cancer Genome Atlas Network. 2012. Comprehensive molecular portraits of human breast tumours. Nature 490:61–70. http://dx.doi.org /10.1038/nature11412.
30. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S, Backlund MG, Yin Y, Khramtsov AI, Bastein R, Quackenbush J, Glazer RI, Brown PH, Green JE, Kopelovich L, Furth PA, Palazzo JP, Olopade OI, Bernard PS, Churchill GA, Van Dyke T, Perou CM. 2007. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol. 8:R76. http://dx .doi.org/10.1186/gb-2007-8-5-r76.
31. Masi G, Mercati D, Vannuccini E, Paccagnini E, Riparbelli MG, Lupetti P, Pelicci PG, Baldari CT, Ulivieri C. 2014. p66Shc regulates vesicle- mediated secretion in mast cells by affecting F-actin dynamics. J. Leukoc. Biol. 95:285–292. http://dx.doi.org/10.1189/jlb.0313178.
32. Patrussi L, Capitani N, Cannizzaro E, Finetti F, Lucherini OM, Pelicci PG, Baldari CT. 2014. Negative regulation of chemokine receptor signaling and B-cell chemotaxis by p66Shc. Cell Death Dis. 5:e1068. http://dx .doi.org/10.1038/cddis.2014.44.
33. Giannoni E, Parri M, Chiarugi P. 2012. EMT and oxidative stress: a bidirectional interplay affecting tumor malignancy. Antioxid. Redox Signal. 16:1248–1263. http://dx.doi.org/10.1089/ars.2011.4280.
34. Min C, Eddy SF, Sherr DH, Sonenshein GE. 2008. NF-kappaB and epithelial to mesenchymal transition of cancer. J. Cell. Biochem. 104:733– 744. http://dx.doi.org/10.1002/jcb.21695.

35. Muraoka RS, Koh Y, Roebuck LR, Sanders ME, Brantley-Sieders D, Gorska AE, Moses HL, Arteaga CL. 2003. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. Mol. Cell. Biol. 23:8691–8703. http://dx.doi.org/10.1128 /MCB.23.23.8691-8703.2003.
36. Garofalo M, Romano G, Di Leva G, Nuovo G, Jeon YJ, Ngankeu A, Sun J, Lovat F, Alder H, Condorelli G, Engelman JA, Ono M, Rho JK, Cascione L, Volinia S, Nephew KP, Croce CM. 2012. EGFR and MET receptor tyrosine kinase-altered microRNA expression induces tumorigenesis and gefitinib resistance in lung cancers. Nat. Med. 18:74–82. http: //dx.doi.org/10.1038/nm.2577.
37. Knight JF, Lesurf R, Zhao H, Pinnaduwage D, Davis RR, Saleh SM, Zuo D, Naujokas MA, Chughtai N, Herschkowitz JI, Prat A, Mulligan AM, Muller WJ, Cardiff RD, Gregg JP, Andrulis IL, Hallett MT, Park M. 2013. Met synergizes with p53 loss to induce mammary tumors that possess features of claudin-low breast cancer. Proc. Natl. Acad. Sci. U. S. A. 110:E1301–E1310. http://dx.doi.org/10.1073/pnas.1210353110.
38. Ponzo MG, Lesurf R, Petkiewicz S, O'Malley FP, Pinnaduwage D, Andrulis IL, Bull Chughtai SBN, Zuo D, Souleimanova M, Germain D, Omeroglu A, Cardiff RD, Hallett M, Park M. 2009. Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. Proc. Natl. Acad. Sci. U. S. A. 106:12903– 12908. http://dx.doi.org/10.1073/pnas.0810402106.
39. Ignatiadis M, Sotiriou C. 2013. Luminal breast cancer: from biology to treatment. Nat. Rev. Clin. Oncol. 10:494–506. http://dx.doi.org/10.1038 /nrclinonc.2013.124.
40. Davol PA, Bagdasaryan R, Elfenbein GJ, Maizel AL, Frackelton AR, Jr. 2003. Shc proteins are strong, independent prognostic markers for both node-negative and node-positive primary breast cancer. Cancer Res. 63: 6772–6783.
41. Grossman SR, Lyle S, Resnick MB, Sabo E, Lis RT, Rosinha E, Liu Q, Hsieh CC, Bhat G, Frackelton AR, Jr, Hafer LJ. 2007. p66Shc tumor levels show a strong prognostic correlation with disease outcome in stage IIA colon cancer. Clin. Cancer Res. 13:5798–5804. http://dx.doi.org/10 .1158/1078-0432.CCR-07-0073.
42. Jackson JG, Yoneda T, Clark GM, Yee D. 2000. Elevated levels of p66Shc are found in breast cancer cell lines and primary tumors with high meta- static potential. Clin. Cancer Res. 6:1135–1139.
43. Lee MS, Igawa T, Chen SJ, Van Bemmel D, Lin JS, Lin FF, Johansson SL, Christman JK, Lin MF. 2004. p66Shc protein is upregulated by steroid hormones in hormone-sensitive cancer cells and in primary prostate carcinomas. Int. J. Cancer 108:672–678. http://dx.doi.org/10.1002/ijc .11621.
44. Ralph SJ, Rodriguez-Enriquez S, Neuzil J, Saavedra E, Moreno-Sanchez R. 2010. The causes of cancer revisited: "mitochondrial malignancy" and ROS-induced oncogenic transformation—why mitochondria are targets for cancer therapy. Mol. Aspects Med. 31:145–170. http://dx.doi.org/10 .1016/j.mam.2010.02.008.
45. Bubici C, Papa S, Dean K, Franzoso G. 2006. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. Oncogene 25:6731–6748. http://dx.doi.org/10 .1038/sj.onc.1209936.
46. Nowsheen S, Aziz K, Kryston TB, Ferguson NF, Georgakilas A. 2012. The interplay between inflammation and oxidative stress in carcinogenesis. Curr. Mol. Med. 12:672–680. http://dx.doi.org/10.2174/156652412800792642.

47. Prasad S, Ravindran J, Aggarwal BB. 2010. NF-kappaB and cancer: how intimate is this relationship. Mol. Cell. Biochem. 336:25–37. http://dx.doi .org/10.1007/s11010-009-0267-2.
48. Zhang YW, Su Y, Volpert OV, Vande Woude GF. 2003. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. Proc. Natl. Acad. Sci. U. S. A. 100:12718–12723. http://dx.doi.org/10.1073/pnas.2135113100.

2.8 Supplementary Figures

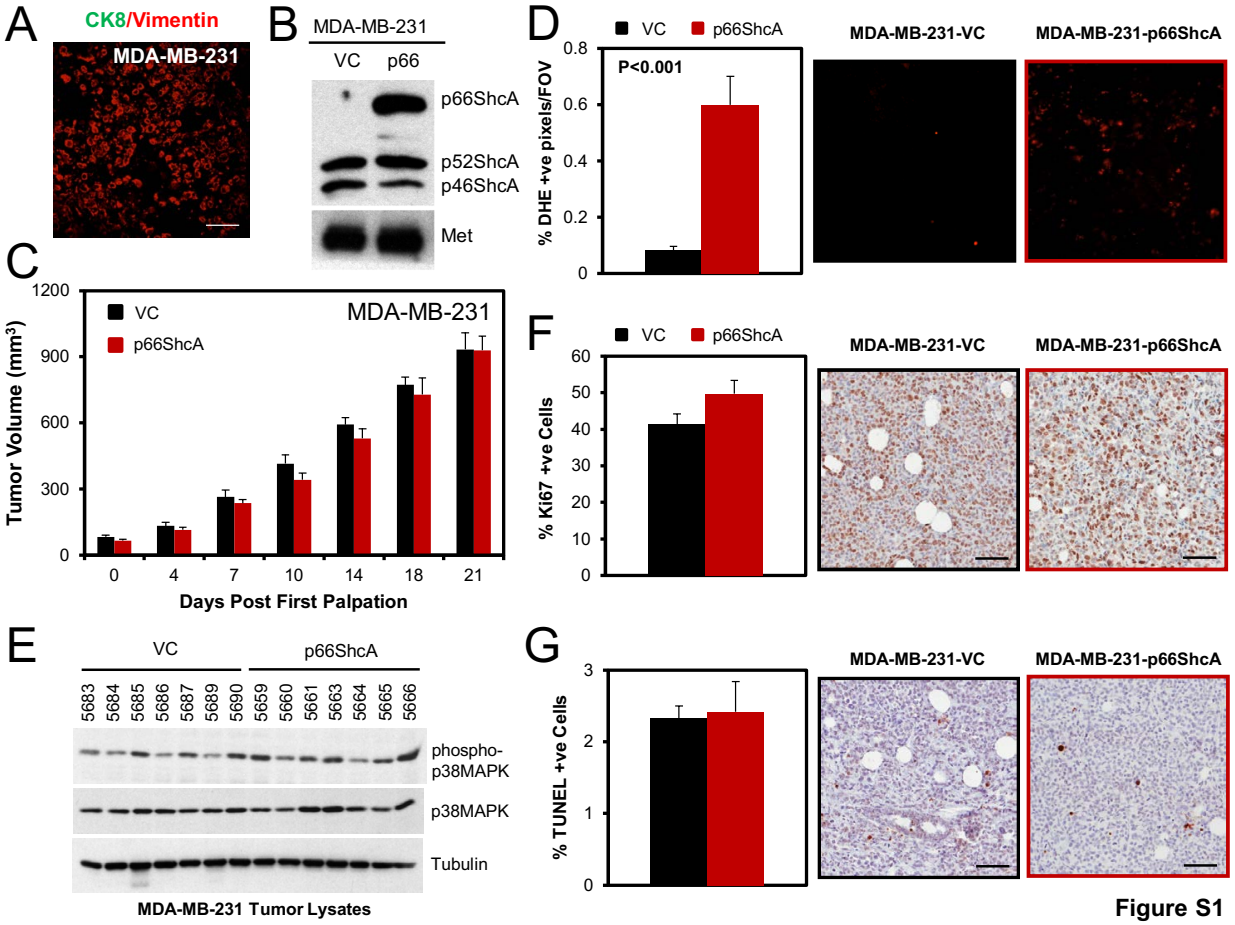


Figure S1: p66ShcA overexpression does not alter the growth potential of Claudin-low breast cancer cells. (A) Immunohistofluorescent staining of MDA-MB-231 mammary tumors using Cytokeratin 8/18 (CK8) and Vimentin-specific antibodies. Scale bar=30 microns. **(B)** Immunoblot of vector control (VC) and p66ShcA overexpressing MDA-MB-231 cell lysates using ShcA and Tubulin specific antibodies. **(C)** Mammary fat pad injection of VC and p66ShcA-overexpressing MDA-MB-231 cells (1 x 106). The rate of tumor growth following first palpation was determined by caliper measurements. The data is recorded as tumor volume (mm3) ± SEM and is representative of 12 independent mice per cell line. **(D)** The percentage of dihydroethidium (DHE) positive cells present in cryosections from the indicated mammary tumors is shown. The data is representative of 55-58 fields (20X) and 7 independent tumors per cell line and is shown as % DHE positive cells/field of view ± SEM. **(E)** Mammary tumor lysates were probed with phospho-p38MAPK, p38MAPK and Tubulin-specific antibodies. **(F)** Ki67 immunohistochemical staining of paraffin embedded sections from the indicated mammary tumors. The data is representative of 7 independent tumors per cell line and a minimum of 20,000 cells per tumor section and is depicted as % Ki67 positive cells ± SEM. All statistical analyses were performed using a two tailed students T test. Scale bar=40 microns. **(G)** TUNEL staining of paraffin-embedded sections from vector control and p66ShcA-expressing tumors. For each section, a minimum of 20,000 nuclei were counted using Image Scope software. The data is presented as the percentage TUNEL positive cells ± SEM (n=7 tumors each). Scale bar=40 microns.

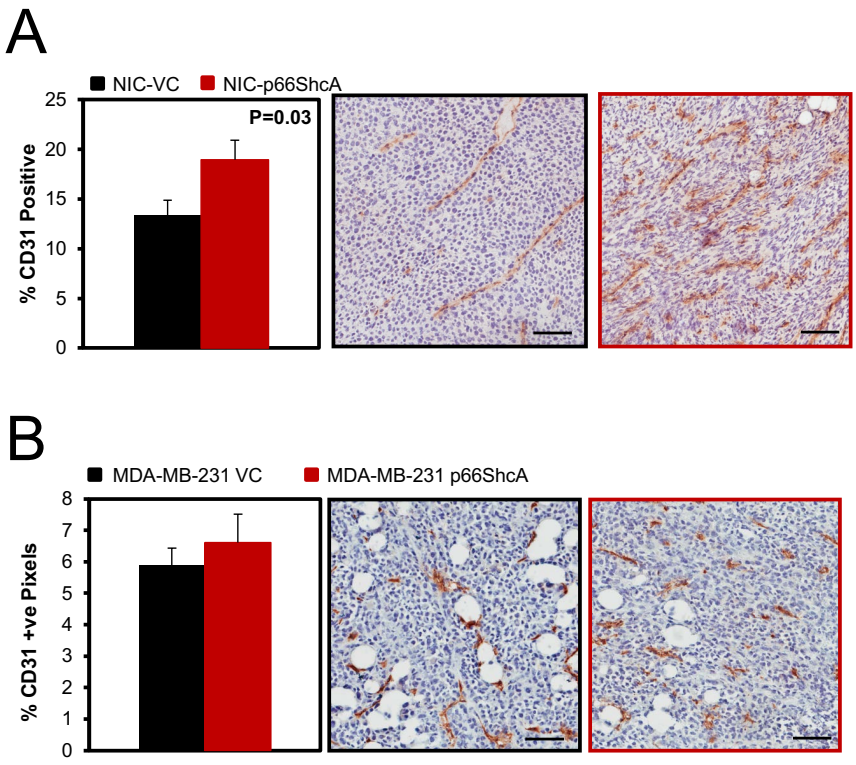


Figure S2

Figure S2: p66ShcA overexpression stimulates vascular endothelial cell recruitment in NIC (luminal) breast tumors but not MDA-MB-231 (basal) breast tumors. CD31 immunohistochemical staining of OCT-embedded sections from NIC-VC and NIC-p66ShcA **(A)** along with MDA-MB-231 VC and MDA-MB-231 p66ShcA **(B)** mammary tumors. For each tumor section, a minimum of 10 20X fields were analyzed using Image Scope software. The data is representative of 6-7 tumors per cell line and is presented as the percentage of CD31 positive pixels ± SEM. Statistical analysis was performed using a two tailed students T test. Scale bar=40 microns.

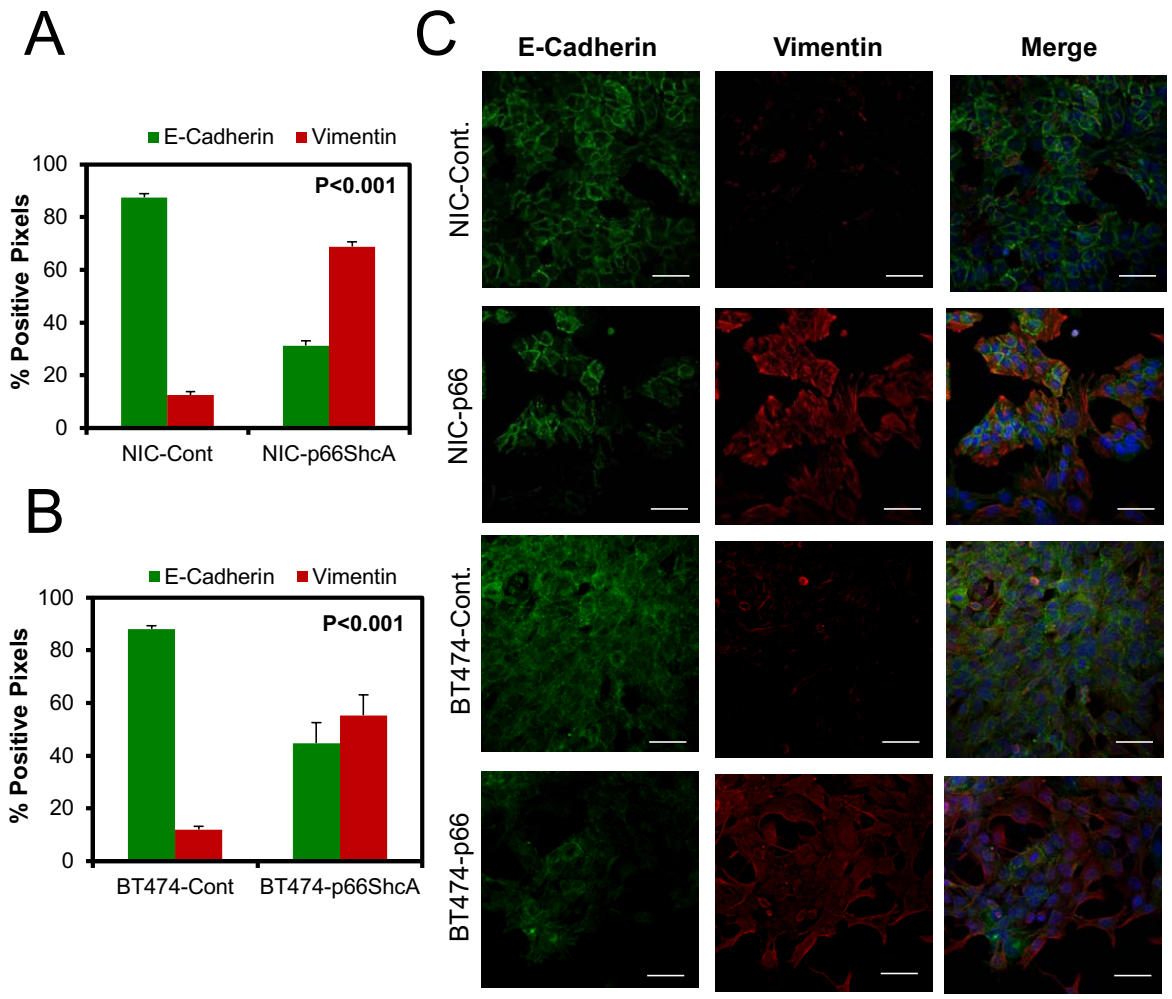


Figure S3

Figure S3: E-Cadherin and Vimentin co-immunofluorescent staining of control and p66ShcA-overexpressing NIC and BT474 cells. (A, B) The percentage E-Cadherin (green) and Vimentin (red) positive pixels over the total number of pixels per 20X field were determined using Image J software. The data is representative of seven (NIC) and 12 (BT474) 20X fields per cell line. Statistical analysis was performed using a two-tailed student's t test. For the BT474 cells, the parental cell line was used as the control as the empty vector already expresses GFP. **(C)** Representative images (20X) of the E-Cadherin (green)/Vimentin (red) co-immunofluorescent staining of control and p66ShcA-expressing NIC and BT474 cells. Scale bar=40 microns.

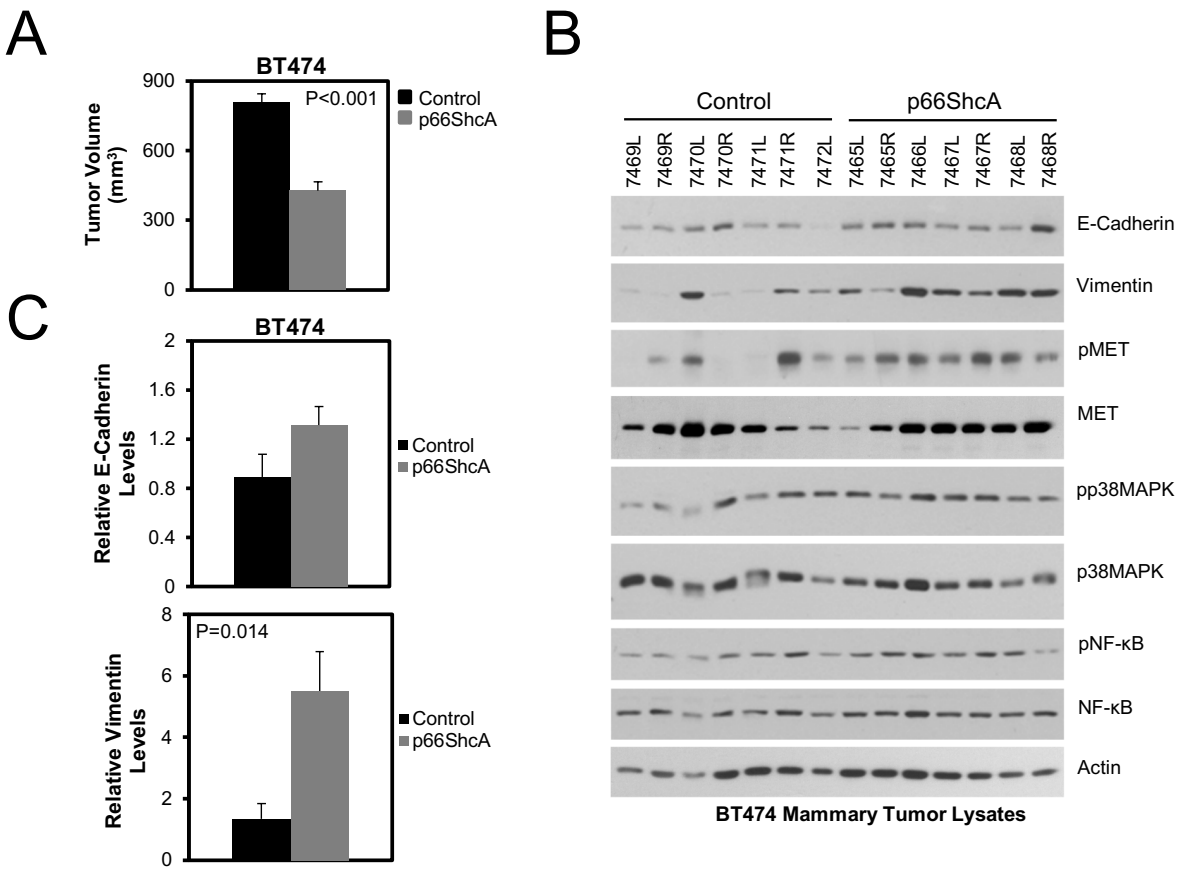


Figure S4

Figure S4: p66ShcA increases the mesenchymal properties of BT474 mammary tumors in vivo. (A) Mammary fatpad injection of control and p66ShcA-expressing BT474 cells (n=7 mice per cell line). The average tumor volume (mm3 ± SEM) at the experimental endpoint was determined by caliper measurements for each cell line. **(B)** Mammary tumor lysates were subjected to immunoblot analysis using the indicated antibodies. The numbers represent individual mice. **(C)** Densitometric analysis of relative E-Cadherin and Vimentin levels in mammary tumors from control and p66ShcA-expressing BT474 cells by Image J software. The data is normalized to Tubulin levels.

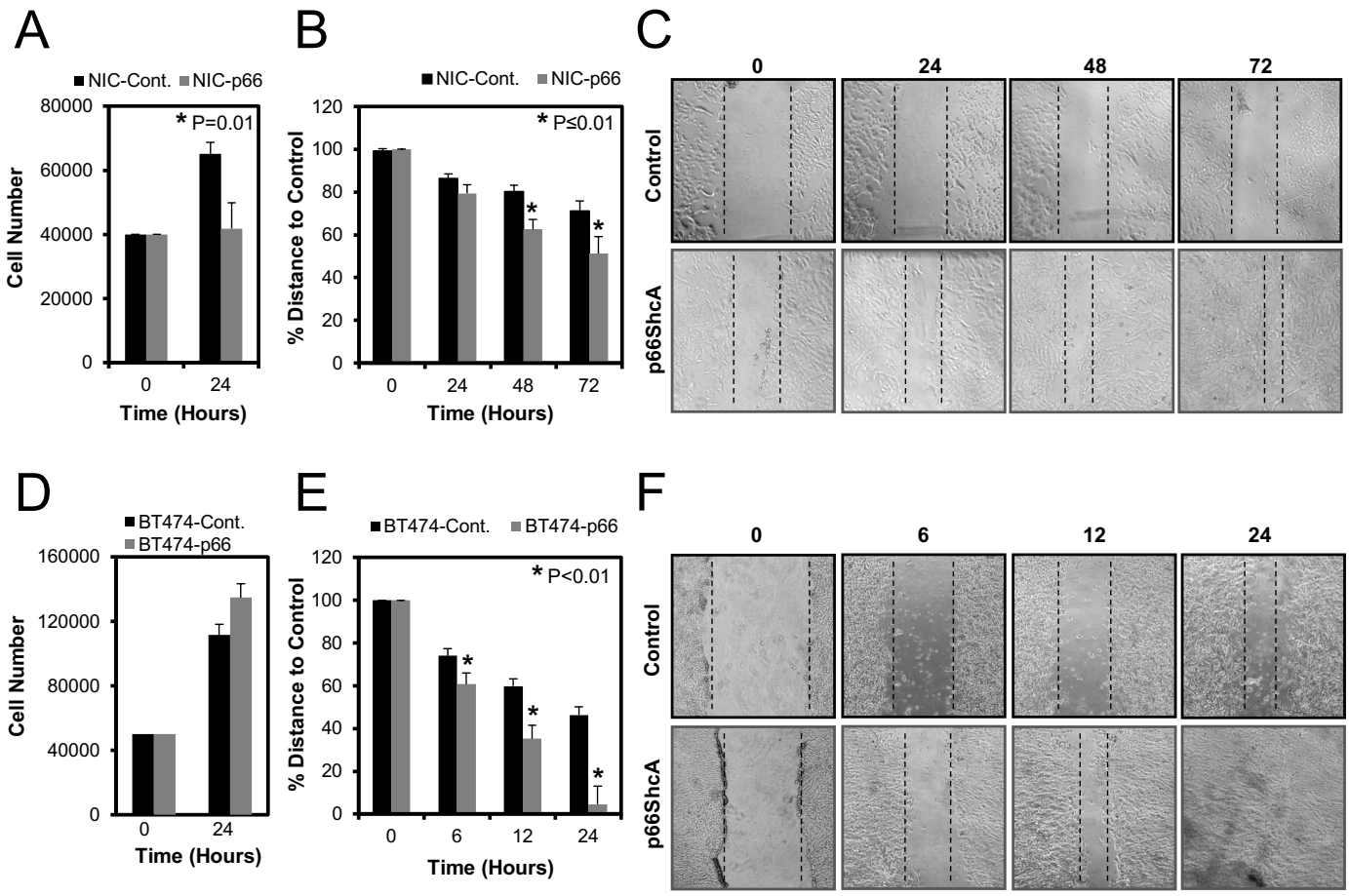


Figure S5

Figure S5: p66ShcA increases the migratory properties of NIC and BT474 luminal breast cancer cells in vitro. (A, B) The growth rate of NIC **(A)** and BT474 **(B)** cells was determined by counting the number of viable cells by trypan blue exclusion after a 24 hour time period. The data is indicative of three independent wells. **(C, E)** Control and p66ShcA-expressing NIC **(C)** and BT474 (E) cells were assessed for their migratory properties using a scratch assay. For the NIC cells, images were captured at the 0, 24, 48 and 72 hour time points while for the BT474 cells, the wells were photographed at the 0, 6, 12 and 24 hour time points. For each time point, the distance between the borders of the scratch was measured by Image J software in three areas (top, middle and bottom). The average distance of the scratch was quantified and expressed as a ratio compared to the 0 hour time point to determine the percent wound closure. The data is representative of 8 independent wells per cell line. **(D, F)** Representative images are shown for the control and p66ShcA expressing NIC and BT474 cells for the various time points.

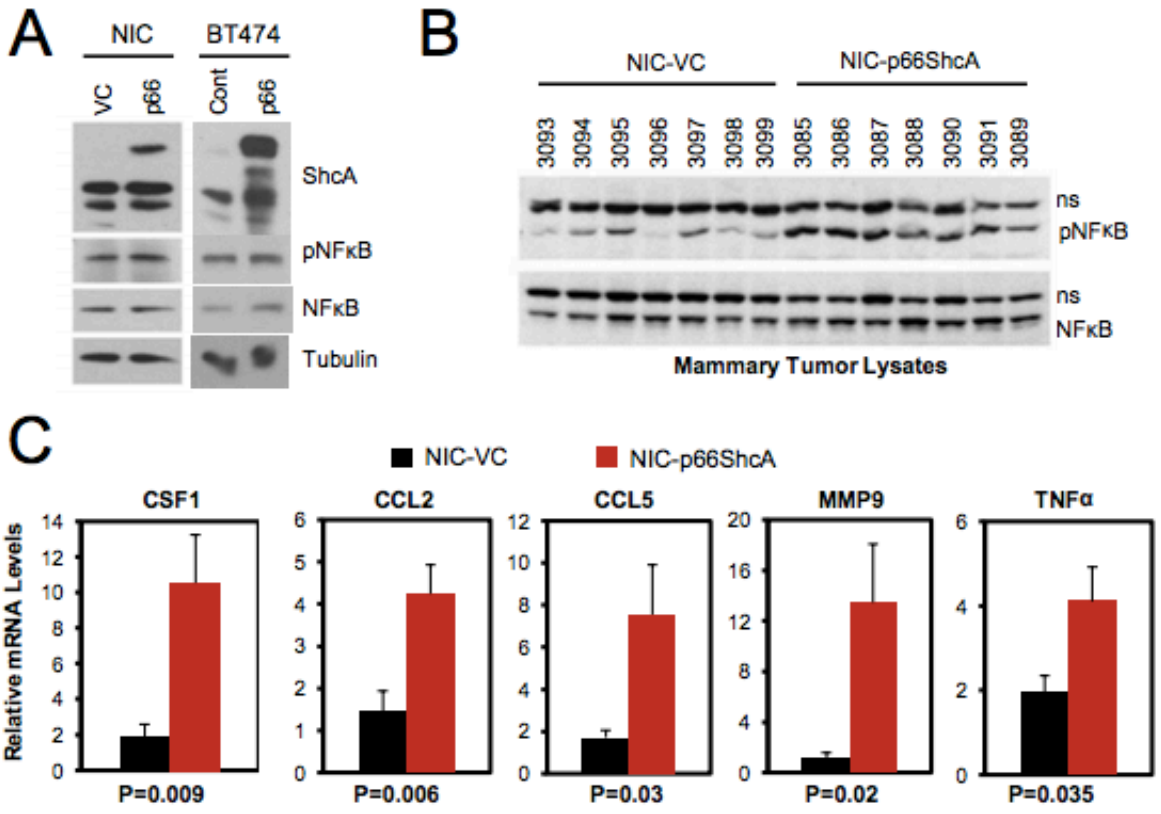


Figure S6

Figure S6: p66ShcA activates NF-κB signaling in mammary tumors in vivo. (A) Immunoblot analysis of total cell lysates from control and p66ShcA expressing NIC and BT474 cells were probed with ShcA, pNF-κB, NF-κB and Tubulin-specific antibodies. **(B)** Immunoblot analysis of NIC-VC and NIC-p66ShcA tumor lysates using pNK-κB, NF-κB and Tubulin specific antibodies. NS refers to a non-specific band. **(C)** RT-qPCR analysis of NIC-VC and NIC-p66ShcA tumors (n=7 tumors each) to quantify CSF1, CCL2, CCL5, MMP9 and TNFα levels (normalized to GAPDH).

Table S1: List of antibodies used in this study.

Antibody Specificity	Company	Dilution (WB)	Dilution (IHC/IHF)	Catalogue Number
ShcA	BD Biosciences	1:2500		610081
pS36-ShcA	ABCAM	1:1000		54518
pS239/240-ShcA	Cell Signaling	1:1000		2434
pERK (T202/Y204)	Cell Signaling	1:2000		9106
ERK	Cell Signaling	1:4000		9102
pAKT (S473)	Cell Signaling	1:1000		9271
AKT	Cell Signaling	1:1000		9272
pJNK (T183/Y185)	Cell Signaling	1:1000		9251
JNK	Cell Signaling	1:1000		9452
pp38MAPK (T180/Y182)	Cell Signaling	1:1000		9215
p38MAPK	Cell Signaling	1:1000		9212
pSMAD2 (S465/S467)	Cell Signaling	1:1000		3101
SMAD2/3	Cell Signaling	1:1000		3102
pMET (Y1234/5)	Cell Signaling	1:1000		3126
MET	R&D	1:1000		AF527
pNF-κB (p65-S536)	Cell Signaling	1:1000		3033
NF-κB (p65)	Cell Signaling	1:1000		3034
ErbB2	Santa Cruz	1:1000		sc284
EGFR	Santa Cruz	1:1000		sc03
E-Cadherin	BD Biosciences	1:10000	1:200	610181
Vimentin	Abcam	1:1000	1:500	ab92547
CK8/18	Fitzgerald Labs		1:200	20R-CP004
SMA	Sigma		1:500	A5228
CK14	Covance		1:500	CLPRB-155P
Ki67	ABCAM		1:500	AB15580
α-Tubulin	Sigma	1:5000		T5168

Supplementary Table 2: Primer sequences used for quantitative real-time polymerase chain reactions.

Gene	Forward Primer Sequence	Reverse Primer Sequence
p66ShcA	TCCGGAATGAGTCTCTGTCA	GAAGGAGCACAGGGTAGTGG
SNAIL	CTTGTGTCTGCACGACCTGT	GCAGTGGGAGCAGGAGAAT
SNAIL2	GGCTGCTTCAAGGACACATT	GATGTGCCCTCAGGTTTGAT
TWIST1	CTCGGACAAGCTGAGCAAG	CAGCTTGCCATCTTGGAGTC
TWIST2	ATGTCCGCCTCCCACTAGC	GTCATGAGGAGCCACAAGGT
ZEB1	TGAAGGTGATCCAGCCAAAC	GGCGTGGAGTCAGAGTCATT
ZEB2	TGGCCTATACCTACCCAACG	GTGCTCCATCCAGCAAGTCT
CDH1	GACGCTGAGCATGTGAAGAA	CAGGACCAGGAGAAGAGTGC
CLDN3	CCAACTGCGTACAAGACGAG	CCAGGACACCGGTACTAAGG
CLDN4	ACCTCGTAGCAACGACAAGC	CAAAGGCAATGTGGACAGAG
CLDN7	TGTACAAGGGGCTCTGGATG	CACCATTAAGGCTCGAGTGG
GAPDH	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC

Chapter 3 - Mitochondrial and cytoplasmic p66ShcA are required for different stages of the metastatic cascade to support triple negative breast cancer lung metastasis

Jesse Hudson*,[a] Kyle Lewis*,[a] Alexander Kiepas*,[b,d] Julien Senécal,[b,d] Matthew G. Annis,[b,d] Jacqueline R. Ha,[a] Valerie Sabourin,[a] Sébastien Tabariès,[b,d] Ryuhjin Ahn,[a] Matthew Siegel, [a] Rachel La Selva,[a] Eduardo Cepeda,[a] Peter Siegel,[b,d] and Josie Ursini-Siegel[a,c,d]

Lady Davis Institute for Medical Research, Montreal, Quebec, Canada[a];

Goodman Cancer Research Centre, Montreal, Quebec, Canada[b];

Department of Oncology, McGill University, Montreal, Quebec, Canada[c];

Department of Biochemistry, McGill University, Montreal, Quebec, Canada[d]

* These authors contributed equally to this work

Manuscript in preparation for submission, December 2018

3.1 Preface

This chapter explores the dual role of the adaptor protein p66ShcA on tumor growth and metastatic progression in triple negative breast cancer (TNBC), as well as understanding the function of different pools of p66ShcA in these processes. In addition, delineating key components of the metastatic cascade regulated by p66hcA was a major interest. Outlining subtype specific roles for p66ShcA in breast cancer was another area of importance as chapter 2 identified both anti- and pro-tumorigenic roles for p66ShcA in ErbB2+ luminal breast cancer as a suppressor of tumor growth, while simultaneously promoting tumor plasticity through induction of an EMT (Hudson et al. 2014). Of significance, we are the first to examine the role of p66ShcA in TNBC breast cancer metastasis and delineated mitochondrial-p66ShcA and cytoplasmic-p66ShcA dependent mechanisms within the metastatic cascade. Considering ROS has been shown be a double-edged sword in cancer depending on cancer type/context and metastasis is the major cause of death in the majority of patients, these studies have high clinical relevance. Finally, several theories exist on the mechanistic nature of the emergence of metastatic traits. Having the tools on hand to study these aspects, we were able to identify the contribution of clonal evolution theory in lung metastasis using *in vivo* selected metastatic variant breast cancer cells.

3.2 Abstract

Over the past few years, widespread screening has allowed for early detection of breast cancer and reduced the mortality rate in developed countries. In addition, the advent of molecular classifications based on gene expression profiling has allowed for the development of personalized medicine and adjuvant treatments. Despite these advances, breast cancer is still the most common cancer in women and one third of patients will develop metastases and die from the disease. The metastatic cascade involves a number of stochastic events, including: invasion of cancer cells into the surrounding tissue and entry into the circulation, survival in the bloodstream, arrest at a distant organ, extravasation into the secondary parenchyma and colonization leading to the formation of overt metastases. In this study, we evaluated the sufficiency and requirement of p66ShcA in TNBC versus ErbB2+ luminal tumors for tumor growth and lung metastasis at different stages of the metastatic cascade. We provide the first *in vivo* evidence that p66ShcA is required for efficient lung metastasis in aggressive TNBC. We utilized the 4T1 mouse breast tumor model (and metastatic variants) with stable overexpression of wild- type p66ShcA or a nonphosphorylatable mutant (p66-S36A) that cannot translocate into the mitochondria. We selected 4T1 cells due to their ability to form tumors quickly in an immunocompetent background (Balb/c) and metastasize to various organs, including lung, liver and bone. We show that mitochondrial versus cytoplasmic pools of p66ShcA regulate different stages of the metastatic cascade. In parental 4T1 cells, cytoplasmic-p66ShcA was sufficient to promote tumor outgrowth through reduced apoptosis and these effects are maintained in breast cancer lung metastases. However, p66ShcA is not sufficient to increase the lung metastatic potential of parental 4T1 cells. Intriguingly, in 4T1 variants that were in vivo selected to preferentially metastasize to the lung, we show that p66ShcA is required to promote lung metastasis, both through mitochondrial-dependent and independent mechanisms.

Mitochondrial p66ShcA is required for entry/survival in the circulation due to elevated CTCs compared to VC and a p66ShcA (S36A) mutant, the latter of which is restricted to the cytoplasm. In contrast, cytoplasmic-p66ShcA was necessary for elevated TNBC cell migration to enter the vasculature and increased focal adhesion dynamics to facilitate lung colonization after entry into the circulation. Furthermore, through screening of parental 4T1 clones (ancestors of lung metastatic variants), we identify high expression of p66ShcA in 1/3 a of pre-existing clones, indicating high p66ShcA levels are enriched in TNBCs through metastatic *in vivo* selection to the lung and liver and suggests a role for p66ShcA as a metastatic initiation and/or progression gene. This evidence supports previous studies indicating metastases often resemble the primary tumor, that driver mutations are a rare event in breast cancer and metastatic progression genes often are already present in the primary tumor and are selected for through environmental factors such as stress and the microenvironment. Finally, p66ShcA is a subtype specific regulator of tumor growth and progression. We show that p66ShcA is sufficient to modestly inhibit tumor growth in ErbB2+ luminal breast cancer through mitochondrial ROS formation. Furthermore, while p66ShcA is not sufficient for enhanced lung metastasis, non-mitochondrial p66ShcA pools suppress dissemination to the lungs in this model.

3.3 Introduction

Metastasis is responsible for 90% of cancer-related deaths and includes efficient and inefficient steps, with the later stages being rate-limiting to successfully establishing metastases (Luzzi et al. 1998). This is due to the fact that a diverse number of cellular functions must be acquired through clonal selection in order to complete the metastatic cascade. During this process, tumor cells must break cell-to-cell contacts in order to detach from the primary tumour and degrade the basement membrane to intravasate into the circulation. Next, within the bloodstream tumor cells must withstand the force of shear stress, avoid the surveillance of circulating immune cells and cope with the loss of cell contacts in order to survive and eventually arrest at a new organ. Afterward, they must extravasate into the surrounding tissue to ultimately colonize and reinitiate growth cues, both in terms of proliferation and survival pathways, to regenerate the tumor mass (Chambers, Groom, and MacDonald 2002).

The metastatic niche consists of resident cells that are essential for physiological functions of the tissue. Extracellular matrix that is deposited and modulated by various cell types during progression, stromal populations, including: endothelial cells that produce new vasculature to feed the tumor, and infiltration and repolarization of immune types that can promote or eliminate the tumor mass. Combined, these diverse cell types make up the tumor microenvironment and there is a great deal of crosstalk through intercellular interactions (Josie Ursini-Siegel and Siegel 2016). Tumor dormancy can describe either tumor cells exiting the cell cycle (G_0–G_1 arrest) or those promoting differentiation as a means to avoid replication in the absence of appropriate growth and survival cues in the pre-metastatic niche of foreign tissues (cellular dormancy). However, dormancy can also be stimulated at the population level due to a lack of perfusion (angiogenic

dormancy) and/or immunosurveillance (immune dormancy) that increase apoptosis and counters the proliferation of disseminated tumor cells (DTC) (Dasgupta, Lim, and Ghajar 2017).

Breast cancer cells have a preference for the bone and lymph nodes during metastasis (Müller et al. 2001), however lung, liver, brain and pleura are also common sites of dissemination. The competence of a tumor to infiltrate and colonize will dictate organ tropism and the period of latency for metastatic progression. Several factors influence dissemination competence, including: circulation patterns, endothelial barriers and survival niches. In contrast, colonization competence is dictated by reactivation signals, stromal partners and therapeutic response (Vanharanta and Massagué 2013). Notably, breast cancer can have a short, medium or long latency in between initial diagnoses and relapse depending on the molecular subtype. The majority of ER negative tumors tend to have a short or medium latency, prior to recurrence, peaking at two years after primary diagnosis and up to five years after initial diagnosis (Breast, Trialists, and Group 2005; Hess et al. 2003). In contrast, the ER positive subtype has a much broader range peaking at around five years after initial diagnosis, shows a preference for bone metastasis and the period of latency can last up to 20 years after initial diagnosis of the primary tumor (Breast, Trialists, and Group 2005; Hess et al. 2003).

Src homology and collagen A (ShcA) is an adaptor protein that relays extracellular signals by coupling to receptor and cytoplasmic tyrosine kinases to control cell proliferation, survival, invasion, and angiogenesis (Josie Ursini-Siegel and Muller 2008b). The ShcA allele encodes three proteins that originate through alternative promoter usage (p66) or alternate translation initiation (p46, p52) (Giuliana Pelicci et al. 1992; Ventura 2002). ShcA adaptor proteins possess two phospho-tyrosine binding motifs; an amino terminal PTB domain and a carboxy terminal SH2 domain in addition to a proline-rich CH1 domain (Peter van der Geer et al. 1995; Migliaccio et al.

1997; Giuliana Pelicci et al. 1992; Ravichandran et al. 1997; Josie Ursini-Siegel and Muller 2008a). p66ShcA is unique in that it contains an N-terminal CH2 domain that functions in mediating an oxidative stress response. In response to stress stimuli, including ROS and UV light, p66ShcA is phosphorylated on serine 36 by stress kinases and this allows for binding of Pin1, which induces a conformational change in p66ShcA that allows for its dimerization and mitochondrial transport. Once in the mitochondria, p66ShcA can catalyze the transfer of electrons from cytochrome c onto molecular oxygen, leading to ROS formation, to induce apoptosis through the opening of the permeability transition pore (Giorgio et al. 2005a). Stable overexpression with a plasmid either empty or encoding p66WT or the serine-to- alanine mutated residue p66Shc (p66S36A) protein in cell lines allows for the study of different pools of p66ShcA. p66WT is able to translocate to the mitochondria, while the serine-to-alanine mutated residue p66Shc (p66S36A) is restricted to the cytoplasm, due to the lack of phosphorylation and inability of Pin1 to bind and induce a conformational change.

Previously, we provided the first *in vivo* evidence of the role of p66ShcA in ErbB2+ breast cancer in inducing an EMT to promote cellular plasticity (Hudson et al. 2014). Reversible conversion between epithelial and mesenchymal states has been associated with aggressiveness, increased tumor heterogeneity and resistance to therapy (Wahl and Spike 2017). However, the role of p66ShcA in recurrence is conflicting and poorly understood in breast cancer (Frackelton et al. 2006; Grossman et al. 2007; Jackson et al. 2000), and even across cancer types, as p66ShcA has been reported to have pro or anti-metastatic features depending on the context (X. Li et al. 2014; Lin 2010). Reactive oxygen species (ROS) are important in the regulation of MAPK signalling, Akt and NF-Kb signalling to control cell proliferation, apoptosis and cell survival. The major source of ROS in the cell is from the mitochondria and the formation of ROS can also enhance the

metastatic potential of breast cancers by perturbing mitochondrial respiration and enhancing migration and/or invasion (Kundu, Zhang, and Fulton 1995; Pelicano et al. 2009). There have not been any *in vivo* studies performed in breast cancer elucidating the role of p66ShcA in this setting and we set out to answer these questions.

3.4 Materials and Methods

Cell Lines

4T1 cells were obtained from the ATCC. 4T1 cells were cultured in 10% FBS DMEM, 1% penicillin/streptomycin, and 0.1% gentamycin (Wisent Bioproducts). 4T1 metastatic variant cells were obtained by in vivo selecting breast cancer cells through the mammary fatpad, lungs, liver or bone and maintained similar to parental cells (10% FBS DMEM, 1% penicillin/streptomycin, and 0.1% gentamycin (Wisent Bioproducts)) (Tabariès et al. 2015). The NIC tumor cell line was established from a MMTV/Neu-IRES-Cre transgenic mouse mammary tumor and maintained in DMEM/5% FBS supplemented with mammary epithelial growth supplement (Invitrogen) (Josie Ursini-Siegel et al. 2008). A C-terminal, FLAG-tagged mouse p66ShcA cDNA was sub-cloned into the XhoI/EcoRI sites of pMSCV-puro (NIC cells) (Clontech) or the NotI/EcoRI sites of pQXCIP-blast (4T1 cells) or pQXCIP-puro (4T1-537 cells) (Addgene) and used for the generation of stable cell lines. NIC cells were transfected with the pMSCV-puro vector and maintained in 2 μg/ml puromycin, while the 4T1 cells were transduced with the pQXCIB/p66ShcA vector and maintained in 10 μg/ml blasticidin and 4T1-537 cells were transduced with the pQXCIP/p66ShcA vector and maintained in 2 μg/ml puromycin.

Primary tumor growth, metastasis assays and analysis of tumor tissue

For in vivo tumor outgrowth studies, 50,000 (4T1) or 1 million (NIC) breast cancer cells were injected into the fourth gland of the mammary fat pad of Balb/c mice (4T1). Tumor volumes were

determined by caliper measurement and tumor volume was calculated according to the following formula: $4/3\pi(\text{length}/2)(\text{width}/2)2$ where L refers to the length and W to the width of the tumor as previously described (Josie Ursini-Siegel et al. 2008). At endpoint, tumor tissue was harvested for immunoblot or immunohistochemical analysis.

For spontaneous metastasis assays, 10,000 4T1 cells, 5,000 4T1-537 cells or 500,000 NIC tumor cells were resuspended in a 50:50 mixture of 1x phosphate-buffered saline/matrigel and were injected into the fourth mammary gland of 6-8-week-old female Balb/c mice (for 4T1/537 cell) or SCID-Beige (for NIC cells) (Taconic). Following first palpation, tumor volumes were measured on a bi- or tri-weekly basis using calipers, as described (Josie Ursini-Siegel et al. 2008). Mammary tumors were resected at a volume of $500mm^3$, and tumor tissue was harvested for immunoblot or immunohistochemical analysis. Animals were killed 2-3 weeks post resection and lung tissue was processed and analyzed as previously described (Josie Ursini-Siegel et al. 2008). Hematoxylin and eosin lung sections were quantified using Aperio ImageScope software (Leica Biosystems, Concord, ON, Canada). Metastatic tissue was quantified from a step section and expressed as a percentage of total lung area.

Experimental metastasis assays were performed by injecting 100,000 4T1-537 cells directly into the lateral tail veins of Balb/c mice. All mice were sacrificed 3-4 weeks after tail vein injection to determine the extent of lung metastasis. In all cases, lungs were removed, embedded in paraffin, and subjected to hematoxylin/eosin staining to evaluate the metastatic burden. For scoring the presence of lung metastases, step sections of the entire lung were taken.

Animal studies were approved by the Animal Resources Centre at McGill University and comply with guidelines set by the Canadian Council of Animal Care.

Immunoblotting and RT-qPCR studies

Immunoblots were performed as described (Josie Ursini-Siegel et al. 2008) using the antibodies listed in Table S1. Semi-quantitative analysis of immunoblots was determined using Image J software. For the RT-qPCR studies, total RNA was isolated using RNeasy midi-kits (Qiagen) and cDNA was generated using Superscript Reverse Transcriptase II (Invitrogen). Quantitative PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen). (The only qPCRs mentioned in this paper at the moment are the data not shown comments for CCL5 and PGC1-alpha in the discussion section, I'll remove this section if they're not kept).

Immunohistochemistry

Immunohistochemical staining of paraffin embedded tissue was performed as described (Josie Ursini-Siegel et al. 2008). The antibodies are listed in Table S1. Quantification of stained sections was performed using Aperio Imagescope software. To quantify Ki67, cleaved caspase 3 and 4-HNE, vimentin and E-cadherin stained sections, the positive pixel count was determined using Imagescope software (Aperio). Ten 20X images were analyzed for every stained tumor sample (from the fatpad or lungs) and positive pixels were expressed as a percentage of total pixels/ field.

Live cell imaging and Focal Adhesions

Cells were plated the evening before imaging and images were acquired every 10 minutes for 24h. For the live cell imaging analysis, we manually tracked every cell for a minimum of 36 time points (6h) up until cell division. Average migration speed of breast cancer cells, live cell migration tracks and focal adhesion aspects were determined by fluorescence intensity, while assembly and disassembly rates were determined from changes in mean fluorescence intensity.

Boyden Chamber Assays

Boyden chamber assays were performed as described (Northey et al. 2008) using 200,000 4T1-537 cells and cell migration was monitored for 24H.

Metastasis colonization assay

4T1-537 breast cancer cells were labelled using Vybrant CFSE (Invitrogen:V12883). Cells were washed with PBS (twice) and $5x10^6$ cells (in 1mL) were incubated with 5uM CFSE in PBS for 15 minutes on ice. Then 15 minutes at 37°C.

Experimental metastasis assays were performed by injecting 200,000 cells directly into the lateral tail veins of Balb/c mice. All mice were sacrificed 1H or 24H following tail vein injection to determine the extent of lung metastasis. Lungs were perfused with 4% PFA prior to removal. Lobes were separated and whole mounted. Fluorescent images were captured using a confocal microscope (Zeiss LSM510-META).

Circulating tumour cells assay

Tumour-bearing mice were placed under terminal anaesthesia, whole blood was drawn by cardiac puncture and the interphase containing mononuclear cells was isolated following gradient separation. The cell pellet was then incubated with red blood cell lysis buffer and washed as per manufacturer's protocol (cat#: 555899, BD Pharm Lyse). The final pellet was resuspended in the appropriate cell media and plated onto fibronectin-coated plates (cat# FC010, Millipore). The next day, cell media was changed and 2 μg/ml puromycin was added to promote CTC selection. After 14 days, the adherent CTC-derived cell colonies were fixed in formalin and stained with crystal violet. Images were captured with a Zeiss microscope (AxioZoom v16) and the number of colonies per image were determined using a Cell Counter.

Statistical Analysis

All statistical analysis was performed using a one-way Anova with a Tukey's multiple comparisons test with the exception of Figure 1, where a two-tailed Students T test was used.

3.5 Results

p66ShcA is sufficient to elevate primary tumor growth, but not lung metastasis in TNBC

In order to interrogate the sufficiency of p66ShcA to regulate breast cancer growth and progression in TNBC, we utilized the 4T1 mouse breast tumor model with stable overexpression of vector control or wild-type p66ShcA (Fig. 1, panel A) due to their ability to form tumors quickly in an immunocompetent background (Balb/c) and metastasize to various organs, including lung, liver, and bone (Lu and Kang 2007; Pulaski and Ostrand-Rosenberg 2001). Mammary fatpad injection of TNBC cells stably overexpressing p66ShcA (Fig. 1, panel B) significantly increased tumor outgrowth compared to vector control (VC) tumors. Notably, these effects were the reverse of the p66shcA-dependent phenotypes we saw in ErbB2 positive luminal breast cancer, where stable overexpression of p66ShcA (S1, panel A) in the NIC cell line (Josie Ursini-Siegel et al. 2008), lead to significantly reduced tumor outgrowth compared to vector control (VC) tumors (S1, panel B). These effects were dependent on reduced cell proliferation as NIC-VC tumors possessed significantly more Ki67 compared to NIC-p66ShcA tumors, while apoptosis (cleaved-caspase 3) remained unchanged between groups (S1, panels D and E). In order to identify the mechanism of elevated tumor outgrowth in TNBC, the amount of tumor cell proliferation (Ki67) and apoptosis (cleaved caspase 3) were quantified by immunohistochemistry. Notably, overexpression of p66ShcA in TNBC lead to significantly increased cell survival compared to TNBC-VC tumors (Fig. 1, panel D), while cell proliferation remained unchanged (Fig. 1, panel C). These results suggest that p66ShcA may mediate its pro-tumorigenic effects on TNBC growth by reducing apoptosis. Hence, p66ShcA may promote or suppress tumor growth through unique mechanisms, regulating both cell proliferation and apoptosis depending on the context. Next, we interrogated whether p66ShcA could impact lung metastasis in TNBC. Stable overexpression of p66ShcA led

to a modest increase in lung metastatic burden, compared to VC tumors, that did not exceed the differences in tumor growth seen from the mammary fatpad (Fig. 1, panel E). Therefore, p66ShcA is not sufficient for lung metastasis in TNBC and functions primarily as a tumorigenic gene to increase tumor growth. In the ErbB2 positive subtype, stable overexpression of p66ShcA was also not sufficient to promote metastasis relative to vector control tumors (S1, panel C). However, lung metastatic burden was significantly reduced in the NIC-p66ShcAS36A group compared to NIC-VC and NIC-p66ShcA (~30% reduced to 1%), despite both NIC-p66ShcA, NIC-p66ShcAS36A tumors undergoing a partial EMT (S1, panels F and G). These data appear contradictory as p66ShcA has been shown to promote an EMT (Hudson et al. 2014), which is associated with tumor aggressiveness, and EMT induction occurs at the earliest stages of transformation (up to ~50mm^3) (Xue, Plieth, and Venkov 2003). However, ErbB2-driven transgenic mouse models are known to disseminate early (Hüsemann et al. 2008). Hence the advantages provided by an EMT may have already been provided in the NIC model or are not sufficient to further amplify metastatic progression in this model. Notably, NIC-p66ShcS36A tumors did not display reduced cell proliferation (Ki67) or increased apoptosis (Cleaved-caspase 3) from the primary site, indicating the reduced metastatic potential did not stem from primary phenotypes.

p66ShcA is required for efficient lung metastasis in aggressive TNBC

Breast cancers are capable of metastasizing to different organs, including: bone (most common), and organotropism exists even across subtypes. Screening for p66ShcA expression

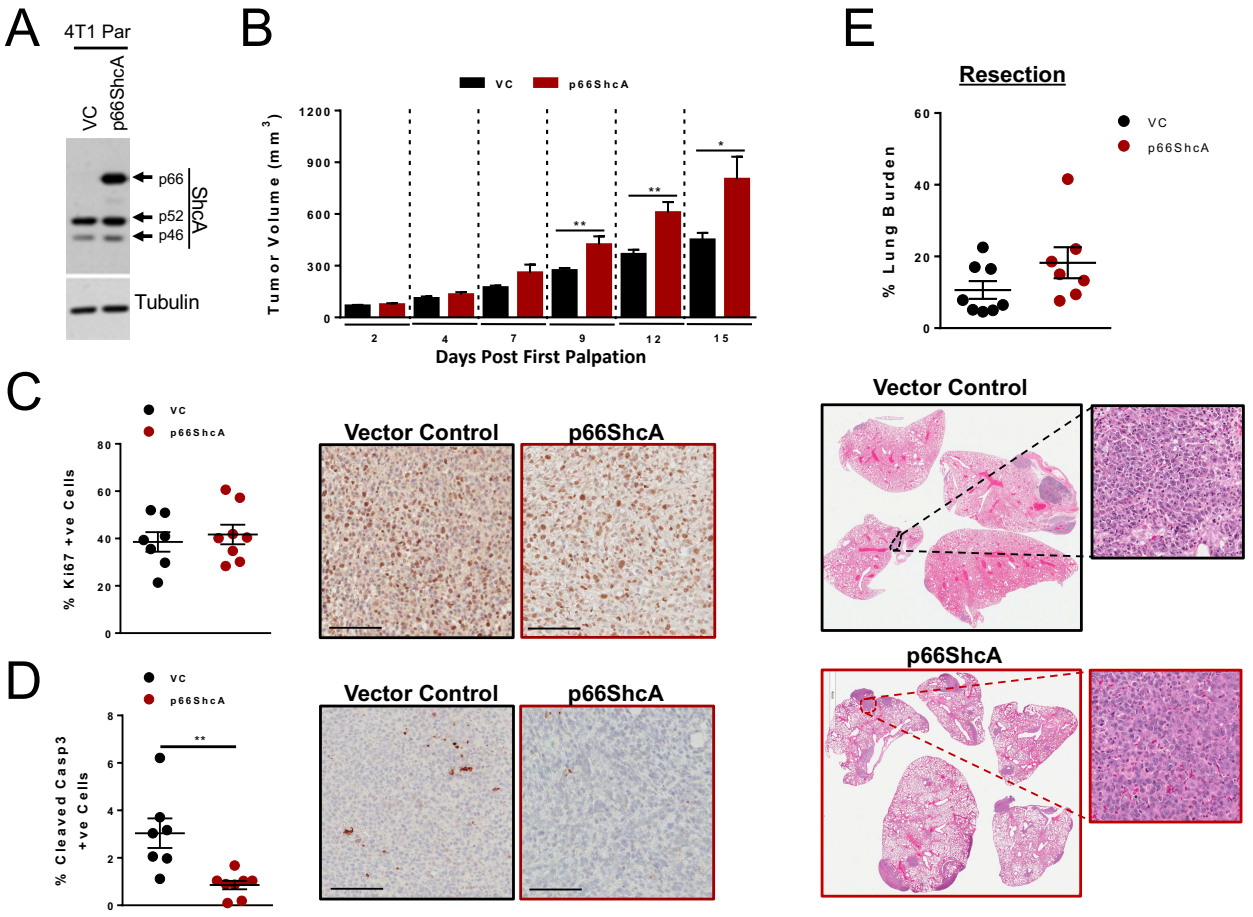


Figure 1 - p66ShcA expression is not sufficient to enhance TNBC lung metastasis (A) Immunoblot analysis of ShcA in vector control (VC) and p66ShcA-overepressing parental 4T1 breast cancer cells. An immunoblot for α-Tubulin served as a loading control. **(B)** Mammary fat pad (MFP) injections of VC and p66ShcA-overexpressing parental 4T1 cells. The data is shown as average tumor volume (mm^3) ± SEM (n=13 tumors/group). **(C)** Ki67 immunohistochemical (IHC) staining of VC and p66ShcA-overexpressing 4T1 mammary tumors. The data is shown as the average number of positive cells ±SEM (n=8 tumors/group). **(D)** Cleaved caspase-3 IHC of VC and p66ShcA-overexpressing 4T1 mammary tumors. The data is shown as average number of positive cells ±SEM (n=8 tumors/group). **(E)** Metastatic tumor burden in the lungs of mice bearing VC- and p66ShcA-overexpressing 4T1 mammary tumors. Mammary tumors were resected at 500 mm^3 and the development of lung metastases was quantified 14 days later. The data is shown as the average percentage of lung metastasis area to lung tissue area ±SEM (n=8 tumors/group). Representative images are shown. Statistical analysis was performed using a two-tailed unpaired student's t test (*, $P < 0.05$; **, $P < 0.01$).

levels across a panel of in vivo selected metastatic TNBC cell lines through the mammary fatpad, bone, liver and lung, we found p66ShcA to be significantly overexpressed in breast cancer cell lines that are metastatic to the lung and liver (S2, panel A). Of note, p66ShcA was selectively enriched only upon in vivo selection of breast cancer cells from these distinct metastatic sites and not through selection in the mammary fatpad. This indicates that p66ShcA may contribute to the metastatic cascade in metastatic breast cancers. Notably, screening the TNBC parental clones it was discovered that p66ShcA is upregulated to levels seen in the lung metastatic variants in about 1/3 of clones, indicating these cells are a minor, but significant population that are already present in the primary tumor and likely are selected for during the metastatic cascade (S2, panel B).

As lung metastasis is the most common site of recurrence in TNBC (Smid et al. 2008; Wei and Siegal 2017), we sought to test the requirement of p66ShcA on tumor growth and lung metastatic progression by using a lung metastatic 4T1 variant (4T1-537) that were genetically engineered to delete endogenous p66ShcA (Crispr/Cas technology) and then re-expressed with vector control (VC), wild-type p66ShcA(WT) or a nonphosphorylatable p66ShcA(S36A) mutant. The CRISPR guide targets exon2 within the CH2 domain of p66ShcA (S2, panel C) to specifically remove p66ShcA while leaving the p46/p52ShcA isoforms untouched. We then pooled clones and exogenously rescued with p66ShcAWT or p66ShcAS36A (S2, panel D). Mammary fatpad injections revealed loss of endogenous p66ShcA did not alter the growth potential of lung aggressive TNBC tumors. Furthermore, neither mitochondrial or cytoplasmic pools of p66ShcA were required for tumor growth in aggressive TNBC compared to VC tumors as there were no significant differences between groups (Fig. 2, panel A). Next, we interrogated whether tumor growth correlated with the regulation of cell proliferation, cell death and lipid peroxidation levels within the primary tumor. 4-HNE is a reliable marker of lipid peroxidation and a measure of

oxidative stress (Z. Feng, Hu, and Tang 2004). Despite this, no significant differences were seen between groups in lung metastatic TNBC cells for Ki67, cleaved caspase 3 or 4-HNE (S3, panels A, B and C). This is in contrast to our findings in parental 4T1 tumors whereby p66ShcA overexpression increased primary tumor growth. This indicates that p66ShcA is dispensible for primary tumor growth of TNBC tumors that have already acquired aggressive metastatic properties.

Next, we sought to determine the requirement of p66ShcA in lung metastasis from the primary site by performing mammary fatpad injections followed by surgical resections. Metastasis is a largely inefficient process. Hence, removal of the primary tumor allows for disseminated tumor cells lodged at secondary organs to form overt metastases over a period of latency. Indeed, p66-CR(WT) was necessary for aggressive 4T1-537 tumors to disseminate to the lung as loss of p66-CR(VC) in aggressive TNBC tumors led to a ~10-fold reduction in lung metastatic burden compared to parental controls. Next, we tested whether rescue of mitochondrial p66-CR(WT) or cytoplasmic p66-CR(S36A) would be required to rescue the metastatic potential of these 4T1-537 cells. Intriguingly, this mechanism was dependent on mitochondrial-p66ShcA, as rescue of p66-CR(WT) but not p66-CR(S36A) resulted in a significant, but partial (4-fold increase) rescue in lung metastatic burden compared to VC tumors (Fig. 2, panel C). In addition, p66-CR(WT) rescue lead to significantly more mice with at least one detectable metastasis compared to control tumors p66-CR(VC) (Fig. 2, panel B). Hence, we hypothesize that p66ShcA aids breast cancer cell entry and survival within the circulation or to extravasate and colonize the lung.

To test this, we looked at the requirement of p66ShcA to exit the circulation, which would delineate a role for p66ShcA late in the metastatic cascade in either extravasation or lung colonization. To do this, we injected parental 4T1-537 cells, or lung variants p66-CR(VC), re-

expressing p66ShcA p66-CR(WT) or S36A mutant p66-CR(S36A) directly into the bloodstream. Notably, p66ShcA was required for lung colonization upon entering the bloodstream. Loss of p66ShcA (parental TNBC cells compared to p66-CR(VC) significantly reduced metastasis (Fig. 2, panels D), however these effects were independent of the p66ShcA S36 phosphorylation status (Fig. 2, panels D and E), as both 4T1-537 cells expressing p66-CR(WT) or p66-CR(S36A) mutant restored lung metastatic burden to levels seen in parental tumors. Of note, genetic deletion of p66ShcA may have caused off-target effects that influenced metastasis as re-expression of p66ShcA only partially rescued metastatic potential in spontaneous and experimental metastasis systems (Anderson et al. 2018). A second possibility is that p66 is no longer under the transcriptional control of the endogenous promoter and that elevated p66ShcA levels only allow a partial rescue. Hence, cytoplasmic-p66ShcA is necessary for colonization once in the circulation, while mitochondrial-p66ShcA enhances dissemination early in the metastatic cascade.

Non-mitochondrial functions of p66ShcA support breast cancer cell migration

As p66ShcA has previously been shown to induce an EMT, we compared the expression levels of p66ShcA, the epithelial marker E-cadherin or the mesenchymal marker Vimentin to Tubulin or p52ShcA as relative controls to determine if p66ShcA regulates EMT in 4T1-537 tumors and whether this might play a role in the metastatic phenotypes we were seeing (S4, panel A). Across groups in the lung variants (S4, panels B and C), there was no significant difference between Vimentin or E-cadherin expression when compared to Tubulin or p52 loading controls despite significant differences in p66ShcA expression (S4, panels B and C). Therefore, the

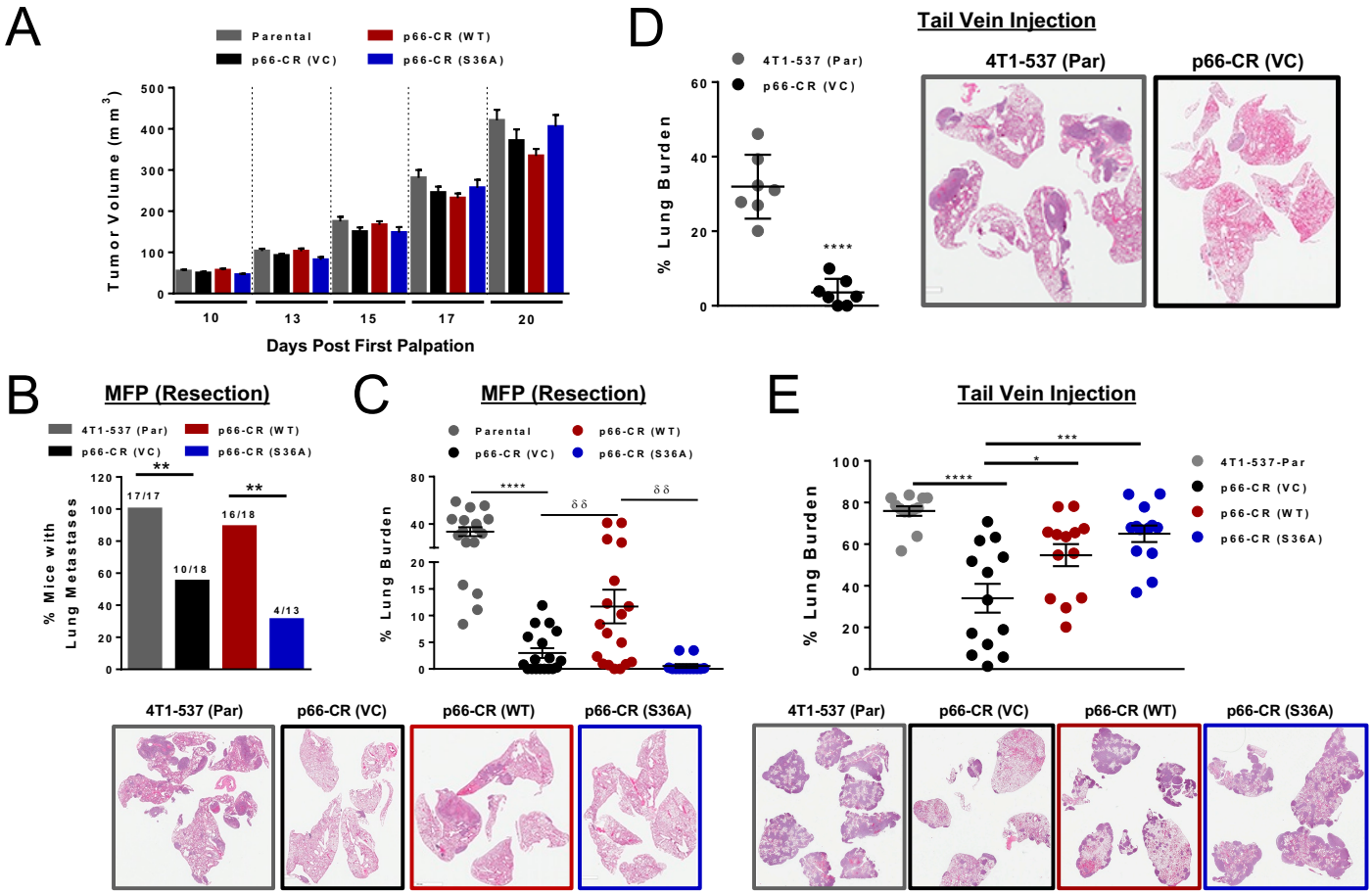


Figure 2 - p66ShcA is required for efficient triple negative breast cancer lung metastasis
(A) Mammary fat pad (MFP) injection of parental, p66-CR(VC), p66-CR(WT) and p66-CR (S36A) expressing lung metastatic 4T1 cells (537 population). The data is shown as average tumor volume (mm^3) ± SEM (n=18 tumors/group). **(B)** Percentage of mice with lung metastases following primary tumor resection. Mice were sacrificed 21 days post-resection of the primary tumor. Statistical analysis was performed using a Fishers exact test (**P<0.01). **(C)** Metastatic burden, following primary tumor resection, in the lungs of mice bearing the indicated 537 breast cancer cell populations. The data is shown as average lung tumor burden ±SEM (parental: n=17; p66-CR(VC): n=18; p66-CR(WT): n=18; p66-CR(S36A): n=13). Representative images are shown. (D) Metastatic burden in the lungs of mice following tail vein injection of the indicated 537 lung-metastatic breast cancer cells. Mice were sacrificed 28 days post-injection. The data is shown as average lung tumor burden ±SEM (n=7 mice per cohort). (E) Metastatic burden in the lungs of mice following tail vein injection of the indicated 537 lung-metastatic breast cancer cells. Mice bearing parental 537 cells were necropsied 21 days post-injection, whereas the remaining mice were necropsied 26 days post-injection. Representative images are shown. The data is shown as average lung tumor burden ±SEM (n=13 mice per cohort). For panel B, statistical analysis was performed using a Fishers exact test (**P<0.01). For panels C-E, statistical analysis was performed using a one-way Anova with a Tukey's multiple comparisons test (*P<0.05; **P<0.01; ***P<0.001).

increased metastatic potential of p66ShcA-expressing cells is not the result of an EMT. Given that p66ShcA was necessary for metastasis from the primary tumor, in vivo, we questioned whether mitochondrial-p66ShcA provided 4T1-537 cells with an increased migratory capacity to enter the circulation. Loss of p66 expression from aggressive parental 4T1-537 cells reduced cell migration by 50% (Fig. 3, panel A), and intriguingly p66ShcA was necessary for cell migration independent of serine 36 phosphorylation. We further show that restoring p66ShcA(WT) or p66ShcA(S36A) expression into these p66ShcA-null cells rescued the migratory potential back to those observed with parental 4T1-537 cells. Complementing this data, loss of p66ShcA reduced the distribution of cells migrating at a speed of 25-55um/h (parental) to between 15-35um/h p66-CR(VC), which was rescued back to 25-60um/h p66-CR(WT) or 25-65um/h with rescue of p66-CR(S36A) (Fig. 3, panel B). Next, loss of p66ShcA reduced the migratory spread of 4T1-537 cells on a X/Y positional map from over 40um/h (parental) to 20um/h (p66-CR(VC)) that was rescued both by p66-CR(WT) and p66-C65fR(S36A) groups (Fig. 3, panel C). Thus, these data indicate that cytoplasmic p66ShcA is required to migrate at an elevated average speed and to cover a greater surface area. Moreover, using boyden chamber assays to complement our live imaging results, we also found p66ShcA was required for increased migration, independent of p66S36A, as loss of p66ShcA reduced migration by 2-fold and rescue of p66ShcA in p66-CR(WT) and p66-CR(S36A) groups restored migration to parental levels (Fig. 3, panel D). These findings suggest p66ShcA may be important for exiting the primary site and entering the circulation, however, additional mitochondrial-p66ShcA dependent mechanisms are required for survival in the blood.

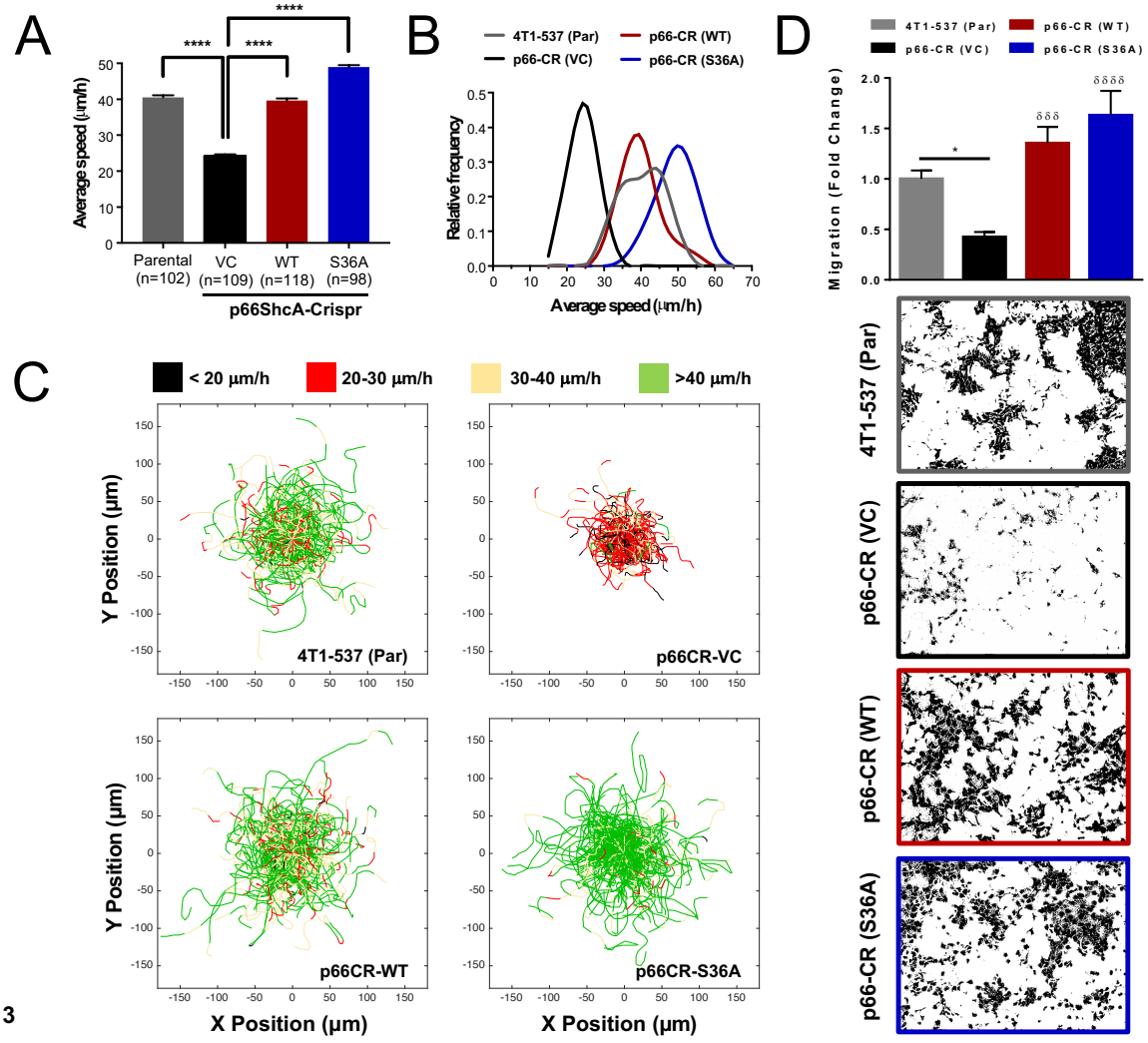


Figure 3

<u>Figure 3</u> - Non-mitochondrial functions of p66ShcA support breast cancer cell migration. **(A)** Average migration speed of the indicated cell lines was determined by live cell imaging. The data is representative of average speed (µm/h) ± SEM from three independent experiments. The number of cells analyzed per cell lines is indicated. **(B)** Frequency distribution of migration speeds from panel A. Data values were binned into 5µm/h segments and smoothed using Lowess. **(C)** Live cell migration tracks of the indicated cell lines on fibronectin-coated plates. Each line represents the migration path of a single cell over a 6-hour period (parental: n=102; p66-CR(VC): n=109; p66-CR(WT): n=118; p66-CR(S36A): n=98). The starting point of each cell was superimposed onto the origin (0, 0). Tracks were color-coded based on cell speed (calculated for each 40-minute interval: black, < 20 µm/h; red, 20-30 µm/h; yellow 30-40 µm/h; green >40 µm/h). Data represents tracks from three independent experiments. **(D)** Boyden chamber assays to determine the migratory properties of the indicated cell lines. The data is shown as fold change in cell migration relative to the parental 4T1-537 cell line ± SEM and is representative of 9 wells over three independent experiments. Representative images are shown. For panels A and D, statistical analysis was performed using a one-way Anova with a Tukey's multiple comparisons test (*P<0.05; ***P<0.001; ****P<0.0001).

Non-mitochondrial p66ShcA accelerates the dynamics of fibrillar adhesion formation

Cell migration involves assembly of focal adhesion kinase (FAK), paxillin and other proteins at focal adhesions within leading protrusions of cells and disassembly at the rear and the base of cell protrusions (Webb et al. 2004). These adhesions are closely linked to the actin cytoskeleton and the rate of adhesion (known as adhesion turnover) dictates protrusion generation and traction forces to regulate cell migration (Mitra, Hanson, and Schlaepfer 2005). We stably overexpressed mCherry paxillin in aggressive 4T1-537 cells with or without deletion of p66ShcA (p66-CR(VC)), followed by rescue of p66ShcA(WT) and p66-CR(S36A) to test whether p66ShcA increases cell motility through altered adhesion turnover (Fig. 4, panel A). Indeed, focal adhesion size and the abundance of larger adhesions were significantly elevated with genetic deletion of p66ShcA compared to parental cells and these effects were reversed upon restoration of p66null cells with p66ShcAWT or p66ShcA-S36A constructs (Fig. 4, panel C and D). Hence, loss of p66ShcA leads to larger focal adhesions, which correlates with reduced migration. Furthermore, p66ShcA was required for elevated focal adhesion assembly and disassembly as rescue of p66ShcAWT or p66ShcAS36A expression in 4T1-537 cells restored focal adhesion assembly and disassembly rates to those seen in parental cells relative to p66null controls (Fig. 4, panel E). Hence, cytoplasmic-p66ShcA appears to play a pro-metastatic role in TNBC by enhancing focal adhesion turnover. These results indicate that elevated focal adhesion turnover might also play an important role in colonization and the late stages of metastasis where adhesion to the lung parenchyma and microenvironment is a crucial component to reactivating pathways necessary for growth cues.

p66ShcA increases breast cancer spread into the bloodstream and lung colonization

To support our metastatic findings, we compared the expression levels of p66ShcA, the

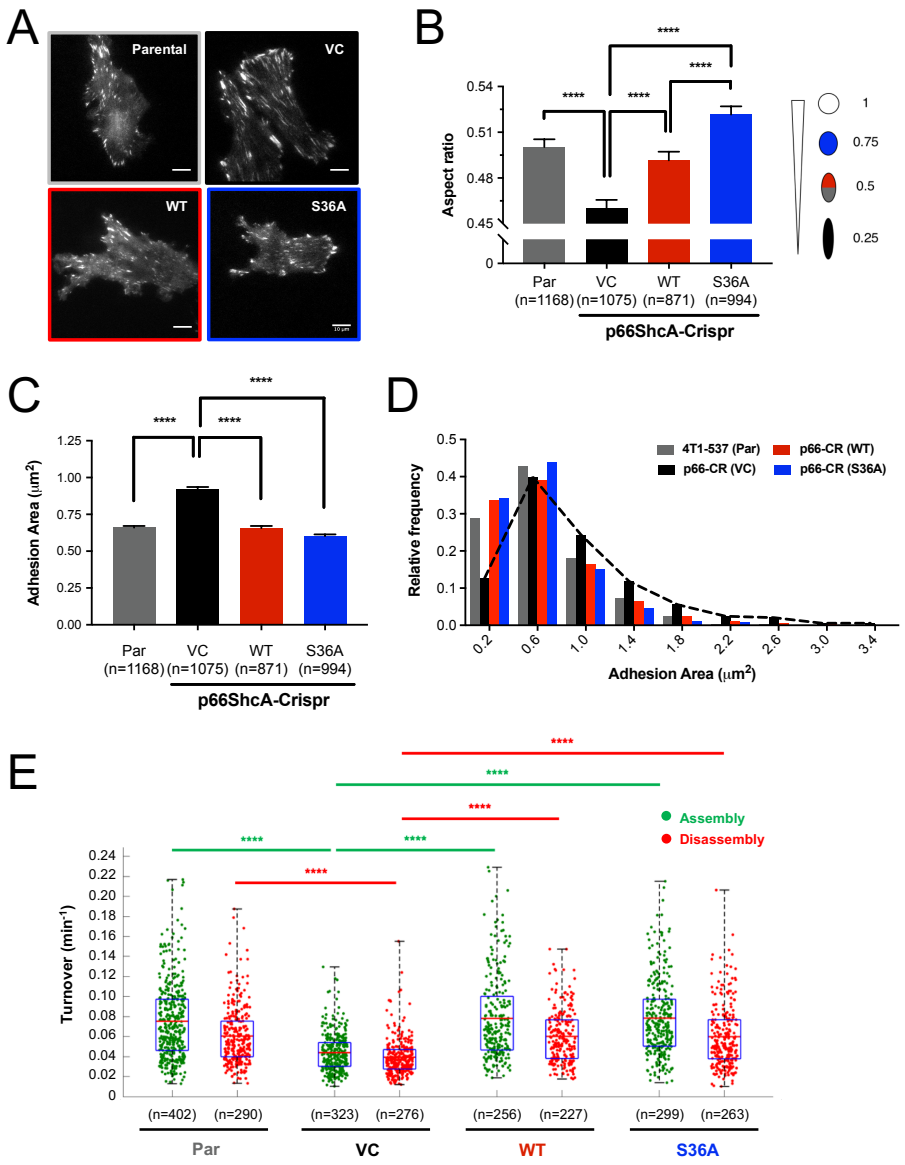


Figure 4

Figure 4 - Non-mitochondrial p66ShcA accelerates the dynamics of fibrillar adhesion formation. (A) Representative images of parental, p66-CR(VC), p66-CR(WT) and p66-CR (S36A) expressing lung metastatic 4T1 cells (537 population) transfected with mCherry paxillin. Scale bar is 10 μm. **(B)** Adhesions were segmented based on fluorescence intensity and analyzed for shape. Aspect ratio was determined by finding the ratio between semi-minor and -major axes. **(C)** Adhesions were segmented based on fluorescence intensity and analyzed for size. **(D)** Frequency distribution of adhesion areas from (C). Data values were binned into 1μm^2 segments. **(E)** Adhesions in protrusive cell regions were tracked over time to determine average assembly and disassembly rates from changes in mean fluorescence intensity. Cells were imaged every 20 seconds for a total of 25 minutes (parental: $n = 12$; VC,: $n = 11$; WT: $n = 10$; S36A: $n = 11$). Data represent assembly and disassembly events for adhesions from three independent experiments. Top and bottom lines of the box indicate the 3rd and 1st quartile, respectively, while the bold central lines indicate mean. The whiskers extend up to 1.5 times the interquartile range (**** $P < 0.0001$).

epithelial marker E-cadherin or the mesenchymal marker Vimentin to Tubulin or p52ShcA as relative controls to determine if expression levels of these proteins correlated with aggressiveness. Notably, tumors with the highest metastatic burden correlated with the uppermost levels of p66ShcA expression (S5, panels A and B), independent of p52ShcA levels (S5, panel C), and this was not seen when screening E-cadherin and Vimentin expression (S5, panels D and E). Hence, maintenance of high p66ShcA expression in vivo is a readout for tumor aggressiveness and may be indicative of underlying tumor interactions with the local microenvironment.

As serine36 of p66ShcA was required for metastasis from the primary site, but not for cell migration or metastasis following entry into the bloodstream, we sought to measure the ability of p66ShcA to enter and survive within the circulation from the mammary fatpad to determine if serine36 dependent effects on dissemination might occur at this stage. Notably, isolation of circulating tumor cells (CTCs) from the blood of tumor-bearing mice indicated that loss of p66ShcA (p66-CR(VC)) virtually ablated the number of CTCs present compared to parental tumor cells. Furthermore, rescue of p66ShcA(WT), but not p66ShcA(S36A) mutant was able to partially, but significantly rescue circulating tumor cell levels (>5-fold increase) and mimicked our spontaneous lung metastasis data. Hence, the increased number of CTCs is dependent on serine36 status and outlines a novel mechanism for mitochondrial-p66ShcA in promoting lung metastasis from the primary site through CTC regulation (Fig. 5, panel A). Therefore, mitochondrial ROS may be an important promoter of metastasis, specifically to the lung.

In addition, once in the bloodstream tumor cells must arrest at the metastatic site and extravasate into the secondary organ. Injection of tumor cells labeled with cell tracker red allowed us to perform lung colonization assays and study the late phases of metastasis. We show that there were significantly less tumor cells at the lung following injection in p66ShcAWT and

p66ShcAS36A rescue groups compared to p66 null cells. As there was no difference between parental and p66null groups immediately following injection, this may suggest a difference in inoculum, rather than increased apoptosis or entry into dormancy. Loss of p66ShcA p66-CR(VC) significantly reduced lung colonization 24 hours after injection compared to parental cells. However, rescue with p66ShcAWT or p66ShcAS36A restored the capacity to colonize the lungs after 24 hours to levels seen by parental cells (Fig. 5, panel C). These results support the experimental metastasis data (Fig. 2, panels D and E) and indicate that p66ShcA is required, independently of serine36, for lung colonization at the late stages of the metastatic cascade, with the formation of large clinically detectable overt metastases. However, from the primary site, effective dissemination requires mitochondrial p66ShcA-dependent mechanisms to either invade the primary site or to survive within the circulation, which results in elevated CTCs and increased metastasis following tumor resections. Collectively, these results indicate that both mitochondrial-p66ShcA and cytoplasmic-p66ShcA are required during different stages of the metastatic cascade.

Non-mitochondrial role for p66ShcA in increasing the growth of macroscopic breast tumor lung metastases.

To better understand how p66ShcA might regulate lung metastases, immunohistochemical staining was performed to characterize the lungs for levels of cell proliferation, cellular apoptosis and lipid peroxidation. Notably, p66ShcA was required to promote cell proliferation upon colonization regardless of site of injection (Fig. 6, panel A), as loss of p66ShcA (p66-CR(VC)) significantly reduced the number of proliferating cells within the lungs following spontaneous

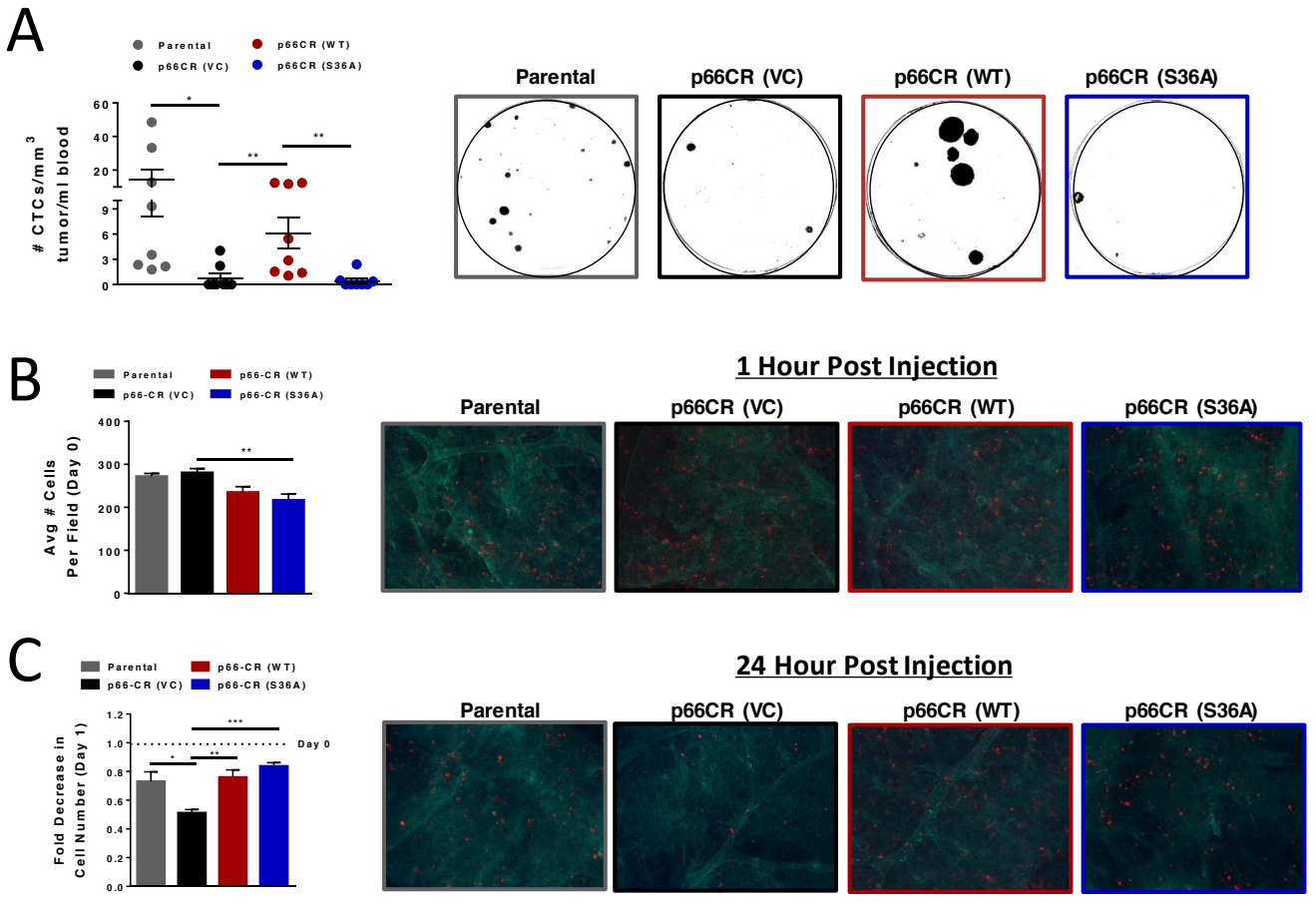


Figure 5

Figure 5 - p66ShcA increases breast cancer cell dissemination into the bloodstream and subsequent lung colonization. (A) Number of circulating tumor cells (CTC) in mice bearing parental, p66-CR(VC), p66-CR(WT) and p66-CR(S36A) mammary tumors normalized both to tumor volume at necropsy and the volume of blood collected. The data is shown as average number of CTCs/mm^3 tumor/ml blood ± SEM and is representative of 8 mice per group. **(B)** Number of breast tumor cells present in the lung 1 hour following tail vein injection. Breast cancer cells were labeled with Cell Tracker Red CMPTX dye and visualized in the lungs by fluorescent microscopy. The data is shown as average # cells per field of view ± SEM. For each cell line, the data is representative of 5 mice and 5 fields of view per mouse. Statistical analysis was performed using a one-way Anova with a Tukey's multiple comparisons test (**, $P < 0.01$). **(C)** Number of breast tumor cells present in the lung 24 hours following tail vein injection. Breast cancer cells were labeled and visualized as in panel B. The data is shown as the fold decrease in cell number relative to day 0 ± SEM. For each cell line, the data is representative of 5 mice and 5 fields of view per mouse. Statistical analysis was performed using a one-way Anova with a Tukey's multiple comparisons test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

metastasis from ~40% in parental cells to ~30%, which was rescued back to >40% through re-expression of p66ShcAWT p66-CR(WT). Furthermore, following tail vein injection, rescue of p66ShcAWT p66-CR(WT) and p66ShcAS36A p66-CR(S36A) both significantly restored levels of proliferation within the lung tissue to >60%, significantly higher than p66ShcA null tumors ~40% (p66-CR(VC)). Thus, p66ShcA is important for reactivation of cell proliferation cues at the secondary site following both spontaneous and experimental metastasis and independent of serine36. In contrast, p66ShcA was not necessary to alter cellular apoptosis (Fig. 6, panel B), as cleaved caspase 3 levels remained unchanged (~1%) following spontaneous metastasis, between parental, p66-CR(VC) and p66-CR(WT) groups. However, rescue of p66ShcAWT p66-CR(WT) resulted in significantly more cell death within the lungs compared to rescue with p66ShcAS36A p66-CR(S36A), but this did not appear to alter metastatic potential. Hence, despite p66ShcA's characterization as a mediator of cellular apoptosis, it does not seem to play a role in this setting. Finally, while p66ShcAWT p66-CR(WT) tumors display elevated 4-HNE positivity compared to p66-CR(S36A) tumors, this does not appear to correlate with metastatic potential (Fig. 6, panel C). While mitochondrial-p66ShcA p66-CR(WT) increases oxidative damage compared to p66-CR(S36A), the functional consequences remain unknown and unrelated to dissemination.

Overall, we show that different pools of p66ShcA act as novel regulators of breast cancer lung metastasis. Cytoplasmic-p66ShcA is required early during metastasis for increased cell migration and also for colonization and reactivation of cell proliferation cues during the late stages of the metastatic cascade, potentially through enhanced focal adhesion dynamics. In contrast, mitochondrial-p66ShcA and ROS formation is necessary to promote entry and/or survival in the circulation as shown by the presence of elevated numbers of CTCs in the blood following experimental metastasis from the primary site.

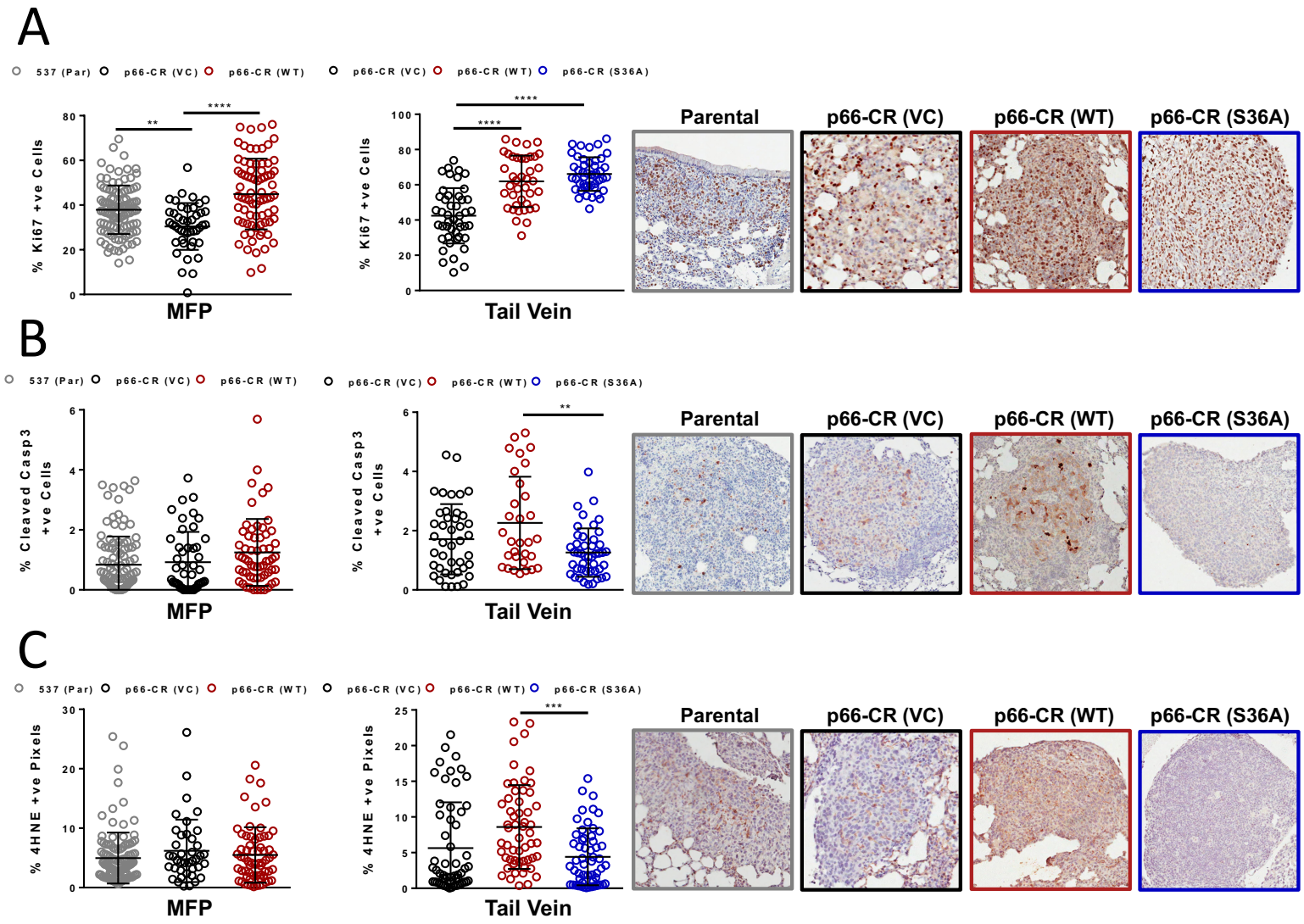


Figure 6. Non-mitochondrial role for p66ShcA in increasing the growth of macroscopic breast tumor lung metastases. (A) Percentage of Ki67-positive cells, **(B)** cleaved Caspase-3 positive cells and **(C)** 4HNE positive pixels in individual lung metastatic lesions derived from 4T1-537 Parental, p66-CR (VC), p66-CR (WT) and p66-CR (S36A) breast cancer cells, both following tumor resection and following tail vein injection. Panel A Resection: Parental, n=109 mets/7 mice; p66-CR (VC). n=44 mets/8 mice; p66-CR (WT). n=76 mets/8 mice. Panel A Tail Vein: p66-CR (VC), n=46 mets/8 mice; p66-CR (WT), n=40 mets/8 mice; p66-CR (S36A), n=45 mets/8 mice. Panel B Resection: Parental, n=109 mets/7 mice; p66-CR (VC), n=47 mets/8 mice; p66-CR (WT), n=59 mets/8 mice. Panel B Tail Vein: p66-CR (VC), n=42 mets/8 mice; p66-CR (WT), n=32 mets/8 mice; p66-CR (S36A), n=45 mets/8 mice. Panel C Resection: Parental, n=109 mets/7 mice; p66-CR (VC), n=40 mets/8 mice; p66-CR (WT), n=66 mets/8 mice. Panel B Tail Vein: p66-CR (VC), n=56 mets/7 mice; p66-CR (WT), n=56 mets/8 mice; p66-CR (S36A), n=55 mets/8 mice. Statistical analysis was performed using a one-way Anova with a Tukey's multiple comparisons test (**P<0.01; ***P<0.001; ****P<0.0001).

3.6 Discussion

Subtypes, TNBC and clonal evolution in metastasis

Breast cancers are stratified into distinct subtypes, which influences organ-specific metastasis and patient outcome. Breast cancer often metastasizes to bone, liver, lung and brain and other organs outside of these are relatively rare. Indeed, TNBC frequently metastasizes to the lung compared to the Luminal A subtype that preferentially leads to bone metastases. Hence, both the intrinsic properties of breast cancer cells and the host organ microenvironment are important in determining the efficiency of organ-specific metastasis. In this study we identified elevated levels of the p66ShcA adaptor protein specifically in lung and liver metastatic variants in TNBC, which closely resembles basal breast cancer. The clonal selection of tumors from successive metastases has been shown to increase tumour survival and overall fitness to establish secondary growths (Weihs 1973). Our functional data reinforce these findings as mitochondrial p66ShcA was required to increase the % of mice possessing spontaneous lung metastases (Fig2, panel B). Clonal evolution theory posits that metastasis involves the selection of rare tumour cells capable of completing the metastatic cascade and only these cells are capable of successful secondary growth. Notably, clones from parental TNBC cells possessing high levels of endogenous p66ShcA represent a minor, but significant population (1/3 clones, (S2, panel B). Hence, this study supports the model of clonal evolution whereby primary tumors already possess the majority of alterations seen in metastases, yet these tumour cells are few in number and can be enriched for through selection.

Furthermore, we outline a novel requirement for distinct pools of p66ShcA in promoting lung metastatic progression at differing stages of the metastatic cascade. Starting with local migration and entry into the circulation, cytoplasmic-p66ShcA is required for elevated migration,

but only mitochondrial-p66ShcA increases the abundance of CTCs in the bloodstream (Fig5, panel A), and is required for spontaneous metastasis from the primary site (Fig2, panel C). Supporting these findings, levels of circulating tumor cells were the most predictive of overall survival in metastatic breast cancer patients before and after treatment of all variables tested (Cristofanilli and Budd 2004). Of relevance, in vitro selection of highly invasive breast cancer cells was previously demonstrated to increase metastatic potential in vivo and was dependent on elevated mitochondrial activity (Porporato et al. 2014). Yet the role of oxidative stress in metastasis is conflicting in the literature. In breast cancer, inhibition of mtROS with antioxidants through stable overexpression of catalase inhibited invasiveness and metastatic progression (Goh et al. 2011). In contrast, antioxidant supplementation promotes metastasis in melanoma, where CTCs possess elevated oxidative stress levels (Gal et al. 2015; Piskounova et al. 2015). Screening breast tumours for levels of a panel of antioxidant enzymes, we see no significant differences in mRNA expression across groups (data not shown). In addition, changes in the levels of lipid peroxidation do not correlate with metastatic potential in aggressive TNBC from primary (S3, panel C) or secondary tumours (Fig. 6, panel C). Hence, p66ShcA-dependent CTC formation may not require elevated ROS defense systems to cope with oxidative stress or be fueled by oxidatively damaged proteins while in the circulation. Another mechanism that promotes survival within the circulation involves platelets coating metastasizing CTCs with their own class I MHC. This allows tumors to downregulate endogenous levels and to avoid T-cell–mediated immunity without activating NK cell killing (Placke et al. 2012). The role of platelets in p66ShcA-dependent spontaneous metastasis may warrant investigation.

Metabolic plasticity in metastasis

Organ specific metastasis involves transient metabolic changes that can influence both early and late stages of the metastatic cascade. Lung metastases shift toward oxidative phosphorylation and upregulate the expression of PGC-1α compared to liver metastases that favour glycolysis and induce PDK-1 expression (Andrzejewski et al. 2017; Dupuy et al. 2015). This metabolic shift can influence the early stages of metastasis as PGC-1α has been shown to enhance mitochondrial biogenesis, the oxygen consumption rate and oxidative phosphorylation of breast cancer cells to increase their local invasiveness and lung metastatic potential (Lebleu et al. 2014). PGC-1a also confers cellular protection from oxidative stress by regulating antioxidant genes (St-Pierre et al. 2006), and its engagement may protect circulating cancer cells from apoptosis as its expression is upregulated in CTCs (Lebleu et al. 2014). Given this evidence, it is plausible that p66ShcA might regulate PGC-1a expression to increase cell motility, CTC formation and promote lung dissemination. However, screening TNBC breast tumors deficient in p66ShcA or those re-expressing exogenous p66ShcA rescue for mRNA expression of PGC-1a revealed no significant differences across groups (data not shown). Recent work has demonstrated that EMT induction enhances glycolytic metabolism in breast cancer cells (Kondaveeti, Guttilla Reed, and White 2015). Notably, p66ShcA does not induce an EMT in TNBC (S5, panels D and E), despite providing TNBC with enhanced metastatic ability, (Fig2, panel C). Therefore, whether p66ShcA contributes to the metabolic plasticity of cancer cells during discrete steps of the metastatic process would require further investigation.

Tumor microenvironment

These data also indicate that the unique interactions with the tumor microenvironment are key to modulate gene expression involved with metastasis as selection through the mammary

fatpad was not sufficient to significantly enrich for p66ShcA (S2, panel A). This is in agreement with our findings indicating p66ShcA is not required for primary tumor growth (Fig. 2, panel A) and functions mainly to increase the metastatic potential of tumours at both the early and late stages (Fig. 2, panel C, D and E). Breast cancer cells induce the secretion of the chemokine CCL5 (also called RANTES) in stromal mesenchymal stem cells derived from the bone marrow, which then acts in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis (Karnoub et al. 2007). Despite this, p66ShcA did not regulate CCL5 mRNA expression in aggressive TNBC tumors (data not shown). CXCR4 is a chemokine receptor that has been shown to promote lung metastasis and can increase invasiveness through the secretion of MMP9 (Müller et al. 2001; Zuo et al. 2017). ROS has also been shown to induce MMP9 expression in breast cancer (Mori et al. 2018). Combined, these data indicate mitochondrial p66ShcA may increase the number of CTCs present in the bloodstream by increasing tumour invasiveness. Notably, CXCR4 signaling has been shown to support colonization by activating Akt and Src to promote cell proliferation (Kayali et al. 2003) and p66ShcA is necessary for increased metastasis by elevating tumor cell proliferation within the lungs of tumor-bearing mice (Fig. 6, panel A). Hence, both pools of p66ShcA could potentially engage MMPs and increase the invasive properties of TNBC cells to enhance lung metastasis by degrading the ECM at the primary and/or secondary site. Thus, interactions with the local tumor microenvironment may explain the differential effects between p66-dependent metastasis from the primary site versus entry from the bloodstream.

In terms of stromal factors and cell types, CAFS have been shown to play an important role in promoting metastasis in the 4T1 TNBC model by modulating the immune microenvironment (Liao et al. 2009). Further research is required to determine if CAFs, immune suppression and/or immune surveillance and their associated immune types (MDSCs, CTLs, NK

cells, etc.) may play a role in p66ShcA-dependent metastasis in breast cancer. The pre-metastatic niche can also be controlled by systemic factors including exosomes and cytokines. In addition, subtype specific factors include: circulatory routes that guide the distribution of cancer cells and the seeding of compatible tissues, which influences colonization.

Non-mitochondrial p66ShcA in Metastasis

The metastatic data herein indicates p66ShcA is required for colonization independent of mitochondrial-ROS in experimental models of metastasis in aggressive TNBC, however, mitochondrial-p66ShcA promotes spontaneous metastasis. Cytoplasmic-p66ShcA was also required to increase the migratory potential of aggressive TNBCs. This suggests cytoplasmic-p66ShcA is required both during the early and late stages of the metastatic cascade. This can be broken down into migration and invasion of the primary tumor, degradation of the basement membrane, intravasation and survival within the circulation, arrest at the secondary organ, extravasation into the foreign parenchyma, coping with environmental stressors within the secondary tumor microenvironment and avoiding tumor dormancy by engaging proliferation and survival pathways to enhance growth of the secondary tumour mass. Of note, focal adhesion genes were differentially regulated in lung relapse in a study on subtype specific organotropism in breast cancer (Smid et al. 2008). Indeed, non-mitochondrial p66ShcA was required for increased migration and paxillin-dependent focal adhesion turnover (assembly and disassembly) in vitro. FAK is known to interact with paxillin within focal adhesions and FAK signalling has been shown to regulate cell migration and invasion through the expression of MMPs 2 and 9 to promote breast cancer metastasis to the lung (Wangpu et al. 2016; Woo et al. 2017). Hence, it would be interesting to test the importance of MMPs and cellular invasion in vitro to see if p66ShcA influences metastasis at this early stage of the metastatic cascade. Notably, FAK has also been shown to

recruit p66ShcA, through the PTB domain to adhesion complexes and regulate tension-induced p66ShcA-dependent RhoA activation, which can then transactivate YAP/TAZ signalling downstream (Wu et al. 2016). YAP/TAZ transcription factors relay proliferative, death and differentiation signals, in response to mechanical stimuli. Indeed, non-mitochondrial p66ShcA is required for dissemination to the lungs during experimental metastasis, is necessary for lung colonization and reactivates cell proliferation in overt lung metastases. Notably, metastatic breast cancer cells require the expression of beta1 integrins and activation of FAK for actin stress fiber formation to promote proliferation of micrometastases (escape from dormancy) following extravasation and colonization within the lungs (Barkan et al. 2008; Quah et al. 2009). Hence, it is tempting to suggest non-mitochondrial p66ShcA engages FAK/Paxillin signalling to enhance adhesion turnover, which would allow for increased migration, enhanced adhesion to the lung parenchyma and ultimately promote colonization, potentially through YAP/TAZ activation which could provide growth cues and increase cell proliferation. Therefore, these data show that non-mitochondrial p66ShcA (independent of serine36 within the CH2 domain) is essential to promote both integrin dependent and anchorage-independent cell proliferation by regulating the actin cytoskeleton and provide a strong rationale for p66ShcA's effects in TNBC lung dissemination during the late stages of the metastatic cascade. Potentially this could occur through elevated integrin signalling with the lung ECM which would avoid tumour dormancy by providing cues to reactivate proliferation pathways at the secondary site. Studies show that a high ratio of ERK MAPK/p38 MAPK can reactivate dormant cancer cells to proliferate while the reverse promotes dormancy (Gao et al. 2017). Src is a known interactor with FAK and paxillin at focal adhesions. Co-inhibition of ERK and Src prevents metastatic outgrowth of dormant tumour cells (Touny et al. 2014). Another mechanism could involve inhibitors of differentiation proteins, Id1 and Id3,

which facilitate sustained proliferation during the early stages of metastatic lung colonization, subsequent to extravasation in TNBC (Gupta et al. 2007). The extracellular protein TNC has also been shown to promote lung colonization in breast cancer by supporting the CSC niche (Oskarsson et al. 2011). Additional studies are required to delineate p66ShcA's role late in the metastatic cascade, however, we provide strong evidence that p66ShcA is required for elevated focal adhesion turnover, through regulation of paxillin and a review of the literature indicates other cytoskeletal players like FAK, RhoA and Rac1 may be involved in extravasation, adhesion at the lung and/or in reactivation of tumor growth cues during lung colonization.

p66ShcA supports TNBC tumor growth

Another important finding from our work is that p66ShcA is sufficient for TNBC tumor growth independent of mitochondrial-ROS and this phenotype is maintained during metastasis to the lung (Fig. 1, panel B and C). Surprisingly, these effects appear to be driven by reduced apoptosis (Figure 13, panel B), despite the fact p66ShcA is best characterized as a redox protein that induces cell death under stress conditions (Giorgio et al. 2005b) and inhibits breast cancer growth in ErbB2-Driven breast cancer (Hudson et al. 2014). Yet, these data are in line with published work indicating p66ShcA promotes tumor growth in steroid hormone sensitive cancer and in breast cancer *in vitro*, however, p66ShcA dependent growth effects were mediated through ROS signaling in these settings (Bhat et al. 2014; M.-S. Lee et al. 2004). Collectively, p66ShcA's effects on tumor growth appear to be subtype specific and/or potentially dependent on the oncogenic driver as p66ShcA modestly inhibited tumor outgrowth in ErbB2+ luminal breast cancer *in vivo* and we now show that these effects are dependent on mitochondrial-ROS (Figure 24, panel B) (Hudson et al. 2014). Hence, p66ShcA is sufficient for increased tumor outgrowth independent of serine36 within the CH2 domain of p66ShcA only in TNBC. In addition, the

differences in metastatic potential are comparable to the differences seen in tumor outgrowth, indicating p66ShcA's role is primarily in tumor initiation and not metastatic initiation, progression or virulence as these effects are only maintained at the secondary site.

Mitochondrial-p66ShcA is necessary for lung metastasis, but not sufficient in ErbB2 positive tumors

Surprisingly, p66ShcA was not sufficient to increase the lung metastatic potential of NIC tumours (S1, panel B), despite promoting EMT induction (S1, panels C and D);(Hudson et al. 2014). ErbB2 blocks anoikis through aggregation of cells, which is lost upon disaggregation through destabilization of EGFR and downstream ERK/MAPK survival signalling (Rayavarapu et al. 2015). Hence, in NIC-p66WT tumors, the advantages of enhanced single cell migratory and mesenchymal properties provided by an EMT, including avoiding loss of ECM-induced cell death may be mitigated by the fact that ErbB2 activation and aggregate was already allowing for anoikis evasion. Another factor is that the tumour resections were performed at a relatively late stage (large volume) given an EMT provides tumors with access to the circulation at a relatively early stage. Intriguingly, non-mitochondrial p66ShcA is responsible for EMT induction (S4, panels A, D and E). The cytoskeletal protein RhoA, has been shown to be regulated by p66ShcA through the PTB domain to regulate cellular migration (Zhenyi; Ma et al. 2007). Hence, there is a precedent for non-mitochondrial pools of p66ShcA to control cell motility and its effect on cellular plasticity through EMT induction also appear to be independent of serine36. However, given p66ShcA only induces an EMT in ErbB2+ luminal breast cancer in vivo compared to TNBC, we conclude the role of p66ShcA is context and subtype specific. Therefore, understanding the molecular drivers, even within subtypes, is key to identify potential therapeutic avenues in breast cancer.

3.7 References

Anderson, K. R., Haeussler, M., Watanabe, C., Janakiraman, V., Lund, J., Modrusan, Z., … Warming, S. (2018). CRISPR off-target analysis in genetically engineered rats and mice. *Nature Methods*, *15*(7), 512–514. https://doi.org/10.1038/s41592-018-0011-5

Andrzejewski, S., Klimcakova, E., Johnson, R. M., Tabariès, S., Annis, M. G., McGuirk, S., … St-Pierre, J. (2017). PGC-1α Promotes Breast Cancer Metastasis and Confers Bioenergetic Flexibility against Metabolic Drugs. *Cell Metabolism*, *26*(5), 778–787.e5. https://doi.org/10.1016/j.cmet.2017.09.006

Barkan, D., Kleinman, H., Simmons, J. L., Asmussen, H., Kamaraju, A. K., Hoenorhoff, M. J., … Green, J. E. (2008). Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. *Cancer Research*, *68*(15), 6241–6250. https://doi.org/10.1158/0008-5472.CAN-07-6849

Bhat, H. F., Baba, R. A., Adams, M. E., & Khanday, F. A. (2014). Role of SNTA1 in Rac1 activation, modulation of ROS generation, and migratory potential of human breast cancer cells. *British Journal of Cancer*, *110*(3), 706–714. https://doi.org/10.1038/bjc.2013.723

Breast, E., Trialists, C., & Group, C. (2005). Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet*, *366*, 2087–2106. https://doi.org/10.2169/internalmedicine.51.6871 LK - http://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=09182918&id=doi:10.2169%2Finternalmedicine.51.6871&atitle=Interleukin-18+in+adult-onset+still%27s+disease%3A+Treatment+target+or+disease+activity+indicator%3F&stitle=Intern.+Med.&title=Internal+Medicine&volume=51&issue=4&spage=451&epage=&aulast=Sato&aufirst=Kojiro&auinit=K.&aufull=Sato+K.&coden=IEDIE&isbn=&pages=451-&date=2012&auinit1=K&auinitm=

Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer*, *2*, 563. Retrieved from http://dx.doi.org/10.1038/nrc865

Cristofanilli, M., & Budd, G. (2004). Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *England Journal of Medicine*, *351*(8), 781–791. https://doi.org/10.1056/NEJMoa040766

Dasgupta, A., Lim, A. R., & Ghajar, C. M. (2017). Circulating and disseminated tumor cells: harbingers or initiators of metastasis? *Molecular Oncology*, *11*(1), 40–61. https://doi.org/10.1002/1878-0261.12022

Dupuy, F., Tabariès, S., Andrzejewski, S., Dong, Z., Blagih, J., Annis, M. G., … Siegel, P. M. (2015). PDK1-dependent metabolic reprogramming dictates metastatic potential in breast cancer. *Cell Metabolism*, *22*(4), 577–589. https://doi.org/10.1016/j.cmet.2015.08.007

Feng, Z., Hu, W., & Tang, M.-S. (2004). Trans-4-hydroxy-2-nonenal inhibits nucleotide excision repair in human cells: a possible mechanism for lipid peroxidation-induced carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(23), 8598–8602. https://doi.org/10.1073/pnas.0402794101

Frackelton, A. R., Lu, L., Davol, P. A., Bagdasaryan, R., Hafer, L. J., & Sgroi, D. C. (2006). p66 Shc and tyrosine-phosphorylated Shc in primary breast tumors identify patients likely to relapse despite tamoxifen therapy. *Breast Cancer Research*, *8*(6), 1–8. https://doi.org/10.1186/bcr1631

Gal, K. Le, Ibrahim, M. X., Wiel, C., Sayin, V. I., Akula, M. K., Karlsson, C., … Bergo, M. O. (2015). Antioxidants can increase melanoma metastasis in mice. *Science Translational Medicine*, *7*(308), 1–8. https://doi.org/10.1126/scitranslmed.aad3740

Gao, X. L., Zhang, M., Tang, Y. L., & Liang, X. H. (2017). Cancer cell dormancy: Mechanisms and implications of cancer recurrence and metastasis. *OncoTargets and Therapy*, *10*, 5219–5228. https://doi.org/10.2147/OTT.S140854

Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi, C., … Pelicci, P. G. (2005a). Electron Transfer between Cytochrome c and $p66^{Shc}$ Generates Reactive Oxygen Species that Trigger Mitochondrial Apoptosis. *Cell*, *122*(2), 221–233. https://doi.org/10.1016/j.cell.2005.05.011

Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi, C., … Pelicci, P. G. (2005b). Electron transfer between cytochrome c and p66Shcgenerates reactive oxygen species that trigger mitochondrial apoptosis. *Cell*, *122*(2), 221–233. https://doi.org/10.1016/j.cell.2005.05.011

Goh, J., Enns, L., Fatemie, S., Hopkins, H., Morton, J., Pettan-Brewer, C., & Ladiges, W. (2011). Mitochondrial targeted catalase suppresses invasive breast cancer in mice. *BMC Cancer*, *11*. https://doi.org/10.1186/1471-2407-11-191

Grossman, S. R., Lyle, S., Resnick, M. B., Sabo, E., Lis, R. T., Rosinha, E., … Hafer, L. J. (2007). p66 Shc tumor levels show a strong prognostic correlation with disease outcome in stage IIA colon cancer. *Clinical Cancer Research*, *13*(19), 5798–5804. https://doi.org/10.1158/1078-0432.CCR-07-0073

Gupta, G. P., Perk, J., Acharyya, S., de Candia, P., Mittal, V., Todorova-Manova, K., … Massague, J. (2007). ID genes mediate tumor reinitiation during breast cancer lung metastasis. *Proceedings of the National Academy of Sciences*, *104*(49), 19506–19511. https://doi.org/10.1073/pnas.0709185104

Hess, K. R., Pusztai, L., Buzdar, A. U., & Hortobagyi, G. N. (2003). Estrogen receptors and distinct patterns of breast cancer relapse. *Breast Cancer Res Treat*, *78*(1), 105–118. https://doi.org/10.1023/A:1022166517963

Hudson, J., Ha, J. R., Sabourin, V., Ahn, R., La Selva, R., Livingstone, J., … Ursini-Siegel, J. (2014). p66ShcA Promotes Breast Cancer Plasticity by Inducing an Epithelial-to-Mesenchymal Transition. *Molecular and Cellular Biology*, *34*(19), 3689–3701. https://doi.org/10.1128/MCB.00341-14

Hüsemann, Y., Geigl, J. B., Schubert, F., Musiani, P., Meyer, M., Burghart, E., … Klein, C. A. (2008). Systemic Spread Is an Early Step in Breast Cancer. *Cancer Cell*, *13*(1), 58–68. https://doi.org/10.1016/j.ccr.2007.12.003

Jackson, J. G., Yoneda, T., Clark, G. M., Yee, D., Jackson, J. G., Yoneda, T., … Yee, D. (2000). Elevated Levels of p66 Shc Are Found in Breast Cancer Cell Lines and Primary Tumors with High Metastatic Potential Elevated Levels of p66 Shc Are Found in Breast Cancer Cell Lines and Primary Tumors with High Metastatic Potential 1, *6*(March), 1135–1139.

Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., … Weinberg, R. A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*, *449*(7162), 557–563. https://doi.org/10.1038/nature06188

Kayali, A. G., Van Gunst, K., Campbell, I. L., Stotland, A., Kritzik, M., Liu, G., … Sarvetnick, N. (2003). The stromal cell-derived factor-1α/CXCR4 ligand-receptor axis is critical for progenitor survival and migration in the pancreas. *Journal of Cell Biology*, *163*(4), 859–869. https://doi.org/10.1083/jcb.200304153

Kondaveeti, Y., Guttilla Reed, I. K., & White, B. A. (2015). Epithelial-mesenchymal transition induces similar metabolic alterations in two independent breast cancer cell lines. *Cancer Letters*, *364*(1), 44–58. https://doi.org/10.1016/j.canlet.2015.04.025

Kundu, N., Zhang, S., & Fulton, A. M. (1995). Sublethal oxidative stress inhibits tumor cell adhesion and enhances experimental metastasis of murine mammary carcinoma. *Clinical & Experimental Metastasis*, *13*(1), 16–22. https://doi.org/10.1007/BF00144014

Lebleu, V. S., O'Connell, J. T., Gonzalez Herrera, K. N., Wikman, H., Pantel, K., Haigis, M. C., … Kalluri, R. (2014). PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nature Cell Biology*, *16*(10), 992–1003. https://doi.org/10.1038/ncb3039

Lee, M.-S., Igawa, T., Chen, S.-J., Van Bemmel, D., Lin, J. S., Lin, F.-F., … Lin, M.-F. (2004). p66Shc protein is upregulated by steroid hormones in hormone-sensitive cancer cells and in primary prostate carcinomas. *International Journal of Cancer. Journal International Du Cancer*, *108*(5), 672–678. https://doi.org/10.1002/ijc.11621

Li, X., Xu, Z., Du, W., Zhang, Z., Wei, Y., Wang, H., … Liu, Z. (2014). Aiolos promotes anchorage independence by silencing p66Shctranscription in cancer cells. *Cancer Cell*, *25*(5), 575–589. https://doi.org/10.1016/j.ccr.2014.03.020

Liao, D., Luo, Y., Markowitz, D., Xiang, R., & Reisfeld, R. A. (2009). Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model. *PLoS ONE*, *4*(11). https://doi.org/10.1371/journal.pone.0007965

Lin, M. (2010). p66Shc—a longevity redox protein in human prostate cancer progression and metastasis. *Cancer*, *29*(1), 207–222. https://doi.org/10.1007/s10555-010-9213-8.p66Shc

Lu, X., & Kang, Y. (2007). Organotropism of breast cancer metastasis. *Journal of Mammary Gland Biology and Neoplasia*, *12*(2–3), 153–162. https://doi.org/10.1007/s10911-007-9047-3

Luzzi, K. J., MacDonald, I. C., Schmidt, E. E., Kerkvliet, N., Morris, V. L., Chambers, A. F., & Groom, A. C. (1998). Multistep nature of metastatic inefficiency: Dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *American Journal of Pathology*, *153*(3), 865–873. https://doi.org/10.1016/S0002-9440(10)65628-3

Ma, Z., Myers, D. P. ., Ru, F. W., Nwariaku, F. E. ., & Terada, L. S. (2007). p66Shcmediates anoikis through RhoA. *Journal of Cell Biology*, *179*(1), 23–31. https://doi.org/10.1083/jcb.200706097

Migliaccio, E., Mele, S., Salcini, A. E., Pelicci, G., Lai, K. V., Superti-furga, G., … Pelicci, P. G. (1997). Opposite effects of the p52 shc / p46 shc and p66 shc splicing isoforms on the EGF receptor – MAP kinase – fos signalling pathway. *The EMBO Journal*, *16*(4), 706–716.

Mitra, S. K., Hanson, D. A., & Schlaepfer, D. D. (2005). Focal adhesion kinase: In command and control of cell motility. *Nature Reviews Molecular Cell Biology*, *6*(1), 56–68. https://doi.org/10.1038/nrm1549

Mori, K., Uchida, T., Yoshie, T., Mizote, Y., Ishikawa, F., Katsuyama, M., & Shibanuma, M. (2018). A mitochondrial ROS pathway controls matrix metalloproteinase 9 levels and invasive properties in RAS-activated cancer cells. *The FEBS Journal*. https://doi.org/10.1111/febs.14671

Müller, a, Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., … Zlotnik, a. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, *410*(6824), 50–56. https://doi.org/10.1038/35065016

Northey, J. J., Chmielecki, J., Ngan, E., Russo, C., Annis, M. G., Muller, W. J., & Siegel, P. M. (2008). Signaling through ShcA Is Required for Transforming Growth Factor - and Neu/ErbB-2-Induced Breast Cancer Cell Motility and Invasion. *Molecular and Cellular Biology*, *28*(10), 3162–3176. https://doi.org/10.1128/MCB.01734-07

Oskarsson, T., Acharyya, S., Zhang, X. H. F., Vanharanta, S., Tavazoie, S. F., Morris, P. G., … Massagué, J. (2011). Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nature Medicine*, *17*(7), 867–874. https://doi.org/10.1038/nm.2379

Pelicano, H., Lu, W., Zhou, Y., Zhang, W., Chen, Z., Hu, Y., & Huang, P. (2009). Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism. *Cancer Research*, *69*(6), 2375–2383. https://doi.org/10.1158/0008-5472.CAN-08-3359

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., … Giuseppe Pelicci, P. (1992). A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*, *70*(1), 93–104. https://doi.org/https://doi.org/10.1016/0092-8674(92)90536-L

Piskounova, E., Agathocleous, M., Murphy, M. M., Hu, Z., Huddlestun, S. E., Zhao, Z., … Morrison, S. J. (2015). Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature*, *527*(7577), 186–191. https://doi.org/10.1038/nature15726

Placke, T., Örgel, M., Schaller, M., Jung, G., Rammensee, H. G., Kopp, H. G., & Salih, H. R. (2012). Platelet-derived MHC class I confers a pseudonormal phenotype to cancer cells that subverts the antitumor reactivity of natural killer immune cells. *Cancer Research*, *72*(2), 440–448. https://doi.org/10.1158/0008-5472.CAN-11-1872

Porporato, P. E., Payen, V. L., Pérez-Escuredo, J., De Saedeleer, C. J., Danhier, P., Copetti, T., … Sonveaux, P. (2014). A mitochondrial switch promotes tumor metastasis. *Cell Reports*, *8*(3), 754–766. https://doi.org/10.1016/j.celrep.2014.06.043

Pulaski, B. A., & Ostrand-Rosenberg, S. (2001). Mouse 4T1 Breast Tumor Model. *Current Protocols in Immunology*, *39*(1), 20.2.1-20.2.16. https://doi.org/10.1002/0471142735.im2002s39

Quah, J. C., Barlow, V. P., Mcphun, V., Klaus, I., Hulett, M. D., Parish, C. R., … Hoeflich, K. P. (2009). Correction for Yang et al., Regulation of the protein disulfide proteome by mitochondria in mammalian cells. *Proceedings of the National Academy of Sciences*, *106*(34), 14734–14734. https://doi.org/10.1073/pnas.0908506106

Ravichandran, K. S., Zhou, M. M., Pratt, J. C., Harlan, J. E., Walk, S. F., Fesik, S. W., & Burakoff, S. J. (1997). Evidence for a requirement for both phospholipid and phosphotyrosine binding via the Shc phosphotyrosine-binding domain in vivo. *Mol Cell Biol*, *17*(9), 5540–5549. https://doi.org/10.1128/MCB.17.9.5540

Rayavarapu, R. R., Heiden, B., Pagani, N., Shaw, M. M., Shuff, S., Zhang, S., & Schafer, Z. T. (2015). The role of multicellular aggregation in the survival of ErbB2-positive breast cancer cells during extracellular matrix detachment. *Journal of Biological Chemistry*, *290*(14), 8722–8733. https://doi.org/10.1074/jbc.M114.612754

Smid, M., Wang, Y., Zhang, Y., Sieuwerts, A. M., Yu, J., Klijn, J. G. M., … Martens, J. W. M. (2008). Subtypes of breast cancer show preferential site of relapse. *Cancer Research*, *68*(9), 3108–3114. https://doi.org/10.1158/0008-5472.CAN-07-5644

St-Pierre, J., Drori, S., Uldry, M., Silvaggi, J. M., Rhee, J., Jäger, S., … Spiegelman, B. M. (2006). Suppression of Reactive Oxygen Species and Neurodegeneration by the PGC-1

Transcriptional Coactivators. *Cell*, *127*(2), 397–408. https://doi.org/10.1016/j.cell.2006.09.024

Tabariès, S., Ouellet, V., Hsu, B. E., Annis, M. G., Rose, A. A. N., Meunier, L., … Siegel, P. M. (2015). Granulocytic immune infiltrates are essential for the efficient formation of breast cancer liver metastases. *Breast Cancer Research*, *17*(1), 1–18. https://doi.org/10.1186/s13058-015-0558-3

Touny, L. H. El, Vieira, A., Mendoza, A., Khanna, C., Hoenerhoff, M. J., & Green, J. E. (2014). Combined SFK/MEK inhibition prevents metastatic outgrowth of dormant tumor cells. *Journal of Clinical Investigation*, *124*(1), 156–168. https://doi.org/10.1172/JCI70259

Ursini-Siegel, J., Hardy, W. R., Zuo, D., Lam, S. H. L., Sanguin-Gendreau, V., Cardiff, R. D., … Muller, W. J. (2008). ShcA signalling is essential for tumour progression in mouse models of human breast cancer. *The EMBO Journal*, *27*(6), 910–920. https://doi.org/10.1038/emboj.2008.22

Ursini-Siegel, J., & Muller, W. J. (2008a). The ShcA adaptor protein is a critical regulator of breast cancer progression. *Cell Cycle*, *7*(13), 1936–1943. https://doi.org/10.4161/cc.7.13.6205

Ursini-Siegel, J., & Muller, W. J. (2008b). The ShcA adaptor protein is a critical regulator of breast cancer progression. *Cell Cycle*, *7*(13), 1936–1943. https://doi.org/10.4161/cc.7.13.6205

Ursini-Siegel, J., & Siegel, P. M. (2016). The influence of the pre-metastatic niche on breast cancer metastasis. *Cancer Letters*, *380*(1), 281–288. https://doi.org/10.1016/j.canlet.2015.11.009

van der Geer, P., Wiley, S., Ka-Man Lai, V., Olivier, J. P., Gish, G. D., Stephens, R., … Pawson, T. (1995). A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides. *Current Biology*, *5*(4), 404–412. https://doi.org/https://doi.org/10.1016/S0960-9822(95)00081-9

Vanharanta, S., & Massagué, J. (2013). Origins of Metastatic Traits. *Cancer Cell*, *24*(4), 410–421. https://doi.org/10.1016/j.ccr.2013.09.007

Ventura, A. (2002). The p66Shc Longevity Gene Is Silenced through Epigenetic Modifications of an Alternative Promoter. *Journal of Biological Chemistry*, *277*(25), 22370–22376. https://doi.org/10.1074/jbc.M200280200

Wahl, G. M., & Spike, B. T. (2017). Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity. *Npj Breast Cancer*, *3*(1), 1–12. https://doi.org/10.1038/s41523-017-0012-z

Wangpu, X., Lu, J., Xi, R., Yue, F., Sahni, S., Park, K. C., … Richardson, D. R. (2016). Targeting the Metastasis Suppressor, N-Myc Downstream Regulated Gene-1, with Novel Di-2-Pyridylketone Thiosemicarbazones: Suppression of Tumor Cell Migration and Cell-Collagen Adhesion by Inhibiting Focal Adhesion Kinase/Paxillin Signaling. *Molecular Pharmacology*, *89*(5), 521–540. https://doi.org/10.1124/mol.115.103044

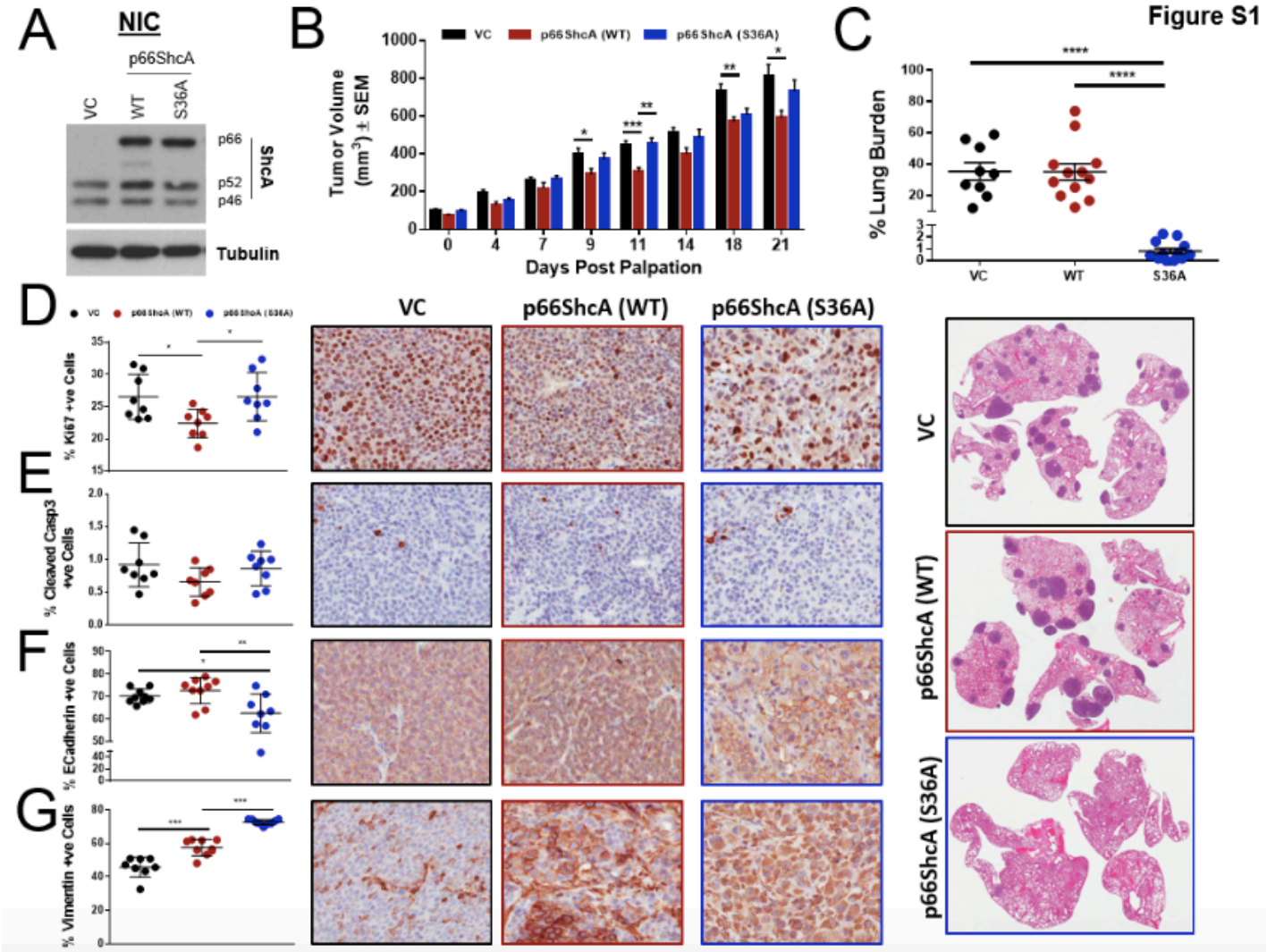
Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., & Horwitz, A. F. (2004). FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nature Cell Biology*, *6*(2), 154–161. https://doi.org/10.1038/ncb1094

Wei, S., & Siegal, G. P. (2017). Metastatic organotropism: An intrinsic property of breast cancer molecular subtypes. *Advances in Anatomic Pathology*. https://doi.org/10.1097/PAP.0000000000000140

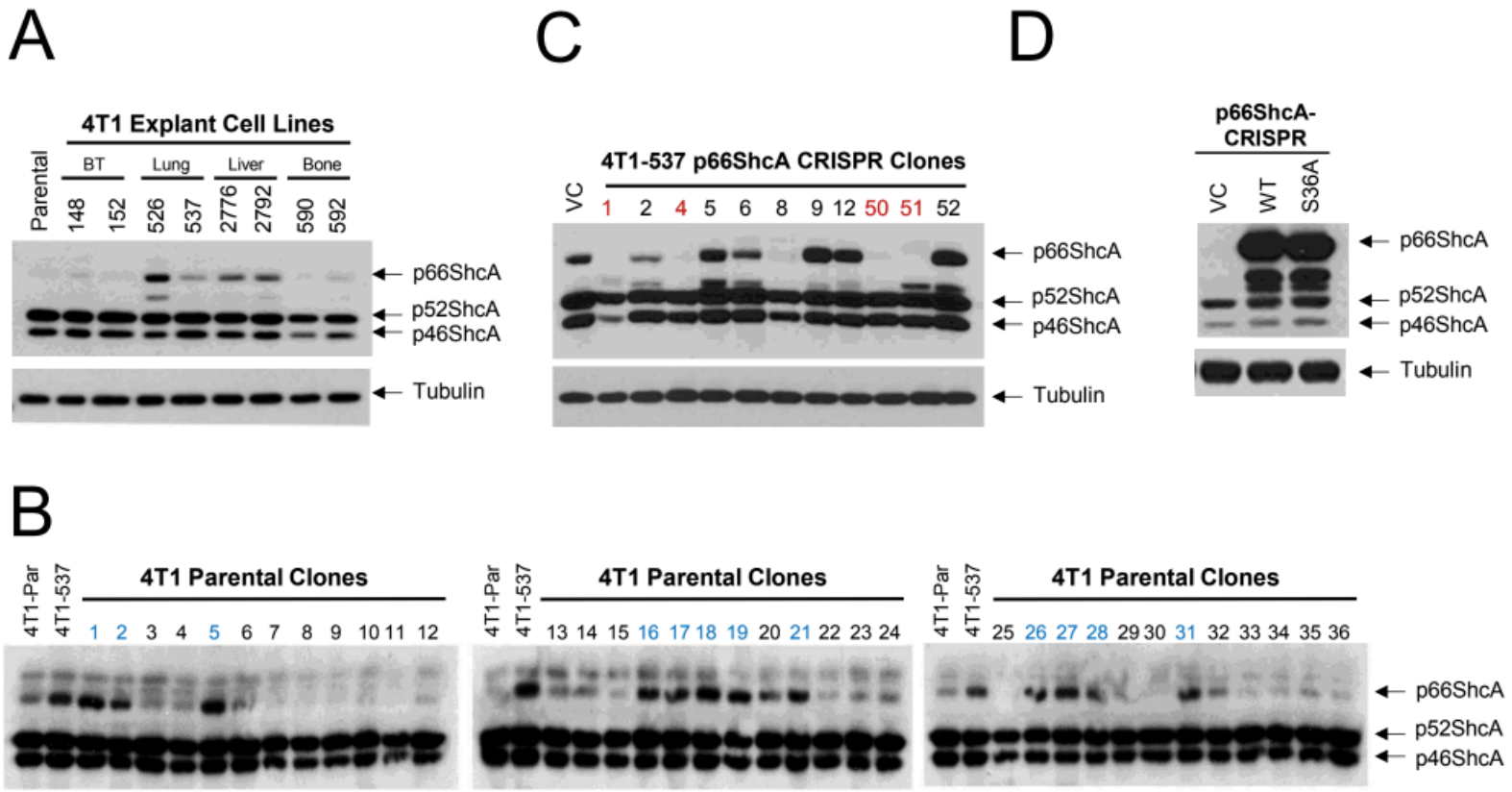
Weihs, D. (1973). Selection of successive tumour lines for metastasis. *Nature*, *242*, 117–118.

https://doi.org/10.1038/227680a0
Woo, J. K., Jung, H. J., Park, J., Kang, J., Lee, B. Il, Shin, D., … Seong, J. K. (2017). Daurinol blocks breast and lung cancer metastasis and development by inhibition of focal adhesion kinase (FAK), *8*(34), 57058–57071. https://doi.org/10.18632/oncotarget.18983
Wu, R.-F., Liao, C., Fu, G., Hayenga, H. N., Yang, K., Ma, Z., … Terada, L. S. (2016). p66 ^{Shc} Couples Mechanical Signals to RhoA through Focal Adhesion Kinase-Dependent Recruitment of p115-RhoGEF and GEF-H1. *Molecular and Cellular Biology*, *36*(22), 2824–2837. https://doi.org/10.1128/MCB.00194-16
Xue, C., Plieth, D., & Venkov, C. (2003). The Gatekeeper Effect of Epithelial-Mesenchymal Transition Regulates the Frequency of Breast Cancer Metastasis The Gatekeeper Effect of Epithelial-Mesenchymal Transition Regulates the Frequency of Breast Cancer Metastasis 1, 3386–3394.
Zuo, J., Wen, M., Li, S., Lv, X., Wang, L., Ai, X., & Lei, M. (2017). Overexpression of CXCR4 promotes invasion and migration of non-small cell lung cancer via EGFR and MMP-9. *Oncology Letters*, *14*(6), 7513–7521. https://doi.org/10.3892/ol.2017.7168

3.8 Supplemental information



Supplementary Figure 1 - Non-mitochondrial p66ShcA restrains metastatic progression in a luminal breast cancer model (A) Immunoblot analysis of whole cell lysates from Erbb2+ luminal NIC breast cancer cells stably overexpressing p66ShcA, p66ShcAS36A or vector control (VC) using ShcA or Tubulin specific antibodies. **(B)** Mammary fat pad (MFP) injection of VC, p66ShcA-WT and p66ShcA-S36A overexpressing NIC cells. The data is shown as average tumor volume (mm^3) ± SEM (n=7 tumors/group). **(C)** Percentage of tumor burden in the lungs of mice bearing VC, p66ShcA-WT and p66ShcA-S36A overexpressing NIC tumors. Mammary tumors were resected at 500 mm^3 and the development of lung metastases was quantified 28 days later. The data is shown as average lung tumor burden ±SEM (n=9-12 mice/group). Representative images are shown. **(D-G)** Immunohistochemical staining of the indicated mammary tumors using **(D)** Ki67, **(E)** cleaved Caspase-3, **(F)** E-Cadherin and **(G)** Vimentin-specific antibodies. Representative images are shown. Statistical analysis was performed using a one-way Anova with a Tukey's multiple comparisons test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).



Supplementary Figure 2 - p66ShcA is overexpressed in lung and liver metastatic triple negative breast cancer cells. **(A)** Whole cell lysates were generated from parental 4T1 cells along with explants isolated either from breast tumors (BT: 148,152) or that are enriched for their metastatic ability to lung (526, 537), liver (2776, 2792) and bone (590, 592). Immunoblot analysis using ShcA- and Tubulin-specific antibodies. **(B)** Individual clones from 4T1 parental tumors were analyzed by immunoblot using a ShcA-specific antibody. **(C)** p66ShcA was deleted from 4T1-537 cells by Crispr/Cas9 genomic editing. Individual clones were screened by immunoblot analysis using ShcA- and Tubulin-specific antibodies. p66ShcA-null clones identified in red font were pooled to generate p66-CR cells used for further analysis. **(D)** 4T1-537, p66-CR cells were transfected with either empty vector (VC) or p66ShcA-WT or p66ShcA-S36A expression vectors. Immunoblot analysis using ShcA- and Tubulin-specific antibodies.

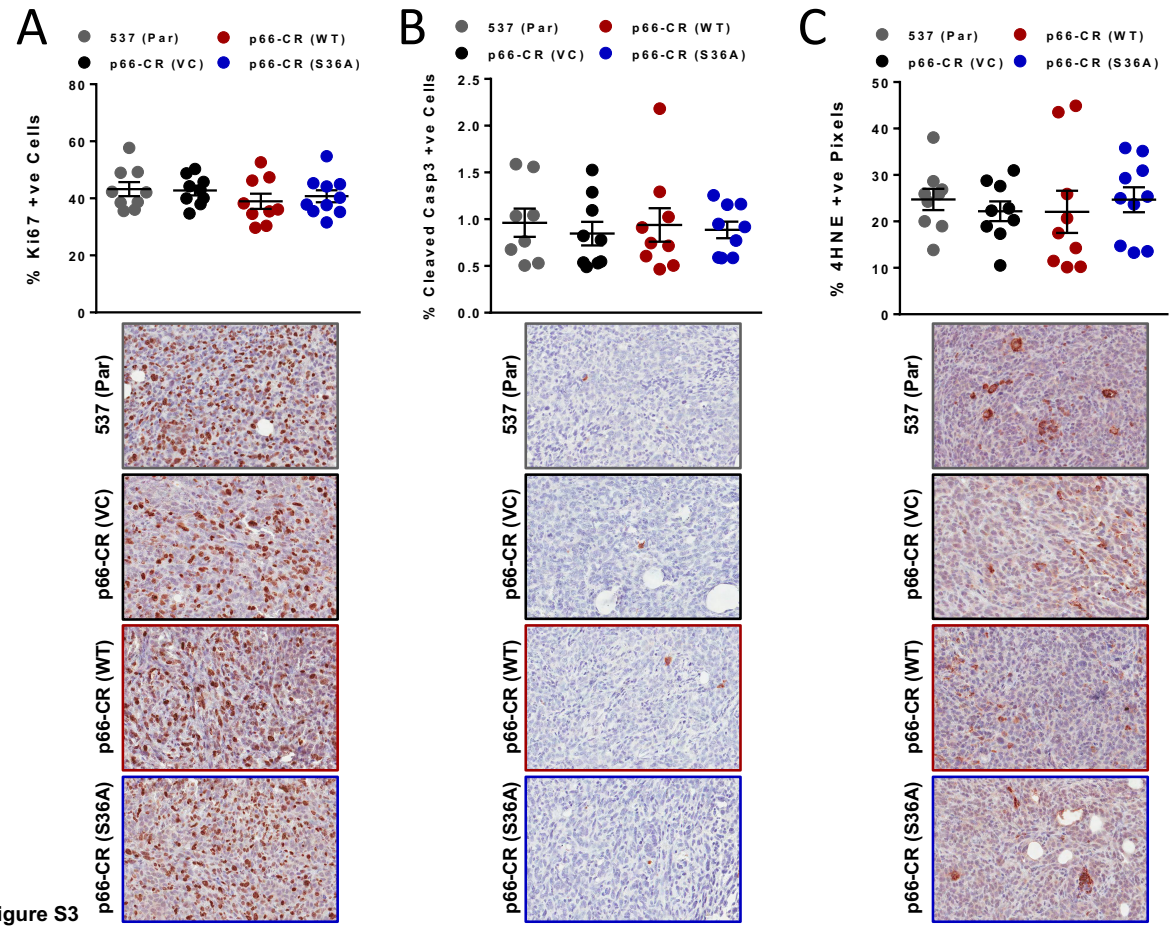
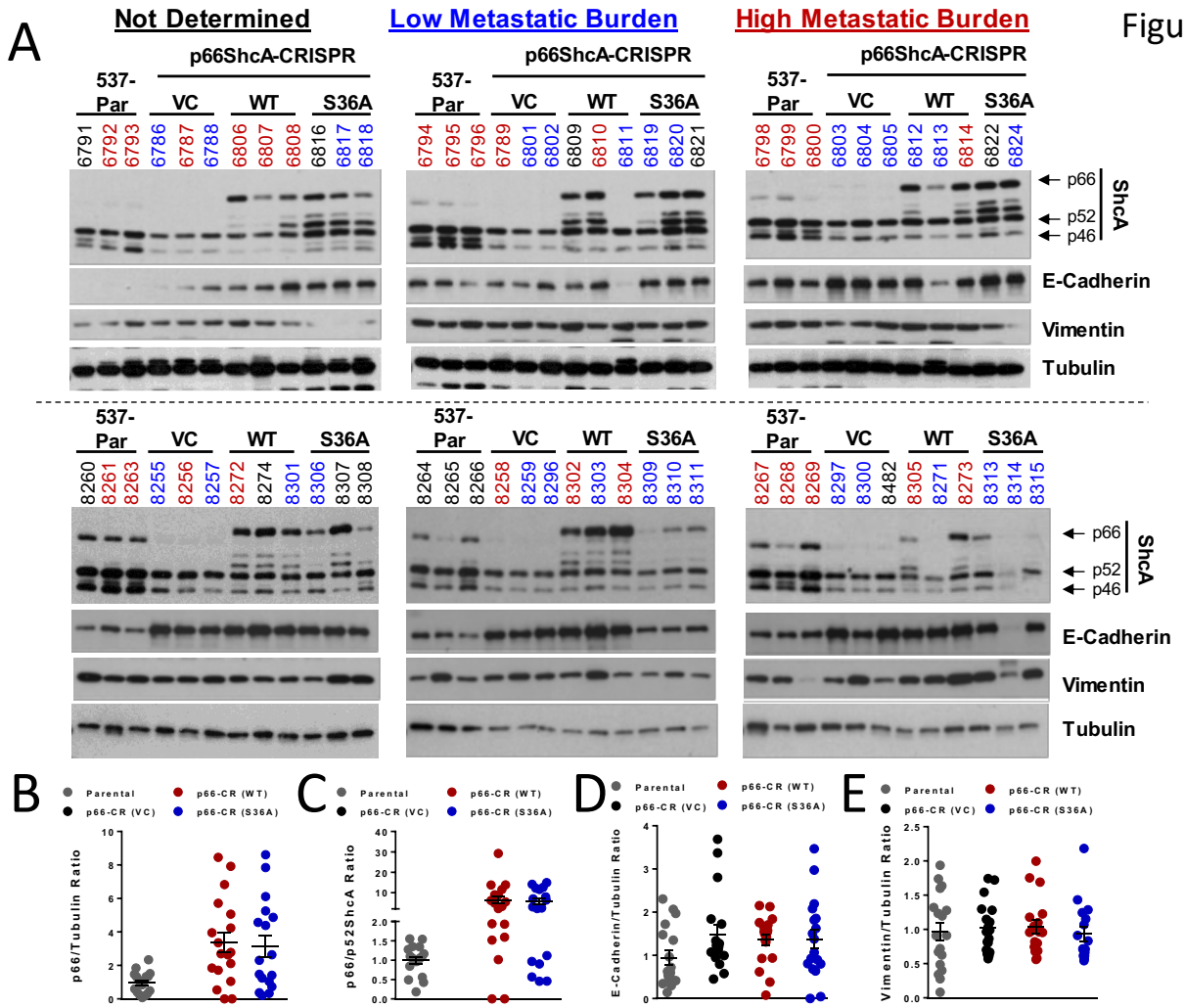


Figure S3

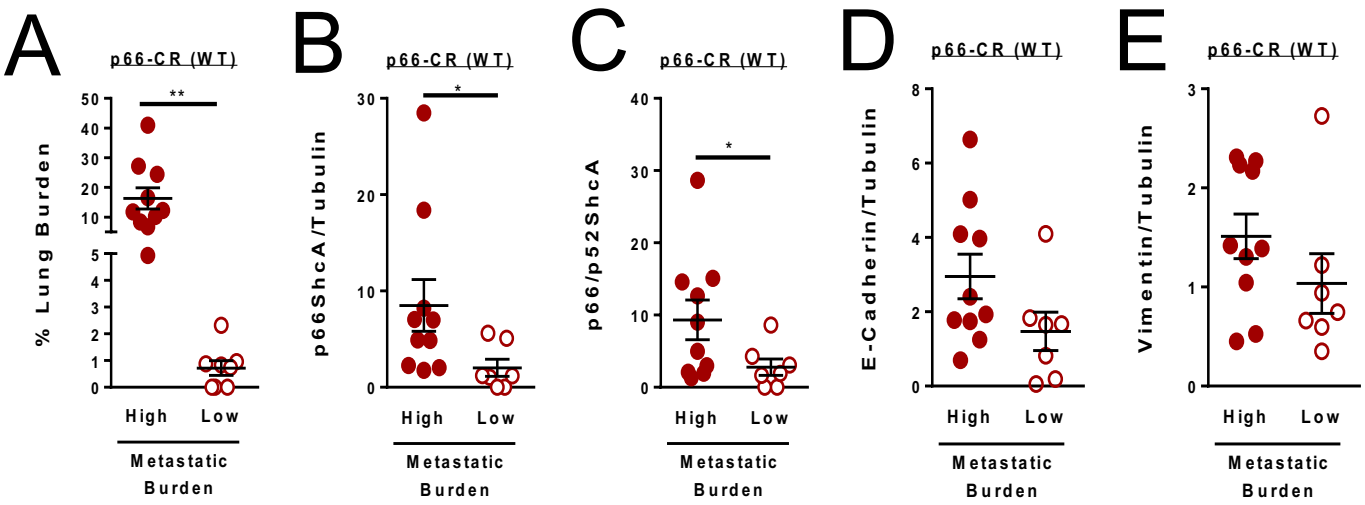
Supplementary Figure 3 - p66ShcA minimally impacts the growth properties of lung metastatic triple negative primary breast tumors. Quantification of the percentage **(A)** Ki67 positive cells, **(B)** cleaved caspase-3 positive cells and **(C)** 4-HNE positive pixels in 4T1-537 parental, p66-CR, p66-CR (WT) and p66-CR (S36A) mammary tumors. The data is shown as positivity ± SEM and is representative of 9-10 tumors per group. Representative images are shown below each graph.

Figure S4



Supplementary Figure 4 - p66ShcA does not alter the mesenchymal properties of 4T1-derived triple negative breast cancers. **(A)** Immunoblot analysis of whole cell lysates isolated from 4T1-537 parental, p66-CR (VC), p66-CR (WT) and p66-CR (S36A) mammary tumors (n=18 each) using ShcA-, E-Cadherin, Vimentin and Tubulin-specific antibodies. **(B-D)** Densitometric quantification of mammary tumors shown in panel A for the **(B)** p66ShcA/Tubulin, **(C)** p66ShcA/p52ShcA, **(D)** E-Cadherin/Tubulin and **(E)** Vimentin/Tubulin ratios. The data is normalized to the parental 537 tumors.

Supplementary figure 5 - High p66ShcA expression correlates with increased metastasis.



(A) 4T1-537 p66-CR (p66ShcA-WT) mammary tumors were stratified into two groups based on the degree of lung metastatic burden following tumor resection (high, n=10 versus low, n=7). These groups were then compared for relative **(B)** p66ShcA/Tubulin, **(C)** p66ShcA/p52ShcA, **(D)** E-Cadherin/Tubulin and **(E)** Vimentin/Tubulin protein levels in mammary tumors as shown in Supplementary Figure 2A. Statistical analysis was performed using a two-tailed, unpaired Welch's student's t test (*P<0.05; **P<0.01).

Supplemental table

Table S1	List of antibodies used in this study			
Antibody Specificity	**Company**	**Dilution (WB)**	**Dilution (IHC)**	**Cat #**
4-HNE	Abcam		1/500	ab46545
Cleaved caspase 3	Cell Signal		1/250	9661
E-cadherin	BD Biosciences	1/1000	1/200	610181
Ki67	Abcam		1/500	ab15580
ShcA	BD Biosciences	1/2500		610081
Tubulin	Sigma	1/5000		T5168
Vimentin	Abcam	1/1000	1/500	ab92547

Chapter 4 – Epigenetic regulation of p66ShcA in breast cancer

Jesse Hudson,[a] Harvey Li,[a] Valerie Sabourin,[a] Tiejun Zhao,[a] Michael Witcher,[a,b] and Josie Ursini-Siegel[a,b]

Lady Davis Institute for Medical Research, Montreal, Quebec, Canada[a];

Department of Oncology, McGill University, Montreal, Quebec, Canada[b]

Manuscript prepared December 2018

4.1 Preface

In this chapter, we explored mechanisms regulating p66ShcA expression in breast cancer cell lines across molecular subtypes and in parental versus metastatic variants. Epigenetic changes result in stable phenotypes that are heritable with cell division without any change to the DNA sequence. Hence, molecular alterations in the histone code or methylation of DNA can lead to changes in transcriptional programs (gene expression signatures) that are beneficial (pro-tumorigenic) and complementary to genetic mutations in tumor cells. These modifications are often influenced by external stimuli, including stress and the microenvironment and can even alter the tumorigenic and metastatic potential of cancer cells. Indeed, there is great excitement about the potential of epigenetic drugs to treat and potentially cure cancer in a number of settings. p66ShcA has previously been shown to be regulated at the epigenetic level through promoter methylation and acetylation, however, the regulation of p66ShcA in breast cancer in the primary and metastatic settings remains unknown.

4.2 Abstract

p66ShcA has been shown to have pro and anti-tumorigenic functions and its role in breast cancer has been conflicting and poorly understood. We previously provided the first in vivo evidence of the significance of p66ShcA in ErbB2+ luminal versus TNBC. In this study, we assessed the regulation of p66ShcA across molecular subtypes in breast cancer cells where endogenous p66ShcA expression was high, intermediate or low. Indeed, our work indicates transcriptional mechanisms govern p66ShcA expression at the epigenetic level. Active chromatin marks (open) were enriched at the p66ShcA promoter of breast cancer cells expressing high p66ShcA, which associated with the basal subtype. Furthermore, similar effects were seen in TNBC parental versus metastatic variant cells to the lung and liver. Within the basal subtype increased euchromatin at the p66ShcA promoter correlated with elevated CTCF binding in comparison to the luminal subtype. CTCF has been shown to regulate chromatin boundaries in tumor cells and PARylation of CTCF by PARP allows for its binding to DNA. Notably, UV damage was sufficient (known to activate PARP1 activity) to induce p66ShcA expression levels. Complementing these findings, inhibition of PARP activity reduced p66ShcA expression in the basal subtype. Finally, the p66ShcA promoter appears to be enriched for active chromatin in lung and liver metastatic variants compared to parental cells. These data suggest p66ShcA is regulated at the transcriptional level by chromatin modifiers, across molecular subtypes and during metastatic progression. This raises the intriguing possibility of an epigenomic basis for breast cancer metastasis that warrants further research to identify therapeutic avenues and biomarkers that may be beneficial for patient outcome.

4.3 Introduction

The best characterized epigenetic marker is DNA methylation; when a methyl group is covalently added to a cytosine residue, that precedes guanine, by a DNA methylase at the 5' regulatory end of genes. The consequences of DNA methylation in cancer were found to include hypomethylated or hypermethylated regions of the genome (Feinberg and Vogelstein 1983; Sakai et al. 1991). These hypo and hypermethylated regions occur in CpG islands and lead to the activation of oncogenes and inactivation of tumor suppressors respectively to promote the development of cancer (Flavahan et al. 2016; Sakai et al. 1991).

It is now known that alterations to histone structure regulate DNA hypermethylation and these interactions occur in complicated chromatin networks (Flavahan et al. 2016). DNA hypomethylation is commonly seen in cancer and recently has become a therapeutic target of interest given its influence on tumor progression and metastasis (Stefanska et al. 2014). Lysine acetylation of histones is associated with transcriptional activation (Hebbes, Thorne, and Crane-Robinson 1988). In contrast, lysine methylation promotes transcriptional activation or repression depending upon which residue is modified and the degree of methylation (Liang et al. 2004). Common histone modifications that occur in cancer include: H3K4me3, H3K9Ac and H3K27Ac near the transcription start site that correlate with low levels of DNA methylation and open, actively transcribed chromatin (Liang et al. 2004; Sharma, Kelly, and Jones 2009). Notably, H3K9me3 and H3K27me3 are repressive chromatin marks and the two main mechanisms associated with gene silencing in mammalian cells (Hon et al. 2012; Margueron et al. 2009). The polycomb repressive complex consisting of PRC1 and PRC2 control methylation of H3K27 (Lund, Lohuizen, and M 2004). G9a has been shown to regulate methylation of H3K9 in HCC and

regulates p16 gene silencing along with DNA methylation (Kondo et al. 2007). In contrast, HAT's p300 and CBP maintain active chromatin by adding acetyl groups (Lund, Lohuizen, and M 2004).

While the p46 and p52 isoforms are expressed ubiquitously in breast cancer cell lines and primary tumors, p66 levels are variable and controlled through an alternative promoter (Stevenson and Frackelton 1998; Ventura 2002). This is in agreement with published work indicating p66ShcA is regulated at the epigenetic level through promoter methylation and deacetylation (Ventura 2002). Intriguingly, endothelial cell exposure to LDL was shown to induce p66ShcA expression through a mechanism dependent on the DNA methyltransferases DNMT1 and DNMT3b (Y.-R. Kim et al. 2012). Indeed, homocysteine was shown to epigenetically regulate p66 expression by controlling methylation of CpG dinucleotides 6 and 7 (CpG6,7) within the p66 promoter (C. S. Kim et al. 2011). Sirtuin1, a class 3 histone deacetylase, has also been shown to epigenetically regulate the p66 promoter through modifications on histone 3 (S. Zhou et al. 2011). In lung cancer, ChIP analyses for histone marks in the $p66^{ShcA}$ promoter region indicated epigenetic silencing of p66ShcA as revealed through decreased association of activating histones (H3K9Ac, H3K4me2, and H3K4me3) and enrichment of repressing histones (H3K9me2) in SCLC cells. Moreover, occupancy of this region was controlled by the lymphocyte lineage-restricted transcription factor, Aiolos (X. Li et al. 2014). Hence, while a number of mechanisms have been shown to regulate p66ShcA expression, little is known in the context of breast cancer.

The zinc finger CCCTC-binding factor (CTCF) functions as an epigenetic regulator of gene transcription by preventing the spread of repressive heterochromatin at promoter elements (Witcher and Emerson 2009a). Basal breast tumors exhibit genomic instability, which activates poly(ADP-ribose) polymerase (PARP). In turn, PARP catalyzes the post-translational addition of poly-ADP ribose (PAR) units onto proteins involved in DNA damage repair, which increases their

function (Smith D.C, Simon M., Alldredge A.L. 1992). PARP-dependent parylation of CTCF is required for CTCF to bind DNA and CTCF was PARylated and dissociated from PARP-1 in cells expressing the p16 tumor suppressor versus in cells where it was silenced (Witcher and Emerson 2009a). Intriguingly, CTCF has even been shown to promote cell survival in breast cancer (Venkatraman and Klenova 2015). Given the wealth of literature on the epigenetic regulation of p66ShcA, we chose to investigate mechanisms controlling p66ShcA expression in p66ShcA-high versus p66ShcA-low expressing breast cancer cells and in vivo selected metastatic variants. These studies could be useful in identifying attractive therapeutic targets and candidate biomarkers.

4.4 Material and Methods

Cell lines

MCF7, MDA-MB-468, MDA-MB-231, Hs578T and BT549 cells were obtained from the ATCC. 4T1 and MDA-MB-231 metastatic variant cell lines were developed by Dr. Peter Siegel through in vivo selection to different organs and were a gift. All cell lines were cultured in 10% fetal bovine serum (FBS)–Dulbecco modified Eagle medium (DMEM).

Immunoblotting

Immunoblots were performed as described (Josie Ursini-Siegel et al. 2008) using the antibodies listed in Table S1.

RT-qPCR studies

For the quantitative PCR (qPCR) studies, total DNA was isolated from ChIP assays for the various cell lines. Quantitative PCR was performed using the QuantiTect SYBR green RT-PCR kit (Qiagen) with primers listed in Table S2 in the supplemental information. For the reverse transcription-quantitative PCR (RT-qPCR) studies, total RNA was isolated using RNeasy midi-kits (Qiagen) and cDNA was generated using Superscript II reverse transcriptase (Invitrogen).

Quantitative PCR was performed using the QuantiTect SYBR green RT-PCR kit (Qiagen) with primers listed in Table S2 in the supplemental material.

Chromatin Immunoprecipitation (ChIP) Assays

Cells were prepared using 4 x 15cm plates. DNA and protein were crosslinked for 10 minutes at room temperature using PBS with 1% formaldehyde (final concentration) and the reaction was ended by adding 125mM Glycine. Plates were washed two times with PBS (Cells can be frozen in liquid N2 at this point). 1ml of lysis buffer was added per 15 cm plate and cells were scraped and pooled. Cells were sonicated to ~300-1000 bp using a Fisher sonicator at 15 bursts of 15 seconds using a power setting of 20. Lysates were spun down (split to 1.5 ml tubes) at 4^0C for 15 minutes at top speed and the supernatant was collected. 1 mg of protein lysate was added per CHIP sample to a 1.5 ml tube and filled up to 1ml using ChIP IP buffer (Final detergent concentration of 0.75-1%). Chromatin solution was precleared for 2 hours using 50µl's of 50% protein G beads suspended in IP buffer to reduce background at 4^0C. To reduce non-specific binding, G beads were blocked with salmon sperm DNA (final concentration of 0.3 mg/ml). To prepare beads (sepharose, agarose or magnetic), IP buffer was mixed with beads in an eppendorf tube and spun down. Excess was suctioned off and beads were washed with 1ml IP Buffer and spun. After suctioning excess an equal volume of IP Buffer was added to produce a 50% slurry. 1% of the precleared lysate was removed for input (frozen). Eppendorf tubes were centrifuged at 4000 rpm for 1 minute to pellet beads. Supernatant was aliquoted into equal volumes into eppendorf tubes (~1.2 ml/tube). 30µl's of primary antibody G slurry mix (50% protein G beads suspended in 50% IP buffer slurry with the requisite primary antibody) was added and incubated overnight at 4^0C. For histone and CTCF CHIPs, 2.5µg's of primary antibody was added per sample (H3K9Ac; 07-352, Millipore), (H3K27Ac; 07-360, Millipore) and (H3K4Ac; 07-473, Millipore) and (CTCF; 07-729, Millipore).

50% G slurry was prepared in advance (and spun for 1 to 2 hours) with 0.3 mg/ml salmon sperm DNA (to block non-specific binding capacity). Negative bead only controls were included. Tubes were spun at 4000 rpm for 1 min to pellet beads. Beads were sequentially washed with 1ml of the following wash solutions for 5 minutes:

Wash#1 0.1%SDS, 1% Triton x-100, 2mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl

Wash#2 0.1% SDS, 1% Triton x-100, 2mM EDTA, 20 mM Tris-HCl pH 8.0, 300 mM NaCl

Wash#3 0.1% SDS, 1% Triton x-100, 2mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl

Wash#4 0.25M LiCl, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 10 mM Tris-HCl pH 8.0

Wash #5&6 TE pH 8.0 for 3 minutes each to remove salt and detergents.

Remove all wash buffers and only beads are left in tube.

Phenol-chloroform DNA purification. Complexes were eluted from beads with 500µl elution buffer for 15 minutes at 65^{o}C (Vortexing tubes occasionally). Beads were spun at 4000 rpm for 3 minutes. Elution buffer (this now has your protein-DNA complex) was transferred to a new tube. 20µl NaCl was added to the 500µl eluate (to 0.2 mM) and allowed DNA to uncrosslink at 65^{0} C overnight. 2µl of 10mg/ml proteinase K was added to the eluate the next day and incubated for 1 hour at 45°C followed by 15 minutes at 65°C. DNA was recovered by adding 500µl phenol/chloroform/isoamyl alcohol mix and spin down for 4 minutes at top speed. Supernatant was transferred to a new tube and a chloroform wash was performed to ensure the complete removal of phenol. To precipitate DNA 900µl of 95% EtOH was added with 2 µl (20 µg) of ytRNA (as a carrier), and 17 µl of 3M NaAcetate. and the mixture was allowed to incubate overnight. Tubes were then spun down at max speed for 15 minutes to pellet DNA. DNA was washed with 70% EtOH and spun for 10 minutes. EtOH was removed and DNA allowed to dry (can dry in

speed vacuum). Pellet was resuspended in 100µl H_2O (or 0.1x TE) and vortexed. 5µl was used per sample for quantitative pcr amplification.

Buffers

Lysis Buffer

0.5% NP-40, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50mM Tris pH 8, 100mM NaCl, 5mM EDTA, add protease inhibitors and NaF directly prior to lysis

IP Buffer

100mM NaCl, 50mM Tris pH 8.0, 5mM EDTA and 1% triton X-100

Elution Buffer for Phenol-chloroform

1% SDS, 1mM EDTA and 50mM Tris pH 8.0

Elution Buffer for column

1% SDS, 50mM tris pH 8.0 and 1mM EDTA

PB Buffer

5M Guanidine-HCl, 30% isopropanol and store at RT

PE Buffer

10 mM Tris-HCl pH 7.5, 80% ethanol and store at RT

For UV Experiments

BT474 (luminal) and MDA-MB-231 (basal) breast cancer cells were treated with UVC irradiation (0-20 J/m2) and whole cell lysates were made 1H, 24H or 48H post UVC treatment and screened for UV damage and p66ShcA expression.

PARP inhibitor experiments

PARP activity was blocked by treating breast cancer cells with 20mM of the PARP inhibitor 3-ABA or DMSO for 24H. (Sigma, cat #: 3544-24-9)

Statistical analysis

All statistical analysis was performed using a two-tailed Student t test

4.5 Results

p66ShcA expression correlates with active chromatin marks

As p66ShcA has previously been shown to be epigenetically regulated in human cells we sought to investigate the mechanism governing high versus low p66ShcA expression in breast cancer. We performed chromatin immunoprecipitation assays to screen the p66ShcA promoter for active histone marks that are indicative of open euchromatin (H3K4me3, H3K9Ac and H3K27Ac) and compared the activation status to that of the p46/p52ShcA promoter. Indeed, we discovered that high p66ShcA expression correlated with increased association of active histone marks (H3K4me3, H3K9Ac and H3K27Ac) within the p66ShcA promoter (Figure1), upstream of the transcriptional start site, while low levels of active chromatin marks were present within the p66ShcA promoter of low p66ShcA expressing cells. In comparison, the activation status of the p46/p52ShcA promoter remained steady, with high levels of acetylated H3 binding seen across all breast cancer cell lines tested.

The epigenetic insulator CTCF binds to the p66ShcA promoter and correlates with high p66ShcA expression levels and active chromatin marks

Next, we sought to determine if the p66ShcA promoter possessed any binding sites for known epigenetic regulators. CTCF has been shown to regulate chromatin boundaries at tumor suppressor genes like p16 by preventing the spread of repressive chromatin marks (Witcher and Emerson 2009a). Thus, we wondered if the p66ShcA promoter might possess a CTCF binding

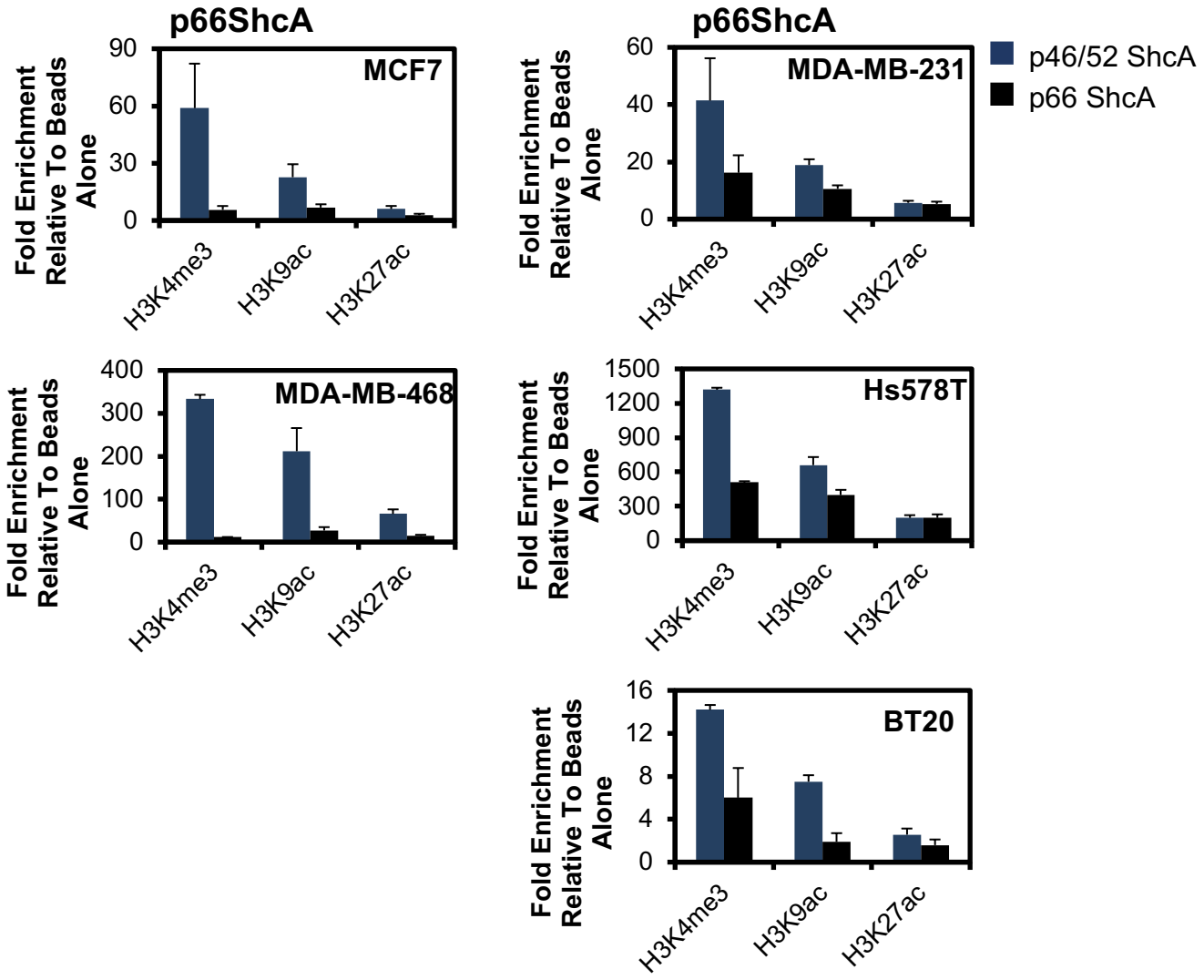


Figure 1 - High p66ShcA expression levels correlate with active chromatin marks across a panel of breast cancer cell lines. Active versus repressed chromatin immunoprecipitation (CHIP) assays were performed on cell lysates from two p66ShcA negative cell lines (MCF7-luminal; MDA-MB-468 (basal) and three basal breast cancer cell lines that show moderate (MDA-MB-231; Hs578T) or strongly elevated (BT20) p66ShcA expression levels as previously described. Using a H3K4me3, H3K9Ac or H3K27Ac-specific antibody, the amount of active histone present at the p66ShcA promoter (-2000 p66ShcA) was determined. The data is normalized to values obtained with control IPs (beads alone) and is representative of three biological repeats (active chromatin mark or beads alone IP reactions) per cell line. For each biological repeat, the qPCR reaction was done in triplicate and the average value of the three technical repeats is reported.

site as it is expressed through an alternative promoter compared to p46/p52 ShcA. Screening several cell lines within the UCSC genome browser for epigenetic binding sites within the p66ShcA promoter, we did indeed discover the presence of a CTCF binding site approximately 250-450bp upstream of the p66ShcA transcriptional start site (Figure2, panel A). Hence, we set out to test this hypothesis in vitro using breast cancer cell lines expressing low, intermediate and high levels of p66ShcA. Using CTCF-specific antibodies and performing chromatin immunoprecipitation assays, we discovered that CTCF is indeed significantly enriched within the p66ShcA promoter and is present at the highest levels in breast cancer cell lines expressing intermediate and high levels of p66ShcA (Figure2, panel B). Therefore, the p66ShcA promoter possesses both active histone marks and increased binding of CTCF upstream of the p66ShcA start site, which may indicate it plays an insulator function in these cells.

UV damage and PARP activity regulate p66ShcA expression in breast cancer cell lines

PARP-dependent PARlation of CTCF is necessary for its insulator function in breast cancer as PARlated CTCF correlates with active expression of p16 (Witcher and Emerson 2009a). In response to UV exposure a number of DNA repair enzymes are upregulated in order to repair damaged DNA, including PARP. Therefore, we wished to test whether breast cancer cells exposed to UV damage might induce the expression and activation of PARP, which could indirectly upregulate p66ShcA expression by promoting PARlation of CTCF and induce its binding to the p66ShcA promoter to promote the formation of open euchromatin. Notably, exposure to UV damage induced the upregulation of p66ShcA expression in breast cancer cell lines of the ErbB2 positive luminal and claudin-low subtypes that normally express low or intermediate levels of p66ShcA (Figure3). Hence, stress stimuli stably induce the expression of p66ShcA (24H or 48H

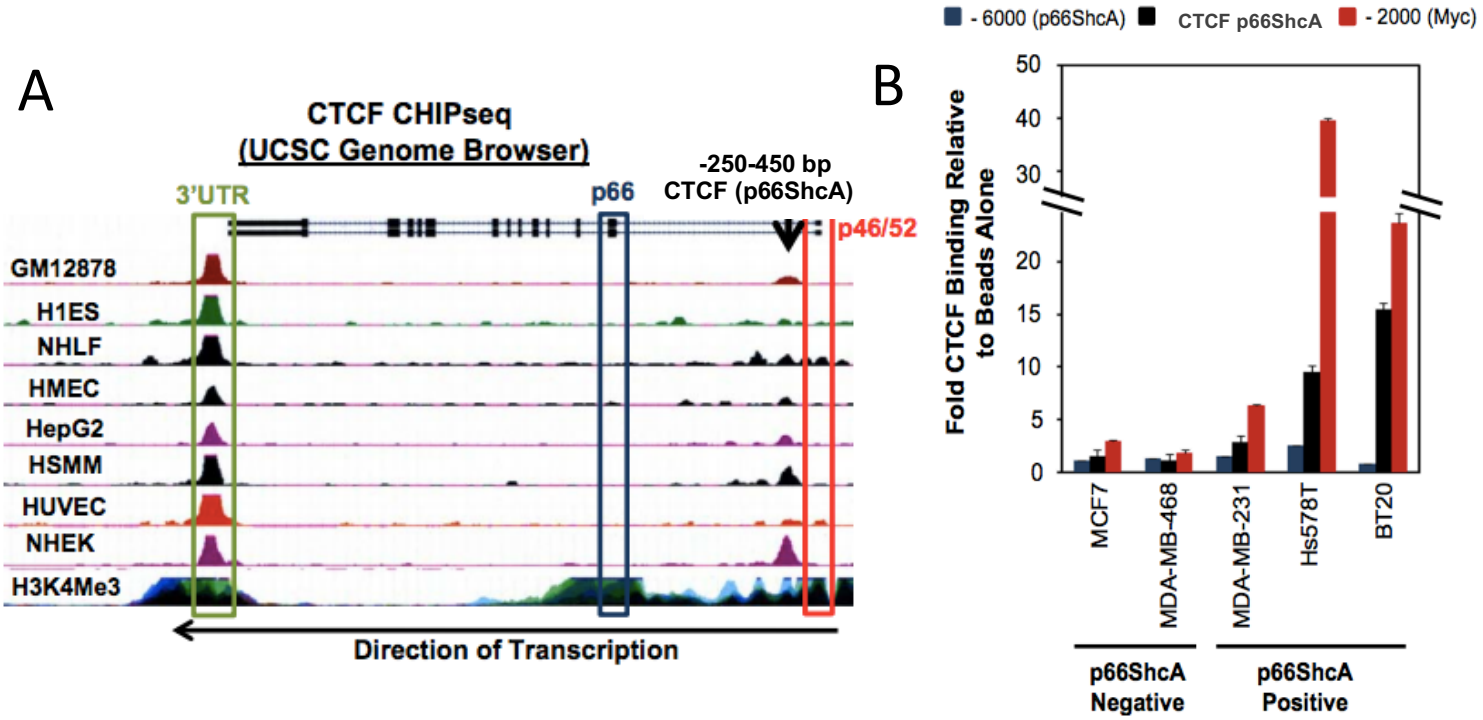


Figure 2 - High p66ShcA expression levels correlates with a CTCF chromatin boundary within the p66ShcA promoter in basal breast cancer. (A) CTCF-CHIPseq analysis for the ShcA locus from 9 normal and cancer human cell lines using the UCSC genome browser (http://genome.ucsc.edu). A CTCF binding site (black arrow) is flanking between the p46/52ShcA (red box) and p66ShcA (blue box) and resides 2000nt upstream of the p66ShcA promoter (blue box). Importantly, published studies have demonstrated that the p66ShcA promoter contains many CpG islands, which are hypermethylated in p66ShcA negative cells. In contrast, the p46/52 ShcA promoter lacks CpG islands (ref). A strong CTCF binding site is present in the 3'UTR of the ShcA gene (green box). **(B)** CTCF chromatin immunoprecipitation (CHIP) assays were performed on cell lysates from two p66ShcA negative cell lines (MCF7-luminal; MDA-MB-468 (basal) and three basal breast cancer cell lines that show moderate (MDA-MB-231; Hs578T) or strongly elevated (BT20) p66ShcA expression levels as previously described. Using a CTCF-specific antibody, the amount of CTCF bound to the CTCF binding site upstream of the p66ShcA locus (-2000 p66ShcA), to a non-CTCF site distal to the ShcA gene (-4600 p66ShcA) and a strong CTCF binding site distal to the Myc promoter (-2000 Myc) as a positive control. The data is normalized to values obtained with control IPs (beads alone) and is representative of three biological repeats (CTCF or beads alone IP reactions) per cell line. For each biological repeat, the qPCR reaction was done in triplicate and the average value of the three technical repeats is reported.

after UV exposure), which may provide indirect evidence that PARP regulates p66hcA protein levels. Next, we treated basal breast cancer cells normally expressing high or intermediate endogenous levels of p66ShcA with a PARP inhibitor (3-Aba) to test for the importance of PARP activity in the regulation of p66ShcA. Intriguingly, direct inhibition of PARP activity was sufficient to reduce the expression levels of p66ShcA in vitro. Thus, UV damage and PARP activity both regulate p66ShcA expression in breast cancer cell lines.

p66ShcA expression is transcriptionally regulated in aggressive TNBC and correlates with increased histone activation status within the p66ShcA promoter

To gain a better understanding of how p66ShcA is regulated in metastatic variants we first looked at mRNA expression across a panel of TNBC cell lines. In line with previous work, p66ShcA appears to be regulated at the transcriptional level at secondary sites as mRNA expression of p66ShcA was significantly enriched in lung and liver metastatic variants compared to parental 4T1 cells and this correlates with protein levels seen in chapter 3 (Figure4, panel A), (Hudson et al. 2014; Ventura 2002). Next, inhibition of transcription (actinomycin D) and translation (cycloheximide), in TNBC lung and liver variants confirmed p66ShcA is indeed controlled at the transcriptional level (Figure4, panel B), as previously described (Ventura 2002).

Notably, p66ShcA expression was also upregulated in claudin-low metastatic variants to the lung, liver and bone (Figure4, panel C). As p66ShcA expression has been shown to be transcriptionally regulated at the epigenetic level (Ventura 2002), we wanted to determine if epigenetic mechanisms might also play a role in upregulating p66ShcA following in vivo selection. Screening the p66ShcA promoter for active chromatin marks, H3K9Ac and H3K27Ac marks were enriched in metastatic variants to the lung and liver (Figure4, panel D).

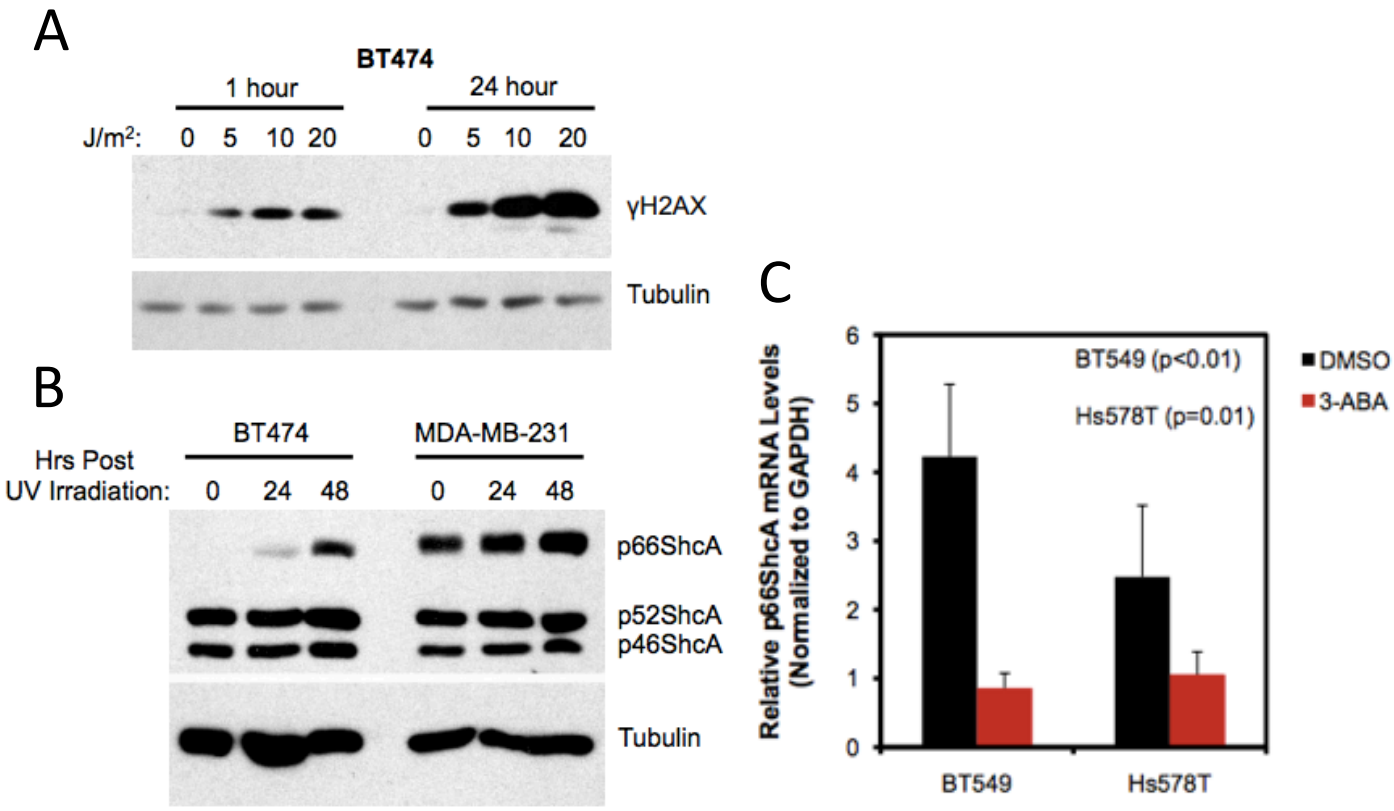
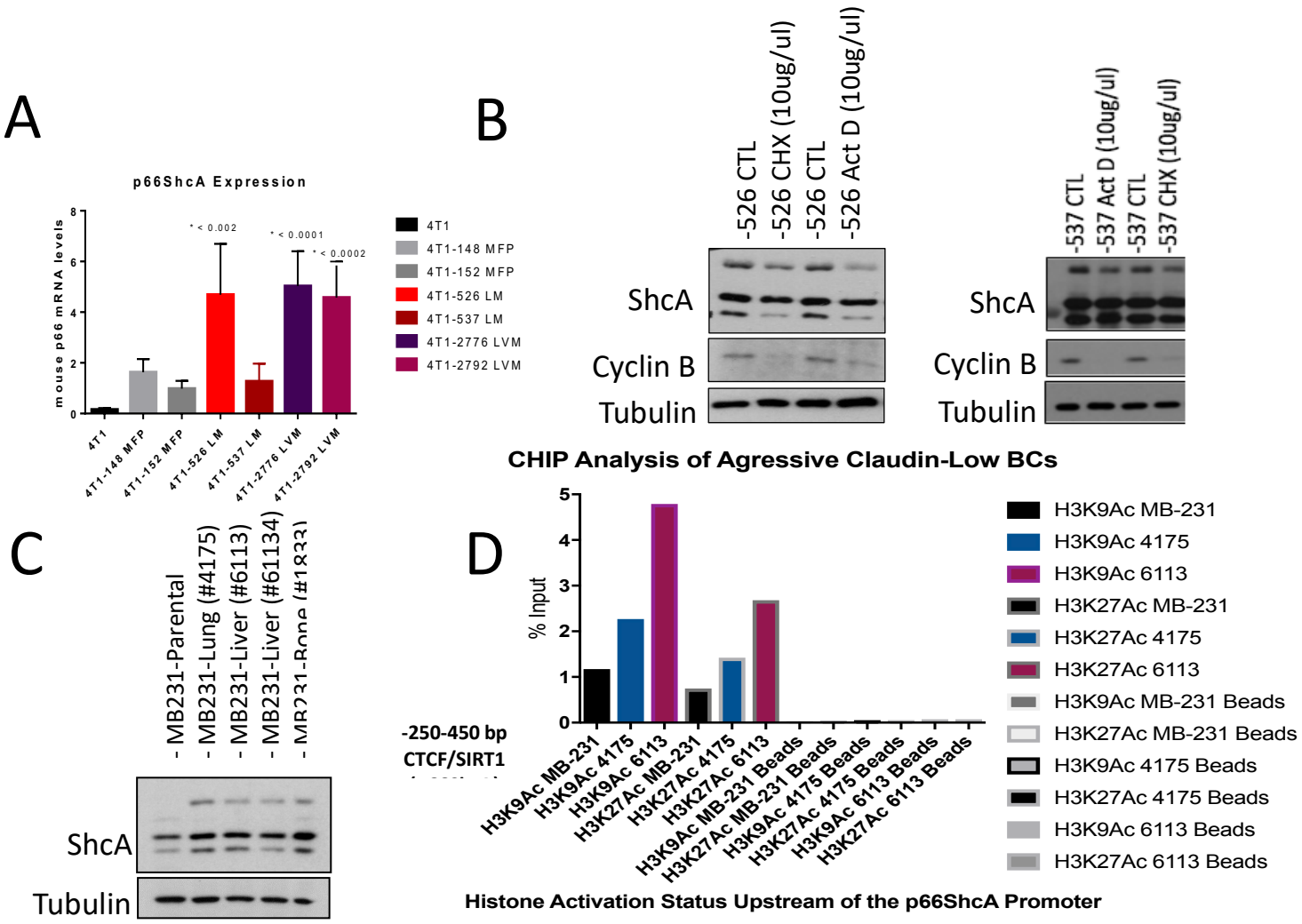


Figure 3 - UV exposure and damage induce high p66ShcA expression in ErbB2+ luminal and basal breast cancer, while PARP inhibition reduces p66ShcA expression in basal breast cancer. (A) BT474 (luminal) cells were treated with increasing amounts of UVC irradiation (0, 5, 10, 20 J/m2). Immunoblot analysis of cell extracts 1 or 24 hours post UVC treatment using γH2AX and Tubulin specific antibodies. **(B)** BT474 (luminal) and MDA-MB-231 (basal) breast cancer cells were treated with 20J/m2 UVC and whole cell lysates were generated 0, 24 or 48 hours later. Immunoblot analysis using ShcA and Tubulin specific antibodies. **(C)** Basal breast cancer cell lines expressed moderate (Hs578T) or high (BT549) p66ShcA levels were cultured for 24 hours with 20mM 3-ABA (a pharmacological PARP inhibitor) or DMSO as a control. Total RNA was extracted and subsequently analyzed by RT-qPCR analysis using p66ShcA-specific primers (residing within the CH2 domain). The data is normalized to GAPDH levels and is representative of two biological and two technical repeats per condition. Statistical analysis was performed using a two tailed student's T test.



<u>Figure 4</u> - p66ShcA expression is transcriptionally regulated in aggressive TNBC and correlates with increased histone activation status within the p66ShcA promoter. (A) mRNA expression of p66ShcA normalized relative to GAPDH controls measured by RT-qPCR. **(B)** Immunoblot analysis of aggressive TNBC cell lysates using ShcA- or Tubulin- specific antibodies. Cells were treated with (10ug/ul) actinomycin D or chlorhexidine (10ug/ul) for 10 hours to inhibit de novo transcription or translation, respectively. **(C)** Immunoblot analysis of a panel of aggressive (*in vivo* selected to the lung, liver or bone) "Claudin-low" breast cancer cell lysates using ShcA or Tubulin specific antibodies. **(D)** Active versus repressed chromatin immunoprecipitation (CHIP) assays were performed on cell lysates from one p66ShcA moderate expression cell lines (MDA-MB-231 (TNBC)) and two aggressive TNBC cell lines that show high (MDA-MB-231 (lung) and (liver)) p66ShcA expression levels. Using H3K9Ac or H3K27Ac-specific antibody, the amount of active histone present at the p66ShcA promoter was determined (-250-450 bp upstream of the p66ShcA start site and at the CTCF/SIRT1 binding site). The data is normalized to values obtained with control IPs (beads alone) and is representative of three biological repeats.

4.6 Discussion

In this study, we identified novel mechanisms regulating p66ShcA expression in established breast cancer cell lines and in vivo selected metastatic variants. p66ShcA expression correlates with active chromatin in breast cancer cell lines across molecular subtypes, with the highest levels being in the "claudin-low" Basal B subtype (Figure1). This is in line with our previous work indicating p66ShcA is enriched in the basal subtype and that the p66ShcA promoter is controlled through promoter methylation and acetylation (Hudson et al. 2014; Ventura 2002). Hence, epigenetic regulators appear to be important for p66ShcA expression in breast cancer and across subtypes. Our work shows that the p66ShcA promoter contains a CTCF binding site in several cell lines (Figure2, panel A). Furthermore, the amount of binding directly correlates with p66ShcA expression levels and with the presence of open chromatin across molecular subtypes (Figure2, panel B). CTCF is known to function as an insulator protein by establishing a chromatin boundary within the p16 promoter that is lost when the gene is silenced in cancer cells (Witcher and Emerson 2009a). In addition, CTCF binding also correlates with RASSF1A and CDH1 transcription, while this interaction is absent when these genes are methylated and silenced. Hence, out data are in line with previous observations outlining a role for CTCF as an insulator protein.

Notably, it was shown that the class 3 histone deacetylase Sirt1 binds to and is enriched at CTCF binding sites in murine embryonic stem cells and loss of SIRT1 correlates with increased histone activation at the CTCF binding region of the Dnmt3l locus (Heo et al. 2017). Indeed, we find that CTCF binding within the p66ShcA promoter occurs at the Sirt1 binding site (S. Zhou et al. 2011). It is tempting to suggest that a complex involving CTCF and Sirt1 (which has been shown to negatively regulate p66ShcA) may dictate p66ShcA gene activation in breast cancer. Both SIRT1 and PARP1 share a common co-factor nicotinamide adenine dinucleotide (NAD+),

making it a rate limiting substrate in cancer cells (Luna, Aladjem, and Kohn 2013). Sirt1 dependent deacetylation of histones uses NAD+, while PARP1 requires it for ADP-ribosylation, including ADP-ribosylation of CTCF for the establishment of chromatin boundaries (Witcher and Emerson 2009b). Thus, a CTCF/PARP1/SIRT1 complex may act as an on/off switch to regulate p66ShcA transcription, with NAD+ availability functioning to tip the balance in either direction. Indeed, UV exposure, which is known to induce PARP activity, significantly upregulates the expression of p66ShcA in luminal and basal breast cancer cell lines (Figure3). In addition, inhibition of PARP activity in TNBC cells that express intermediate or high levels of p66ShcA leads to significantly reduced p66 mRNA expression (Figure4). Further experiments are required to directly test the role of these proteins in the direct regulation of p66ShcA expression.

It is also possible that other players may be involved as several positive and negative regulators of H3K9Ac and H3K27Ac status exist. PRC2 is an H3 Lys 27 deacetylase that represses gene expression and consists of several subunits, including the PRC2 components: EZH2, SUZ12, and EED (Tan et al. 2007). Indeed, p66ShcA expression inversely correlates with EZH2 in breast cancer patients across molecular subtypes, where EZH2 is highest in the basal and ErbB2 subtypes, while p66ShcA is enriched in patients of the luminal A and normal-like (Bockhorn et al. 2014; Hudson et al. 2014). Notably, the luminal A subtype is characterized by ER positivity and good outcome. A recent study reveals that inhibition of HDACs and DNMTs leads to re-expression of ER in ER negative tumors and re-sensitizes these tumors to tamoxifen treatment (Meeran et al. 2012). Hence, p66ShcA may act as a biomarker for ER negative tumors that would be at an increased benefit to a combination of tamoxifen and epigenetic treatments. Further studies are required to test p66ShcA expression and sensitivity to HDACs and DNMTs.

Our work shows that upregulation of p66ShcA is transcriptionally controlled (Figure5, panels A and B) at the epigenetic level (Figure6, panel B). Moreover, the tumor microenvironment may play a prominent role in governing p66ShcA expression in metastatic variant TNBC cells as in vivo selection through the lungs and liver significantly increases p66ShcA protein levels compared to parental cells and those from the mammary fatpad (Chapter 3 - S2, panel A). This is in line with published work indicating p66ShcA is regulated through promoter methylation and acetylation in normal and cancer cells (Ming-Shyue et al. 2003; Ventura 2002). Notably, genes that mediate metastasis to the lung have been shown to be transcriptionally regulated, as protein levels correlate with gene expression profiles (Minn et al. 2005). Indeed, the tumor microenvironment induces a number of transcription factors promote tumor progression and Twist, Zeb, or SLUG to promote an EMT to (Ell and Kang 2013).

In addition, epigenetic regulation can also lead to metastatic spread such as EZH2 dependent silencing of E-cadherin or FOXC1 in breast cancer (Cao et al. 2009; J. Du et al. 2012). In breast cancer, EZH2 was downregulated in PDX metastatic lung variants and shown to regulate migration and metastasis (Bockhorn et al. 2014). Thus, transcriptional, epigenetic programs appear to govern metastasis. G9A, a methyltransferase responsible for H3K9me2, interacts with Snail to repress E-cadherin in breast cancer (Dong et al. 2012). SETBD1 methylates histone H3 on lysine 9 (H3K9), positively maintains stem cell state and was recently shown to regulate Myc to control EMT, invasion and metastasis in breast cancer (Jinling and Hospital 2014). Studies directly testing the functional importance of histone modifications and methylation status within the p66ShcA promoter are necessary to establish a mechanism governing p66ShcA expression both in TNBC and metastatic variants and we have listed some potential candidates. Intriguingly, epigenetic mechanisms were recently shown to induce resistance to therapy by reprogramming cell state

plasticity in breast cancer. However, chromatin modifier inhibitors were able to re-sensitize breast tumor cells in the basal subtype (Risom et al. 2018). This study underscores the importance of epigenomics in promoting tumor plasticity, heterogeneity and resistance in cancer and the potential of targeting the histone code as an effective anti-cancer therapeutic avenue. Further investigation is required to determine whether epigenetic therapies may differentially effect p66ShcA expressing tumors during tumorigenesis and progression.

4.7 References

Bockhorn, J., Prat, A., Chang, Y. F., Liu, X., Huang, S., Shang, M., … Liu, H. (2014). Differentiation and loss of malignant character of spontaneous pulmonary metastases in patient-derived breast cancer models. *Cancer Research*, *74*(24), 7406–7417. https://doi.org/10.1158/0008-5472.CAN-14-1188

Cao, Q., Yu, J., Dhanasekaran, S. M., Kim, J. H., Tomlins, S. A., Mehra, R., … Arul, M. (2009). Repression of E-cadherin by the Polycomb Group Protein EZH2 in Cancer, *27*(58), 7274–7284. https://doi.org/10.1038/onc.2008.333.Repression

Dong, C., Evers, B. M., Zhou, B. P., Dong, C., Wu, Y., Yao, J., … Rychahou, P. G. (2012). G9a interacts with Snail and is critical for Snail- mediated E-cadherin repression in human breast cancer Find the latest version : G9a interacts with Snail and is critical for Snail-mediated E-cadherin repression in human breast cancer, *122*(4), 1469–1486. https://doi.org/10.1172/JCI57349.microenvironment

Du, J., Li, L., Ou, Z., & Kong, C. (2012). FOXC1 , a target of polycomb , inhibits metastasis of breast cancer cells, 65–73. https://doi.org/10.1007/s10549-011-1396-3

Ell, B., & Kang, Y. (2013). Transcriptional control of cancer metastasis, *23*(12), 603–611.

Fang, F., Turcan, S., Rimner, A., Kaufman, A., Giri, D., Morris, L. G. T., … Chan, T. A. (2011). Breast Cancer Methylomes Establish an Epigenomic Foundation for Metastasis. *Science Translational Medicine*, *3*(75), 75ra25 LP-75ra25. Retrieved from http://stm.sciencemag.org/content/3/75/75ra25.abstract

Feinberg, A. P., & Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, *301*(5895), 89–92. https://doi.org/10.1038/301089a0

Flavahan, W. A., Drier, Y., Liau, B. B., Gillespie, S. M., Venteicher, A. S., Stemmer-Rachamimov, A. O., … Bernstein, B. E. (2016). Insulator dysfunction and oncogene activation in IDH mutant gliomas. *Nature*, *529*(7584), 110–114. https://doi.org/10.1038/nature16490

Hebbes, T. R., Thorne, A. W., & Crane-Robinson, C. (1988). A direct link between core histone acetylation and transcriptionally active chromatin. *The EMBO Journal*, *7*(5), 1395–1402. https://doi.org/10.1007/BF00777468

Heo, J., Lim, J., Lee, S., Jeong, J., Kang, H., Kim, Y. H., … Shin, D. M. (2017). Sirt1 Regulates DNA Methylation and Differentiation Potential of Embryonic Stem Cells by Antagonizing Dnmt3l. *Cell Reports*, *18*(8), 1930–1945. https://doi.org/10.1016/j.celrep.2017.01.074

Hon, G. C., Hawkins, R. D., Caballero, O. L., Lo, C., Lister, R., Pelizzola, M., … Ren, B. (2012). Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer, 246–258. https://doi.org/10.1101/gr.125872.111

Hudson, J., Ha, J. R., Sabourin, V., Ahn, R., La Selva, R., Livingstone, J., … Ursini-Siegel, J. (2014). p66ShcA Promotes Breast Cancer Plasticity by Inducing an Epithelial-to-Mesenchymal Transition. *Molecular and Cellular Biology*, *34*(19), 3689–3701. https://doi.org/10.1128/MCB.00341-14

Jinling, N., & Hospital, Z. (2014). MiR-7 , Inhibited Indirectly by LincRNA HOTAIR , Directly Inhibits SETDB1 and Reverses the EMT of Breast Cancer Stem Cells by Downregulating the STAT3 Pathway, (32), 2858–2868.

Kim, C. S., Kim, Y. R., Naqvi, A., Kumar, S., Hoffman, T. A., Jung, S. B., … Irani, K. (2011). Homocysteine promotes human endothelial cell dysfunction via site-specific epigenetic

regulation of p66shc. *Cardiovascular Research*, *92*(3), 466–475. https://doi.org/10.1093/cvr/cvr250

Kim, Y.-R., Kim, C.-S., Naqvi, A., Kumar, A., Kumar, S., Hoffman, T. A., & Irani, K. (2012). Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction. *AJP: Heart and Circulatory Physiology*, *303*(2), H189–H196. https://doi.org/10.1152/ajpheart.01218.2011

Kondo, Y., Shen, L., Suzuki, S., Kurokawa, T., Masuko, K., Tanaka, Y., … Sekido, Y. (2007). Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. *Hepatology Research*, *37*(11), 974–983. https://doi.org/10.1111/j.1872-034X.2007.00141.x

Li, X., Xu, Z., Du, W., Zhang, Z., Wei, Y., Wang, H., … Liu, Z. (2014). Aiolos promotes anchorage independence by silencing p66Shctranscription in cancer cells. *Cancer Cell*, *25*(5), 575–589. https://doi.org/10.1016/j.ccr.2014.03.020

Liang, G., Lin, J. C. Y., Wei, V., Yoo, C., Cheng, J. C., Nguyen, C. T., … Jones, P. A. (2004). Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. *Proceedings of the National Academy of Sciences*, *101*(19), 7357–7362. https://doi.org/10.1073/pnas.0401866101

Luna, A., Aladjem, M. I., & Kohn, K. W. (2013). SIRT1/PARP1 crosstalk: Connecting DNA damage and metabolism. *Genome Integrity*, *4*(1), 1–11. https://doi.org/10.1186/2041-9414-4-6

Lund, a H., Lohuizen, V., & M. (2004). b. Epigenetics and cancer. *Genes & Dev*, *18*(May 2016), 2315–2335. https://doi.org/10.1101/gad.1232504.Per

Margueron, R., Justin, N., Ohno, K., Sharpe, M. L., Son, J., Drury, W. J., … Gamblin, S. J. (2009). Role of the polycomb protein EED in the propagation of repressive histone marks. *Nature*, *461*(7265), 762–767. https://doi.org/10.1038/nature08398

Meeran, S. M., Patel, S. N., Li, Y., Shukla, S., & Tollefsbol, T. O. (2012). Bioactive dietary supplements reactivate ER expression in ER-negative breast cancer cells by active chromatin modifications. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0037748

Ming-Shyue, L., Tsukasa, I., Siu-Ju, C., Dana, V. B., S., L. J., Fen-Fen, L., … Ming-Fong, L. (2003). p66Shc protein is upregulated by steroid hormones in hormone-sensitive cancer cells and in primary prostate carcinomas. *International Journal of Cancer*, *108*(5), 672–678. https://doi.org/10.1002/ijc.11621

Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., … Massagué, J. (2005). Genes that mediate breast cancer metastasis to lung. *Nature*, *436*(7050), 518–524. https://doi.org/10.1038/nature03799

Risom, T., Langer, E. M., Chapman, M. P., Rantala, J., Fields, A. J., Boniface, C., … Sears, R. C. (2018). Differentiation-state plasticity is a targetable resistance mechanism in basal-like breast cancer. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-05729-w

Sakai, T., Toguchida, J., Ohtani, N., Yandell, D. W., Rapaport, J. M., & Dryja, T. P. (1991). Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *American Journal of Human Genetics*, *48*(5), 880–888. https://doi.org/10.1007/BF02890404

Sharma, S., Kelly, T. K., & Jones, P. A. (2009). Epigenetics in cancer. *Carcinogenesis*, *31*(1), 27–36. https://doi.org/10.1093/carcin/bgp220

Smith D.C, Simon M., Alldredge A.L., A. F. (1992). © 19 9 2 Nature Publishing Group. *Letters to Nature*, *359*, 139–141.

Stefanska, B., Cheishvili, D., Suderman, M., Arakelian, A., Huang, J., Hallett, M., … Szyf, M.

(2014). Genome-wide study of hypomethylated and induced genes in patients with liver cancer unravels novel anticancer targets. *Clinical Cancer Research*, *20*(12), 3118–3132. https://doi.org/10.1158/1078-0432.CCR-13-0283

Stevenson, L. E., & Frackelton, A. R. (1998). Constitutively tyrosine phosphorylated p52 Shc in breast cancer cells: correlation with ErbB2 and p66 Shc expression. *Breast Cancer Research and Treatment*, *49*(2), 119–128. https://doi.org/10.1023/A:1006007227747

Tan, J., Yang, X., Zhuang, L., Jiang, X., Chen, W., Puay, L. L., … Yu, Q. (2007). Pharmacologic disruption of polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes and Development*, *21*(9), 1050–1063. https://doi.org/10.1101/gad.1524107

Ursini-Siegel, J., Hardy, W. R., Zuo, D., Lam, S. H. L., Sanguin-Gendreau, V., Cardiff, R. D., … Muller, W. J. (2008). ShcA signalling is essential for tumour progression in mouse models of human breast cancer. *The EMBO Journal*, *27*(6), 910–920. https://doi.org/10.1038/emboj.2008.22

Venkatraman, B., & Klenova, E. (2015). Role of CTCF poly(ADP-Ribosyl)ation in the regulation of apoptosis in breast cancer cells. *Indian Journal of Medical and Paediatric Oncology : Official Journal of Indian Society of Medical & Paediatric Oncology*, *36*(1), 49–54. https://doi.org/10.4103/0971-5851.151784

Ventura, A. (2002). The p66Shc Longevity Gene Is Silenced through Epigenetic Modifications of an Alternative Promoter. *Journal of Biological Chemistry*, *277*(25), 22370–22376. https://doi.org/10.1074/jbc.M200280200

Witcher, M., & Emerson, B. M. (2009a). Epigenetic Silencing of the p16^{INK4a} Tumor Suppressor Is Associated with Loss of CTCF Binding and a Chromatin Boundary. *Molecular Cell*, *34*(3), 271–284. https://doi.org/10.1016/j.molcel.2009.04.001

Witcher, M., & Emerson, B. M. (2009b). Epigenetic Silencing of the p16INK4a Tumor Suppressor Is Associated with Loss of CTCF Binding and a Chromatin Boundary. *Molecular Cell*, *34*(3), 271–284. https://doi.org/10.1016/j.molcel.2009.04.001

Zhou, S., Chen, H. Z., Wan, Y. Z., Zhang, Q. J., Wei, Y. S., Huang, S., … Liang, C. C. (2011). Repression of P66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction. *Circulation Research*, *109*(6), 639–648. https://doi.org/10.1161/CIRCRESAHA.111.243592

4.8 Supplemental information

Table S1	List of antibodies used in this study			
Antibody Specificity	Company	Dilution (WB)	Dilution (CHiP)	Cat #
CTCF	Millipore		1/400	07-729
H3K4me3	Millipore		1/400	07-473
H3K9Ac	Millipore		1/400	07-352
H3K27Ac	Millipore		1/400	07-360
PARP1	Millipore	1/1000		610181
γ-H2AX	Millipore	1/1000		05-636
ShcA	BD Biosciences	1/2500		610081
Tubulin	Sigma	1/5000		T5168

Table S2	List of primers for histone RT-qPCRs
Promoter Primers for Histone CHIP	Sequence
P46/52 PROMOTER-F1:	TAGTGAGGCCGGAAGTGAGT
P46/52 PROMOTER-R1:	CAGCTTAGGTTACCGCTCCA
P66 PROMOTER-F1:	ATTCGAGTGTCCAACCAGGA
P66 PROMOTER-R1:	GAAGCCAGGCAGGAGTACAG

Chapter 5 - Discussion

5.1 Overall Discussion

Bringing it all together

The combined body of work presented in this thesis sheds light on a number of emerging concepts in cancer research, including: cellular plasticity, tumor heterogeneity, understanding the molecular drivers of tumor growth and metastatic progression, the role of epigenomics in cancer and the influence of ROS on all of these processes. Genetic and epigenetic mechanisms, but also inflammation and cell state reprogramming contribute to tumor heterogeneity. We studied the role of p66ShcA in the context of these emerging fields in breast cancer.

Cellular plasticity

In chapter two, our research shows that a high degree of tumor heterogeneity exists even within breast cancer subtypes. Luminal A tumors, that are normally well-differentiated and epithelial-like are enriched for the p66ShcA adaptor protein. Furthermore, these tumors correlate with the acquisition of mesenchymal features normally seen by myoepithelial cells within the mammary gland, during development and in basal breast cancers. This is intriguing as ER$\propto$ and GATA3 collaborate to activate signalling that stimulates proliferation and differentiation, while simultaneously repressing EMT TFs (Guttilla, Adams, and White 2012). However, microRNAs can overcome these inhibitions to promote plasticity. Indeed, our work shows luminal A tumors expressing high levels of endogenous p66ShcA are enriched in primary “claudin-low” breast tumors. In fact, the development of novel integrated molecular classification systems to further distinguish unique tumor features has allowed for more personalized diagnoses and may uncover potential therapeutic avenues (Dawson et al. 2013). These findings reinforce the importance of personalized medicine in cancer treatment, as the bulk tumor may be composed of a heterogenous

group of cell types that can influence therapeutic response and recurrence. In fact, supporting this concept, p66ShcA promotes an EMT in ErbB2 positive luminal breast cancer that contributes to the cellular plasticity and heterogeneity seen within breast cancer subtypes. Induction of an EMT stimulates plasticity by promoting the emergence of reversible cell states, including a partial- or full-EMT. Indeed, there has been a new found interest in the effects of a partial EMT on cancer progression and as legitimate cell state on the spectrum of cellular programming (Brabletz et al. 2018). In addition, a multitude of transcription factors have been discovered that govern the EMT program. Some induce stem-like features, cancer initiating abilities and increase the number of circulating tumor cells in the bloodstream, while in other situations there is no effect on aggressiveness. One of the biggest challenges that remains both in terms of developing a complete understanding of the number of processes regulated by EMT and in monitoring specific components of the metastatic cascade is the ability to constantly monitor tumor cells as they disseminate from the primary site to secondary organs. Advances in technology will be helpful in expanding the field in these key areas.

EMT Promotes Tumor Heterogeneity

Growth factors and cytokines within the tumor microenvironment and epigenetic regulators within the tumor cell have all been shown to regulate the EMT program. Common mechanisms center on the inhibition of E-cadherin via DNA methylation of its promoter leading to gene silencing (Dong et al. 2012). In a follow up study, FBP1 was shown to be epigenetically silenced by Snail1, however this inhibited EMT, promoting glycolysis and reduced the production of ROS by suppressing mitochondrial complex I activity, leading to increased CSC-initiating ability (Dong et al. 2013). Even MMPs can induce EMT through a mechanism that is dependent

on ROS (M. A. Cichon and Radisky 2014). Hence, the literature indicates various mediators can control EMT, including epigenetic regulators and ROS, and these modulators can activate or inhibit EMT depending on the cellular context. Indeed, TWIST, a master EMT TF is essential to promote breast cancer metastasis (J. Yang et al. 2004). In contrast, EMT was found to be dispensable in pancreatic cancer (X. Zheng et al. 2015). Our work also suggests context matters in terms of p66ShcA-dependent EMT induction as p66ShcA was able to promote an EMT in ErbB2 positive breast cancer that was not seen in a basal breast cancer cell line. This plasticity is important within subtypes as well as across subtypes to promote cellular plasticity.

Tumor Heterogeneity and Epigenetics

Plasticity allows for bivalent transitions in state (reversible). Epigenetic changes in state require an initiating event, such as exposure to environmental stimuli (inflammatory conditions, a hypoxic environment or oxidative stress) or genetic events and result in stable, inheritable phenotypes in the new cell (Iliopoulos, Hirsch, and Struhl 2009). Epigenetic changes can function to silence tumor suppressor genes or activate oncogenes (Yoshiura et al. 1995). Indeed, MYC was recently shown to reprogram breast cancer cells through the epigenetic activation of MYC-dependent oncogenic enhancers. Overexpression of MYC induces transcriptional repression of lineage-specifying transcription factors, causing decommissioning of luminal-specific enhancers and promotes the emergence of basal-like breast cancer, a stem-like state and increases metastatic potential (Poli et al. 2018). This work provides powerful evidence supporting the notion that cancer is driven through oncogenic-drivers.

Epigenetic regulation of cellular reprogramming provides cellular plasticity and increases aggressiveness and provides a strong rationale for therapeutic avenues to treat basal breast cancers.

Our work identifies p66ShcA as a promoter of cellular plasticity by inducing the differentiation of luminal breast tumors through an EMT. The role of p66ShcA in regulating breast cancer stemness, across subtypes and the importance of ROS requires further investigation. This plasticity may have therapeutic consequences that remain to be investigated. Furthermore, our work indicates p66ShcA is epigenetically regulated through chromatin modifications in p66ShcA high expressing cells versus low expressing cells. In addition, lung metastatic variant breast cancer cells also possess enhanced active chromatin marks within the p66ShcA promoter. Integrating these findings, future studies investigating whether p66ShcA predicts breast tumors at increased likelihood of responding to histone deacetylation and/or methylation inhibitors warrant further investigation.

Molecular drivers in cancer

Other findings from our work looked at p66ShcA levels in the "claudin-low" subtype. We discovered p66ShcA is significantly enriched in "claudin-low" basal breast tumors that are highly plastic in nature (enriched in mesenchymal features and stem-cell properties), our work suggests that drivers of cellular plasticity can shape the emergence of different breast cancer subtypes. Complete loss of p53 in Balb/c mice leads to sarcomas and lymphomas most frequently, whereas 55% of the female BALB/c-p53(+/-) mice developed mammary carcinomas that resemble Li-Fraumeni syndrome (Kuperwasser et al. 2000). Hence, p53 typically requires additional drivers in breast cancer to promote aggressive breast cancers. Our collaborators have previously shown that stable overexpression of constitutive c-Met leads to the emergence of aggressive basal breast tumors of mixed pathology and solid tumors that associate with Her2 positive luminal tumors (Ponzo et al. 2009). Only through the combined loss of wild-type p53 function and overexpression

of c-Met do they see the emergence of “claudin-low” tumors that resemble human breast tumors (Jennifer F Knight et al. 2013).

In addition, MET signalling is required in these tumors to maintain the “claudin-low” phenotype. Indeed, we show that p66ShcA is enriched in tumor lysates from these mice and that p66ShcA correlates with EMT features across molecular subtypes in primary patients. Therefore, p66ShcA appears to contribute to cellular plasticity in breast cancer and various molecular players are required to drive and fine tune the emergence of heterogenous basal breast cancer. Intriguingly, p53 and p66 proteins compete for HIF-1α stabilization in young and old rat hearts exposed to intermittent hypoxia (Bianchi et al. 2006). Moreover, direct regulation of TWIST by HIF-1α promotes metastasis in response to intratumoral hypoxia. These findings link the tumor microenvironment, hypoxia and EMT to metastatic progression. Indeed, HIF-1a overexpression was detected in only 29% of primary breast cancers but in 69% of breast cancer metastases (Hua Zhong et al. 2000). The role of hxpoxia in p66ShcA-dependent EMT and metastatic phenotypes may warrant further investigation. Prognostic biomarkers that predict tailored therapeutic avenues that would benefit individual patients will pave the road ahead.

Context specific regulators in breast cancer

Upon further examination, however, our work suggests that p66ShcA’s role in breast cancer is context dependent, differing across subtypes and situation. In the luminal subtype, p66ShcA is sufficient to modestly inhibit tumor growth, but is not adequate to increase spontaneous lung metastasis despite promoting the induction of an EMT. Despite this, serine36 of p66ShcA, and mitochondrial-ROS facilitate ErbB2-dependent lung dissemination as stable overexpression of the p66ShcAS36A mutant had a dominant-negative effect and significantly

reduced lung dissemination in vivo. Hence, ROS signalling downstream of RTKs may be crucial to promote cancer aggressiveness. Indeed, ROS has been shown to induce ErbB2/ErbB3 expression through miRNAs (He et al. 2012; Scott et al. 2007). Furthermore, p66ShcA has been shown to enhance the migratory properties of luminal breast cancers through Rac1 activation and ROS production (Bhat et al. 2014).

In contrast, p66ShcA has also been shown to induce anoikis through activation of RhoA through the PTB domain (independent of serine36) (Zhenyi; Ma et al. 2007). Hence, the status of the cytoskeleton may dictate pro- versus anti-metastatic roles for p66ShcA. Complementing these findings, ErbB2 has been shown to enhance evasion of anoikis, however these effects are dependent on cellular aggregation (Rayavarapu et al. 2015). Therefore, as cytoplasmic p66ShcA induces an EMT in this subtype, loss of cell contacts through an EMT may put a high degree of stress on breast cancer cells and induce anoikis upon ECM detachment.

Finally, ErbB2 was shown to enhance anchorage independence through anti-oxidants that restore ATP deficiency and increases cell survival through EGFR and PI3K activation (Schafer et al. 2009). Therefore, we hypothesize that p66ShcA-dependent regulation of ROS downstream of RTKs are essential to promote cell signaling and induce the expression of antioxidants to protect cancer cells from high levels of stress during the early stages of metastasis and entry into the bloodstream. In contrast, in the basal subtype p66ShcA is able to promote tumor growth in TNBC. Furthermore, in aggressive, lung metastatic variant TNBC cells, p66ShcA is required for lung dissemination at several stages of the metastatic cascade. Cytoplasmic versus mitochondrial pools of p66ShcA appear to govern these effects at different stages of metastasis.

In basal breast cancer, the lack of a singular molecular driver increases tumor heterogeneity and resistance to therapy. This may also modulate the role of p66ShcA depending on the

abundance of various protein interactors. During metastasis to the lung, proteins may be selected that enhance signaling and enhance p66ShcA-dependent metastatic functions. In contrast, within the primary site, different players may be involved that funnel signaling toward growth activation. Rasal2 is another protein that can function to promote or suppress cancer depending on the context. Rasal2 inhibits EMT and stemness in bladder cancer and functions as a tumor and metastasis suppressor in luminal breast cancer (Hui et al. 2017). Intriguingly, Rasal2 promotes mesenchymal invasion and metastasis through mir-203 independent of EMT (M. Feng et al. 2014).

Addressing critical research gaps

Recently, elucidating the biological mechanisms and genetic factors related to recurrence and adverse effects has been identified among the 5 highest research priorities to addressing the gap in cancer survivorship (Goyette et al. 2018). Furthermore, a separate panel recognized the importance of enhancing knowledge of molecular drivers behind breast cancer subtypes, progression and metastasis and understanding the molecular mechanisms of tumour heterogeneity, dormancy, *de novo* or acquired resistance and how to target key nodes in these dynamic processes (Tomuschat 2005). In chapter three, our work elucidated the requirement of distinct pools of p66ShcA in multiple steps of the metastatic cascade. Indeed, the RTK Axl was recently shown to regulate several stages of metastatic progression, including intravasation, extravasation and survival at the metastatic site (Goyette et al. 2018). These effects were shown in HER2 positive breast cancer and occurred independent of Axl ligand Gas6 by complexing with the ErbB2 receptor. Hence, intracellular signalling downstream of RTKs contributes to cancer aggressiveness.

In TNBC, we show that p66ShcA is necessary for cellular migration and survival within the bloodstream early in the metastatic cascade. During the later stages, p66ShcA is required for colonization and reactivation of cellular proliferation to form overt metastases. Both cytoplasmic-p66ShcA and mitochondrial-p66ShcA are necessary at different stages. At the earliest stages, cytoplasmic-p66ShcA increases the migratory capacity of TNBC cells, potentially to increase access to the vasculature. During extravasation, cytoplasmic pool aids in the turnover of focal adhesions, increasing their assembly/disassembly, reducing their size and surface area to potentially aid in lung colonization. These data raise the intriguing possibility that integrins and/or additional cytoskeletal proteins (such as FAK/RhoA/Rac1 which have previously been shown to interact with p66ShcA/ShcA proteins) are involved in p66ShcA-dependent colonization to aid in adhesion/tension-induced mechanotransduction. Mitochondrial-p66ShcA, however, is necessary for increasing CTCs in the bloodstream from the primary site and this correlates with lung metastasis from the primary site. Notably, cytoplasmic-p66ShcA was unable to rescue lung metastatic burden from the primary site compared to breast tumors lacking p66ShcA, indicating mitochondrial p66ShcA is necessary for intravasation or increased survival once in the bloodstream.

Paget developed the “seed and soil” concept over 100 years ago, which states that the selection of a site for secondary tumor development is influenced by the properties of the target organ, or “soil” and not only made by the tumor cell, or the “seed,”. In recent years, the ability of disseminated tumor cells to reach and interact with the foreign microenvironment to successfully colonize has become the cornerstone of metastasis and dictates whether they grow to form secondary tumors (Riggi, Aguet, and Stamenkovic 2018). A plethora of unique mechanisms have been discovered indicating distinct tumor cells are able to form secondary tumors from the primary

tumor mass. Intense focus on identifying putative drivers has greatly enhanced our understanding, however, little progress has been made in successfully targeting these avenues toward patient benefit. Hence, future work should identify the network of proteins implicated in p66-dependent metastasis in order to identify potential targets.

Precision medicine

Precision medicine involves tailoring treatment to the individual characteristics of the patient and some of its best applications have been from cancer research through targeted treatments and immunotherapies in several forms of cancer. Cancers possessing the highest levels of somatic mutations appear to be the most likely to benefit from immunotherapy (Sturm et al. 2012). These include: bladder cancer, melanoma, lung cancer and blood cancers (Greenman et al. 2007; Sturm et al. 2012). Through the use of gene expression profiling clinicians can identify unique molecular alterations that drive tumorigenesis, even on the single cell level, and this is at the heart of the potential of personalized medicine. Given that breast tumors may be less likely to respond to immunotherapies compared to other forms of cancer (until present day, future research may overcome current barriers), there is an even greater emphasis in utilizing genetic and epigenetic signatures in addition to identifying molecular drivers and genes that predict therapeutic benefit and those most likely to relapse. Recently, it's application in a large clinical trial revealed ~70% of patients can be spared highly toxic adjuvant therapy with virtually no risk, a staggering finding (Mishra et al. 2016). Another recent study acknowledged genetic testing for key driver mutations could aid in treatment, increase quality years of life options and would be cost-effective (Y. Li et al. 2017). Our work identified p66ShcA as a biomarker of breast tumors possessing mesenchymal features in the ErbB2 positive luminal subtype. Hence, p66ShcA may be useful in predicting breast cancers likely to relapse.

Epigenomic basis for breast cancer metastasis

Intriguingly, recent work implicates a foundational role for the epigenomic contribution to metastasis, whereby a lack of CpG island methylator phenotype (B-CIMP) correlates with recurrence and reduced survival in breast cancer (Fang et al. 2011b). Of note, p66ShcA expression is upregulated in both TNBC and "claudin-low" metastatic variants to the lung and liver. In addition, p66ShcA is regulated transcriptionally in TNBC, raising the possibility that p66ShcA may be regulated epigenetically during breast cancer metastasis. Indeed, an increase in active chromatin was seen at the p66ShcA promoter in "claudin-low" metastatic variants. These data support the potential role for an epigenomic program in breast cancer metastasis that may include p66ShcA, a novel promoter of breast cancer metastasis. Hence, p66ShcA may serve as a biomarker of aggressive breast cancers at increased likelihood of metastasis to the lung and liver. Albeit, our work has focused on active chromatin marks, while most predictive metastatic signatures have utilized bisulphite sequencing to verify DNA methylation status. Hence, future work should verify that p66ShcA is indeed epigenetically regulated in breast cancer cells and aggressive metastatic variants through DNA promoter methylation and identify the molecular players involved.

Notably, methylation of CpGs within the CTCF-binding site prevents binding of CTCF, integrating DNA methylation and insulator function in gene regulation (Bell and Felsenfeld 2000). Recently, CTCF was shown to regulate FOXM1, and a CTCF-FOXM1 axis regulates tumour growth and metastasis in hepatocellular carcinoma and ovarian cancer (B. Zhang et al. 2017; Zhao et al. 2017). The forkhead transcription factor FOXM1 is a key regulator of the cell cycle, DNA damage response and EMT (Barger et al. 2015; C. Yang et al. 2013; Yu et al. 2017; Zona et al. 2014). It is frequently overexpressed in cancer and a gene expression signature of FOXM1 predicts

the prognosis of hepatocellular carcinoma and luminal breast cancer patients (Sanders et al. 2013; Song and Chu 2018). FOXM1 was also found to co-bind with estrogen receptor alpha to regulate luminal breast cancer. Finally, FOXM1 was shown to promote stemness and radio resistance in glioblastoma and increased mitochondrial function and tumorigenesis through expression of PRX3 in colon CSCs (Y. Lee et al. 2015; C. Zhang et al. 2016). Hence, these findings integrate various processes including EMT, mitochondrial function, stemness and epigenetic regulation by CTCF to coordinate a program promoting metastatic progression. It will be interesting to determine whether a CTCF/p66ShcA-driven axis exists and contributes to metastasis. Furthermore, future studies should delineate the importance of p66-dependent regulation on metastasis, potentially focusing on acetylation and methylation and their associated epigenetic players such as Sirt1 and and DMNT 1 and 3b.

ROS

ROS can have pleiotropic effects on tumor growth and metastasis. The majority of ROS are generated from the ETC within the mitochondria or in the cytoplasm by NADPH. Growth factors and cytokines can promote tumor growth and metastasis through the formation of ROS (Bruna et al. 2012; Ferraro et al. 2006; Masui et al. 1984). Furthermore, ROS can promote metastasis at different stages of the metastatic cascade. At early stages, PDGF can stimulate H_20_2 production to increase the expression of MMP2, leading to ECM degradation and increased invasion (Yoon et al. 2002). In addition, mitochondrial DNA damage has been shown to increase ROS levels resulting in elevated migratory and metastatic ability (Kaori Ishikawa et al. 2008). Our work indicate mitochondrial ROS is important early on in the metastatic cascade, promoting invasion, cell survival and/or intravasation of breast cancer cells. However, some tumors

upregulate the antioxidant machinery to cope with elevated ROS, including the nuclear respiratory factor 2 (NRF2) that acts as a master transcription factor of antioxidant genes (Shibata et al. 2008). During cell detachment from the ECM ROS are produced which leads to anoikis that can be prevented through antioxidant supplementation (Schafer et al. 2009). The matricellular protein SPARC enhances vascular leakiness, extravasation and lung metastasis in part through ROS due to high levels of shear stress in the circulation (Shijun Ma et al. 2017). Indeed, upregulation of NRF2 can even promote tumor resistance to chemotherapy (Shibata et al. 2008). While ROS can promote metastasis in certain contexts, reduced levels are seen in CSCs where excess DNA damage can be harmful to the tumor. These CSCs also induce free radical scavenger systems and combined with reduced ROS are able to resist the cytotoxic effects of radiation and chemotherapy (Diehn et al. 2009). Notably, breast cancers possess elevated levels of oxidative damage (8-OHdG) compared to the normal breast tissue and ER positive tumors have significantly higher levels of 8-OHdG compared to ER negative tumors (Musarrat and Wani 1996). These findings support the notion that ROS is likely important early on during tumorigenesis, however the degree of ROS is tightly regulated, particularly in aggressive breast cancers and in CSCs. Inflammatory cells are an external source of ROS within the tumor microenvironment (Ruggiero, Pryjma, and Zembala 1999). Low oxygen levels due to insufficient angiogenesis and blood supply can lead to a hypoxic microenvironment. ROS produced from complex 3 of the ETC has been shown to stabilize HIF-α (Guzy et al. 2005). Future studies should focus on establishing whether antioxidant supplementation or genetic manipulation to antioxidant genes is sufficient to effectively prevent the early stages of p66-dependent metastasis.

5.2 Summary and Future Directions

Our work provides in vivo evidence that p66ShcA's phenotypic and functional importance in breast cancer is context dependent. In ErbB2-driven luminal breast cancer, p66ShcA leads to the phenotypic acquisition of mesenchymal features, through an EMT, which are associated with increased migratory capacity. Further work revealed that these effects appear to be independent of mitochondrial-p66ShcA. Functionally this is also associated with increased cellular plasticity. Indeed, we observed enrichment of p66ShcA in patients of the luminal A subtype in addition to "claudin-low" tumors that are known to be highly plastic and possess elevated stem-cell features. We discovered p66ShcA induced an EMT by activating Met signaling, which is a known inducer of EMT and tumor progression. From patient samples we also uncovered that p66ShcA acts as a biomarker of breast tumors possessing mesenchymal features regardless of molecular subtype.

By elucidating the role of p66ShcA in both ErbB2-driven and TNBC models, we discovered p66ShcA can be pro or anti-tumorigenic depending on the context. In two ErbB2 positive luminal breast cancer cell lines, p66ShcA modestly reduced tumor outgrowth by inhibiting cell proliferation, while simultaneously promoting an EMT. In contrast, in 4T1 parental TNBC tumors, p66ShcA promoted tumor outgrowth by reducing apoptosis. Finally, in aggressive 4T1-537 cells that metastasize to the lungs, we discovered p66ShcA is required for dissemination to the secondary site. Hence, the role of p66ShcA in breast cancer is strictly context dependent. We determined that different pools of p66ShcA regulate early and late stages of the metastatic cascade during breast cancer metastasis to the lung. Mitochondrial-p66ShcA is important for intravasation and/or survival within the circulation. In contrast, cytoplasmic-p66ShcA controls migration from the primary site early on and enhances focal adhesion turnover to promote lung colonization and reactivate cell proliferation pathways during the late stages of the metastatic cascade. Hence, our

work indicate p66ShcA can be both pro- or anti-tumorigenic in breast cancer and that p66ShcA is able to influence metastasis through mechanisms that are dependent and independent of mitochondrial ROS. Finally, we show that p66ShcA is epigenetically regulated in breast cancer. Elevated p66ShcA expression correlates with the binding of acetylated histone marks within the p66shcA promoter and with binding of the chromatin boundary forming protein CTCF. Furthermore, inhibition of PARP activity reduces p66shcA expression and CTCF is known to regulate gene expression through PARP-dependent PARlation of CTCF. We also discovered, p66ShcA is transcriptionally regulated in lung metastatic variant breast cancer cells expressing high endogenous p66ShcA and that these aggressive breast cancer cells possess elevated levels of active chromatin within the p66ShcA promoter compared to parental cells.

Our work suggest that a great deal of heterogeneity exists even within breast cancer subtypes. Genetic and epigenetic change, environmental differences and reversible changes in cell properties all contribute to phenotypic and functional heterogeneity. One mechanism that promotes reversible heterogeneity within a cell population is induction of the EMT differentiation program. Indeed, predictive mathematical modelling shows cells undergoing a partial- or full-EMT have distinctive functions and phenotypes (Gould et al. 2016). Our data support these mathematical findings as p66ShcA promotes cellular plasticity by inducing an EMT in breast cancer. p66ShcA leads to the phenotypic acquisition of mesenchymal features while simultaneously reducing epithelial characteristics in ErbB2-driven luminal breast tumors that are normally well-differentiated. These changes are associated with functional gains in migratory capacity. Hence, future work should test the importance of p66ShcA and the induction of an EMT on the response to therapies as an EMT has been associated with resistance. Our data suggest p66ShcA-dependent

EMT is independent of mitochondrial-p66ShcA. Hence, as relative levels of ROS are important for determining the balance between cellular signalling and stimulation of apoptosis, future work is of interest to determine the output of these processes on therapeutic resistance in different contexts. These various contexts may include how p66ShcA integrates across: different subtypes, variations in genetic or epigenetic change and environmental alterations such as exposure to hypoxia, inflammation and oxidative stress. We also observed that p66ShcA regulates different stages of the metastatic cascade. Future work should delineate the exact stages p66ShcA is required for and identify downstream factors that mediate these effects in order to identify potential therapeutic targets. FAK, paxillin, Rac1, RhoA and Akt signalling are excellent candidate genes downstream of p66-dependent signalling to validate. This is particularly important given p66-dependent metastasis is primarily non-mitochondrial in nature, an intriguing observation that should be resolved with future work.

5.3 References

Barger, C. J., Zhang, W., Hillman, J., Stablewski, A. B., Higgins, M. J., Vanderhyden, B. C., … Karpf, A. R. (2015). Genetic determinants of FOXM1 overexpression in epithelial ovarian cancer and functional contribution to cell cycle progression. *Oncotarget*, *6*(29), 27613–27627. https://doi.org/10.18632/oncotarget.4546

Bell, A. C., & Felsenfeld, G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature*, *405*(6785), 482–485. https://doi.org/10.1038/35013100

Bhat, H. F., Baba, R. A., Adams, M. E., & Khanday, F. A. (2014). Role of SNTA1 in Rac1 activation, modulation of ROS generation, and migratory potential of human breast cancer cells. *British Journal of Cancer*, *110*(3), 706–714. https://doi.org/10.1038/bjc.2013.723

Bianchi, G., Di Giulio, C., Rapino, C., Rapino, M., Antonucci, A., & Cataldi, A. (2006). P53 and P66 Proteins Compete for Hypoxia-Inducible Factor 1 Alpha Stabilization in Young and Old Rat Hearts Exposed To Intermittent Hypoxia. *Gerontology*, *52*(1), 17–23. https://doi.org/10.1159/000089821

Brabletz, T., Kalluri, R., Nieto, M. A., & Weinberg, R. A. (2018). EMT in cancer. *Nature Reviews Cancer*, *18*(2), 128–134. https://doi.org/10.1038/nrc.2017.118

Bruna, A., Greenwood, W., Le Quesne, J., Teschendorff, A., Miranda-Saavedra, D., Rueda, O. M., … Caldas, C. (2012). TGFβ induces the formation of tumour-initiating cells in claudin low breast cancer. *Nature Communications*, *3*, 1012–1055. https://doi.org/10.1038/ncomms2039

Cichon, M. A., & Radisky, D. C. (2014). ROS-induced epithelial-mesenchymal transition in mammary epithelial cells is mediated by NF-κB-dependent activation of Snail. *Oncotarget*, *5*(9), 2827–2838. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4058048/

Dawson, S. J., Rueda, O. M., Aparicio, S., & Caldas, C. (2013). A new genome-driven integrated classification of breast cancer and its implications. *EMBO Journal*, *32*(5), 617–628. https://doi.org/10.1038/emboj.2013.19

Diehn, M., Cho, R. W., Lobo, N. A., Kalisky, T., Dorie, M. J., Kulp, A. N., … Clarke, M. F. (2009). Association of reactive oxygen species levels and radioresistance in cancer stem cells, *458*(April), 6–11. https://doi.org/10.1038/nature07733

Dong, C., Evers, B. M., Zhou, B. P., Dong, C., Wu, Y., Yao, J., … Rychahou, P. G. (2012). G9a interacts with Snail and is critical for Snail- mediated E-cadherin repression in human breast cancer Find the latest version : G9a interacts with Snail and is critical for Snail-mediated E-cadherin repression in human breast cancer, *122*(4), 1469–1486. https://doi.org/10.1172/JCI57349.microenvironment

Dong, C., Yuan, T., Wu, Y., Wang, Y., Fan, T. W. M., Miriyala, S., … Evers, B. M. (2013). Article Loss of FBP1 by Snail-Mediated Repression Provides Metabolic Advantages in Basal-like Breast Cancer, 316–331.

Fang, F., Turcan, S., Rimner, A., Kaufman, A., Giri, D., Morris, L. G. T., … Chan, T. A. (2011). Breast Cancer Methylomes Establish an Epigenomic Foundation for Metastasis. *Science Translational Medicine*, *3*(75), 75ra25 LP-75ra25. Retrieved from http://stm.sciencemag.org/content/3/75/75ra25.abstract

Feng, M., Bao, Y., Li, Z., Li, J., Gong, M., Lam, S., … Yu, Q. (2014). RASAL2 activates RAC1 to promote triple-negative breast cancer progression. *Journal of Clinical Investigation*, *124*(12), 5291–5304. https://doi.org/10.1172/JCI76711

Ferraro, D., Corso, S., Fasano, E., Panieri, E., Santangelo, R., Borrello, S., … Galeotti, T. (2006). Pro-metastatic signaling by c-Met through RAC-1 and reactive oxygen species (ROS). *Oncogene*, *25*(26), 3689–3698. https://doi.org/10.1038/sj.onc.1209409

Gould, R., Bassen, D. M., Chakrabarti, A., Varner, J. D., & Butcher, J. (2016). Population Heterogeneity in the Epithelial to Mesenchymal Transition Is Controlled by NFAT and Phosphorylated Sp1. *PLoS Computational Biology*, *12*(12), 1–29. https://doi.org/10.1371/journal.pcbi.1005251

Goyette, M. A., Duhamel, S., Aubert, L., Pelletier, A., Savage, P., Thibault, M. P., … Côté, J. F. (2018). The Receptor Tyrosine Kinase AXL Is Required at Multiple Steps of the Metastatic Cascade during HER2-Positive Breast Cancer Progression. *Cell Reports*, *23*(5), 1476–1490. https://doi.org/10.1016/j.celrep.2018.04.019

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., … Stratton, M. R. (2007). Patterns of somatic mutation in human cancer genomes. *Nature*, *446*(7132), 153–158. https://doi.org/10.1038/nature05610

Guttilla, I. K., Adams, B. D., & White, B. A. (2012). ERα, microRNAs, and the epithelial-mesenchymal transition in breast cancer. *Trends in Endocrinology and Metabolism*, *23*(2), 73–82. https://doi.org/10.1016/j.tem.2011.12.001

Guzy, R. D., Hoyos, B., Robin, E., Chen, H., Liu, L., Mansfield, K. D., … Schumacker, P. T. (2005). Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing, *1*(June), 401–408. https://doi.org/10.1016/j.cmet.2005.05.001

He, J., Xu, Q., Jing, Y., Agani, F., Qian, X., Carpenter, R., … Jiang, B. H. (2012). Reactive oxygen species regulate ERBB2 and ERBB3 expression via miR-199a/125b and DNA methylation. *EMBO Reports*, *13*(12), 1116–1122. https://doi.org/10.1038/embor.2012.162

Hui, K., Gao, Y., Huang, J., Xu, S., Wang, B., Zeng, J., … Wu, K. (2017). RASAL2, a RAS GTPase-activating protein, inhibits stemness and epithelial-mesenchymal transition via MAPK/SOX2 pathway in bladder cancer. *Cell Death and Disease*, *8*(2), e2600-9. https://doi.org/10.1038/cddis.2017.9

Iliopoulos, D., Hirsch, H. A., & Struhl, K. (2009). An Epigenetic Switch Involving NF-κB, Lin28, Let-7 MicroRNA, and IL6 Links Inflammation to Cell Transformation. *Cell*, *139*(4), 693–706. https://doi.org/10.1016/j.cell.2009.10.014

Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., … Hayashi, J.-I. (2008). ROS-Generating Mitochondrial DNA Mutations Can Regulate Tumor Cell Metastasis. *Science*, *320*(5876), 661 LP-664. Retrieved from http://science.sciencemag.org/content/320/5876/661.abstract

Knight, J. F., Lesurf, R., Zhao, H., Pinnaduwage, D., Davis, R. R., Saleh, S. M. I., … Park, M. (2013). Met synergizes with p53 loss to induce mammary tumors that possess features of claudin-low breast cancer. *Proceedings of the National Academy of Sciences*, *110*(14), E1301 LP-E1310. Retrieved from http://www.pnas.org/content/110/14/E1301.abstract

Kuperwasser, C., Hurlbut, G. D., Kittrell, F. S., Dickinson, E. S., Laucirica, R., Medina, D., … Joseph Jerry, D. (2000). Development of spontaneous mammary tumors in BALB/c p53 heterozygous mice: A model for Li-Fraumeni syndrome. *American Journal of Pathology*, *157*(6), 2151–2159. https://doi.org/10.1016/S0002-9440(10)64853-5

Lee, Y., Kim, K. H., Kim, D. G., Cho, H. J., Kim, Y., Rheey, J., … Nam, D. H. (2015). FoxM1 promotes stemness and radio- resistance of glioblastoma by regulating the master stem cell regulator Sox2. *PLoS ONE*, *10*(10), 1–18. https://doi.org/10.1371/journal.pone.0137703

Li, Y., Arellano, A. R., Bare, L. A., Bender, R. A., Strom, C. M., & Devlin, J. J. (2017). A

Multigene Test Could Cost-Effectively Help Extend Life Expectancy for Women at Risk of Hereditary Breast Cancer. *Value in Health*, *20*(4), 547–555. https://doi.org/10.1016/j.jval.2017.01.006

Ma, S., Fu, A., Giap, G., Chiew, Y., & Qian, K. (2017). Hemodynamic shear stress stimulates migration and extravasation of tumor cells by elevating cellular oxidative level. *Cancer Letters*, *388*, 239–248. https://doi.org/10.1016/j.canlet.2016.12.001

Ma, Z., Myers, D. P. ., Ru, F. W., Nwariaku, F. E. ., & Terada, L. S. (2007). p66Shcmediates anoikis through RhoA. *Journal of Cell Biology*, *179*(1), 23–31. https://doi.org/10.1083/jcb.200706097

Masui, H., Kawamoto, T., Sato, J. D., Wolf, B., Sato, G., & Mendelsohn, J. (1984). Growth Inhibition of Human Tumor Cells in Athymic Mice by Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies. *Cancer Research*, *44*(3), 1002–1007.

Mishra, D. K., Kalmath, B. C., Gautam, S., Kawar, R., & Goyal, B. K. (2016). Adjuvant Chemotherapy Guided by a 21-Gene Expression Assay in Breast Cancer. *Journal of Association of Physicians of India*, *64*(JUNE), 90–91. https://doi.org/10.1056/NEJMoa1804710

Musarrat, J., & Wani, A. A. (1996). Original Paper Prognostic and Aetiological Relevance of SHydroxyguanosine in Human Breast Carcinogenesis, *32*(7), 1209–1214.

Poli, V., Fagnocchi, L., Fasciani, A., Cherubini, A., Mazzoleni, S., Ferrillo, S., … Zippo, A. (2018). MYC-driven epigenetic reprogramming favors the onset of tumorigenesis by inducing a stem cell-like state. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-03264-2

Ponzo, M. G., Lesurf, R., Petkiewicz, S., O'Malley, F. P., Pinnaduwage, D., Andrulis, I. L., … Park, M. (2009). Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(31), 12903–12908. https://doi.org/10.1073/pnas.0810402106

Rayavarapu, R. R., Heiden, B., Pagani, N., Shaw, M. M., Shuff, S., Zhang, S., & Schafer, Z. T. (2015). The role of multicellular aggregation in the survival of ErbB2-positive breast cancer cells during extracellular matrix detachment. *Journal of Biological Chemistry*, *290*(14), 8722–8733. https://doi.org/10.1074/jbc.M114.612754

Riggi, N., Aguet, M., & Stamenkovic, I. (2018). Cancer Metastasis: A Reappraisal of Its Underlying Mechanisms and Their Relevance to Treatment. *Annual Review of Pathology: Mechanisms of Disease*, *13*(1), 117–140. https://doi.org/10.1146/annurev-pathol-020117-044127

Ruggiero, I., Pryjma, J., & Zembala, M. (1999). Induction of reactive oxygen intermediates in human monocytes by tumour cells and their role in spontaneous monocyte cytotoxicity, *79*, 737–743.

Sanders, D. A., Ross-Innes, C. S., Beraldi, D., Carroll, J. S., & Balasubramanian, S. (2013). Genome-wide mapping of FOXM1 binding reveals co-binding with estrogen receptor alpha in breast cancer cells. *Genome Biology*, *14*(1), R6. https://doi.org/10.1186/gb-2013-14-1-r6

Schafer, Z. T., Grassian, A. R., Song, L., Jiang, Z., Gerhart-Hines, Z., Irie, H. Y., … Brugge, J. S. (2009). Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature*, *461*(7260), 109–113. https://doi.org/10.1038/nature08268

Scott, G. K., Goga, A., Bhaumik, D., Berger, C. E., Sullivan, C. S., & Benz, C. C. (2007). Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-

125a or miR-125b. *Journal of Biological Chemistry*, *282*(2), 1479–1486. https://doi.org/10.1074/jbc.M609383200

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., … Hirohashi, S. (2008). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proceedings of the National Academy of Sciences*, *105*(36), 13568–13573. https://doi.org/10.1073/pnas.0806268105

Song, B.-N., & Chu, I.-S. (2018). A gene expression signature of FOXM1 predicts the prognosis of hepatocellular carcinoma. *Experimental & Molecular Medicine*, *50*(1), e418. https://doi.org/10.1038/emm.2017.159

Sturm, D., Witt, H., Hovestadt, V., Khuong-Quang, D. A., Jones, D. T. W., Konermann, C., … Pfister, S. M. (2012). Hotspot Mutations in H3F3A and IDH1 Define Distinct Epigenetic and Biological Subgroups of Glioblastoma. *Cancer Cell*, *22*(4), 425–437. https://doi.org/10.1016/j.ccr.2012.08.024

Tomuschat, C. (2005). Critical research gaps and translational priorities for the successful prevention and treatment of breast cancer. *The Fundamental Rules of the International Legal Order: Jus Cogens and Obligations Erga Omnes*, 425–436. https://doi.org/10.1163/ej.9789004149816.i-472.101

Yang, C., Chen, H., Tan, G., Gao, W., Cheng, L., Jiang, X., … Tan, Y. (2013). FOXM1 promotes the epithelial to mesenchymal transition by stimulating the transcription of Slug in human breast cancer. *Cancer Letters*, *340*(1), 104–112. https://doi.org/10.1016/j.canlet.2013.07.004

Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., … Lamarque, A. (2004). Twist , a Master Regulator of Morphogenesis , Plays an Essential Role in Tumor Metastasis Ben Gurion University of the Negev, *117*, 927–939.

Yoon, S. O., Park, S. J., Yoon, S. Y., Yun, C. H., & Chung, A. S. (2002). Sustained production of H(2)O(2) activates pro-matrix metalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-kappa B pathway. *The Journal of Biological Chemistry*, *277*(33), 30271–30282. https://doi.org/10.1074/jbc.M202647200

Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T., & Hirohashi, S. (1995). Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proceedings of the National Academy of Sciences*, *92*(16), 7416–7419. https://doi.org/10.1073/pnas.92.16.7416

Yu, C. P., Yu, S., Shi, L., Wang, S., Li, Z. X., Wang, Y. H., … Liang, J. (2017). FoxM1 promotes epithelial-mesenchymal transition of hepatocellular carcinoma by targeting snail. *Molecular Medicine Reports*, *16*(4), 5181–5188. https://doi.org/10.3892/mmr.2017.7223

Zhang, B., Zhang, Y., Zou, X., Chan, A. W. H., Zhang, R., Lee, T. K. W., … Ko, B. C. B. (2017). The CCCTC-binding factor (CTCF)–forkhead box protein M1 axis regulates tumour growth and metastasis in hepatocellular carcinoma. *Journal of Pathology*, *243*(4), 418–430. https://doi.org/10.1002/path.4976

Zhang, C., Samanta, D., Lu, H., Bullen, J. W., Zhang, H., Chen, I., … Semenza, G. L. (2016). Hypoxia induces the breast cancer stem cell phenotype by HIF-dependent and ALKBH5-mediated m ^{6} A-demethylation of NANOG mRNA. *Proceedings of the National Academy of Sciences*, *113*(14), E2047–E2056. https://doi.org/10.1073/pnas.1602883113

Zhao, L., Yang, Y., Yin, S., Yang, T., Luo, J., Xie, R., … Zhu, B. (2017). CTCF promotes epithelial ovarian cancer metastasis by broadly controlling the expression of metastasis-associated genes. *Oncotarget*, *8*(37), 62217–62230.

https://doi.org/10.18632/oncotarget.19216
Zheng, X., Carstens, J. L., Kim, J., Scheible, M., Kaye, J., Sugimoto, H., … Kalluri, R. (2015). Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature*, *527*(7579), 525–530. https://doi.org/10.1038/nature16064
Zhong, H., Marzo, A. M. De, Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., … Simons, J. W. (2000). Overexpression of Hypoxia-inducible Factor 1 ␣ in Common Human Cancers and Their Metastases 1, (22), 5830–5835.
Zona, S., Bella, L., Burton, M. J., Nestal de Moraes, G., & Lam, E. W. F. (2014). FOXM1: An emerging master regulator of DNA damage response and genotoxic agent resistance. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, *1839*(11), 1316–1322. https://doi.org/10.1016/j.bbagrm.2014.09.016