The Role of the p66^{Shc} Adaptor Protein in Mammary Tumourigenesis

By

Lauren Podmore

Division of Experimental Medicine, Faculty of Medicine
McGill University
Montreal, Canada

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement of the degree of Master of Science

Submitted July 2013

Abstract

The ShcA adaptor protein is a key signaling molecule in the regulation of survival, angiogenesis, immune suppression and metastasis in breast cancer cells. The mammalian ShcA gene encodes three isoforms which are produced through alternative translation initiation ($p46^{Shc}$, $p52^{Shc}$) or different promoter usage ($p66^{Shc}$). Although $p46^{Shc}$ and $p52^{Shc}$ are constitutively expressed and confer pro-tumorigenic signals, $p66^{Shc}$ levels vary in cancer cell lines. While activation of $p66^{Shc}$ through phosphorylation of its serine-36 (S36) is known to induce reactive oxygen species (ROS) formation, the role it plays in tumourigenesis is poorly understood. Our objective was to characterize the impact of $p66^{Shc}$ expression on mammary tumourigenesis both *in vitro* and *in vivo*. To this end, $p66^{Shc}$-expressing vectors were stably transfected into two cell lines low in endogenous $p66^{Shc}$; one driven by oncogenic ErbB2 activation (NIC-4360) and one lacking ErbB2 expression altogether (MDA-MB-231). These cells were characterized *in vitro* before being injected into the mammary fat pad (MFP) of immune-compromised mice for characterization *in vivo*. Our data revealed that while increased $p66^{Shc}$ expression promotes tumour initiation by way of increased angiogenesis, sustained $p66^{Shc}$ activation serves to decrease tumour cell proliferation. Our observation that $p66^{Shc}$ delayed tumour outgrowth in ErbB2-expressing cells also indicates a link between $p66^{Shc}$ activation and ErbB2 expression. The results of this study demonstrate that $p66^{Shc}$ confers both pro- and anti-tumourigenic properties, and that its role in mammary tumourigenesis is largely dependent on its ability to induce production of ROS.

Abrégé

La protéine adaptatrice ShcA est une molécule clé dans la régulation de la survie, de l'angiogénèse, de la suppression tumorale et des métastases des cellules cancéreuses du sein. Le gène ShcA, chez les mammifères, encode trois isoformes qui sont produites par site d'initiation alternatif pour la transcription ($p46^{Shc}$, $p52^{Shc}$) ou l'utilisation d'un différent promoteur ($p66^{Shc}$). Malgré le fait que $p46^{Shc}$ et $p52^{Shc}$ soient constitutivement exprimés et confèrent un signal bénéfique aux tumeurs, les niveaux de $p66^{Shc}$ varient dans les lignées cellulaires cancéreuses. L'activation de $p66^{Shc}$ via la phosphorylation de Sérine36 (S36) est connue pour induire la formation de dérivés réactifs de l'oxygène (ROS), toutefois le rôle que cela joue dans la tumorigénèse est mal compris. Notre objectif était de caractériser l'impact de l'expression de $p66^{Shc}$ sur la tumorigénèse mammaire, à la fois *in vitro* et *in vivo*. À cette fin, des vecteurs exprimant $p66^{Shc}$ ont été transfectés, de façon stable, dans deux lignées cellulaires ayant une expression endogène faible en $p66^{Shc}$: une mû par l'activation de l'oncogène ErbB2 (NIC-4360), l'autre complètement exempte de ErbB2 (MDA-MB-231). Ces cellules ont été caractérisées *in vitro* avant leur injection dans les tissus graisseux mammaires de souris immunodéficientes pour la caractérisation *in vivo*. Nos données démontrent qu'une augmentation de l'expression de $p66^{Shc}$ promouvoit l'initiation tumorale via une augmentation de l'angiogénèse, toutefois une activation soutenue de $p66^{Shc}$ entraîne une diminution de la prolifération des cellules tumorales. Notre observation que $p66^{Shc}$ retarde la croissance tumorale dans les cellules exprimant ErbB2 dénote un lien entre l'activation de $p66^{Shc}$ et l'expression de ErbB2. Les résultats de cette étude démontrent que $p66^{Shc}$ confèrent des propriétés à la fois bénéfiques et néfastes pour les tumeurs, et que son rôle dans la tumorigénèse mammaire est largement dépendante de son habilité à induire la production de ROS.

Table of Contents

Acknowledgements

First and foremost, I would like to sincerely thank my supervisor, Dr. Josie Ursini-Siegel, for taking me on as her first Master's student and giving me the chance to research such an interesting project. The guidance, support and encouragement she provided were invaluable to me throughout my graduate studies. I am grateful for having had the opportunity to work with her, as well as the knowledge and experience I gained as being a part of her research team, all of which will undoubtedly benefit me greatly in my future endeavours.

I would also like to thank my lab colleagues, without whom I would have been unable to complete this thesis. To our lab manager, Valérie Sabourin, for her valued guidance and assistance with the animal work, as well as always being willing to lend a helping hand. To Angela Ahn, for her help with Western blotting, and whose positive attitude, organization, resourcefulness and friendship were very much appreciated. To Jesse Hudson, for the insight he often provided to help me better understand my results and make connections, as well as the many laughs we shared on a daily basis. And thank you to the rest of my fellow graduate students, Young Im, Laura Forrest and Jacqueline Ha, as well as our animal technician Julie Lamarche, for both your support in the lab and the good times outside of it.

From neighbouring laboratories, I would like to thank Dr. Rongtuan Lin's laboratory for providing us with many of the primers used in my qRT-PCR analysis. Also thanks to Andreas Papadakis from Dr. Koromilas's laboratory for providing assistance and advice many times throughout my graduate education.

Thanks to my research advisory committee members; Dr. Antonis Koromilas, Dr. Michael Witcher, and Dr. Lorraine Chalifour for all of you valuable scientific input. Thank you to Dr. Koren Mann for agreeing to be my external examiner; your feedback on my initial thesis submission was very helpful and much appreciated.

Thanks to the McGill Integrated Cancer and Research Training Program for providing funding for this project as well as allowing me to gain valuable insight into the multi-faceted world of cancer research and treatment. Also thanks to the Department of Experimental Medicine at the University of McGill for accepting me into your program and providing assistance whenever needed.

Finally, I would like to thank my parents for their unwavering love and support, both during my master's education and throughout all my life. I would also like to thank my best friend and roommate Annie, whose friendship, patience and understanding gave me the strength to finish this degree. I most certainly couldn't have done this without any of you.

Contribution of Authors

The transfections on NIC-4360 and MDA-MB-231 breast cancer cell lines to establish colonies of $p66^{Shc}$-expessing variants were performed by my supervisor Dr. Giuseppina Ursini-Siegel. Dr. Ursini-Siegel also performed the analysis on data collected from immunohistochemical staining, as well as providing editing and guidance with regards to all aspects of this thesis.

All mammary fat pad (MFP) injections were performed by Valérie Sabourin, as well as tumour outgrowth measurements and endpoint tumour sample collection of the MDA-MB-231 experiment. Valerie Sabourin also performed DHE assays on OCT-embedded tumours, as well as translating the abstract of this thesis into French.

Western blot analysis of tumour lysates from both NIC and MDA-MB-231 *in vivo* experiments was performed by Angela Ahn.

All other aspects of this project, including tissue culture, Western blot analysis of in vitro experiments, tumour outgrowth measurements and endpoint tumour sample collection of the NIC experiment, all qRT-PCR and IHC analysis, as well as the preparation of this completed thesis were performed by Lauren Podmore.

List of Figures

List of Tables

List of Abbreviations

ABC	avidin/biotinylated enzyme complex
ASK1	apoptotic signal-regulating kinase 1
ATCC	American type culture collection
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2 protein
Bcr-Abl	breakpoint cluster region-abelson leukemia virus chromosomal translocation region
BRCA	breast cancer susceptibility protein
BSA	bovine serum albumin
CCL2	chemokine ligand 2
CCL5	chemokine ligand 5
CD31	cluster of differentiation 31, also known as platelet endothelial cell adhesion molecule
CDK	cyclin dependent kinase
cDNA	complementary DNA
CH	collagen homology domain
COSMIC	Catalogue of Somatic Mutations in Cancer
COX2	cyclooygenase 2
CSF-1	colony stimulating factor 1
CTL	cytotoxic T lymphocytes
DAB	Diaminobenzidine
DCIS	ductal carcinoma in situ
DHE	Dihydroethidium
DMEM	Dulbecco's Modified Eagle Medium
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
ErbB	avian erythroblastosis virus gene
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
F4/80	mouse homolog for human EGF-like module containing mucin-like hormone receptor (EMR)1
FBS	fetal bovine serum
FOXO	forkhead box O
GAB	Grb2-associated-binder
GDP	guanosine diphosphate
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
Hif1-α	hypoxia inducible factor 1 alpha

HSP70	heat shock protein 70
IDC	invasive ductal carcinoma
IFN-γ	interferon γ
IHC	Immunohistochemistry
IKK	IκB kinase
IL-1β	interleukin 1β
IL-6	interleukin 6
ILC	invasive lobular carcinoma
IMM	inner mitochondrial membrane
iNOS	inducible nitric oxide synthase
IRF7	interferon regulatory factor 7
IκBα	NF-κB inhibitor α
JAK	janus kinase
JNK	jun N-terminal kinase, also known as stress activated protein kinase (SAPK)
KO	knock out
LCIS	lobular carcinoma in situ
MAPK	mitogen activated protein kinase
MEGS	mammary epithelial growth supplement
MFP	mammary fat pad
miRNA	micro RNA
MMP-9	matrix metalloproteinase-9
MMTV	mouse mammary tumour virus
mRNA	messenger RNA
MT	polyomavirus middle T antigen
NADPH	nicotinamide adenine dinucleotide phosphate
Neu	neuro/glioblastoma derived oncogene homolog
NF-κB	nuclear factor kappa-light-chain enhancer of activated B cells
NIC	neu-IRES-cre
NK	natural killer
OAS1	2'-5'-oligoadenylate synthetase 1
OCT	optimal cutting temperature compound
OMM	outer mitochondrial membrane
p46Shc	ShcA isoform p46
p52Shc	ShcA isoform p52
p66Shc	ShcA isoform p66
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered sulphate
PFA	Paraformaldehyde
PI3K	phosphatidilinositide 3-kinase
PIG	p53-induced genes
Pin1	peptidyl isomerise

PIP3	phosphatidylinositol-triphosphate
PKC	protein kinase C
PLCγ	phospholipase c gamma lysis buffer
pMSCV	murine stem cell virus plasmid
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog
PTP	permeability transition pore
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real time polymerase chain reaction
RET	rearranged during transfection proto-oncogene
RIG1	retinoic acid-inducible gene 1
ROS	reactive oxygen species
RTK	receptor tyrosine kinases
S36	serine residue at position 36
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SH2	Src homology region 2
ShcA	Src homology and collagen domain-containing protein A
SOD	superoxide dismutase
SOS	Son of Sevenless guanine nucleotide exchange factor
STAT	signal transducer and activation of transcription
TAM	tumour associated macrophage
TBS	Tris-buffered saline
TBS-T	TBS with tween
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor β
TIM/TOM	transporter inner membrane/transporter outer membrane complex
TKD	tyrosine kinase domain
TNF-α	tumour necrosis factor α
TRPM2	transient receptor potential cation channel M2
TUNEL	TdT-mediated dNTP nick end labelling
VEGF	vascular endothelial growth factor
VHL	Von Hippel-Lindau tumour suppressor

Chapter One: Review of the Literature

1.1 Human Breast Cancer

Breast cancer is the most frequently diagnosed cancer in Canadian women, representing 25% of all newly diagnosed cancer cases, more than half of which occur in women between the ages of 50 and 69 [1]. The majority of deaths that result from breast cancer occur in females aged 80 years and above, and breast cancer is the leading cause of cancer-related deaths in women aged 30 to 39. Although increased screening by mammography between the mid-1980s to early 1990s resulted in an increase in the incidence of breast cancer, screening in combination with more effective adjuvant therapies following surgery has resulted in a steady decrease in breast cancer related deaths, with breast cancer mortality currently the lowest it has been since the 1950s. Approximately 10-15% of patients develop aggressive disease with distant metastases developing within 3 years of initial detection, but once diagnosed are at risk for developing metastases for their entire lifetime [2].

Breast cancer is a heterogeneous disease at both the molecular and clinical level, classified into five major molecular subtypes: basal-like, luminal A, luminal B, $Her2^{+}/ER^{-}$, and normal breast-like [3-6]. Clinically, patients with basal-like tumours have the worst prognosis, whereas those with luminal A-type tumours have the best [4]. While most breast cancers are sporadic, both environmental and genetic factors contribute to the incidence of breast cancer [7]. It is estimated that 5-10% of breast cancers are due to hereditary susceptibility, with the majority of hereditary cases attributed to mutations in the BRCA 1/2 repair genes and typically exhibiting features of the basal subtype [8, 9]

1.1.1 Breast Cancer Progression

A normal breast duct consists of a luminal epithelial layer encircled by myoepithelial cells attached to a basement membrane, and the duct in turn is surrounded by a microenvironment composed of numerous stromal cell types [10]. The mammary epithelium also contains secretary alveoli, a series of side branches resembling bunches of grapes, collectively known as lobules, which function to produce milk [11]. Structurally, the alveoli consists of an inner layer of milk-producing luminal cells surrounded by an outer layer of myoepithelial cells, the latter of which contract to squeeze milk out of the alveoli into the ducts.

Encompassing nearly 80% of diagnosed breast cancers are those that originate in the duct, and these are followed by lobular breast cancers at roughly 10% [12, 13]. Ductal breast cancers first arise when a normal duct's luminal epithelium undergoes hyperproliferation and evolves to ductal carcinoma *in situ* (DCIS), a heterogeneous, non-invasive disease in which the epigenetically and phenotypically altered luminal and myoepithelial cells are strictly confined within the milk ducts (**Figure 1**) [10, 14-16]. The loss of myoepithelial cells and basement membrane transitions DCIS to invasive ductal carcinoma (IDC), where tumour cells can invade into the local environment and surrounding tissues. IDC becomes metastatic when these cells are able to migrate to distant organs and form a new tumour. A similar progression is observed in lobular breast cancers, where normal alveolar luminal epithelium transition into lobular carcinoma *in situ* (LCIS), and then progress to invasive lobular carcinoma (ILC). In both cases, the transition from benign to malignant neoplasm is facilitated in part by the stroma, where angiogenesis is

enhanced, and an increase in the numbers of fibroblasts and infiltrated leukocytes leads to elevated secretion of a variety of mediators that promote tumour progression.

1.2 Receptor Tyrosine Kinases

Humans have 58 known receptor tyrosine kinases (RTKs), and while roughly only half of these are well understood, their overall topology, mechanisms of activation and downstream signalling components are all highly conserved in evolution [17, 18]. Structurally, RTKs typically contain a ligand binding domain in the extracellular region, a single transmembrane helix, and a cytoplasmic region consisting of a protein tyrosine kinase domain (TKD), a carboxy terminal and juxta-membrane regulatory regions.

RTKs are activated when bivalent ligand molecules, usually growth factors, interact with monomeric receptor molecules, causing them to dimerize (**Figure 2**) [18]. In a process known as trans-phosphorylation, the tyrosines on the TKD of one receptor molecule phosphorylate tyrosines on the TKD of the other, and this phosphorylation is returned in kind, resulting in an activated RTK [19].

Adaptor or scaffolding proteins, which can interact with multiple signalling molecules, dock to the activated TKD of RTKs, allowing them to serve as a site for the assembly and activation of intracellular signalling proteins. Src homology region 2 (SH2) and phosphotyrosine-binding (PTB) domains are small protein modules that can recognize phosphorylated tyrosines in many signalling proteins including RTKs, and these modules mediate many of the protein-protein interactions in signal transduction [20-23] While the binding of SH2 to target proteins is almost exclusively regulated by tyrosine

phosphorylation, PTB domains have a higher binding affinity for certain types of tertiary amino acid structures, and binding to phosphorylated tyrosine is a secondary priority.

Active RTKs act as nodes in complex signalling networks which facilitate the transmission of information from outside to inside the cell. Two major pathways downstream of RTKs are the phosphatidylinositide 3-kinase (PI3K)/Akt and the Ras/mitogen activated protein kinase (MAPK) pathways [24-26]. Active RTKs can bind the Grb2 adaptor protein. When Grb2 forms a complexes with the guanine nucleotide exchange factor Son of Sevenless (SOS), inactive Ras-GDP is converted to active Ras-GTP, therefore activating the Ras/MAPK pathway, leading to downstream mitogenic signaling [27]. When Grb2 forms a complex with the Grb2-associated-binder (GAB), this recruits the p85 regulatory subunit of PI3K to the membrane, leading to its activation and production of phosphatidylinositol-triphosphate (PIP_3) and subsequent activation of Akt, leading to downstream proliferative and survival signaling [22]. Unsurprisingly, both the Ras/MAPK and PI3K/Akt pathways are frequently deregulated in breast cancer [25, 26].

A common early response in the activation of cell-surface receptors like RTKs is down-regulation, in which endocytosis of ligand-occupied receptors followed by intracellular degradation is stimulated in a negative feedback loop by active RTKs [28-30]. This is a cellular mechanism that has evolved to oppose potentially deregulated and dysfunctional RTKs, as mutations and aberrant activation of their downstream signalling pathways are implicated in a variety of human diseases including diabetes, arteriosclerosis and cancer [18]. Aberrant RTK activation in human cancers is mediated by four principal mechanisms: autocrine activation, chromosomal translocations, RTK overexpression, or

gain-of-function mutations. Many of these are collected in the Catalogue of Somatic Mutations in Cancer (COSMIC) [31].

As RTKs are a common site or source of aberrant signalling in tumorigenic cells, they are also a common drug target in cancer therapies. Drugs against activated RTKs fall into one of two categories, the first of which are small molecule inhibitors that target the ATP-binging site of the intracellular tyrosine kinase domain, such as Imatinib, which has shown clinical activity against the aberrant Bcr-Abl fusion tyrosine kinase that arises in chronic myelogenous leukemia [32]. The second category consists of monoclonal antibodies, such as the HER2/neu-binding antibodies 4D5 or 2C4, which have the ability to both interfere with RTK activation, as well as target RTK-expressing cells for destruction by the immune system [32, 33]. However, drug resistance inevitably develops in most cancer patients treated with drugs from either category due to selective pressure. This often results in either drug resistant variants of the targeted proteins, or compensation by other molecules elsewhere in the pathway which allows the cells to overcome the inhibition of the targeted RTKs [34, 35].

1.2.1 ErbB2

The ErbB family of RTKs consist of four transmembrane growth factor receptor proteins which share structural and functional similarities [36]. Members of this family include the epidermal growth factor receptor (EGFR, also known as ErbB1), ErbB2 (also known as human epidermal growth factor receptor 2, or HER2, in humans, or Neu in rodents), ErbB3 (HER3) and ErbB4 (HER4). Multiple ligands bind to and activate these monomers; for example with EGFR, the two most important ligands are the epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), both of which play

important mitogenic roles, whereas ErbB3 and ErB4 are the principal receptors for neuregulins, which are essential for development of the nervous system and heart [37-39]. Ligands which bind to EGFR, ErbB3 and ErbB4 monomers promote substantial conformational changes in the extracellular region of the protein, allowing it to interact with a second ligand-bound monomer to form an activated homo- or heterodimer. This is followed by receptor internalization, autophosphorylation of the TKD, and finally results in ErbB-mediated signalling transduction which leads to cell migration, survival and proliferation [40] .

ErbB2 promotes cellular proliferation and opposes apoptosis by activating signalling cascades such as the Ras/MAPK, PI3K/Akt, phospholipase C, protein kinase C (PKC), and signal transducer and activation of transcription (STAT) pathways (**Figure 3**) [41]. While ErbB monomers are known to heterodimerize with in every combination with other member of the ErbB family, ErbB2 is the preferred dimerization partner in for all four proteins [42]. This can be attributed to the fact that, in contrast with the other ErbB family members, no direct ligand of ErbB2 has been identified; instead, it exists in an extended 'active' conformation state, rendering it constitutively available for dimerization [43].

The dimerization of ErbB receptors represents the fundamental mechanism driving transformation [39]. ErbB2 is amplified/overexpressed in 18-30% of human breast cancers and its overexpression predicts a poor prognosis [44-48]. Transgenic models have established that ErbB2 overexpression is sufficient to transform the mammary epithelium [49]. The monoclonal antibody Trastuzumab/Herceptin, which binds to the extracellular domain of ErbB2 to block its function and signal ErbB2 overexpressing cancer cells for

destruction, has been used successfully in the clinic to treat many cancer types including mammary carcinoma [33]. However, although Trastuzumab in addition to chemotherapy has improved the prognosis of patients with $Her2^+$ breast tumours, both de novo and acquired mechanisms of drug resistance are implicated in many of these same patients experiencing recurrence of the disease [50-52]. Therefore, further investigation into the biology of these types of tumours is required to develop more effective therapeutics.

1.3 Reactive Oxygen Species

Reactive oxygen species (ROS) are molecules or ions formed by the incomplete one-electron reduction of oxygen (**Figure 4**) [53]. These reactive oxygen intermediates include singlet oxygen (O_2), superoxide (O_2^-), peroxide (H_2O_2), hydroxyl radical (OH^-) and hypochlorous acid (HClO). Superoxide dismutases (SOD) convert O_2^- to H_2O_2, whereas glutathione peroxidase or catalase converts H_2O_2 to H_2O. In turn H_2O_2 can react with iron to generate OH^- [54].

The majority of ROS generated are a by-product of cellular respiration at the electron transport chain (ETC); approximately 1-4% of electrons passing through the ETC react with oxygen prematurely, where it is incompletely reduced to O_2^- instead of forming H_2O [55]. Another important source of ROS in the cell are NADPH oxidases, enzymes which catalyze the production of superoxide by transferring electrons from NADPH to molecular oxygen [56]. Originally found in phagocytes, where the overabundance of ROS produced is used to kill invading microorganisms, the membrane bound NAPDH oxidase has been identified in other cell and tissue types, most commonly the epithelium [57-59]. NADPH oxidases can be activated by growth factors, cytokines, stress, hypoxia and G-

protein coupled agonists, and they produce low levels of ROS for local control of redox signaling cascades by both activating kinases and inactivating protein phosphatases, resulting in increased tyrosine and serine/threonine phosphorylation [60, 61]. ROS derived from NADPH oxidases therefore mediate a wide variety of signaling factors, such as Ras/MAPK, PI3K/Akt, PKC, nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), hypoxia-inducible factor 1 alpha (Hif1-α) as well as several others, and subsequently have a role in regulating many cellular functions including inflammation, vascular remodeling, cell growth and migration, endothelial dysfunction and permeability, apoptosis and senescence [60]. ROS can also be stimulated through ionizing radiation, UV light, pollutants and smoke [62].

The generation of ROS can lead to oxidative stress, an imbalance which results from exposure to high levels of ROS not detoxified by cellular anti-oxidative agents [54]. Prolonged oxidative stress leads to oxidative damage of proteins, lipids and DNA [63]. For example, in the presence of high ROS, the amino acids methionine and cysteine are oxidized on sulphur, preventing proper protein folding [64]. For lipids, OH^- induced lipid peroxidation results in increased membrane rigidity and permeability, as well as lipid-protein and lipid-lipid crosslinking [63]. Finally, in nucleic acids, the most damaging result of prolonged ROS exposure is guanine converted to 8-oxo-guanine, which has an equal affinity for cytosine and adenosine, leading to transversions in both nuclear and mitochondrial DNA [65].

However, low levels of ROS are important for activating a great variety of intracellular signaling pathways, such as the EGF, Insulin, MAPK, Protein Kinase C, NF-κB signaling cascades, as well as regulating Ca^{2+} and glutathione levels [5, 66-72].

Therefore, as high levels of ROS leads to senescence and cell death, and low levels of ROS leads to inactive biological pathways, an intermediate level of ROS must be maintained for a healthy cell (**Figure 5**) [53]. This can be exploited in cancer; ROS have the potential to be carcinogenic by causing genomic instability and promote tumour progression by activating pathways that regulate proliferation, angiogenesis and metastasis. However, these features can also be exploited in cancer treatment; ROS can be anti-tumourigenic in that they can activate the stress response and eventually result in apoptosis and senescence.

1.3.1 Pro-Tumourigenic Properties of ROS

The context in which ROS are most prevalently considered pro-tumourigenic are stated above in 1.3; as DNA-damaging agents causing mutagenesis which promote oncogenic transformation [73]. However, several other pro-tumourigenic properties of ROS are known to exist. For example, prolonged chronic inflammation has been shown to instigate progression to dysplasia [74]. The Ca^{2+} -permeable transient receptor potential cation channel M2 (TRPM2) controls the ROS-induced signaling cascade responsible for chemokine production, which aggravates inflammation. H_2O_2 evokes Ca^{2+} influx through TRPM2 to activate Ca^{2+} dependent tyrosine kinases and amplify extracellular signal-regulated kinase (ERK) signaling [75]. The Ras/ERK pathway is involved in proliferation, as its sustained activation during late G1 phase is necessary for G1/S phase transition resulting in committed progression of the cell cycle, although phosphorylated ERK1/2 proteins are rapidly inactivated before S phase under normal cellular conditions [76-78]. Ras/ERK deregulation can result in either uncontrolled cellular proliferation or cell cycle arrest, depending on the amplitude and duration of ERK1/2 activity; strong early ERK1/2 signaling followed by moderate sustained signaling leads to transient induction of p21 and

accumulation of cyclin D1, allowing for continued G1 progression, whereas robust and prolonged ERK1/2 activation causes G1 arrest due to long term p21 induction and cyclin dependent kinase inhibition[79].

Frequent targets of ROS modification and inactivation are the catalytic cysteine residues of protein tyrosine phosphatases, such as the phosphatase and tensin homolog (PTEN), which negatively regulates the PI3K/Akt signaling pathway by dephosphorylating Akt's upstream activator PIP_3 [80]. Under normal conditions, the phosphorylation and translocation of Akt to the plasma membrane results in increased cell growth and survival; however overexpression of Akt, frequently observed in cancers, is associated with resistance to apoptosis as well as uncontrolled cell growth and proliferation [81]. H_2O_2 has been shown to target and inactivate PTEN by causing the formation of a disulphide bond between PTEN's cysteine residues 124 and 74, and as Cys124 is essential to the PTEN active site, this effectively inhibits the phosphatase that inhibits Akt [82, 83]. Therefore increased ROS can contribute to tumourigenesis through deregulation of the PI3K/Akt pathway.

p53 is a tumour suppressor which is the most frequently mutated gene in human cancers [63]. Known as the "guardian of the cell," it transactivates or transrepresses numerous genes to regulate cell cycle arrest, cellular senescence and apoptosis. It is known to play an anti-oxidant role in oxidative stress. At physiological levels, p53 positively regulates the expression of anti-oxidant genes, protecting the cells from the accumulation of damaging levels of ROS [84]. At low levels of p53 there is a significant decrease in antioxidant genes, leading to an increase in ROS and resulting in oxidative damage of DNA. However, at high levels of p53, oxidative stress results from an unbalanced

induction of antioxidant enzymes by p53; the high cellular stress that results in overexpression of p53 transactivates a series of p53-induced genes (PIGs), which encodes pro-oxidant or redox-active proteins, including ROS-generating enzymes. Therefore, upregulation of p53 leads to oxidative stress and apoptosis. Much like ROS itself, the context under which p53 is expressed depends on whether it is pro- or anti-tumourigenicigenic.

1.3.2 Anti-Tumourigenic Properties of ROS

Together, the p38 MAPK pathway and the Jun N-terminal kinase (JNK) pathway are known as the stress-activated protein kinase pathways and are often deregulated in cancers [85]. Both pathways are induced by environmental and genotoxic stresses, as well as by growth factor and inflammatory cytokine stimulation, and their downstream targets have a regulatory effect on cell proliferation, survival, differentiation, cellular migration and inflammation.

The JNK family of proteins, which consists of 10 isoforms encoded by 3 genes, JNK1, JNK2, and JNK3, are activated when upstream stress stimulation leads to activation of at least one of several mitogen-activated protein kinase kinase kinases (MAPKKKs) that activate MAP kinase kinases 4 and 7 (MKK4/7), which in turn activate and phosphorylated the JNK kinase [86]. When JNK (also known as stress-activated protein kinase, SAPK) is active as a dimer, it translocates to the nucleus to interact with a variety of transcription factors [87-89]. For example, members of the JNK family plays opposing roles in proliferation; c-Jun, a transcriptional regulator of cell cycle progression, is phosphorylated and stabilized by JNK1 under stress conditions, and targeted for degradation by JNK2

under non-stress conditions [90]. JNK also has pro-apoptotic functions which are mediated by the mitochondrial pathway and include phosphorylation of Bcl-2 and 14-3-3 proteins, releasing pro-apoptotic transcription factors like Bcl-2 associated X protein (BAX) and forkhead box O (FOXO) from inactive complexes[91, 92]. JNK has also been linked to the expression of cytokines that control inflammation, such as tumour necrosis factor alpha (TNF-α), though to a lesser extent than p38 MAPK [93].

Similar to JNK, the p38 MAPK family consists of four isoforms, p38α, p38β, p38γ, and p38δ, and are similarly activated by cellular stresses in a MAPKKK signaling cascade that results in phosphorylation of p38 at Thr180 and Tyr182, and in turn regulates a plethora of transcription factors. [94-96]. Active p38 negatively regulates cell cycle progression by down-regulating cyclins and upregulating cyclin dependent kinase (CDK) inhibitors, and can trigger permanent proliferative arrest known as senescence by inducing p53 phosphorylation and upregulation of p16 [97]. Apoptotic induction also involves p38 through transcriptional and post-transcriptional mechanisms, which affect either death receptors, survival pathways or pro- and anti-apoptotic Bcl-2 proteins in a stimulus/context-dependent manner [85]. p38 activation is also known to have important pro-inflammatory effects; including the induction of cyclooxygenase 2 (COX2) , the production of cytokines TNF-α, interleukin 1β (IL-1β) and interleukin 6 (IL-6), and the modulation of the NF-κB transcription factors [98-100].

In cancer, both stress activated pathways are known to negatively regulate cell proliferation and tumourigenesis, as their activation can lead to oncogene-induced senescence, replicative senescence, contact inhibition, DNA-damage responses and apoptosis [53]. Human cancer cell lines with high ROS levels display enhanced

tumourgenicity and impaired p38 activation by ROS [101]. In addition, components of the p38 pathway can phosphorylate p53, the master regulator of the cell, by increasing the transcriptional activity of p53 and inducing a transcriptional target of p53 and p21 [97]. p38 can also induce the expression of p16 INK4A and p14/p19 ARF, which leads to premature senescence, a tumour-suppressing defense mechanism.

ROS may also activate the apoptotic signal-regulating kinase 1 (ASK1), which in turn activates MAP kinase kinases (MKKs) for both the JNK (MKK4/7) and p38 (MKK3/6) pathways, leading to activation of JNK and p38 [102-104]. As mentioned above, one mechanism by which JNK induces cell death is the abrogation of BCl-2, a protein that protects the cell from mitochondrial-related apoptosis; however, as the initial ROS-independent JNK activations are normally not of sufficient magnitude to abrogate enough Bcl-2 to trigger apoptosis [105]. To overcome this, ASK1 can create a positive feedback loop for the stress response when activated p38 stimulates the expression of MK2, inducing increased TNF-α production, which positively regulates ASK1 activity, thus leading to further activation of both p38 and JNK [106, 107]. Finally, the FOXO pathway is activated in oxidative stress responses, and regulates cell cycle arrest, cell death and protection from stress stimuli [108].

1.4 Inflammation in Cancer

Underlying infections and inflammatory responses have been linked to 15-20% of all deaths from cancer worldwide [109]. Cancer-related inflammation is known to influence malignancy, including the survival and proliferation of malignant cells, as well as promotion of cancer progression through tissue remodeling, angiogenesis, metastasis, and

suppression of both the innate anticancer immune response and tumour response to cancer therapy (**Figure 6**) [110, 111]. Treatment with non-steroidal anti-inflammatory agents decreases tumour incidence and mortality [112-114]. Inflammation and cancer are linked through two pathways; the extrinsic mechanism, where inflammation conditions increase cancer risk, and an intrinsic mechanism, where genetic alterations maintain and promote tumour progression [110].

Tumor-infiltrating leukocytes and inflammatory mediators that stimulate cytokine signalling pathways are crucial components in the development of the inflammatory tumour microenvironment [110, 115, 116]. Leukocytes and other phagocytic cells in the tumour infiltrate also increases cancer risk through the production of ROS, a method they normally employ to fight infection, which can result in mutagenic, toxic and genome-destabilizing DNA lesions [117-124]. Abundant production of ROS by inflammatory factors can inhibit base-excision repair, as well as induce aberrant DNA methylation, histone modification and miRNA expression [125-129].

The relative levels of many inflammatory mediators have been linked specifically to breast cancer malignancy. IL-6, which acts as a pro- inflammatory cytokine under conditions of chronic inflammation, is present in high levels of the blood of metastatic cancer patients, and has been shown to be a negative prognosticator in breast cancer [130-132]. Chemokine ligands 2 and 5 (CCL2, CCL5), which stimulate monocytes and T cell migration to inflamed tissues, have been show to actively support breast cancer malignancy [133-137]. High levels of macrophage colony-stimulating factor (CSF-1), which stimulates macrophage differentiation from monocytes, correlates with rapidly progressive metastatic breast carcinoma and poor patient prognosis [133-136]. Several oncogenic signalling

pathways are linked to cancer related inflammation. Activating the RET signalling pathway by chromosomal rearrangement is sufficient to trigger the transformation of a thyrocyte to carcinoma, and can activate expression of many inflammatory proteins [138]. The Ras/Raf signalling pathway, the most frequently mutated and aberrantly activated pathway in human cancers, cooperates with extrinsic chronic inflammation to facilitate cell transformation, and can induce the expression of tumor promoting chemokines and cytokines [139]. The constituent activation of the STAT3/ transforming growth factor β (TGF-β) signalling pathway in tumour cells is known to be involved in both oncogenesis and inhibition of apoptosis, and activation of STAT3 specifically has been implicated in inhibition of dendritic cell maturation, which suppresses the immune response [140, 141]. Tumour suppressor genes PTEN, p16, p53 and Von Hippel-Lindau (VHL) have also been implicated in the induction of inflammatory mediators that contribute to tumour progression [117, 142, 143].

Despite all of these well-documented pro-tumourigenic properties, the immune system is known to play a significant role in combating tumour progression. Anti-tumour immune response comprises of both humoral and cell-mediated branches [144]. Humoral immunity's role in tumour suppression is mediated by macromolecules found in extracellular fluids such as antibodies and complement proteins which recognize tumour-specific antigens and lead to the lysis of these cells, whereas cell-mediated immunity involves the T and B cell response to antigen-specific presentation resulting in multiple immune functions, including the activation of macrophages [144-146]. Tumour destruction results from three major mechanisms: destruction of the tumour by necrosis mediated by polymorphonuclear leukocytes; activation of leukocyte subsets capable of pro-

inflammatory cytokines, CTLs and anti-tumour antibodies; and indirect inhibition of angiogenesis by secondary interferon γ (IFN-γ), TNF-α and chemokines [147]. Interferons are cytokines with antiviral, antibacterial and anti-tumourigenic properties, and interferon regulatory factor 7 (IRF7) has been shown to be silenced in some metastatic breast cancer cell lines, which may help these cells avoid the immune suppression [148].

1.4.1 Angiogenesis

Angiogenesis is the process by which new blood vessels are generated from existing vasculature, which is normally restricted in human adults to wound healing and reproduction, but is aberrantly activated in cancer [149]. When oxygen and nutrient availability becomes a limiting factor for rapidly proliferating tumours, as they reach roughly 2mm in diameter, activation of the angiogenic switch allows them to escape from tumour dormancy [150, 151]. Vessels formed by tumour-driven angiogenesis are abnormal, leaky and have intermittent flow, leading to poor drug and oxygen delivery, exacerbating hypoxia and increasing microenvironmental stresses such as ROS [152, 153].

Vascular endothelial growth factor (VEGF), the best characterized angiogenic factor, stimulates endothelial cell proliferation and migration through binding to various RTKs and activating angiogenic pathways [151]. Tumour associated macrophages (TAMs), an important component in the leukocyte infiltrate, promote angiogenesis by secreting VEGF, as well as several inflammatory mediators including TNF-α and matrix metalloproteinase-9 (MMP-9) [154-159]. Active macrophages also produce the cytokine IL-1β, a key mediator of the inflammatory response and involved in cellular activities such as proliferation, differentiation and apoptosis, as well as inducing COX2 to contribute to

inflammatory pain hypersensitivity, and has been indicated in promoting disease relapse in breast cancer patients [160, 161].

1.4.2 NF-κB

The NF-κB signalling pathway plays a critical roles in cell survival, proliferation, innate immunity and inflammation, and is important endogenous tumour promoter [100]. The canonical NF-κB pathway is triggered by pro-inflammatory cytokines such as TNF-α or IL-1β, leading to phosphorylation and activation of the IKK complex (consisting of IKKα, IKKβ and two IKKγ), which in turn phosphorylates IκBα, an inhibitory molecule that sequesters the canonical NF-κB heterodimer p50/p65 in the cytoplasm [162, 163]. Phosphorylation of IκBα results in its ubiquitination and subsequent degradation, allowing the p50-p65 complex to translocate to the nucleus. This activates expression of genes encoding inflammatory cytokines, adhesion molecules, enzymes involved in prostaglandin-synthesis (such as COX2), inducible nitric oxide synthase (iNOS), anti-apoptotic genes (such as Bcl-2) and angiogenic factors [164]. Deregulation of this pathway results in the persistent nuclear localization of the NF-κB proteins, and constituent activation of the NF-κB is frequently found in breast cancer tumours [162, 165]. NF-κB's role as a tumour promoter depends on balance of inhibitors and activators in the inflammatory tumour microenvironment, and it is believed that NF-κB could be targeted to reprogram tumour promoting macrophages towards an anti-tumour function [110, 166-169].

1.5 ShcA

The Src homology and collagen domain-containing protein A (ShcA) is an adaptor protein whose gene encodes three protein isoforms, the expression of which are regulated either through different promoter usage ($p66^{Shc}$) or alternative translational initiation ($p46^{Shc}$, $p52^{Shc}$) (**Figure 7**) [170, 171]. All three isoforms share two phosphotyrosine binding motifs, an amino-terminal PTB domain and a carboxy-terminal SH2 domain, as well as a collagen homology (CH1) domain with three tyrosine phosphorylation sites at residues 239/240 and 317 (analogous to residue 313 in mice) [170, 172-175]. ShcA binds receptor and cytoplasmic tyrosine kinases, relaying extracellular signals that regulate cell proliferation, survival, invasion and angiogenesis [176]. Phosphorylation of Y239 and Y317 is known to activate mitogenic signaling through the Ras/MAPK pathway, whereas phosphorylation of Y239/240 has been shown to transduce intracellular survival signals [177-182].

Increased ShcA tyrosine phosphorylation is a strong predictor for nodal status, disease stage and relapse in breast cancer patients [183, 184]. Mutation of the ShcA binding site on RTKs has resulted in delayed induction of mammary epithelial hyperplasia and a prolonged latency period before tumour formation [143]. For example, it was shown that ErbB2 must retain its ShcA binding site to efficiently transform the mammary gland; reconstitution of a ShcA-binding site to a mutant receptor, lacking its major tyrosine phosphorylation residues, was sufficient to restore the kinetics of mammary tumour development to levels observed with wild-type ErbB2 [185-187].

Previous work in our laboratory using transgenic mouse models have elucidated important roles for ShcA during mammary tumourigenesis. We have shown that ShcA is critical for mammary tumour onset and progression [173]. Mammary specific expression of the Polyomavirus middle T antigen (MT) results in the rapid induction of multi-focal mammary tumours [188]. Using mouse mammary tumour virus/MT (MMTV/MT) transgenic mice bred with animals expressing targeted ShcA knock-in (KI) alleles harbouring phenylalanine substitutions of the Y313 or Y239/240 phosphotyrosine residues (313F and 2F, respectively), MT mice were bred that were both homozygous and heterozygous for these mutant alleles, as well as heterozygous for all three (3F). Studies from these mice revealed that ShcA signalling is important during the transition from hyperplasia to neoplasia and finally to invasive carcinoma. It was also established that distinct, non-overlapping roles exist for ShcA phosphotyrosine residues; signals emanating from Y313 are important for cell survival, whereas Y239/240 transduce signals promoting tumour vascularization.

In this same study, ShcA knock out (KO) mice were also generated when MMTV/NIC mice were interbred with animals expressing floxed ShcA-3xFLAG cDNA. NIC is a construct encoding a bicistronic transcript expressing an oncogenic ErbB2 (Neu) allele followed by the Cre-recombinase under the translational control of an IRES element, and the mammary epithelial cell-specific expression of Cre-recombinase results in the excision of the floxed ShcA allele, effectively eliminating ShcA expression in these cells. It was first observed that mammary tumour development was completely ablated in these ShcA KO mice, as compared to wild-type MMTV/NIC mice which retained ShcA, suggesting sustained ShcA expression in mammary epithelial cells is critical for

proliferation, survival and transformation by ErbB2. However, in a subsequent study with an expanded cohort of these mice, tumours did arise in some of the mice after a significantly long latency period and 4-fold more frequently with parity, though they lacked ShcA [189]. Therefore tumours lacking ShcA adopt alternative strategies to activate signalling pathways normally dependent on ShcA to support tumour cell proliferation, survival and angiogenesis.

The MMTV/NIC ShcA KO mice in this study also demonstrated that ShcA plays a role in regulating the adaptive immune response; loss of ShcA resulted in increased T-cell and B-cell infiltration during early mammary tumourigenesis, suggesting that ShcA signalling is required for immunosuppression during transformation. The study also found that high ShcA levels and ShcA signalling favours immunosuppression in HER2 and basal breast cancers, potentially contributing to the poor patient outcome associated with these subtypes.

Further transgenic mouse studies were performed by our lab to elucidate the role of the SH2 domain of ShcA [190]. MMTV/MT mice were bred to harbour a point mutation in the SH2 domain (R397K), rendering it unable to bind to phosphotyrosine. The observed increase in latency of primary tumour and lung metastases, as well as the reduction in size of both the tumour and metastatic lesions, demonstrated that the ShcA SH2 domain has a critical role during tumour outgrowth. Both reduced Akt phosphorylation and significant decreases in ShcA/14-3-3ζ and ShcA/p85α co-immunoprecipitation in tumours of the mutant demonstrated that the SH2 domain of ShcA is critical for breast cancer outgrowth and survival, and this is accomplished through its activation of the Akt pathway by recruitment of the 14-3-3ζ/p85α-PI3K complex.

1.5.1 $p46^{Shc}/p52^{Shc}$

The majority of the investigation into the function of ShcA, both in normal and cancer cells, has been targeted to the $p46^{Shc}/p52^{Shc}$ isoforms. These have been shown to have an important role in mitogenic signaling through activation of the Ras/MAPK pathway, which leads to cell proliferation [176]. They have also been implicated in the activation of intracellular pathways, like the PI3K/Akt pathway, that promote cell survival. $p46^{Shc}/p52^{Shc}$ signaling, downstream of Met and ErbB2 signalling, induces VEGF expression and promotes tumour angiogenesis and is important for maintaining endothelial cell survival during the angiogenic process [191, 192]. With regards to metastasis, $p46^{Shc}/p52^{Shc}$ signaling is involved in tumour cell intravasation, which is important for the metastatic spread of breast cancer, and is also required to mediate the synergistic effects of the TGF-β and ErbB2 signaling pathways on breast cancer cell motility and invasion [173, 193].

1.5.2 $p66^{Shc}$

The longest ShcA isoform is $p66^{Shc}$, which has an additional N-terminal CH domain (CH2) with a phosphorylatable serine residue at position 36 (S36) [194]. While $p46^{Shc}/p52^{Shc}$ is ubiquitously expressed, $p66^{Shc}$ is absent or expressed in very low levels in hemopoeietic cell lines, normal blood lymphocytes and brain, as well as a subset of breast cancer cell lines [170, 195-197]. This cell-type specific low-expression is has been shown to be cause by the silencing of the $p66^{Shc}$ promoter by epigenetic modifications, namely histone deacetylation and cytosine methylation; an inverse correlation was demonstrated to exist between $p66^{Shc}$ promoter methylation and $p66^{Shc}$ expression, and treatment with

demethylating agents or histone deacetylase inhibitors results in $p66^{Shc}$ expression in cells normally lacking the $p66^{Shc}$ protein [198]. While deletion of the ShcA gene is embryonic lethal, germline deletion of $p66^{Shc}$ results in increased longevity and resistance to apoptosis following tissue oxidative stress [199, 200].

By playing a pivotal role in the oxidative stress response, $p66^{Shc}$ is considered a master regulator of cell survival and ageing [194]. Endogenous ROS production, a known trigger for apoptosis initiation, is upregulated by $p66^{Shc}$ [201]. Apoptogenic stimuli, such as oxidative stressors like H_2O_2 or UV radiation, trigger S36 phosphorylation by ERK, JNK or p38, resulting in $p66^{Shc}$ conformational changes mediated by peptidyl isomerase (Pin1) and subsequent translocation to the mitochondrial intermembrane space (**Figure 8**) [200, 202-204]. Using electrons from the ETC, $p66^{Shc}$ oxidizes reduced cytochrome c, catalyzing the reduction of O_2 to H_2O_2 and leading to mitochondrial swelling by the opening of the permeability transition pore [205]. Increased mitochondrial permeability releases cytochrome c into the cytoplasm, activating caspase-3 and resulting in apoptosis [206-208]. A fraction of $p66^{Shc}$ is constitutively localized to the mitochondria, where its apoptotic activity is inhibited by sequestration with the mitochondrial chaperonin heat shock protein 70 (Hsp70) until this complex is released by oxidative stimuli [209]. Interestingly, $p66^{Shc}$ null mice were shown to have a ~30% longer lifespan compared to their normal counterparts, as well as significantly less accumulated oxidative damage in their mitochondrial DNA after treatment with UV radiation and ROS [200, 210].

$p66^{Shc}$ has also been shown to negatively affect mitogenic signalling through competitive inhibition with $p52^{Shc}$. Phosphorylation of S36 results in $p66^{Shc}$ binding and sequestering Grb2/SOS in a signaling-incompetent complex, preventing $p52^{Shc}$-mediated

activation of the Ras/MAP kinase pathway [171, 202, 211]. This antagonism of mitogenic signaling has been demonstrated in $p66^{Shc}$-deficient T-cells, which have been reported to proliferate faster than their normal counterpart [202].

However, $p66^{Shc}$ signaling is not exclusively anti-tumourigenic. As elevated ROS can promote an aggressive phenotype in cancer cells by inducing a chronic inflammatory state, $p66^{Shc}$ has been shown to increase the proliferation of prostate cancer cells in an ROS-dependent manner [212, 213]. Additionally, while $p66^{Shc}$ is linked to anoikis, a type of apoptotic cell death that occurs when tissue cells that require integrin-dependent attachment are forced into suspension, a $p66^{Shc}$S36E mutant which simulates constitutive S36 activation was shown to escape anoikis and induce anchorage-independent cell growth [214]. This combined with the fact that only abortive colonies were formed with both wild-type $p66^{Shc}$ and $p66^{Shc}$S36A mutant, the latter simulating constitutive S36 inactivation, suggests that S36 phosphorylation does not exclusively transmit pro-apoptotic signalling. Furthermore, while some clinical studies suggest that high $p66^{Shc}$ expression levels are associated with good outcome in cancer patients, others have proposed that high $p66^{Shc}$ correlates with increased lymph node positivity, grade and recurrence in breast, prostate and colon cancer patients [183, 197, 215, 216].

1.6 Rationale and Objectives

The transformation of normal breast tissue into neoplasia is a complex process that requires a variety of factors both internal and external to the tumourigenic cells. Uncontrolled survival, growth and proliferation, as well as invasion into the breast tissue and eventual metastasis, is mediated by interaction between the cancerous cells and the

tumour microenvironment that hosts them. Inflammatory cells and mediators in this microenvironment further contribute to tumourigenesis in a variety of ways, including activating signaling cascades through RTKs, recruiting vasculature to bring oxygen and nutrients to the growing tumour via stimulation of angiogenesis, and sustaining oncogenic mutagenesis and potentiating growth signals through the production of ROS.

Transgenic studies previously performed by our laboratory have demonstrated that mammary-epithelial ShcA is essential for tumour onset, progression and metastasis [173, 189]. Specifically, ShcA has been shown to regulate survival, angiogenesis, immune suppression and metastasis in breast cancer cells. However, while the ubiquitously expressed $p46^{Shc}$ and $p52^{Shc}$ isoforms have been shown to confer these pro-tumourigenic signals, the $p66^{Shc}$ isoform is variably expressed in cancer cell lines and tumours, and its importance in cancer development is poorly understood [170, 171, 177, 198, 217].

In vitro studies suggest opposing roles for $p66^{Shc}$ in regulating cell proliferation and survival. While it's known that $p66^{Shc}$ contributes to the mitochondrial regulation of apoptosis, it has also been implicated in mediating growth signals in human prostate cancer cells [209, 213]. Similarly, conflicting clinical studies report that $p66^{Shc}$ expression levels in tumours correlate with both good and bad outcomes [183, 197, 215, 216]. This duality makes sense in light of the fact that stress-induced $p66^{Shc}$ activation results in increased ROS production, and ROS exhibit both pro- and anti-tumourigenic properties. Although high levels of ROS can induce programmed cell death and cellular senescence, lower levels can stimulate oncogenic mutagenesis, growth signaling and tumour-promoting inflammatory pathways.

However, while both *in vitro* studies and clinical reports present $p66^{Shc}$ in both oncogenic and tumour suppressing contexts, the effect of $p66^{Shc}$ *in vivo* has yet to be explored. The purpose of this study was to examine the role of $p66^{Shc}$ *in vivo* in order to determine its pro- and anti-tumourigenic properties during mammary tumourigenesis.

1.6.1 Aim 1

Characterization of the role of $p66^{Shc}$ expression in vitro. Before investigating the role of $p66^{Shc}$ in mammary tumourigenesis *in vivo*, it was important to characterize the impact of $p66^{Shc}$ expression in a tumour-microenvironment free context as to establish the cell-autonomous effects this protein has on growth, survival and apoptotic signalling. To this end, two established breast cancer cell lines (the murine NIC 4360, and the human MDA-MB-231), both low in endogenous $p66^{Shc}$ and retrovirally transfected to stably overexpress $p66^{Shc}$ (as well as mutants $p66^{Shc}$S36A and $p66^{Shc}$S36E in MDA-MB-231 cells), were analyzed via Western Blot for key signalling proteins in growth, proliferation, survival and inflammatory pathways to provide mechanistic insight of the effect of $p66^{Shc}$ expression on both untreated and H_2O_2 treated cells.

1.6.2 Aim 2

Characterization of the role of $p66^{Shc}$ expression in vivo. To gain a comprehensive understanding of the effect of $p66^{Shc}$ expression and signalling in mammary tumourigenesis, the cell lines were injected into the mammary fat-pad (MFP) of mice. The effect $p66^{Shc}$ has on relative tumour onset and growth was monitored by palpation and frequent caliper measurement. At endpoint, tumour samples were collected and analyzed by Western Blot, quantitative real-time polymerase chain reaction (qRT-PCR) and

immunohistochemistry (IHC) to identify the relative effects $p66^{Shc}$ expression and signalling has on a variety of tumourigenic factors, including growth, survival, proliferation, apoptosis, inflammation, angiogenesis, and relative ROS levels. These data combined sought to paint an overall picture of how $p66^{Shc}$ influences mammary tumourigenesis.

Chapter Two: Materials and Methods

2.1 Cell Culture

MDA-MB-231 (MDA-231) cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM, Wisent Bioproducts) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.1% gentamycin (Wisent Bioproducts). MMTV-Neu-IRES-Cre 4360 (NIC-4360) cells were previously derived from MMTV mice interbred with animals expressing a bicistronic transcript of an oncogenic ErbB2 (Neu) allele followed by the Cre-recomibase under the translational control of an IRES element [173]. NIC-4360 cells were cultured in DMEM supplemented with 5% FBS plus mammary epithelial growth supplement (MEGS, Invitrogen), 1% penicillin/streptomycin and 0.1% gentamycin.

Wild-type mouse-p66^{Shc} (p66), mutant p66^{Shc} which mimics constituent serine-36 inactivation (S36A), and mutant p66^{Shc} which mimics constituent serine-36 activation (S36E) were each sub-cloned into a murine stem cell virus plasmid (pMSCV)-puro vector (Clonetech) as XhoI/EcoRI fragments, which stably integrates the gene of interest and the puromycin resistance gene once transfected into cells. At least 16 hours prior to transfection, cells were plated on 6 cm plates at a density of 500,000 cells/dish. At the time of transfection, 2 ug of the sub-cloned vector was added to 4 uL of Fugene HD (Promega) in 3 uL of medium, and added drop-wise to the plated cells. At 24 hours after transfection, cells were split into two 10 cm plates, and then stably transfected cells were selected for using 2 ug/ml puromycin. Using this method, NIC-4360 cells were transfected with the p66

vector and a vector control (VC), and MDA-231 cells were transfected with p66, S36A and S36E vectors as well as VC.

2.2 H_2O_2 Time Course Assay

At 2 days prior to treatment, cells were plated in 6 well plates at 250,000 cells/well in 2 mL media (either MDA-MB-231 or NIC-4360 media described above). At 16 hours prior to treatment, normal media was aspirated and cells were washed with 1X phosphate buffered sulphate (PBS), and cells were incubated with low serum media (0.5% FBS instead of 10%/5% FBS). At time of treatment, 40 μL or 200 μL of freshly prepared 10 mM H_2O_2 was added to 4 wells of each cell line to a final concentration of 200 μM or 1 mM H_2O_2, respectively. At the 1, 2, 4 and 24 hour time points, the media was aspirated, cells were washed 1X with PBS, and lysed using phospholipase c γ (PLCγ) lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton-X, 1 mM EGTA pH 8, 1.5 mM $MgCl_2$, 10 mM NaF, 10 mM Sodium Pyrophosphate, 1 mM Sodium Vanadate, 1 ug/ml Chymostatin, 1 ug/ml Pepstatin, 2 ug/ml Antipain, 2 ug/ml Leupeptin, 2 ug/ml Aprotinin).

2.3 *In Vivo* Tumourigenesis Assay

For both NIC-4360 and MDA-MB-231 cells, 1 million cells were injected into the 4^{th} mammary fat pad of severe combined immune deficiency (SCID)/Beige female mice (Taconic). For both assays, tumour growth was monitored by tri-weekly caliper measurements. Tumour volumes (mm^3) were calculated as follows: $4/3\pi(\text{length}/2)(\text{width}/2)^2$. At necropsy (tumour volume reached 1000 mm^3), mammary

tumours were flash frozen in liquid nitrogen, fixed in 10% paraformaldehyde (PFA) and embedded in paraffin, or cryo-embedded in optimal cutting temperature (OCT) compound (Tissue-Tek). Lungs were also fixed in 10% PFA and embedded in paraffin. All animal studies were approved by the Animal Resources Centre at McGill University and comply with guidelines set by the Canadian Council of Animal Care.

2.4 Immunoblotting

For cell lines, whole cell lysates were obtained by lysing cells in PLCγ lysis buffer. For tumours, flash frozen pieces were ground into powder in liquid nitrogen using a mortar and pestle, which increased the available tumour piece surface area for dissolving in PLCγ lysis buffer after the liquid nitrogen evaporated. For both cell lines and tumour pieces, lysates were sonicated to assure extraction of nuclear proteins, and cellular debris was removed by spinning the samples down using a centrifuge at 16000 rpm. Protein concentration was determined by Bradford Assay using Protein Assay Dye Reagent Concentrate (BioRad). Samples were prepared with 6X SDS-PAGE Loading Buffer (375mM Tris/SDS pH 6.8, 50% glycerol, 10% sodium dodecyl sulphate (SDS), 0.03% bromophenol blue) and separated by polyacrylamide gel electrophoresis (PAGE) on a 10% SDS-polyacrylamide gel. Samples were transferred onto a polyvinylidene fluoride (PVDF, VWR) membrane and blocked using 5% bovine serum albumin in Tris-buffered saline with Tween (TBS-T) (1 mM Tris [pH 8.0], 15 mM NaCl, 0.5% Tween). Membranes were then probed overnight with primary antibodies, washed 3X in TBS-T, and then probed with horseradish peroxidase conjugated secondary antibody and visualized using enhanced chemiluminescence (ECL) substrate (Pierce) on autoradiography film (Kodak).

Antibodies used for immunoblotting were diluted at 1/1000 (unless otherwise stated) and include rabbit anti-AKT (9272, Cell Signalling), rabbit anti-AKT phospho Ser473 (9271; Cell Signalling), rabbit anti-p44/42 MAPK (ERK1/2) (9102; Cell Signalling), mouse anti-44/42 MAPK (ERK1/2) phosphoThr202/Tyr204 (E10) (9106; Cell Signalling), mouse anti-FLAG (F1804; Sigma Aldrich), rabbit anti-IκBα (4812; Cell Signalling), rabbit anti- IκBα phosphoSer32 (2859; Cell Signalling), rabbit anti-IKKα (2682; Cell Signalling), rabbit anti-IKKα/β phosphoSer176/180 (2697; Cell Signalling), rabbit anti-SAPK/JNK (9252; Cell Signalling), rabbit anti-SAPK/JNK phosphoThr183/Tyr185 (9251; Cell Signalling), rabbit anti-Neu (C-18) (sc-284; Santa Cruz), rabbit anti-NFκB p65 (3034; Cell Signalling), rabbit anti-NFκB p65 phosphoSer536 (3033; Cell Signalling), rabbit anti-p38 MAPK (9212; Cell Signalling), rabbit anti-p38 MAPK phosphoThr180/Tyr182 (9215; Cell Signalling), rabbit anti-ShcA (6100081; BD Transduction Laboratories, 1/2000), mouse anti-STAT3 (124H6) (9139; Cell Signalling), rabbit anti-STAT3 phosphoTyr705 (D3A7) (9145; Cell Signalling), and mouse anti-α-tubulin (T5168; Sigma Aldrich, 1/4000).

2.5 Immunohistochemistry for Paraffin Embedded Tumours

Paraffin embedded tumours were sectioned at 6 μm and sections were deparaffinised using 3 changes of xylene, 3 changes of 100% ethanol, one change each of 95%, 75% and 50% ethanol, followed by heat-induced epitope revival using 1X Sodium Citrate Buffer (10mM sodium citrate, 0.05% tween 20, pH 6.0) in a pressure cooker (Cuisinart).

For proliferative staining, sections were permeabilized with two 5 minute washes with 0.05% Tween 20/ 0.025% Triton X-100 in TBS (1 mM Tris [pH 8.0], 15 mM NaCl). To prevent endogenous biotin binding with avidin later on in the protocol, sections were incubated for 10 minutes with unconjugated avidin followed by a 5 minute wash with TBS-T and saturation with unconjugated biotin for another 10 minutes. After a 5 minute wash, sections were then blocked for 30 minutes with 10% bovine serum albumin (BSA) and incubated overnight at 4^{o}C with rabbit anti-Ki67 (1:500; ab15580, Abcam) diluted in 2% BSA/TBS. The following day, slides were subjected to three 5 minute washes with TBS-T, followed by two 5 minute washes with TBS. Slides were incubated for 15 minutes in 3% H_2O_2 in TBS, followed by two 5 minute TBS-T washes and incubation with biotinylated goat anti-rabbit (1:1000; 711-065-152, Jackson Laboratories) in 2% BSA/TBS for 30 minutes. After three 5 minute washes in TBS-T, visualization occurred by adding the avidin/biotinylated enzyme complex (ABC) for 30 minutes, followed by three 5 minute TBS-T washes and one rinse with TBS. Slides were developed using diaminobenzidine (DAB) substrate (Vector) until specific staining was obtained and submerged in water before background staining was visible. Sections were counterstained for 30 seconds with 20% hematoxylin to visualize nuclei before submerging in water for 5 minutes, and hydrophobized for 3 minutes each in one change of 50% ethanol, followed by one change each of 70% and 95% ethanol, two changes in 100% ethanol, and finally three changes of xylene. Slides were then mounted with Clearmount (GeneTex). Slides were scanned using an Aperio slide scanner and data were analyzed using Imagescope software with positive pixel count or nuclear algorithms.

For apoptotic cell detection, sections were subjected to TdT-metiated dNTP nick end labelling (TUNEL) staining (Apoptag Detection kit; Chemicon) according to the manufacturer's instructions, and then counterstained, hydrophobized, mounted, scanned and analyzed as described above.

2.6 Immunohistochemistry for OCT Embedded Tumours

OCT embedded tumours were sectioned at 6 μm and sections were thawed at room temperature for an hour and fixed with 4% PFA. Sections were incubated 3 times for 5 minutes each with 0.05% Tween 20/ 0.025% Triton X-100 in TBS (1 mM Tris [pH 8.0], 15 mM NaCl), permeablized for 10 minutes with 0.2% Triton-100 in TBS, and washed 3 times with TBS-T. This was followed by the unconjugated avidin/biotin saturation, with alternating TBS-T washes and blocking with 10% BSA as described above in 2.5. Sections were incubated overnight at 4^{o}C with rabbit anti-CD31 (1:200; ab28364, Cell Signalling) diluted in 2% BSA/TBS. Washing with TBS-T and TBS, incubation with H_2O_2 and further washing with TBS-T was performed as described above in 2.5. Slides incubated were then incubated for 30 minutes with a biotinylated goat anti-rabbit (1:1000; 711-065-152, Jackson Laboratories) diluted in 2% BSA/TBS. After three 5 minute washes in TBS-T, visualization of desired proteins by ABC treatment and DAB development occurred as described above in 2.5. Slides were also counterstained, hydrophobized, mounted, scanned and analyzed as described in 2.5.

2.7 *In Vivo* ROS Detection Assay Using DHE

OCT embedded tumours were sectioned at 6 μm and sections were thawed at room temperature. Slides were incubated with 2 uM dihydroethidium (DHE) in PBS for one minute to allow DNA intercalation, and the red fluorescence this produced was visualized and captured using a LeicaDM2000 microscope with Infinity Capture software, and data were analyzed using Imagescope software with positive pixel count.

2.8 Quantitative Real Time PCR

RNA from flash frozen tumour pieces was isolated using RNeasy Midi Kit (Qiagen). First strand cDNA synthesis was performed using 500 μg of RNA as a template and carried out as outlined in the protocol for Superscript II Reverse Transcriptase (Invitrogen). qRT-PCR was performed using 7500 Real Time PCR system (Applied Biosystems). For SYBR Green reactions, GoTaq qPCR Master Mix (Promega) was supplemented with 1 μL of a 1:10 dilution of the generated cDNA and 0.4 μM each of the forward and reverse primers. For Taqman reactions, Taqman Fast Universal PCR Mater Mix (Applied Biosystems) was supplemented with 1 μL of a 1:10 dilution of cDNA and pre-designed Taqman primers. Primer sequences used in SYBR Green reactions (**Table 1**) or catalogue numbers of primers used in Taqman reactions (**Table 2**) are listed in Appendix II. Fold change gene expression was calculated using the delta-delta Ct method relative to the housekeeping gene glyceraldehyde 3- dehydrogenase (GAPDH).

Chapter Three: Results

3.1 Generation of p66^{Shc} expressing breast cancer cell line variants

Neu-IRES-cre (NIC) cell lines, containing both an oncogenic Neu allele and Cre recombinase under the translational control of an IRES element, were established from MMTV/NIC transgenic mice previously generated. To determine the role of p66^{Shc} in mammary tumourigenesis, we generated p66^{Shc} expressing variants in NIC 4360 cells, as well as the established human breast cancer cell line MDA-MB-231, both of which were shown to be low in endogenous p66^{Shc}. Briefly, these cells were transfected with pMSCV-puro vectors and pooled stable colonies were selected for using puromycin. NIC cells were transfected with either wild-type murine p66^{Shc} (NIC-p66ShcA) or an empty vector control (NIC-VC). MDA-MB-231 cells, in addition to vector control (VC) and murine p66^{Shc} wild-type (p66ShcA-WT), were also transfected with murine p66^{Shc} S36 phospho-mimetic mutants, representing phospho-mimetic (p66ShcA-S36E) and dominant negative (p66ShcA-S36A) p66ShcA alleles. To distinguish between endogenous and endogenous p66^{Shc} expression, both wild-type and mutant DNA inserts were designed to express recombinant p66^{Shc} protein tagged with the FLAG polypeptide. Immunoblot analysis on in vitro cell lysates confirmed expression of the p66^{Shc} cDNAs, as well as the relative lack of p66^{Shc} expression in vector controls from both cell lines (NIC, **Figure 9a**; MDA-MB-231, **Figure 10a**).

3.2 *In vitro* characterization of p66^{Shc} expressing breast cancer cell line variants

Figures 9a and **10a** also revealed the effect of relative p66^{Shc} levels on the expression and activation status of key regulators of major growth, survival, stress and inflammatory pathways under normal cell culture conditions. In both NIC and MDA-MB-231 cells, we observed a decrease in Akt phosphorylation in cells expressing both the wild-type (NIC-p66ShcA, p66ShcA-WT) and constitutively active mutant (p66ShcA-S36E), indicating that increased p66^{Shc} expression and activation inhibits AKT activation. An increase in JNK phosphorylation and p38 expression in NIC-p66ShcA cells suggests p66^{Shc} expression contributes to increased stress signalling, however neither of these observations were repeated in either the wild-type or constitutively active p66^{Shc} MDA-MB-231 variants. Also notable was the lack of effect p66^{Shc} expression appeared to have on NF-κB p65 and STAT3 expression and signalling *in vitro*. All of these observations provide insight into the effects of p66^{Shc} outside of a tumour environment.

3.2.1 Evaluation of the effect of p66^{Shc} expression under cellular stress

As p66^{Shc} is widely known to be activated in response to cellular stress, we wanted to observe what effect differential p66^{Shc} expression has in the presence of stress-inducing ROS. For this purpose, both NIC and MDA-MB-231 cells underwent a time course assay where cells were treated with 1000 μM of H_2O_2, a concentration we determined to induce a differential signalling response without cytotoxicity at 24 hours (data not shown). These cells were subsequently lysed at 1, 2, 4 and 24 hour time points, and immunoblot analysis was performed using antibodies against major inflammatory and stress effector proteins (NIC, **Figure 9b**; MDA-MB-231, **Figure 10b**).

In both NIC and MDA-MB-231 cell lines, expression and phosphorylation of NF-κB was stronger and was maintained for longer in the $p66^{Shc}$ wild-type expressing variants. Most strikingly, at 24 hours after treatment, high NF-κB phosphorylation levels were maintained in the wild-type and p66ShcA-S36E variants, as opposed to returning to the low, pre-treatment levels in the VC and p66ShcA-S36A variants. These data suggest increased $p66^{Shc}$ expression has a positive effect on activating the classical NF-κB pathway under cellular stress.

However, NIC and MDA-MB-231 cells behaved differently in all other measured contexts. In both NIC cell lines, STAT3 phosphorylation was drastically reduced at 1,2, and 4 hours post- treatment, but returned to near pre-treatment levels after 24 hours. This effect was not repeated in MDA-MB-231 cells, where STAT3 phosphorylation was not strongly observed pre- or post-treatment. Another effect observed only in the NIC cell line was the increase of JNK phosphorylation in NIC-p66ShcA at 1, 2, and 4 hours post treatment, echoing the earlier results in this cell line under normal culture conditions (**Figure 9b**). Finally, while p38 phosphorylation levels noticeably increased at 1, 2, and 4 hours post-treatment in both NIC variants, a decrease in p38 phosphorylation was observed in MDA-MB-231 at 1 hour post-treatment, most drastically in the p66ShcA-S36E variant. Overall, under stress conditions, the NIC-p66ShcA variant activates stress signalling pathways much more readily than its counterpart in the MDA-MB-231 cells. This makes sense in light of the fact that NIC cells inherently overexpress ErbB2, a TKR which MDA-MB-231 cells lack [218]. As ErbB2 activation is upstream of both STAT3 and cell-survival signalling, these data suggest that the effects of $p66^{Shc}$ levels on these pathways occur in conjunction with ErbB2 expression.

Vector-expressed $p66^{Shc}$ is FLAG-tagged, making it heavier and decreasing its mobility on an SDS-PAGE gel relative to endogenous $p66^{Shc}$. The intermediate band between $p66^{Shc}$ and $p52^{Shc}$, which is most prominently observed in **Figure 10b**, is most likely endogenous $p66^{Shc}$. This demonstrates that while $p66^{Shc}$ levels are low in MDA-MB-231 VC cells, the protein is still expressed. Therefore, it should be noted that any differential effects observed between VC and p66Shc-WT cells are with respect to relatively high or low $p66^{Shc}$ levels, as opposed to the presence or absence of $p66^{Shc}$.

3.3 *In vivo* characterization of $p66^{Shc}$ expression on mammary tumourigenesis

To investigate the effect of $p66^{Shc}$ expression in mammary tumourigenesis, we injected 1 million cells of all NIC and MDA-MB-231 variants into the mammary fat pad of female SCID/Beige mice, measured tumour growth over time, and collected tumour samples and lungs at endpoint for analysis.

3.3.1 Effect of $p66^{Shc}$ expression on tumour initiation and outgrowth

In contrast with mice injected with NIC cells, none of which remained tumour free by 21 days post-injection (**Figure 11a**), the earliest palpable MDA-MB-231 tumours were detected at 22 days post injection and some mice remained tumour free for nearly two weeks after (**Figure 12a**). This suggests that MDA-MB-231 cells have an overall decreased tumour initiating capacity compared to NIC cells. In both NIC and MDA-MB-231 experiments, mice injected with cells expressing either wild-type or mutant $p66^{Shc}$ develop palpable tumours faster than VC. However, after first palpation, while NIC-p66ShcA

tumours grew slower than NIC-VC tumours (**Figure 11b)**, there was no significant difference in growth between any of the MDA-MB-231 tumours (**Figure 12b**). As was mentioned in 3.2.1, the fact that ErbB2 is overexpressed in NIC cells and diminished in MDA-MB-231 cells likely accounts for the relative delay in MDA-MB-231 tumour initiation. Similarly, since ErbB2 is known to play a significant role in growth and breast cancer tumourigenesis, it's possible that the role $p66^{Shc}$ expression plays in mammary tumour initiation and outgrowth is ErbB2-dependent and therefore only observable in ErbB2-expressing contexts, such as the NIC tumours.

Interestingly, immunoblot analysis on tumour lysates collected at endpoint revealed that 5 out of the 7 NIC-p66ShcA tumours had reduced or completely silenced $p66^{Shc}$ expression (**Figure 11c**). Similarly, while $p66^{Shc}$ expression remained relatively strong in both of the MDA-MB-231 p66ShcA-WT and p66ShcA-S36A tumours, it was visibly reduced or silenced in all 7 p66ShcA-S36E tumours (**Figure 12d**). The $p66^{Shc}$ mRNA expression data provided by qRT-PCR analysis (NIC, **Figure 11d**; MDA-MB-231, **Figure 12c**) on these same tumour samples corroborated the immunoblot data; while all $p66^{Shc}$ expressing variants showed increased $p66^{Shc}$ expression relative to VC tumours, MDA-MB-231 p66ShcA-S36E tumours expressed significantly less $p66^{Shc}$ than either p66ShcA-WT or p66ShcA-S36A. These data indicate that $p66^{Shc}$ activation is deleterious to tumourigenesis and that tumours may reduce its expression as a means of adaptation to allow for tumour growth.

Although $p66^{Shc}$ expression may be reduced or silenced in many of the tumour samples, it is possible that observed differences in tumour initiation between $p66^{Shc}$ expressing and non-expressing cells could indicate differences in other signalling pathways

in the tumours by endpoint. To this end, the tumour samples from both NIC and MDA-MB-231 experiments were subjected to further immunoblot, qRT-PCR and IHC analysis.

3.3.2 Effect of $p66^{Shc}$ expression on tumour ROS levels

To evaluate the relative effect $p66^{Shc}$ expression had on tumour ROS levels, OCT-embedded tumours underwent DHE immunofluorescent staining. DHE can freely permeate cell membranes and react with superoxide anions to form a red fluorescent product, either ethidium or 2-hydrocyethidium, which intercalates with DNA [219]. In both NIC and MDA-MB-231 tumours, $p66^{Shc}$ expressing tumour types displayed increased ROS levels relative to VC tumours (NIC, **Figure 13b**; MDA-MB-231, **Figure 14b**), although NIC ROS levels were more variable across individual tumours (data not shown). As $p66^{Shc}$-induced ROS is known to be facilitated through S36 phosphorylation, these data makes sense in light of the previously discussed results, in which $p66^{Shc}$ was shown to be reduced in most NIC-p66ShcA tumours but not in MDA-MB-231p66ShcA-WT tumours. S36-mediated ROS induction also accounts for the fact that while $p66^{Shc}$ levels remained high in both p66ShcA-WT and p66Shc-S36A tumours, ROS levels in the latter were significantly decreased relative to the wild-type. Interestingly, despite the earlier establishment of reduced $p66^{Shc}$ expression in p66Shc-S36E tumours, ROS levels in these tumours are high and statistically similar to those in p66ShcA-WT tumours. Collectively, these data suggest that increased $p66^{Shc}$ expression results in increased tumour ROS levels, and that this is strongly facilitated through S36 phosphorylation.

3.3.3 Effect of $p66^{Shc}$ expression on survival and proliferative signalling

With regards to survival and proliferative signalling, immunoblot analysis revealed that both stress-activated p38 and growth-promoting ERK were consistently phosphorylated in NIC-p66ShcA tumours, as opposed to the variable p38 and ERK phosphorylation levels observed in NIC-VC tumours (**Figure 13a**). Curiously, IHC revealed a significant decrease in Ki67 positive cells in NIC-p66ShcA tumour samples, indicating $p66^{Shc}$ expression may have a negative effect on tumour cell proliferation (**Figure 13c**), an observation which corresponds with their delayed tumour outgrowth reported above. In contrast, ERK and p38 phosphorylation was inconsistent across all MDA-MB-231 variants (**Figure 14a**) and no significant difference was observed in MDA-MB-231tumour cell proliferation (**Figure 14c**). Both these data correspond with the MDA-MB-231 tumour outgrowth results. IHC using the TUNEL assay demonstrated that relative $p66^{Shc}$ expression had no effect on tumour cell apoptosis (NIC, **Figure 13d**; MDA-MB-231, **Figure 14d**). Combined, these data suggest that in an ErbB2-expressing context, increased $p66^{Shc}$ expression *in vivo* leads to both sustained stress signalling and decreased proliferation, but with no discernible effect on apoptosis.

3.3.4 Effect of $p66^{Shc}$ expression on tumour inflammatory signalling

As we demonstrated in 2.2.1 that increased $p66^{Shc}$ expression was shown to activate the classical NF-κB pathway under stress *in vitro*, we next looked at the effect of relative $p66^{Shc}$ expression on inflammation, one of the major downstream effectors of this pathway *in vivo*. Immunoblot analysis revealed NF-κB p65 to be consistently phosphorylated in NIC-p66ShcA tumours as compared to the variable phosphorylation levels in NIC-VC (**Figure 15a**). Interestingly, NIC-p66ShcA tumours also demonstrated a slight decrease in STAT3 expression while no noticeable difference in STAT3 phosphorylation was

observed. In the immunoblot analysis of MDA-MB-231 tumours (**Figure 16a**), while there appeared to be no difference in expression or phosphorylation of either NF-κB or STAT3, phosphorylation of IκBα, an upstream NF-κB pathway inhibitor, was observed to be decreased in p66ShcA-S36E tumours.

To determine the relative effect of $p66^{Shc}$ expression on NF-κB target gene expression, qRT-PCR analysis was performed on tumour mRNA samples for a variety of genes. In NIC tumours (**Figure 15c**), $p66^{Shc}$ expression significantly increased the expression of several NF-kB target genes known to be involved in tumour inflammation, including those involved in the recruitment of leukocytes such as monocytes (CCL2, CCL5), monocyte differentiation into macrophages (CSF1), macrophage-secreted pro-inflammatory mediators involved in the NF-κB pathway (TNFα, IL-6), including a matrix metallopeptidase involved in the breakdown of the extracellular matrix (MMP-9). Interestingly, IL-6 was only increased in MDA-MB-231 cells in the p66ShcA-S36A tumours relative to all other variants (**Figure 16c**, left panel), suggesting that $p66^{Shc}$-mediated IL-6 expression is facilitated in the absence of S36 phosphorylation. While expression of OAS1, a gene involved in innate immunity against viral infections, was significantly reduced in MDA-MB-231 p66ShcA-WT tumours relative to both mutants, no significant difference in NIC tumour OAS1 levels were observed. Interferon levels were unaffected by $p66^{Shc}$ expression in both NIC and MDA-MB-231 experiments, as were IL-1β levels in NIC tumours. Taken together, these data indicate that increased $p66^{Shc}$ expression leads to increased expression of NF-κB p65 target genes specifically involved with monocyte recruitment and macrophage stimulation, and this in turn leads to increased expression of macrophage-secreted inflammatory mediators. More directly, this suggests

that increased p66^{Shc} expression leads to increased recruitment of TAMs to the tumour environment.

3.3.5 Effect of p66^{Shc} expression on angiogenic signalling

In addition to secreting factors that lead to increased tumour inflammation and extracellular matrix remodelling, which assist in angiogenesis, TAMs are known to secrete VEGF, which directly promotes angiogenic signalling [154-159]. Therefore, we sought to examine the effect of relative p66^{Shc} expression on angiogenesis by performing IHC on tumour samples with CD31 antibody, which is used to visualize and quantify the relative recruitment of tumour vasculature. In the NIC experiment, angiogenesis was significantly increased in NIC-p66ShcA tumours (**Figure 15b**). Interestingly, in the MDA-MB-231 experiment (**Figure 16b**), while there was no statistical difference in angiogenesis between VC and p66ShcA-WT, there was a significant increase in angiogenesis in p66ShcA-S36E relative to both. Taken together, these data echo those of the ROS assay results discussed in 2.3.2, in that increased p66^{Shc} expression results in increased angiogenesis, and this is likely facilitated through S36 phosphorylation.

Chapter Four: Discussion

In the current study, we aimed to determine the role of $p66^{Shc}$ in either promoting or suppressing mammary tumourigenesis. In particular, we established colonies expressing wild-type, constitutively active and constitutively inactive $p66^{Shc}$ variants in both human and mouse breast cancer cell lines with low endogenous $p66^{Shc}$ expression, and sought to examine the relative effect this had on growth, proliferation, survival, stress and inflammatory signalling, both *in vitro* and during mammary tumour development. Unsurprisingly, our findings echoed the few, conflicting studies previously discussed in 1.6; specifically, that increased $p66^{Shc}$ expression does confer both pro- and anti-tumourigenic properties. However this study further established that, like the intracellular ROS that $p66^{Shc}$ is known to generate, the question of whether increased $p66^{Shc}$ expression is beneficial or detrimental to tumour development is largely context-dependent [201].

4.1 $p66^{Shc}$-expression is pro-tumourigenic during tumour initiation

When injected into the MFP of immunocompromised mice, we show that breast cancer cell line variants expressing increased $p66^{Shc}$ formed palpable tumours faster than their relatively low $p66^{Shc}$ expressing VC counterparts, indicating that $p66^{Shc}$ expression assists in tumour initiation. The observation that vascularization is significantly increased in both NIC-p66ShcA and MDA-MB-231 p66ShcA-S36E tumours suggests that one role $p66^{Shc}$ plays in tumour initiation is a contribution to angiogenesis. Since rapidly proliferating tumours are limited at roughly 2 mm by a lack of the nutrients and oxygen

that adequate blood flow provides, increased angiogenesis would allow for an earlier escape from tumour dormancy [150, 151]. Furthermore, the fact that in MDA-MB-231 tumours, this increased angiogenesis is only observed to be sustained at endpoint in mice injected with cells expressing the p66Shc-S36E mutant, which mimics constitutive S36 activation, suggests that the contribution of $p66^{Shc}$ to angiogenesis is facilitated through S36 activation.

Additional observations suggest that $p66^{Shc}$ is involved in the recruitment of macrophages from signalling through the classical NF-κB pathway under ROS-induced cellular stress. *In vitro*, while neither NIC nor MDA-MB-231 showed NF-κB p65 activation under normal cell culture conditions, phosphorylation of the protein was observed to be stronger and maintained for longer in $p66^{Shc}$ expressing variants treated with H_2O_2. Mice injected with the $p66^{Shc}$-expressing variants grew tumours that were also shown to have increased cellular ROS levels, although p66ShcA-S36A tumours had decreased ROS levels compared to the wild-type and S36E, indicating a link between S36 activation and ROS induction. While only NIC-p66ShcA tumours were shown to have consistent NF-κB p65 phosphorylation compared to its VC counterpart, NF-κB inhibition was noticeably decreased in MDA-MB-231 p66ShcA-S36E tumours. This aligns with what is already known about NF-κB; specifically, that NF-κB activation can be stimulated by ROS, and ROS can be induced through $p66^{Shc}$ S36 phosphorylation [200, 202-204]. Furthermore, in NIC tumours, the NF-κB target genes which demonstrated increased expression were all involved with the recruitment of and expression by macrophages, which are known to secrete VEGF and subsequently stimulate angiogenesis [154-159]. Future experiments will involve IHC analysis using antibody against F4/80, which

identifies macrophages, on tumour tissue samples to verify increased TAMs in both NIC-p66ShcA and MDA-MB-231 p66Shc-S36E tumour microenvironments.

Combined, these observations suggest that the pro-tumourigenic role $p66^{Shc}$ plays in mammary tumour initiation is driven by S36 activation, leading to ROS-induced Classical NF-κB pathway stimulation, resulting in TAM recruitment and therefore sustained angiogenic signalling that sufficiently provides rapidly proliferating cancerous lesions an escape from tumour dormancy.

Interestingly, the endogenous $p66^{Shc}$ band observed in the MDA-MB-231 *in vitro* H_2O_2 time course assay is noticeably absent in the p66Shc-S36A variant, even though it is present in the VC. This might suggest that the S36A mutation somehow acts to suppress endogenous $p66^{Shc}$ expression, a mechanism that would have to be elucidated in future studies. However, as the $p46^{Shc}$ and $p52^{Shc}$ levels are appreciably lower in p66ShcA-S36A compared with the VC, it is possible that this exposure of the blot is simply not high enough to visualize endogenous $p66^{Shc}$.

4.2 $p66^{Shc}$ expression becomes anti-tumourigenic during outgrowth in ErbB2-driven tumourigenesis

ErbB2 is a RTK involved in signalling cascades which promote cellular proliferation and oppose apoptosis, and its overexpression and constitutive dimerization is a well-established driver of mammary tumourigenesis [39, 41, 43-48]. As NIC cell lines are defined by their overexpression of ErbB2, while MDA-MB-231 cells lack ErbB2 expression, this study provided insight into the role of $p66^{Shc}$ expression in the presence and absence of ErbB2-driven tumourigenesis [173, 218].

We observed that mice injected with the wild-type NIC-p66ShcA variant saw reduced or silenced $p66^{Shc}$-expression by endpoint, a result only mirrored in the ErbB2-free context with the constitutively active mimetic, indicating that ErbB2 signalling results in $p66^{Shc}$ S36 activation. As has been previously discussed, S36-activated $p66^{Shc}$ leads to induction of ROS. At moderate doses, ROS have been established to enhance growth, proliferative and survival signalling, as well as promote genetic instability, all of which benefit tumourigenesis, whereas high doses of ROS lead to oxidative stress and cellular damage which trigger senescent and apoptotic signalling [5, 53, 54, 63, 66-72]. Therefore, while early stages of tumourigenesis may benefit from the angiogenic signalling provided by $p66^{Shc}$, as established in 4.1, the reduced or silenced $p66^{Shc}$ expression may be a survival mechanism that tumour cells either adapt or are selected for during outgrowth in response to constitutively high levels of ROS induced by active $p66^{Shc}$.

While mice injected with $p66^{Shc}$-expressing variants developed palpable tumours faster than their VC counterparts in both breast cancer cell lines, initiation in all MDA-MB-231 tumours was delayed relative to NIC tumours, illustrating that the proliferative and anti-apoptotic signalling resulting from oncogenic ErbB2 expression accelerates tumour initiation. Additionally, once tumours reached a palpable size, $p66^{Shc}$-expressing tumours grew slower than their VC counterpart in NIC cells, while all MDA-MB-231 tumours had similar rates of outgrowth, suggesting that $p66^{Shc}$-expression delays tumour outgrowth in an ErbB2-driven context.

Within the ErbB2-driven context of the NIC tumours, our IHC analysis indicated that the cause of $p66^{Shc}$-related delay in tumour outgrowth is a decrease in cellular proliferation, not an increase in apoptosis. Western blot analysis in NIC tumours further

revealed that both p38 and ERK were consistently phosphorylated in $p66^{Shc}$-expressing cells relative to inconsistent phosphorylation in their VC counterparts. The stress-activated p38 MAPK and mitogenic signalling molecule ERK MAPK are both downstream effectors of ErbB2 activation, are both known to have increased activation in the presence of ROS, and have both been implicated in the phosphorylation of $p66^{Shc}$ on S36 [41, 75, 200, 202-204].

The mechanism we propose is that ErbB2 activation results in phosphorylation of both p38 and ERK, which in turn activates $p66^{Shc}$ and result in ROS production, facilitating a positive feedback loop for both p38 and ERK phosphorylation. However, in the presence of increased expression and sustained activation of $p66^{Shc}$, leading to increased ROS cellular stress, the balance tips in favour of stress signalling and as a result, cellular proliferation is decreased in these tumours.

Interestingly, while neither the phosphorylation of Akt or JNK seemed to be effected by in tumours from mice injected with the $p66^{Shc}$ variant at endpoint, their phosphorylation levels had been shown to be decreased and increased *in vitro*, respectively. Akt is a downstream effector of ErbB2, and increased ROS is known to promote Akt and JNK activation [53, 82, 83, 102-104]. Therefore, it is possible that these molecules may have also had differential effects to $p66^{Shc}$ activation during the early stages of tumour proliferation, but as $p66^{Shc}$ expression was mostly reduced in NIC tumours by endpoint, observable differential effects might have been lost.

4.3 Summary and Future Directions

We demonstrate that $p66^{Shc}$ activation has a dual role in mammary tumourigenesis that is largely context dependent. During breast cancer initiation, when microscopic tumours are rapidly proliferating, $p66^{Shc}$ expression is pro-tumourigenic as $p66^{Shc}$-induced ROS contributes to the angiogenic switch, likely by recruitment of TAMs through increased activation of the NF-κB Classical Pathway. However, as high levels of $p66^{Shc}$-induced ROS are maintained as a result of persistent ErbB2 activation, or some other form of constitutive $p66^{Shc}$ S36 phosphorylation, the role of $p66^{Shc}$ becomes anti-tumourigenic as prolonged ROS activates stress signalling which inhibits proliferation, and tumours must select against active $p66^{Shc}$ expression to allow for growth. The observations in this study provide molecular insight into the already established understanding of the dose-dependent effects of $p66^{Shc}$-induced ROS levels during the proliferation of cancerous cells.

Further experiments could seek to deepen and broaden this understanding, as well as provide clarification on the results already obtained. One proposed experiment is to establish NIC cell lines expressing the constitutively active (S36E) and inactive (S36A) mimetic mutant and repeat the *in vitro* and *in vivo* analysis methods presented in this study to characterize the effects of S36 phosphorylation in an ErbB2-dependent context. During the *in vivo* portion of these studies, we also propose that NIC tumour samples are collected at various stages during tumour outgrowth, as opposed to only at endpoint, to help further elucidate this ErbB2-dependent mechanism and establish a chronology for the role of $p66^{Shc}$-expression on tumourigenesis.

One area that has yet to be explored is the effect of $p66^{Shc}$ expression on metastasis, and future experiments will seek to characterize this by quantifying relative metastasis to

the lungs, which is one of the most common sites of breast cancer metastases [220]. However, one must take into account that observing metastasis from the MFP is difficult without resection because of the tumour growth rates observed in this study. Therefore, proposed future experiments will require either resection or tail vain injection of the variants to specifically study lung metastasis.

Appendix I: Figures

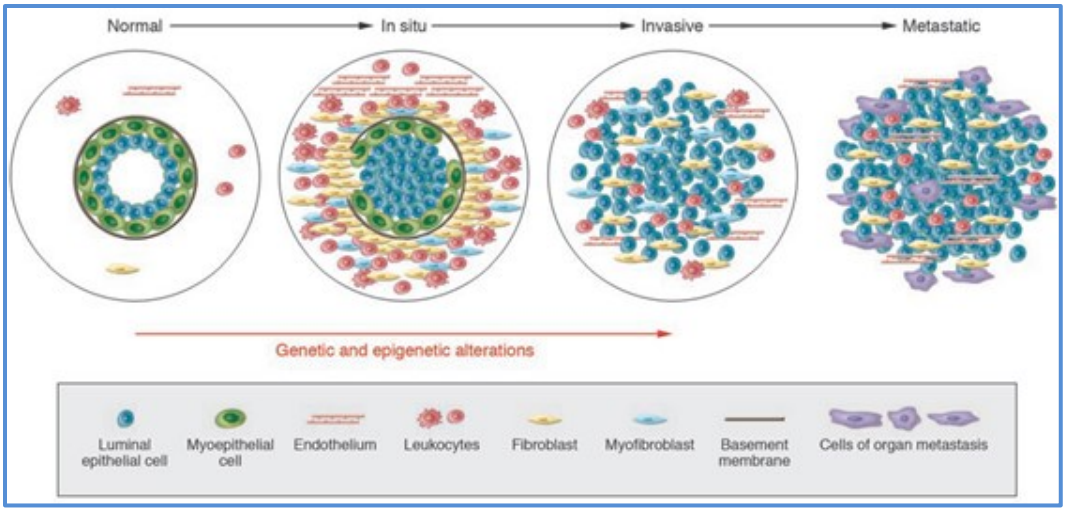

Figure 1: Model of breast cancer progression (adapted from [16]). Normal breast ducts are composed of the basement membrane and a layer of luminal epithelial and myoepithelial cells. Cells composing the stroma include various leukocytes, fibroblasts, myofibroblasts, and endothelial cells. In in situ carcinomas the myoepithelial cells are epigenetically and phenotypically altered and their number decreases, potentially due to degradation of the basement membrane. At the same time, the number of stromal fibroblasts, myofibroblasts, lymphocytes, and endothelial cells increases. Loss of myoepithelial cells and basement membrane results in invasive carcinomas, in which tumour cells can invade surrounding tissues and can migrate to distant organs, eventually leading to metastases.

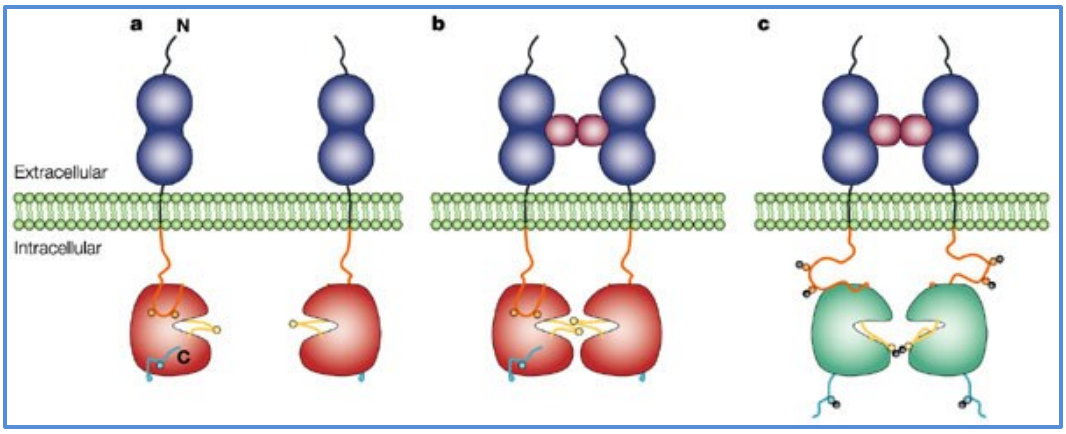

Figure 2: A general autoinhibition/activation model for receptor tyrosine kinases (adapted from [221]). In the absence of ligand, the TKD (red) of a RTK is maintained in a basal, low-activity state through the inhibitory interactions of the juxtamembrane region (orange) and/or the carboxy-terminal tail (blue) with the kinase domain. In addition, the activation segment (yellow) is not optimally positioned for catalysis. After ligand (pink)-mediated dimerization of the extracellular domain (blue), the cytoplasmic domains are juxtaposed, which facilitates the trans phosphorylation of tyrosine residues (shown as circles) in the juxtamembrane region, activation segment and carboxy-terminal tail. After phosphorylation and reconfiguration of the inhibitory segments, the kinase domains become fully active (green) and a subset of phosphotyrosines (black spheres) are available as recruitment sites for proteins that contain SRC homology-2 (SH2) domains or phosphotyrosine-binding domains.

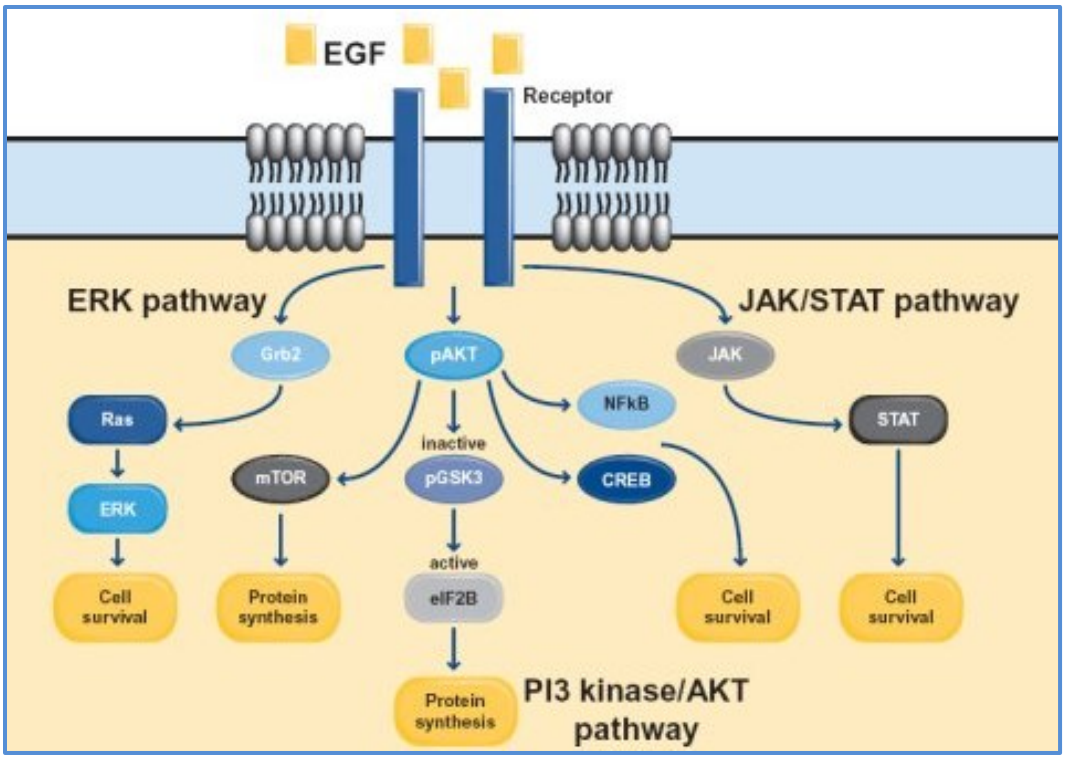

Figure 3: Overview of the EGFR signalling pathway (adapted from [222]). Activation of the EGF receptor results in autophosphorylation of key tyrosine residues. These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains and leads to the activation of downstream signalling cascades including the RAS/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway and the Janus kinase/Signal transducer and activator of transcription (JAK/ STAT) pathway. These pathways act in a coordinated manner to promote cell survival.

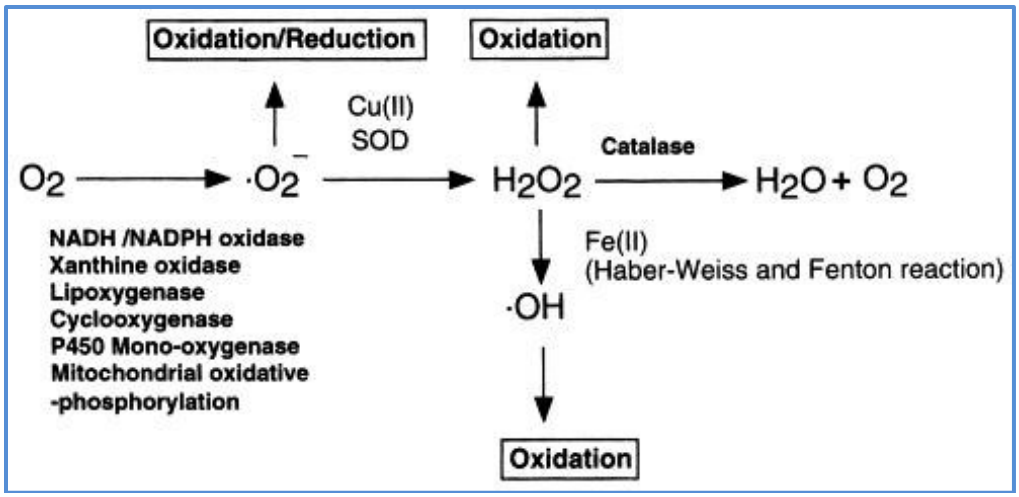

Figure 4: Generation of reactive oxygen species (adapted from [223]). Sources of reactive oxygen species (ROS) produced endogenously key metabolic pathways. Multiple enzymes may stimulate ROS production. These include NADH/NADPH oxidase, xanthine oxidase, lipoxygenases, cyclooxygenase, p450 mono-oxygenases, and the enzymes of mitochondrial oxidative phosphorylation. (·O2, superoxide anion; H2O2, hydrogen peroxide; ·OH−, hydroxyl radical; SOD, superoxide dismutase).

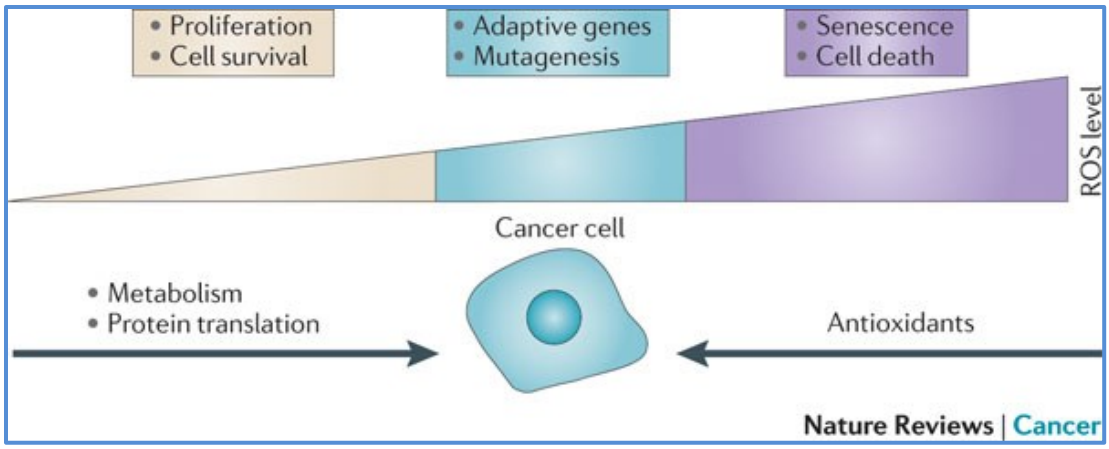

Figure 5: Relationship between ROS and cancer (adapted from [224]). The effect of ROS on cell fate depends on the level at which ROS are present. Low levels provide a beneficial effect, supporting cell proliferation and survival pathways. However, once levels of ROS become excessively high, they cause detrimental oxidative stress that can lead to cell death. To counter such oxidative stress, a cell uses antioxidants that prevent ROS from accumulating at high levels. In a cancer cell, aberrant metabolism and protein translation generate abnormally high levels of ROS. Through additional mutations and adaptations, a cancer cell exerts tight regulation of ROS and antioxidants in such a way that the cell survives and the levels of ROS are reduced to moderate levels (blue). This extraordinary control of ROS and the mechanisms designed to counter it allow the cancer cell to avoid the detrimental effects of high levels of ROS, but also increase the chance that the cell will experience additional ROS-mediated mutagenic events and stress responses that promote tumourigenesis.

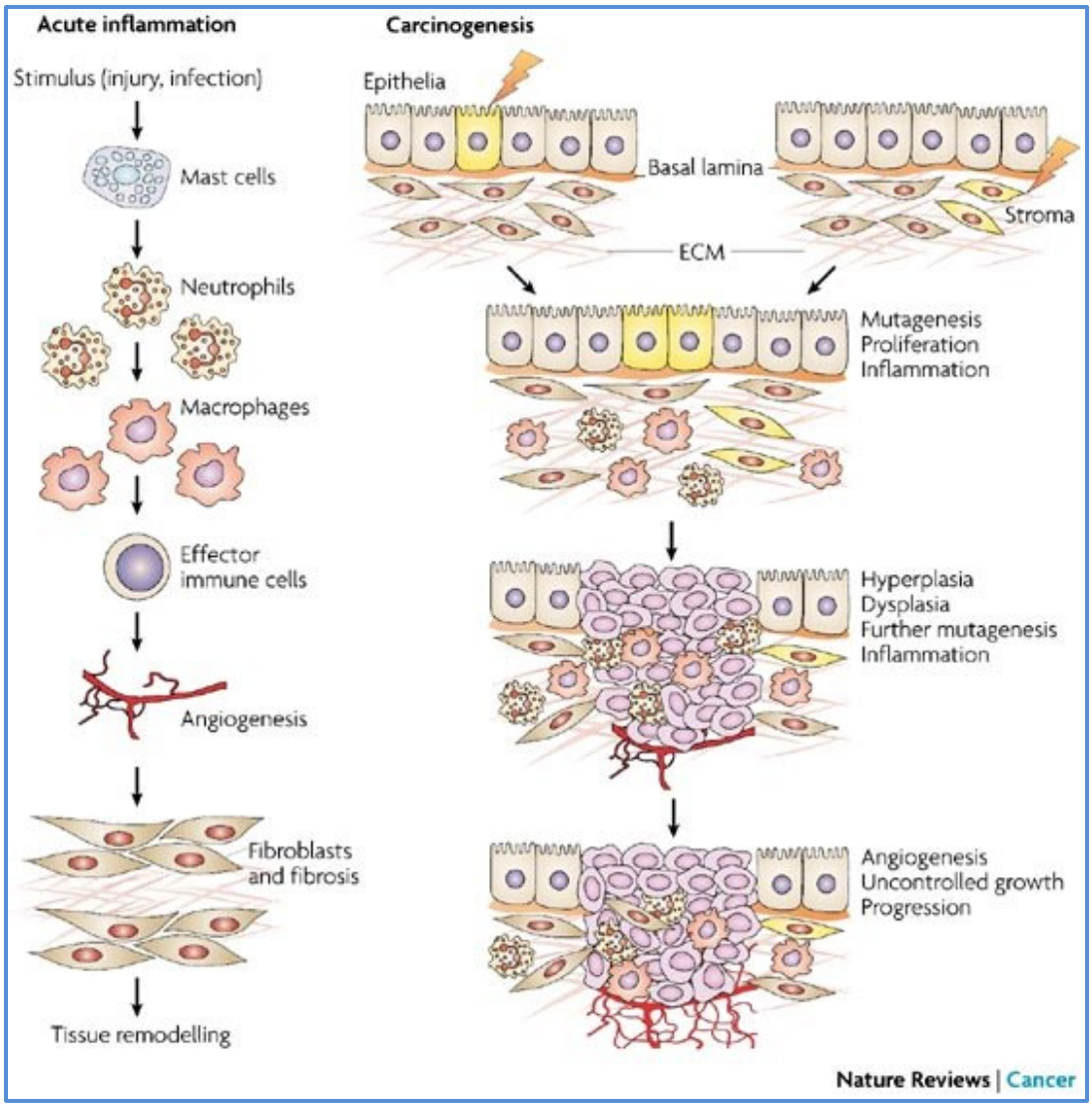

Figure 6: Inflammation in Cancer (adapted from [225]). The process of inflammation initiates a series of catabolic and anabolic processes that occur in a defined order; first, the activation of resident cells (mast cells, resident macrophages and dendritic cells) and rapid entry of granulocytes in response to injury; second, further recruitment of macrophages; third, infiltration of effector immune cells (lymphocytes) to enable specific immune responses; fourth, the recruitment and activation of mesenchymal cells such as endothelial cells and fibroblasts to form new blood vessels and a collagenous matrix; and fifth, tissue remodelling. During carcinogenesis, both epithelial and stromal elements might initially undergo alterations that promote epithelial cell proliferation and mutation. This alteration in tissue homeostasis can in turn lead to an inflammatory response, which then further promotes tumour growth through the activation of the surrounding stroma, especially neovascularization. Continued hyperplasia and dysplasia eventually lead to an invasive neoplastic state.

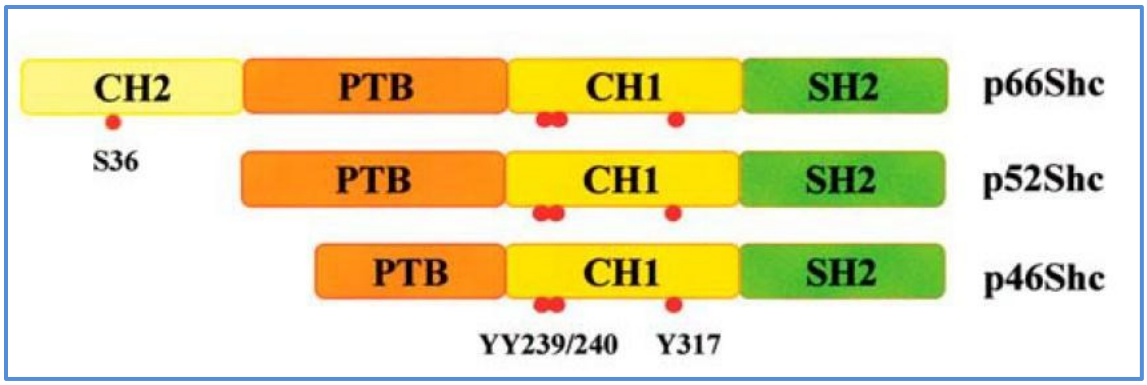

Figure 7: Schematic structure of ShcA isoforms (adapted from [194]). As they are all encoded by the same gene but produced from either different promoter usage or alternative translational initiation, the three ShcA isoforms share an overall similar structure that varies near the N-terminal region. In addition to the PTB, SH2 and CH1 domain containing the phosphorylatable tyrosines (Y239/240 and Y317) that are present in all three isoforms, $p66^{Shc}$ contains a CH2 region with a phosphorylatable serine (S36).

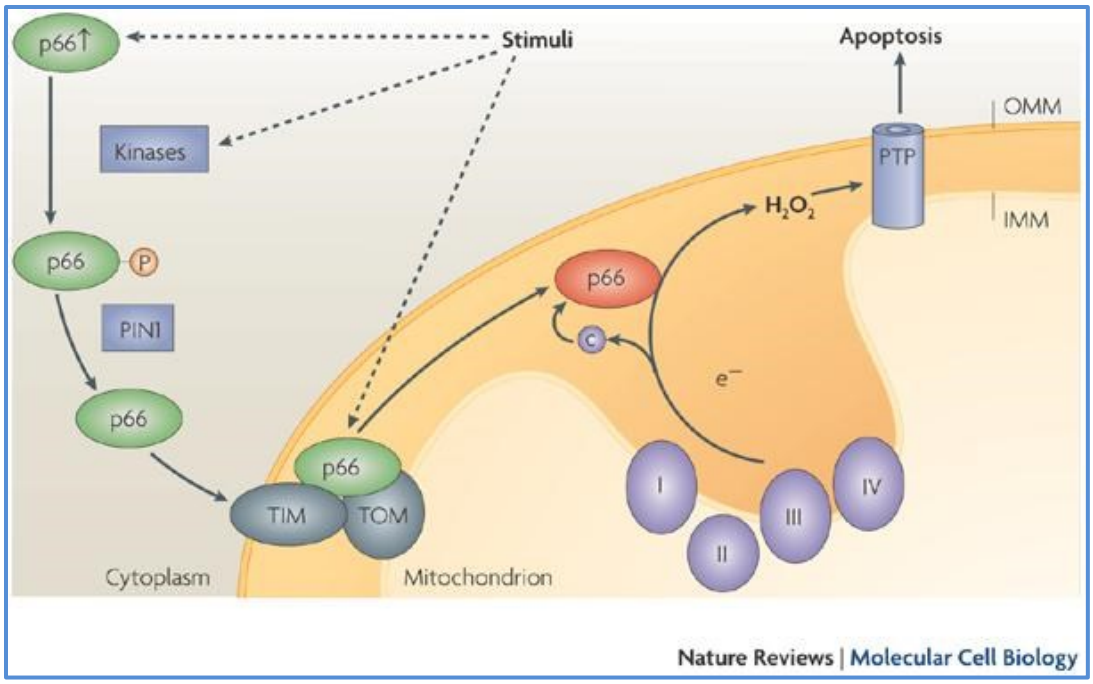

Figure 8: : p66^{Shc} generates pro-apoptotic H_2O_2 (adapted from [205]). An increase in p66^{Shc} expression as well as stimulation leading to phosphorylation of S36 allows binding and conformational transformation by Pin1. This p66^{Shc} is then translocated from the cytoplasm into the intermembrane space of the mitochondrion by the TIM-TOM mitochondrial import complex. Now an activated redox enzyme, p66^{Shc} uses electrons (e^-) from the ETC (complexes I-IV) to oxidize reduced cytochrome c (c; purple) and catalyzes the reduction of O_2 to H_2O_2. Increased H_2O_2 leads to the opening of the permeability transition pore (PTP), mitochondrial swelling and apoptosis. (IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane.)

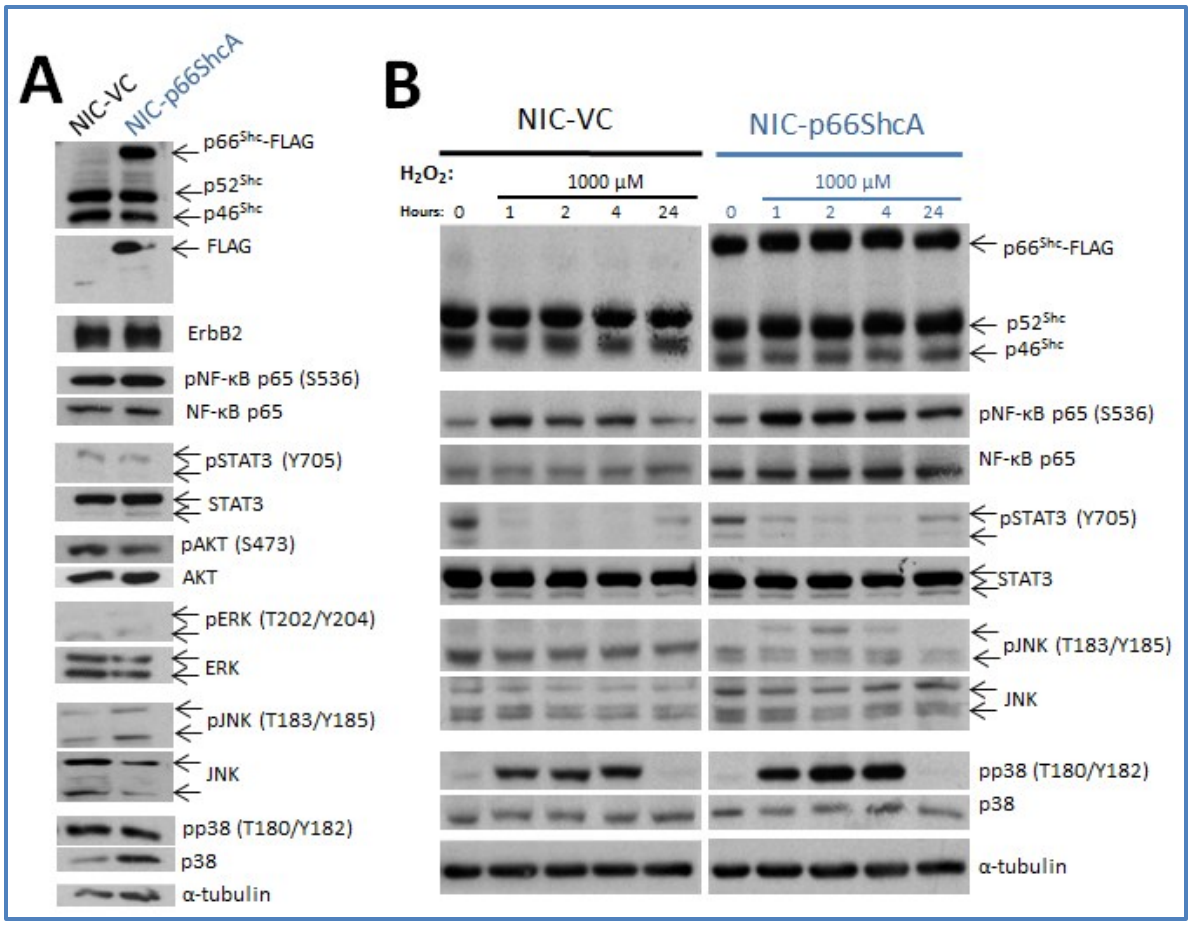

Figure 9: ***In vitro*** **analysis of murine NIC-4360 breast cancer cell line.** (**A**) Immunoblot analysis on NIC-VC and NIC-p66ShcA expressing cells using the indicated antibodies. Relative to NIC-VC, NIC-p66ShcA cells have visibly increased $p66^{Shc}$ and p38 expression, increased JNK phosphorylation and decreased Akt phosphorylation. Additionally, FLAG appears at 66 kDA only in NIC-p66ShcA cells, indicating FLAG-tagged $p66^{Shc}$ expression. (**B)** Immunoblot analysis of H_2O_2 time course assay using the indicated antibodies: cells were treated with 1000 μM H_2O_2 and subsequently lysed at the indicated time points. Relative to NIC-VC, NIC-p66ShcA cells have visibly increased phosphorylation of NF-κB p65 and JNK after H_2O_2 treatment.

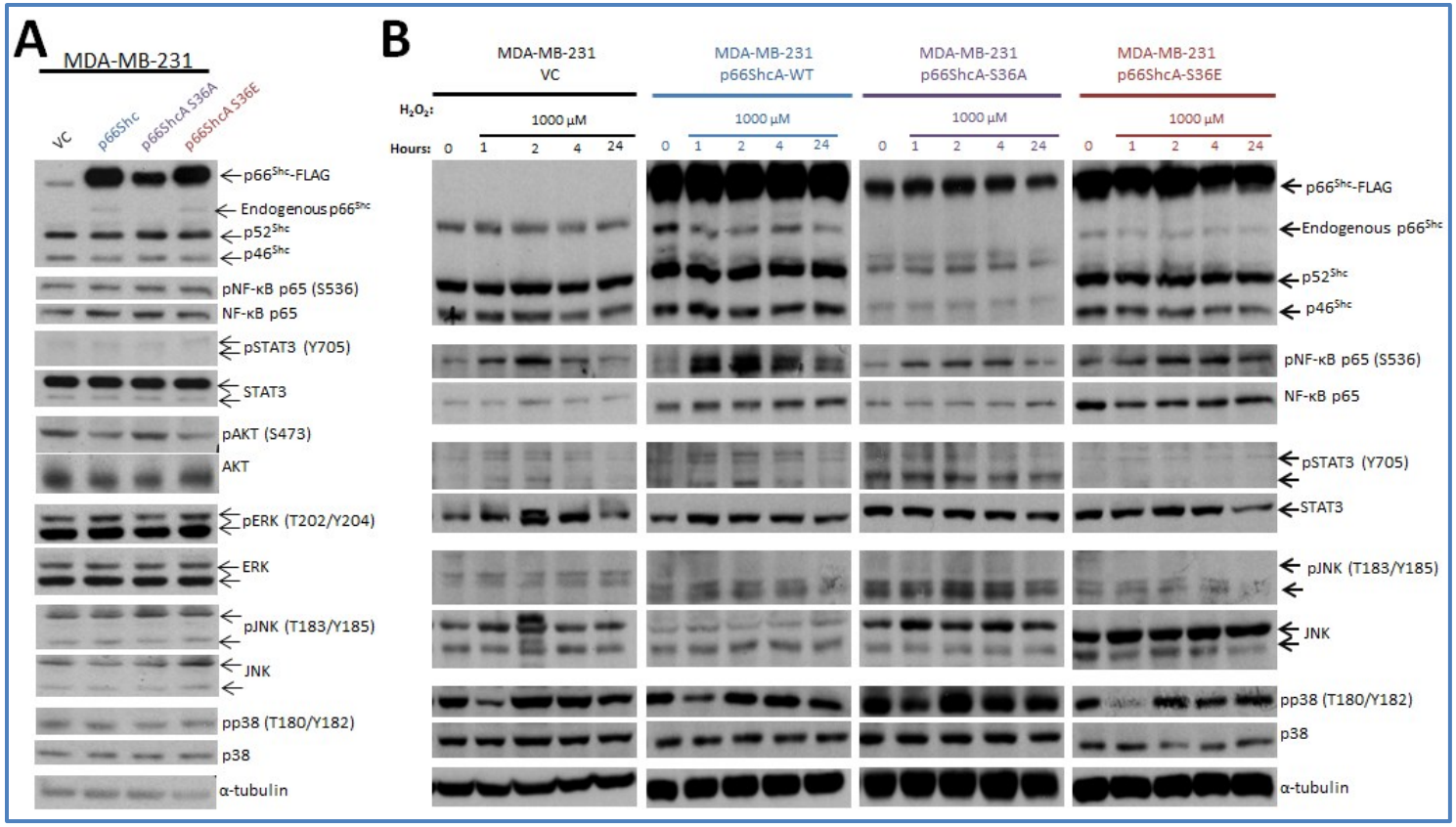

Figure 10: ***In vitro*** **analysis of human MDA-MB-231 breast cancer cell lines.** (**A**) Immunoblot analysis on VC, p66ShcA-WT, p66ShcA-S36A, p66ShcA-S36E expressing MDA-MB-231 cells using the indicated antibodies. Relative to VC and p66ShcA-S36A, both p66ShcA-WT and p66ShcA-S36E cells have visibly increased phosphorylation of the ERK upper (44 kDa) band and decreased Akt phosphorylation. $p66^{Shc}$-FLAG expression is visibly increased in p66ShcA-WT, p66ShcA-S36A and p66ShcA-S36E relative to VC, though expression is highest in p66ShcA-WT and p66ShcA-S36E. (**B**) Immunoblot analysis of H_2O_2 time course assay using the indicated antibodies: cells were treated with 1000 μM H_2O_2 and subsequently lysed at the indicated time points. Phosphorylation of NF-κB is visibly stronger and maintained for longer in the p66ShcA-WT and p66ShcA-S36E cells relative to VC and p66ShcA-S36A. At 1 hour post-treatment, p66ShcA-S36E cells display the most visible decrease in p38 phosphorylation.

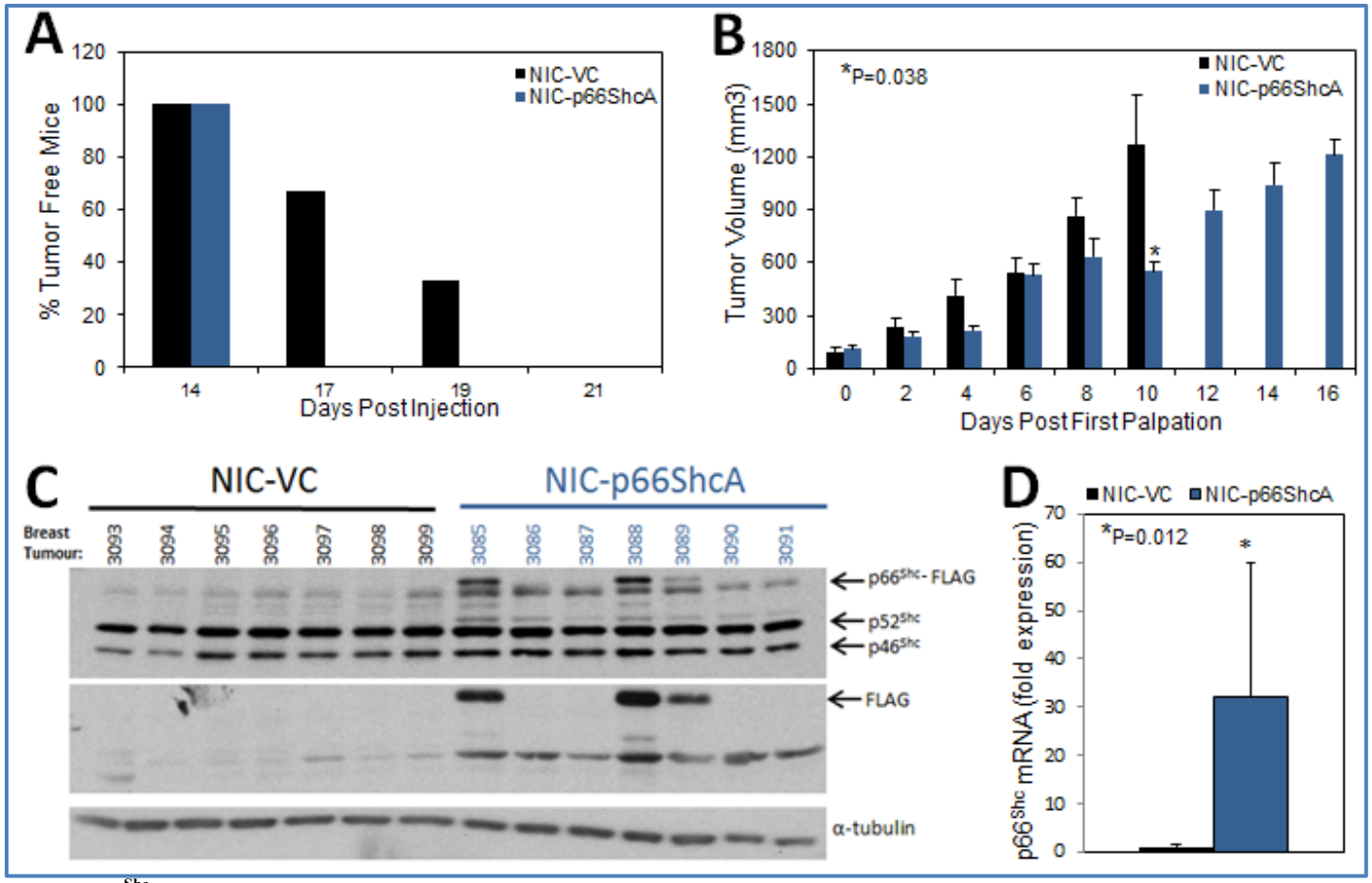

Figure 11: Effect of p66^{Shc} expression on NIC tumour outgrowth after MFP injection in SCID/Beige mice. (**A**)Percentage of tumour free mice over time. (**B**) Following first palpation, average tumour volumes determined by caliper measurement. NIC-p66ShcA tumour growth is significantly delayed starting 10-days post-palpation (*P=0.038) (n=7). (**C**) Immunoblot analysis on tumour lysates using the indicated antibodies. Expression of FLAG-tagged p66^{Shc} is visibly silenced (3086, 3087, 3090, 3091) or reduced (3089) in 5 of the 7 NIC-p66ShcA tumours. (**D**) qRT-PCR analysis of mouse p66^{Shc} mRNA levels reveals p66^{Shc} expression is significantly increased in NIC-p66ShcA tumours (P=0.012) (n=7).

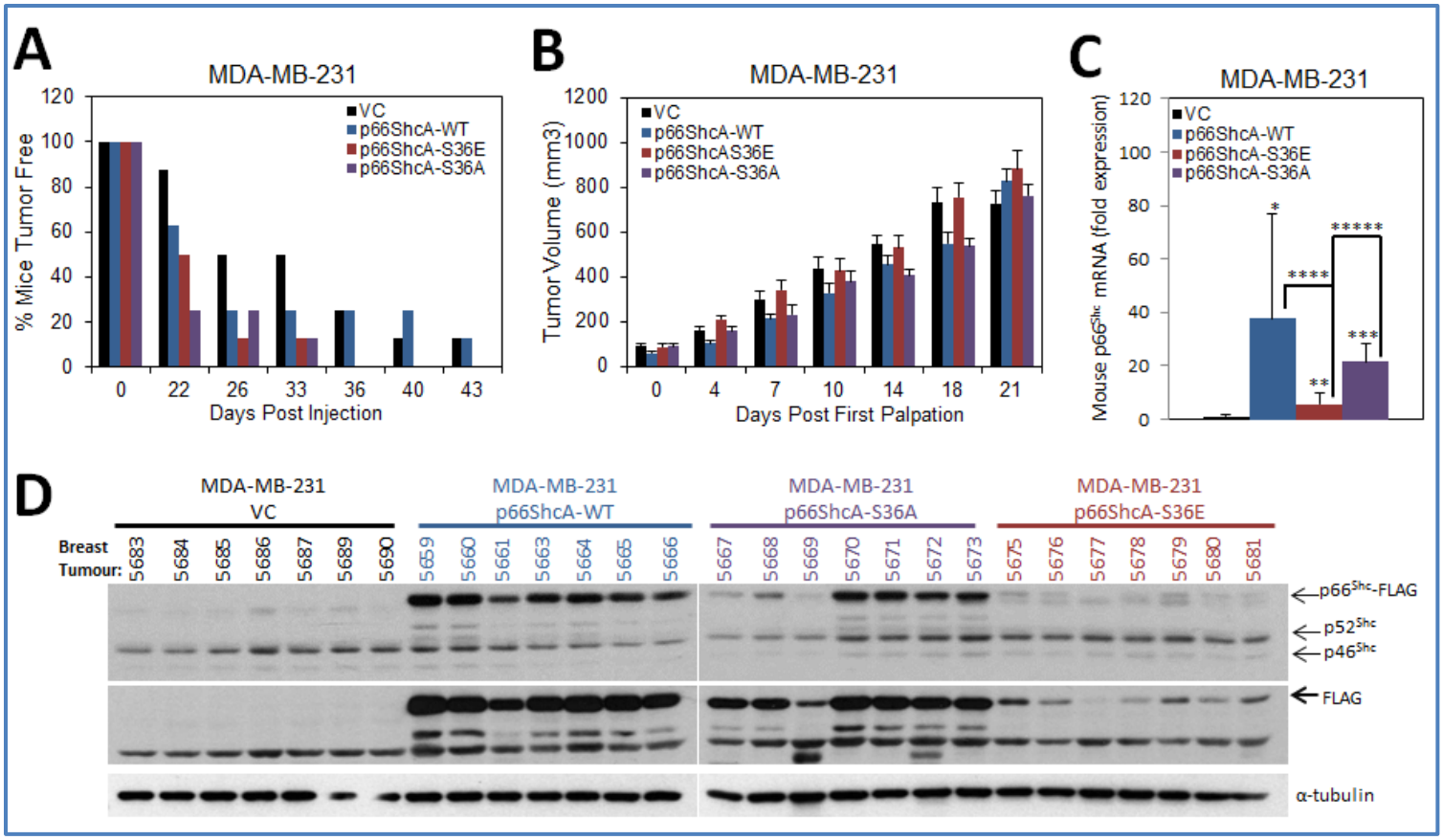

Figure 12: Effect of p66^{Shc} expression on tumour outgrowth in MDA-MB-2311 tumours after MFP injection in SCID/Beige mice. (**A**) Percentage of tumour free mice over time. (**B**) Following first palpation, average tumour volumes determined by caliper measurement (n=8). (**C**) Relative tumour p66^{Shc} mRNA levels; mouse p66^{Shc} mRNA expression is increased in p66ShcA-WT, p66ShcA-S36E and p66ShcA-S36A tumours relative to VC (*P=0.19, **P=0.005, ***P=7.88x10^{-7} respectively). Mouse p66^{Shc} mRNA expression is also increased in p66ShcA-WT and p66ShcA-S36A relative to p66ShcA-S36E (****P=0.038 and *****P=6.46x10^{-5} respectively) (n=8). (**D**) Immunoblot analysis on tumour lysates using the indicated antibodies reveals expression of FLAG-tagged p66^{Shc} is reduced in p66ShcA-S36E tumours relative to p66ShcA-WT and p66ShcA-S36A.

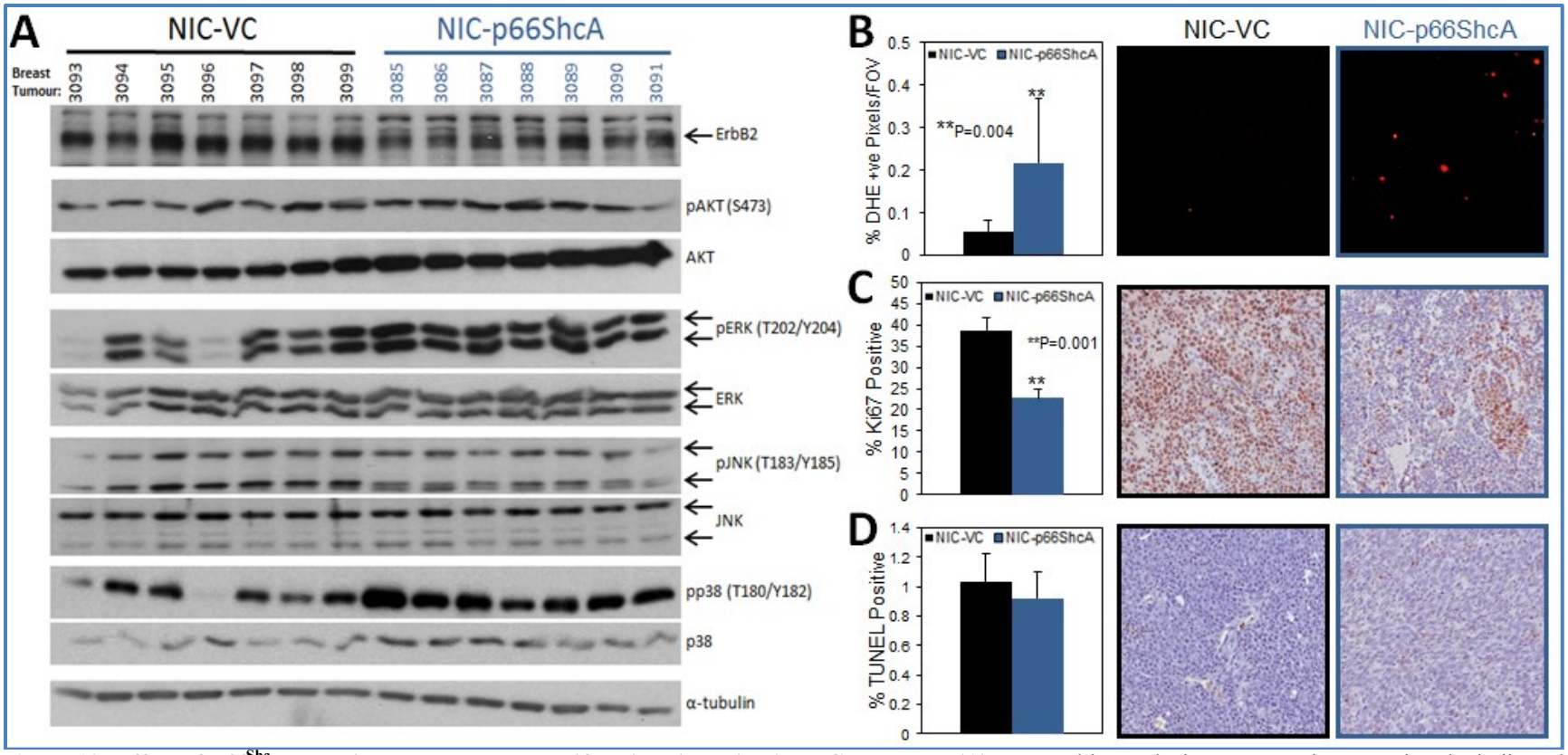

Figure 13: Effect of p66^{Shc} expression on stress and proliferation signalling in NIC tumours. (**A**) Immunoblot analysis on tumour lysates using the indicated antibodies. Relative to NIC-VC, average phosphorylation of ERK and p38 is increased. (**B**) Immunofluorescent staining of OCT-embedded tumours with DHE revealed a significant increase in ROS levels in NIC-p66ShcA tumours (P=0.004) (n=7). (**C**) Immunohistochemical staining of paraffin-embedded tumours with Ki67 antibody revealed proliferation was significantly decreased in NIC-p66ShcA tumours (**P=0.001) (n=7). (**D**) Immunohistochemical staining of paraffin-embedded tumours with TUNEL revealed no significant difference in apoptotic cells (n=7).

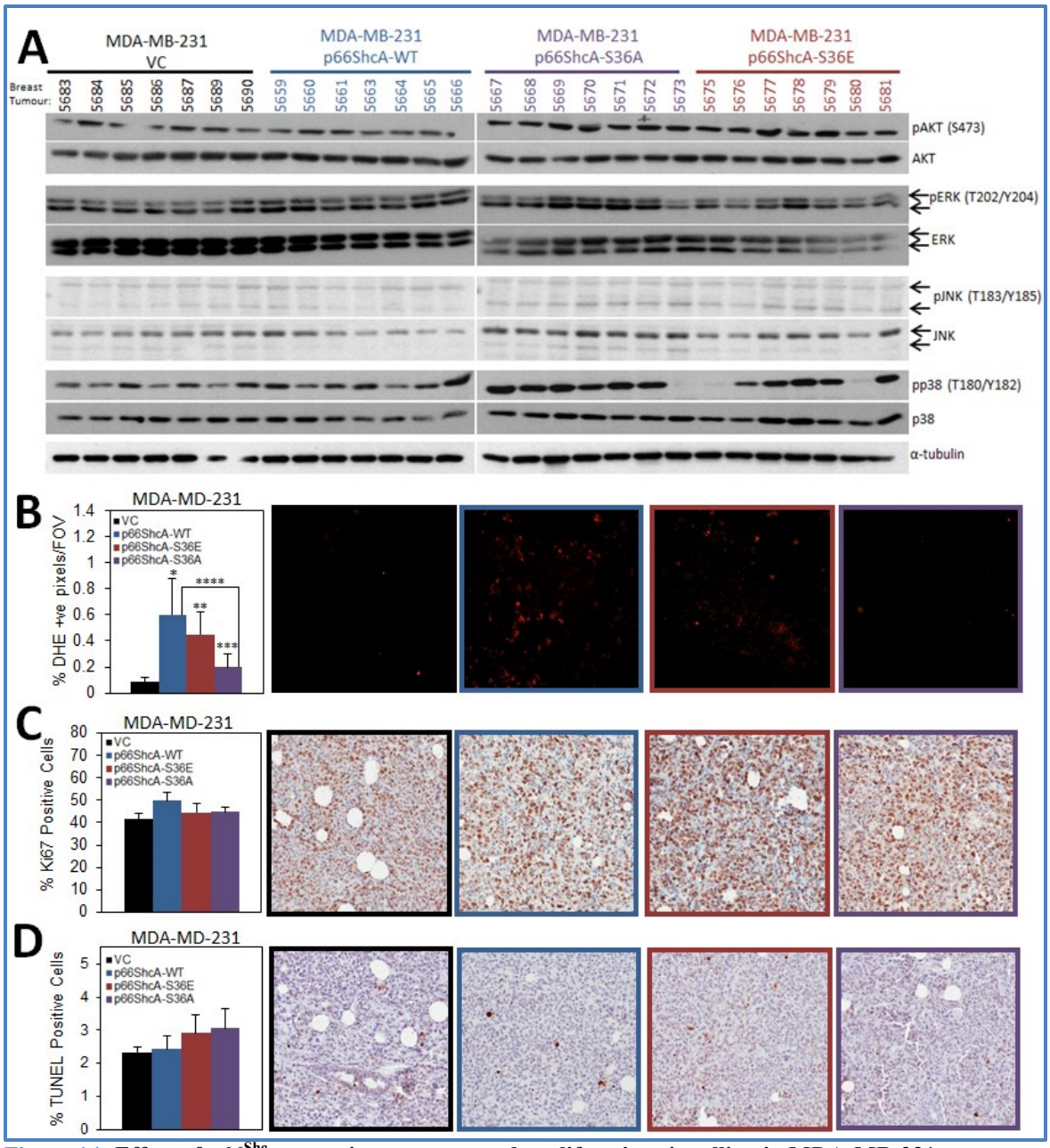

Figure 14: Effect of p66^{Shc} expression on stress and proliferation signalling in MDA-MB-231 tumours.

(A) Immunoblot analysis on tumour lysates using the indicated antibodies. (B) Immunofluorescent staining of OCT-embedded tumours with DHE revealed that ROS levels were increased in p66ShcA-WT, p66ShcA-S36E and p66ShcA-S36A tumours relative to VC (*P=9.97x10^{-7}, **P=0.004, ***P= 1.56x10^{-7} respectively). ROS levels in p66ShcA-S36A tumours were also significantly decreased relative to p66ShcA-WT (****P=9.75x10^{-5}) (n=8). (**C**) Immunohistochemical staining of paraffin-embedded tumours with Ki67 antibody revealed no significant difference in proliferative cells (n=8). (**D**) Immunohistochemical staining of paraffin-embedded tumours with TUNEL revealed no significant difference in apoptotic cells (n=8).

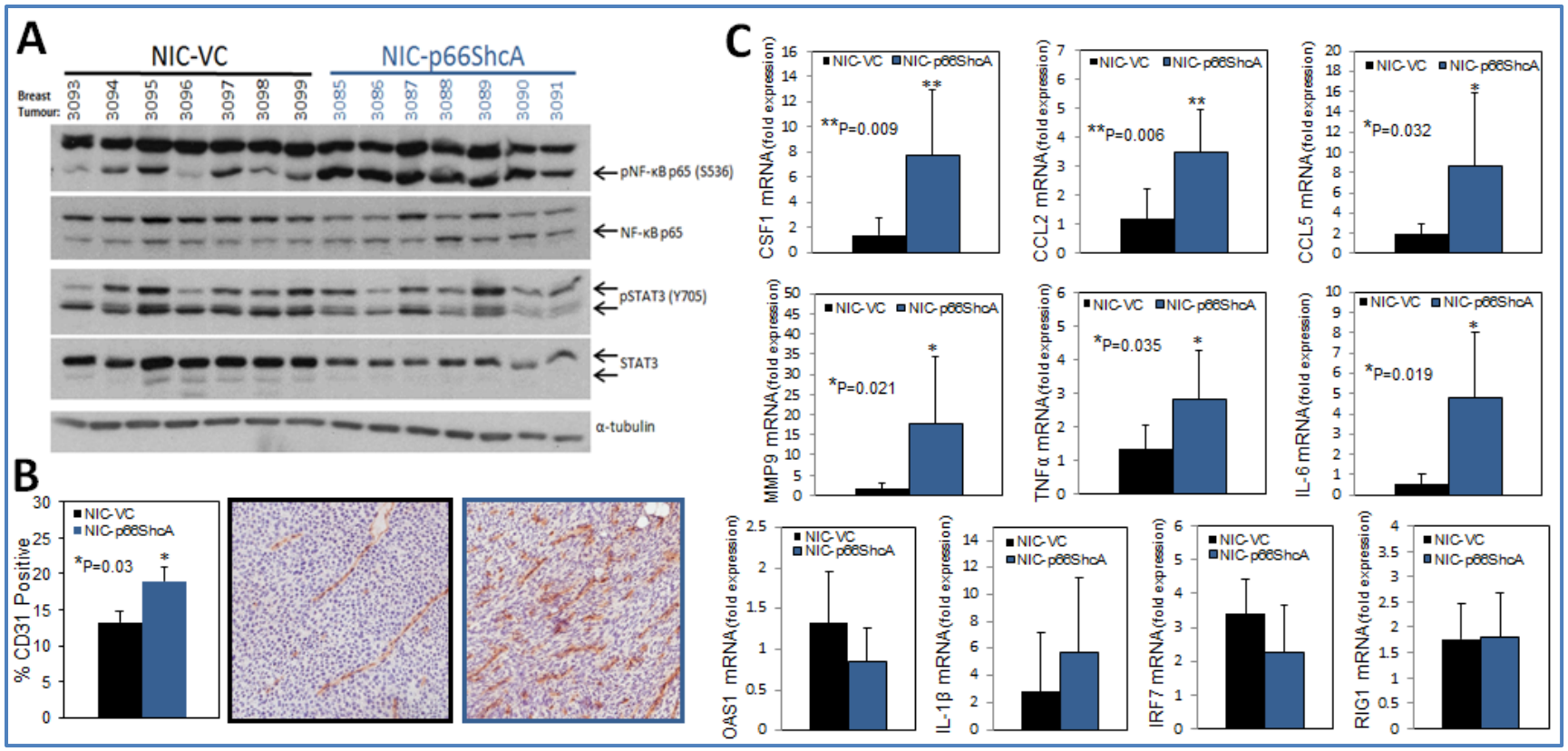

Figure 15: Effect of p66^{Shc} expression on inflammation signalling in NIC tumours. (**A**) Immunoblot analysis on tumour lysates using the indicated antibodies. Relative to NIC-VC, average phosphorylation of NF-κB p65 appears to be increased in NIC-p66ShcA tumours. (**B**) Immunohistochemical staining of OCT-embedded tumours with CD31 antibody revealed a significant increase in angiogenesis in NIC-p66ShcA tumours (*P=0.03) (n=7). (**C**) qRT-PCR analysis on mRNA levels of various inflammation-related genes (n=7). NIC-p66ShcA tumours displayed a significant increase in mRNA expression of CSF1 (**P=0.009), CCL2 (**P=0.006), CCL5 (*P=0.032), MMP9 (*P=0.021), TNFα (*P=0.035), and IL-6 (*P=0.019). No significant difference in expression observed in RIG1, IRF7, OAS1 and IL-1β.

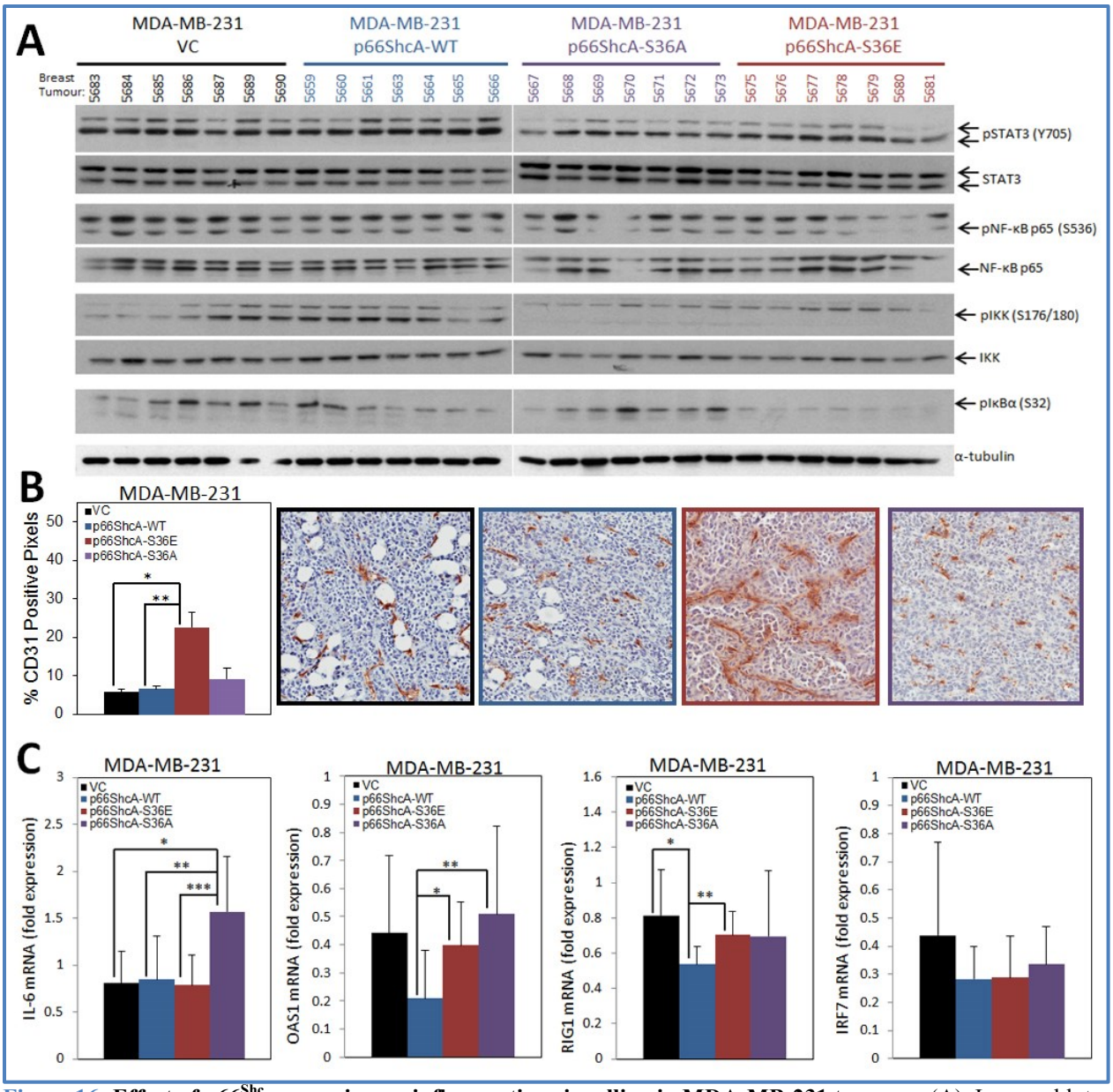

Figure 16: Effect of p66^{Shc} expression on inflammation signalling in MDA-MB-231 tumours. (**A**) Immunoblot analysis on tumour lysates using the indicated antibodies. Expression of phosphorylated IκBα is visibly reduced in p66ShcA-S36E tumour lysates. (**B**) Immunohistochemical staining of OCT-embedded tumours with CD31 antibody reveals angiogenesis is significantly increased in p66ShcA-S36E tumours relative to both VC and p66ShcA-WT (*P=0.001, **P=0.002 respectively) (n=8). (**C**) qRT-PCR analysis on mRNA levels of various inflammation-related genes (n=8). Far left panel: IL-6 mRNA levels were significantly increased in p66ShcA-S36A tumours relative to VC, p66ShcA-WT, p66ShcA-S36E (*P=0.006, **P=0.020, ***P=0.005 respectively). Middle left panel: OAS1 mRNA levels were significantly increased in p66ShcA-S36E and p66ShcA-S36A tumours relative to p66ShcA-WT (*P=0.042 and **P=0.041 respectively). Middle right panel: RIG1 mRNA levels were significantly decreased in p66ShcA-WT tumours relative to VC and p66ShcA-S36E (*P=0.020 and **P=0.018 respectively). Far right panel: no significant difference in IRF7 mRNA levels observed.

Appendix II: Tables

Table 1: Sequence of primers used in SYBR Green qRT-PCR.

Primer	Species	Sequence	Supplier
GAPDH F	Human	5'TGCACCACCAACTGCTTAGC3'	Invitrogen
GAPDH R	Human	5'GGCATGGACTGTGGTCATGAG3'	Invitrogen
IL-6 F	Human	5'GGAGACTTCCTGGTGAAAA3'	Invitrogen
IL-6 R	Human	5'ATCTGAGGTGCCCATGCTAC3'	Invitrogen
IRF7 F	Human	5'CTTCGTGATGCTGCGAGATA3'	Invitrogen
IRF7 R	Human	5'AAGCCCTTCTTGTCCCTCTC3'	Invitrogen
OAS1 F	Human	5'CCTGGTTGTCTTCCTCAGTCC3'	Invitrogen
OAS1 R	Human	5'GTGGAGAACTCGCCCTCTTT3'	Invitrogen
p66 F	Human	5'TCCGGAATGAGTCTCTGTCA3'	Invitrogen
p66 R	Human	5'GAAGGAGCACAGGGTAGTGG3'	Invitrogen
RIG1 F	Human	5'GCAGAGGCCGGCATGAC3'	Invitrogen
RIG1 R	Human	5'TGTAGGTAGGGTCCAGGGTCTTC3'	Invitrogen
IRF7 F	Mouse	5'AAGCATTTCGGTCGTAGGG3'	Invitrogen
IRF7 R	Mouse	5'GAGCCCAGCATTTTCTCTTG3'	Invitrogen
OAS1 F	Mouse	5'GGCTGAAGAGGCTGATGTGT3'	Invitrogen
OAS1 R	Mouse	5'ACCAAGCGTGTGTTCTTTCC3'	Invitrogen
p66 F	Mouse	5'TGAGTTGGGAGAGCAGAGGT3'	Invitrogen
p66 R	Mouse	5'CTCATTCCGAAGTGGGTTGT3'	Invitrogen
RIG1 F	Mouse	5'AAGCAAGGCTGATGAGGATG3'	Invitrogen
RIG1 R	Mouse	5'CTCGCAATGTTGTACCCAAG3'	Invitrogen

Table 2: Catalogue numbers of primers used in Taqman qRT-PCR reaction.

Primer	**Species**	**Catalogue Number**	**Supplier**
CSF1	Mouse	Mm00432686_m1	Invitrogen
CCL2	Mouse	Mm00441242_m1	Invitrogen
CCL5	Mouse	Mm01302427_m1	Invitrogen
GAPDH	Mouse	4352339E	Invitrogen
IL-1β	Mouse	Mm00434228_m1	Invitrogen
IL-6	Mouse	Mm00446190_m1	Invitrogen
MMP-9	Mouse	Mm00442991_m1	Invitrogen
TNF-α	Mouse	Mm00443260_g1	Invitrogen

References

1. Statistics., C.C.S.s.S.C.o.C., *Canadian Cancer Statistics 2012*, C.C. Society, Editor 2012: Toronto, ON.
2. Weigelt, B., J.L. Peterse, and L.J. van't Veer, *Breast cancer metastasis: markers and models.* Nat Rev Cancer, 2005. **5**(8): p. 591-602.
3. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-752.
4. Sørlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proceedings of the National Academy of Sciences, 2001. **98**(19): p. 10869-10874.
5. Abe, J.-i., et al., *Big Mitogen-activated Protein Kinase 1 (BMK1) Is a Redox-sensitive Kinase.* Journal of Biological Chemistry, 1996. **271**(28): p. 16586-16590.
6. Hu, Z., et al., *The molecular portraits of breast tumors are conserved across microarray platforms.* BMC Genomics, 2006. **7**: p. 96.
7. Bissonauth, V., B. Shatenstein, and P. Ghadirian, *Nutrition and breast cancer among sporadic cases and gene mutation carriers: An overview.* Cancer detection and prevention, 2008. **32**(1): p. 52-64.
8. Mincey, B.A., *Genetics and the management of women at high risk for breast cancer.* Oncologist, 2003. **8**(5): p. 466-73.
9. Jönsson, G., et al., *The Retinoblastoma Gene Undergoes Rearrangements in BRCA1-Deficient Basal-like Breast Cancer.* Cancer Research, 2012. **72**(16): p. 4028-4036.
10. Place, A., S. Jin Huh, and K. Polyak, *The microenvironment in breast cancer progression: biology and implications for treatment.* Breast Cancer Research, 2011. **13**(6): p. 227.
11. Smalley, M. and A. Ashworth, *Stem cells and breast cancer: A field in transit.* Nat Rev Cancer, 2003. **3**(11): p. 832-844.
12. Rakha, E.A., et al., *Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation.* The Journal of Pathology, 2006. **208**(4): p. 495-506.
13. Weigelt, B., F.C. Geyer, and J.S. Reis-Filho, *Histological types of breast cancer: How special are they?* Molecular Oncology, 2010. **4**(3): p. 192-208.
14. Wellings, S.R. and H.M. Jensen, *On the origin and progression of ductal carcinoma in the human breast.* J Natl Cancer Inst, 1973. **50**(5): p. 1111-8.
15. Bombonati, A. and D.C. Sgroi, *The molecular pathology of breast cancer progression.* The Journal of Pathology, 2011. **223**(2): p. 308-318.
16. Polyak, K., *Breast cancer: origins and evolution.* J Clin Invest, 2007. **117**: p. 3155 - 3163.
17. Blume-Jensen, P. and T. Hunter, *Oncogenic kinase signalling.* Nature, 2001. **411**(6835): p. 355-65.
18. Lemmon, M.A. and J. Schlessinger, *Cell Signaling by Receptor Tyrosine Kinases.* Cell, 2010. **141**(7): p. 1117-1134.
19. Ullrich, A. and J. Schlessinger, *Signal transduction by receptors with tyrosine kinase activity.* Cell, 1990. **61**(2): p. 203-212.

20. Schlessinger, J. and M.A. Lemmon, *SH2 and PTB Domains in Tyrosine Kinase Signaling.* Sci. STKE, 2003. **2003**(191): p. re12-.
21. Pawson, T., *Specificity in Signal Transduction: From Phosphotyrosine-SH2 Domain Interactions to Complex Cellular Systems.* Cell, 2004. **116**(2): p. 191-203.
22. Schlessinger, J., *Cell Signaling by Receptor Tyrosine Kinases.* Cell, 2000. **103**(2): p. 211-225.
23. Pawson, T., *Protein modules and signalling networks.* Nature, 1995. **373**(6515): p. 573-580.
24. Engelman, J.A., *Targeting PI3K signalling in cancer: opportunities, challenges and limitations.* Nat Rev Cancer, 2009. **9**(8): p. 550-562.
25. Morgensztern, D. and H.L. McLeod, *PI3K/Akt/mTOR pathway as a target for cancer therapy.* Anti-Cancer Drugs, 2005. **16**(8): p. 797-803.
26. Downward, J., *Targeting RAS signalling pathways in cancer therapy.* Nat Rev Cancer, 2003. **3**(1): p. 11-22.
27. Kiyatkin, A., et al., *Scaffolding Protein Grb2-associated Binder 1 Sustains Epidermal Growth Factor-induced Mitogenic and Survival Signaling by Multiple Positive Feedback Loops.* Journal of Biological Chemistry, 2006. **281**(29): p. 19925-19938.
28. Sorkin, A. and L.K. Goh, *Endocytosis and intracellular trafficking of ErbBs.* Exp Cell Res, 2008. **314**(17): p. 3093-106.
29. von Zastrow, M. and A. Sorkin, *Signaling on the endocytic pathway.* Current Opinion in Cell Biology, 2007. **19**(4): p. 436-445.
30. Zwang, Y. and Y. Yarden, *Systems Biology of Growth Factor-Induced Receptor Endocytosis.* Traffic, 2009. **10**(4): p. 349-363.
31. Forbes, S.A., et al., *COSMIC (the Catalogue of Somatic Mutations in Cancer): a resource to investigate acquired mutations in human cancer.* Nucleic acids research, 2010. **38**(Database issue): p. D652-7.
32. Shawver, L.K., D. Slamon, and A. Ullrich, *Smart drugs: Tyrosine kinase inhibitors in cancer therapy.* Cancer Cell, 2002. **1**(2): p. 117-123.
33. Reichert, J.M. and V.E. Valge-Archer, *Development trends for monoclonal antibody cancer therapeutics.* Nat Rev Drug Discov, 2007. **6**(5): p. 349-356.
34. Engelman, J.A. and J. Settleman, *Acquired resistance to tyrosine kinase inhibitors during cancer therapy.* Current Opinion in Genetics & Development, 2008. **18**(1): p. 73-79.
35. Sergina, N.V. and M.M. Moasser, *The HER family and cancer: emerging molecular mechanisms and therapeutic targets.* Trends in Molecular Medicine, 2007. **13**(12): p. 527-534.
36. Herbst, R.S., *Review of epidermal growth factor receptor biology.* International Journal of Radiation Oncology*Biology*Physics, 2004. **59**(2, Supplement): p. S21-S26.
37. Salomon, D.S., et al., *Epidermal growth factor-related peptides and their receptors in human malignancies.* Critical Reviews in Oncology/Hematology, 1995. **19**(3): p. 183-232.
38. Britsch, S., *The neuregulin-I/ErbB signaling system in development and disease.* Adv Anat Embryol Cell Biol, 2007. **190**: p. 1-65.

39. Zhang, H., et al., *ErbB receptors: from oncogenes to targeted cancer therapies.* The Journal of Clinical Investigation, 2007. **117**(8): p. 2051-2058.
40. Oda, K., et al., *A comprehensive pathway map of epidermal growth factor receptor signaling.* Mol Syst Biol, 2005. **1**.
41. Roy, V. and E.A. Perez, *Beyond Trastuzumab: Small Molecule Tyrosine Kinase Inhibitors in HER-2–Positive Breast Cancer.* Oncologist, 2009. **14**(11): p. 1061-1069.
42. Graus-Porta, D., et al., *ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling.* EMBO J, 1997. **16**(7): p. 1647-1655.
43. Baselga, J. and S.M. Swain, *Novel anticancer targets: revisiting ERBB2 and discovering ERBB3.* Nat Rev Cancer, 2009. **9**(7): p. 463-475.
44. Owens, M.A., B.C. Horten, and M.M. Da Silva, *HER2 Amplification Ratios by Fluorescence In Situ Hybridization and Correlation with Immunohistochemistry in a Cohort of 6556 Breast Cancer Tissues.* Clinical Breast Cancer, 2004. **5**(1): p. 63-69.
45. Yaziji H, G.L.C.B.T.S. and et al., *HEr-2 testing in breast cancer using parallel tissue-based methods.* JAMA, 2004. **291**(16): p. 1972-1977.
46. Vermeij, J., et al., *Genomic activation of the EGFR and HER2-neu genes in a significant proportion of invasive epithelial ovarian cancers.* BMC Cancer, 2008. **8**: p. 3.
47. Slamon, D.J., et al., *Studies of the HER-2/neu Proto-Oncogene in Human Breast and Ovarian Cancer.* Science, 1989. **244**(4905): p. 707-712.
48. Slamon, D.J., et al., *Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene.* Science, 1987. **235**(4785): p. 177-182.
49. Ursini-Siegel, J., et al., *Insights from transgenic mouse models of ERBB2-induced breast cancer.* Nat Rev Cancer, 2007. **7**(5): p. 389-97.
50. Puglisi, F., et al., *Overcoming Treatment Resistance in HER2-Positive Breast Cancer.* Drugs, 2012. **72**(9): p. 1175-93.
51. Dawood, S., et al., *Prognosis of Women With Metastatic Breast Cancer by HER2 Status and Trastuzumab Treatment: An Institutional-Based Review.* Journal of Clinical Oncology, 2010. **28**(1): p. 92-98.
52. Danese, M.D., et al., *Estimating recurrences prevented from using trastuzumab in HER-2/neu–positive adjuvant breast cancer in the United States.* Cancer, 2010. **116**(24): p. 5575-5583.
53. Pan, J.S., M.Z. Hong, and J.L. Ren, *Reactive oxygen species: a double-edged sword in oncogenesis.* World J Gastroenterol, 2009. **15**(14): p. 1702-7.
54. Weinberg, F. and N. Chandel, *Reactive oxygen species-dependent signaling regulates cancer.* Cellular and Molecular Life Sciences, 2009. **66**(23): p. 3663-3673.
55. Shertzer, H.G., et al., *TCDD decreases ATP levels and increases reactive oxygen production through changes in mitochondrial F0F1-ATP synthase and ubiquinone.* Toxicology and Applied Pharmacology, 2006. **217**(3): p. 363-374.
56. Babior, B.M., *The NADPH Oxidase of Endothelial Cells.* IUBMB Life, 2000. **50**(4-5): p. 267-269.

57. Zulueta, J.J., et al., *Release of hydrogen peroxide in response to hypoxia-reoxygenation: role of an NAD(P)H oxidase-like enzyme in endothelial cell plasma membrane.* American Journal of Respiratory Cell and Molecular Biology, 1995. **12**(1): p. 41-9.
58. Bayraktutan, U., L. Blayney, and A.M. Shah, *Molecular Characterization and Localization of the NAD(P)H Oxidase Components gp91-phox and p22-phox in Endothelial Cells.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2000. **20**(8): p. 1903-1911.
59. Bayraktutan, U., et al., *Expression of a functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells.* Cardiovascular Research, 1998. **38**(1): p. 256-262.
60. Frey, R.S., M. Ushio-Fukai, and A.B. Malik, *NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology.* Antioxid Redox Signal, 2009. **11**(4): p. 791-810.
61. Griendling, K.K., D. Sorescu, and M. Ushio-Fukai, *NAD(P)H Oxidase: Role in Cardiovascular Biology and Disease.* Circulation Research, 2000. **86**(5): p. 494-501.
62. Burke, K. and H. Wei, *Synergistic damage by UVA radiation and pollutants.* Toxicology and Industrial Health, 2009. **25**(4-5): p. 219-224.
63. Trachootham, D., et al., *Redox regulation of cell survival.* Antioxid Redox Signal, 2008. **10**(8): p. 1343-74.
64. Stadtman, E.R., *Role of oxidant species in aging.* Curr Med Chem, 2004. **11**(9): p. 1105-12.
65. Haghdoost, S., et al., *Extracellular 8-oxo-dG as a sensitive parameter for oxidative stress in vivo and in vitro.* Free Radical Research, 2005. **39**(2): p. 153-162.
66. Dröge, W., *Free Radicals in the Physiological Control of Cell Function.* Physiological Reviews, 2002. **82**(1): p. 47-95.
67. Bae, Y.S., et al., *Epidermal Growth Factor (EGF)-induced Generation of Hydrogen Peroxide.* Journal of Biological Chemistry, 1997. **272**(1): p. 217-221.
68. Czech, M.P., J.C. Lawrence, Jr., and W.S. Lynn, *Evidence for the involvement of sulfhydryl oxidation in the regulation of fat cell hexose transport by insulin.* Proc Natl Acad Sci U S A, 1974. **71**(10): p. 4173-7.
69. Gopalakrishna, R. and W.B. Anderson, *Ca2+- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain.* Proceedings of the National Academy of Sciences, 1989. **86**(17): p. 6758-6762.
70. Baas, A.S. and B.C. Berk, *Differential Activation of Mitogen-Activated Protein Kinases by H2O2 and O2− in Vascular Smooth Muscle Cells.* Circulation Research, 1995. **77**(1): p. 29-36.
71. Manna, S.K., et al., *Overexpression of Manganese Superoxide Dismutase Suppresses Tumor Necrosis Factor-induced Apoptosis and Activation of Nuclear Transcription Factor-κB and Activated Protein-1.* Journal of Biological Chemistry, 1998. **273**(21): p. 13245-13254.
72. Doan, T.N., et al., *Hydrogen peroxide activates agonist-sensitive Ca(2+)-flux pathways in canine venous endothelial cells.* Biochem J, 1994. **297 (Pt 1)**: p. 209-15.

73. Jackson, A.L. and L.A. Loeb, *The contribution of endogenous sources of DNA damage to the multiple mutations in cancer.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2001. **477**(1–2): p. 7-21.
74. Santiago, C., et al., *Prolonged Chronic Inflammation Progresses to Dysplasia in a Novel Rat Model of Colitis-Associated Colon Cancer.* Cancer Research, 2007. **67**(22): p. 10766-10773.
75. Yamamoto, S., et al., *TRPM2-mediated Ca2+ influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration.* Nat Med, 2008. **14**(7): p. 738-747.
76. Meloche, S. and J. Pouyssegur, *The ERK1//2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition.* Oncogene, 0000. **26**(22): p. 3227-3239.
77. Yamamoto, T., et al., *Continuous ERK Activation Downregulates Antiproliferative Genes throughout G1 Phase to Allow Cell-Cycle Progression.* Current biology : CB, 2006. **16**(12): p. 1171-1182.
78. Meloche, S., *Cell cycle reentry of mammalian fibroblasts is accompanied by the sustained activation of P44mapk and P42mapk isoforms in the G1 phase and their inactivation at the G1/s transition.* Journal of Cellular Physiology, 1995. **163**(3): p. 577-588.
79. Roovers, K. and R.K. Assoian, *Integrating the MAP kinase signal into the G1 phase cell cycle machinery.* BioEssays, 2000. **22**(9): p. 818-826.
80. Lee, K. and W.J. Esselman, *Inhibition of PTPs by H2O2 regulates the activation of distinct MAPK pathways.* Free Radical Biology and Medicine, 2002. **33**(8): p. 1121-1132.
81. Watton, S.J. and J. Downward, *Akt/PKB localisation and 3ard, d, , 2002. ctivation of distinct MAPK pathways.tivation of P44mapk and P42mapk isoion.* Current Biology, 1999. **9**(8): p. 433-436.
82. Ushio-Fukai, M., et al., *Reactive Oxygen Species Mediate the Activation of Akt/Protein Kinase B by Angiotensin II in Vascular Smooth Muscle Cells.* Journal of Biological Chemistry, 1999. **274**(32): p. 22699-22704.
83. Lee, S.-R., et al., *Reversible Inactivation of the Tumor Suppressor PTEN by H2O2.* Journal of Biological Chemistry, 2002. **277**(23): p. 20336-20342.
84. Sablina, A.A., et al., *The antioxidant function of the p53 tumor suppressor.* Nat Med, 2005. **11**(12): p. 1306-1313.
85. Wagner, E.F. and A.R. Nebreda, *Signal integration by JNK and p38 MAPK pathways in cancer development.* Nat Rev Cancer, 2009. **9**(8): p. 537-549.
86. Ichijo, H., *From receptors to stress-activated MAP kinases.* Oncogene, 1999. **18**(45): p. 6087-93.
87. Kyriakis, J.M. and J. Avruch, *Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation.* Physiological Reviews, 2001. **81**(2): p. 807-869.
88. Leppa, S. and D. Bohmann, *Diverse functions of JNK signaling and c-Jun in stress response and apoptosis.* Oncogene, 1999. **18**(45): p. 6158-62.
89. Roy, P.K., et al., *Role of the JNK signal transduction pathway in inflammatory bowel disease.* World J Gastroenterol, 2008. **14**(2): p. 200-2.

90. Fuchs, S.Y., et al., *Phosphorylation-dependent targeting of c-Jun ubiquitination by Jun N-kinase.* Oncogene, 1996. **13**(7): p. 1531-5.
91. Tournier, C., et al., *Requirement of JNK for Stress- Induced Activation of the Cytochrome c-Mediated Death Pathway.* Science, 2000. **288**(5467): p. 870-874.
92. Weston, C.R. and R.J. Davis, *The JNK signal transduction pathway.* Current Opinion in Cell Biology, 2007. **19**(2): p. 142-149.
93. Karin, M. and E. Gallagher, *From JNK to Pay Dirt: Jun Kinases, their Biochemistry, Physiology and Clinical Importance.* IUBMB Life, 2005. **57**(4-5): p. 283-295.
94. Rouse, J., et al., *A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins.* Cell, 1994. **78**(6): p. 1027-1037.
95. Lee, J.C., et al., *A protein kinase involved in the regulation of inflammatory cytokine biosynthesis.* Nature, 1994. **372**(6508): p. 739-746.
96. Raingeaud, J., et al., *Pro-inflammatory Cytokines and Environmental Stress Cause p38 Mitogen-activated Protein Kinase Activation by Dual Phosphorylation on Tyrosine and Threonine.* Journal of Biological Chemistry, 1995. **270**(13): p. 7420-7426.
97. Han, J. and P. Sun, *The pathways to tumor suppression via route p38.* Trends in Biochemical Sciences, 2007. **32**(8): p. 364-371.
98. Timoshenko, A.V., et al., *COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer.* Br J Cancer, 2006. **94**(8): p. 1154-1163.
99. Kumar, S., J. Boehm, and J.C. Lee, *p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases.* Nat Rev Drug Discov, 2003. **2**(9): p. 717-726.
100. Karin, M., *Nuclear factor-[kappa]B in cancer development and progression.* Nature, 2006. **441**: p. 431-436.
101. Dolado, I., et al., *p38α MAP Kinase as a Sensor of Reactive Oxygen Species in Tumorigenesis.* Cancer Cell, 2007. **11**(2): p. 191-205.
102. Ichijo, H., et al., *Induction of Apoptosis by ASK1, a Mammalian MAPKKK That Activates SAPK/JNK and p38 Signaling Pathways.* Science, 1997. **275**(5296): p. 90-94.
103. Nishitoh, H., et al., *ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats.* Genes & Development, 2002. **16**(11): p. 1345-1355.
104. Tobiume, K., et al., *ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis.* EMBO Rep, 2001. **2**(3): p. 222-228.
105. Lin, B., et al., *Conversion of Bcl-2 from Protector to Killer by Interaction with Nuclear Orphan Receptor Nur77/TR3.* Cell, 2004. **116**(4): p. 527-540.
106. Noguchi, T., et al., *Requirement of Reactive Oxygen Species-dependent Activation of ASK1-p38 MAPK Pathway for Extracellular ATP-induced Apoptosis in Macrophage.* Journal of Biological Chemistry, 2008. **283**(12): p. 7657-7665.
107. Kuo, P.-L., C.-Y. Chen, and Y.-L. Hsu, *Isoobtusilactone A Induces Cell Cycle Arrest and Apoptosis through Reactive Oxygen Species/Apoptosis Signal-Regulating Kinase 1 Signaling Pathway in Human Breast Cancer Cells.* Cancer Research, 2007. **67**(15): p. 7406-7420.

108. de Keizer, P.L.J., et al., *Activation of Forkhead Box O Transcription Factors by Oncogenic BRAF Promotes p21cip1-Dependent Senescence.* Cancer Research, 2010. **70**(21): p. 8526-8536.
109. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* Lancet, 2001. **357**: p. 539-545.
110. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-444.
111. Wang, F., et al., *Current and potential inflammation targeted therapies in head and neck cancer.* Current Opinion in Pharmacology, 2009. **9**(4): p. 389-395.
112. Koehne, C.H. and R.N. Dubois, *COX-2 inhibition and colorectal cancer.* Semin. Oncol., 2004. **31**: p. 12-21.
113. Flossmann, E. and P.M. Rothwell, *Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies.* Lancet, 2007. **369**: p. 1603-1613.
114. Chan, A.T., S. Ogino, and C.S. Fuchs, *Aspirin and the risk of colorectal cancer in relation to the expression of COX-2.* N. Engl. J. Med., 2007. **356**: p. 2131-2142.
115. Kulbe, H., *The inflammatory cytokine tumor necrosis factor-[alpha] generates an autocrine tumor-promoting network in epithelial ovarian cancer cells.* Cancer Research, 2007. **67**: p. 585-592.
116. Chechlinska, M., M. Kowalewska, and R. Nowak, *Systemic inflammation as a confounding factor in cancer biomarker discovery and validation.* Nat Rev Cancer, 2010. **10**(1): p. 2-3.
117. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nat Rev Cancer, 2003. **3**(4): p. 276-285.
118. Colotta, F., et al., *Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.* Carcinogenesis, 2009. **30**(7): p. 1073-1081.
119. COOKE, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* The FASEB Journal, 2003. **17**(10): p. 1195-1214.
120. Federico, A., et al., *Chronic inflammation and oxidative stress in human carcinogenesis.* International Journal of Cancer, 2007. **121**(11): p. 2381-2386.
121. Nishikawa, M., *Reactive oxygen species in tumor metastasis.* Cancer Letters, 2008. **266**(1): p. 53-59.
122. Goetz, M.E. and A. Luch, *Reactive species: A cell damaging rout assisting to chemical carcinogens.* Cancer Letters, 2008. **266**(1): p. 73-83.
123. Maynard, S., et al., *Base excision repair of oxidative DNA damage and association with cancer and aging.* Carcinogenesis, 2009. **30**(1): p. 2-10.
124. Maeda, H. and T. Akaike, *Nitric oxide and oxygen radicals in infection, inflammation, and cancer.* Biochemistry (Mosc), 1998. **63**(7): p. 854-65.
125. Güngör, N., et al., *Activated neutrophils inhibit nucleotide excision repair in human pulmonary epithelial cells: role of myeloperoxidase.* The FASEB Journal, 2007. **21**(10): p. 2359-2367.
126. Shchors, K., et al., *The Myc-dependent angiogenic switch in tumors is mediated by interleukin 1β.* Genes & Development, 2006. **20**(18): p. 2527-2538.
127. Jones, P.A. and S.B. Baylin, *The Epigenomics of Cancer.* Cell, 2007. **128**(4): p. 683-692.

128. Feinberg, A.P., *EPigenetics at the epicenter of modern medicine.* JAMA, 2008. **299**(11): p. 1345-1350.
129. Franco, R., et al., *Oxidative stress, DNA methylation and carcinogenesis.* Cancer Letters, 2008. **266**(1): p. 6-11.
130. Ferguson-Smith, A.C., et al., *Regional localization of the interferon-β2B-cell stimulatory factor 2/hepatocyte stimulating factor gene to human chromosome 7p15-p21.* Genomics, 1988. **2**(3): p. 203-208.
131. Knupfer, H. and R. Preiss, *Significance of interleukin-6 (IL-6) in breast cancer (review).* Breast Cancer Res Treat, 2007. **102**(2): p. 129-35.
132. Ravishankaran, P. and R. Karunanithi, *Clinical significance of preoperative serum interleukin-6 and C-reactive protein level in breast cancer patients.* World J Surg Oncol, 2011. **9**: p. 18.
133. Stanley, E.R., et al., *Biology and action of colony-stimulating factor-1.* Molecular Reproduction and Development, 1997. **46**(1): p. 4-10.
134. *From decades of growth—toward the new opportunities of the 1990s.* Journal of Cellular Biochemistry, 1991. **45**(1): p. 1-2.
135. Scholl, S.M., et al., *Expression of Colony-Stimulating Factor-1 and Its Receptor (the Protein Product of c-fms) in Invasive Breast Tumor Cells.* Annals of the New York Academy of Sciences, 1993. **698**(1): p. 131-135.
136. Scholl, S.M., et al., *Is colony-stimulating factor-1 a key mediator of breast cancer invasion and metastasis?* Mol Carcinog, 1993. **7**(4): p. 207-11.
137. Soria, G. and A. Ben-Baruch, *The inflammatory chemokines CCL2 and CCL5 in breast cancer.* Cancer Letters, 2008. **267**(2): p. 271-285.
138. Borrello, M.G., *Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene.* Proc. Natl Acad. Sci. USA, 2005. **102**: p. 14825-14830.
139. Guerra, C., *Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice.* Cancer Cell, 2007. **11**: p. 291-302.
140. Bierie, B. and H.L. Moses, *TGF-[beta] and cancer.* Cytokine Growth Factor Rev., 2006. **17**: p. 29-40.
141. Yu, H., M. Kortylewski, and D. Pardoll, *Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment.* Nature Rev. Immunol., 2007. **7**: p. 41-51.
142. Blanco, D., et al., *Molecular analysis of a multistep lung cancer model induced by chronic inflammation reveals epigenetic regulation of p16 and activation of the DNA damage response pathway.* Neoplasia, 2007. **9**(10): p. 840-52.
143. Solinas, G., et al., *Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation.* Journal of Leukocyte Biology, 2009. **86**(5): p. 1065-1073.
144. Mitra, R., S. Singh, and A. Khar, *Antitumour immune responses.* Expert Reviews in Molecular Medicine, 2003. **5**(03): p. 1-22.
145. Rabb, H., *The T cell as a bridge between innate and adaptive immune systems: Implications for the kidney.* Kidney Int, 2002. **61**(6): p. 1935-1946.

146. Broere, F., et al., *A2 T cell subsets and T cell-mediated immunity*, in *Principles of Immunopharmacology*, F.P. Nijkamp and M.J. Parnham, Editors. 2011, Birkhäuser Basel. p. 15-27.
147. Cavallo, F., et al., *Immune Events Associated with the Cure of Established Tumors and Spontaneous Metastases by Local and Systemic Interleukin 12.* Cancer Research, 1999. **59**(2): p. 414-421.
148. Bidwell, B.N., et al., *Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape.* Nat Med, 2012. **18**(8): p. 1224-1231.
149. Fox, S.B., D.G. Generali, and A.L. Harris, *Breast tumour angiogenesis.* Breast Cancer Res, 2007. **9**(6): p. 216.
150. Hanahan, D. and J. Folkman, *Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis.* Cell, 1996. **86**(3): p. 353-364.
151. Reuter, S., et al., *Oxidative stress, inflammation, and cancer: How are they linked?* Free Radical Biology and Medicine, 2010. **49**(11): p. 1603-1616.
152. Vaupel, P., et al., *Hypoxia in breast cancer: Role of blood flow, oxygen diffusion distances, and anemia in the development of oxygen depletion*, in *Oxygen Transport to Tissue Xxvi*, P. Okunieff, J. Williams, and Y. Chen, Editors. 2005, Springer: New York. p. 333-342.
153. Harris, A.L., *Hypoxia [mdash] a key regulatory factor in tumour growth.* Nat Rev Cancer, 2002. **2**(1): p. 38-47.
154. De Palma, M., *Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors.* Cancer Cell, 2005. **8**: p. 211-226.
155. Mantovani, A., et al., *Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.* Trends Immunol., 2002. **23**: p. 549-555.
156. Rothenberg, M.E., *Eosinophilia.* New England Journal of Medicine, 1998. **338**(22): p. 1592-1600.
157. Torisu, H., et al., *Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: Possible involvement of TNFα and IL-1α.* International Journal of Cancer, 2000. **85**(2): p. 182-188.
158. Coussens, L.M., et al., *MMP-9 Supplied by Bone Marrow Derived Cells Contributes to Skin Carcinogenesis.* Cell, 2000. **103**(3): p. 481-490.
159. Mantovani, A., et al., *Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.* Trends in Immunology, 2002. **23**(11): p. 549-555.
160. Zhu, Y., M. Zhu, and P. Lance, *IL1β-mediated Stromal COX-2 signaling mediates proliferation and invasiveness of colonic epithelial cancer cells.* Experimental Cell Research, 2012. **318**(19): p. 2520-2530.
161. Soria, G., et al., *Inflammatory mediators in breast cancer: Coordinated expression of TNFalpha & IL-1beta with CCL2 & CCL5 and effects on epithelial-to-mesenchymal transition.* BMC Cancer, 2011. **11**(1): p. 130.
162. Hayden, M. and S. Ghosh, *Shared principles in NF-kappa B signaling.* Cell, 2008. **132**: p. 344 - 362.
163. Shostak, K. and A. Chariot, *NF-kappaB, stem cells and breast cancer: the links get stronger.* Breast Cancer Res, 2011. **13**(4): p. 214.

164. Courtois, G. and T.D. Gilmore, *Mutations in the NF-[kappa]B signaling pathway: implications for human disease.* Oncogene, 2006. **25**: p. 6831-6843.
165. Karin, M. and A. Lin, *NF-kappa B at the crossroads of life and death.* Nat Immunol, 2002. **3**: p. 221 - 227.
166. Garlanda, C., *Increased susceptibility to colitis-associated cancer of mice lacking TIR8, an inhibitory member of the interleukin-1 receptor family.* Cancer Research, 2007. **67**: p. 6017-6021.
167. Xiao, H., *The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis.* Immunity, 2007. **26**: p. 461-475.
168. Saccani, A., *p50 nuclear factor-[kappa]B overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance.* Cancer Research, 2006. **66**: p. 11432-11440.
169. Hagemann, T., *Re-educating tumor-associated macrophages by targeting NF-[kappa]B.* J. Exp. Med., 2008. **205**: p. 1261-1268.
170. Pelicci, G., et al., *A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction.* Cell, 1992. **70**(1): p. 93-104.
171. Migliaccio, E., et al., *Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway.* EMBO J, 1997. **16**(4): p. 706-716.
172. van der Geer, P. and T. Pawson, *The PTB domain: a new protein module implicated in signal transduction.* Trends in Biochemical Sciences, 1995. **20**(7): p. 277-280.
173. Ursini-Siegel, J., et al., *ShcA signalling is essential for tumour progression in mouse models of human breast cancer.* EMBO J, 2008. **27**(6): p. 910-20.
174. Bonfini, L., et al., *Not all Shc's roads lead to Ras.* Trends Biochem Sci, 1996. **21**(7): p. 257-61.
175. Ravichandran, K.S., *Signaling via Shc family adapter proteins.* Oncogene, 2001. **20**(44): p. 6322-30.
176. Ursini-Siegel, J. and W.J. Muller, *The ShcA adaptor protein is a critical regulator of breast cancer progression.* Cell Cycle, 2008. **7**(13): p. 1936-1943.
177. Rozakis-Adcock, M., et al., *Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases.* Nature, 1992. **360**(6405): p. 689-692.
178. van der Geer, P., et al., *The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein–protein interactions.* Current Biology, 1996. **6**(11): p. 1435-1444.
179. Rozakis-Adcock, M., et al., *The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1.* Nature, 1993. **363**(6424): p. 83-85.
180. Gotoh, N., A. Tojo, and M. Shibuya, *A novel pathway from phosphorylation of tyrosine residues 239/240 of Shc, contributing to suppress apoptosis by IL-3.* Embo Journal, 1996. **15**(22): p. 6197-6204.
181. Gotoh, N., M. Toyoda, and M. Shibuya, *Tyrosine phosphorylation sites at amino acids 239 and 240 of Shc are involved in epidermal growth factor-induced mitogenic signaling that is distinct from Ras/mitogen-activated protein kinase activation.* Molecular and Cellular Biology, 1997. **17**(4): p. 1824-1831.

182. Salcini, A.E., et al., *Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins.* Oncogene, 1994. **9**(10): p. 2827-36.
183. Davol, P.A., et al., *Shc Proteins Are Strong, Independent Prognostic Markers for Both Node-Negative and Node-Positive Primary Breast Cancer.* Cancer Research, 2003. **63**(20): p. 6772-6783.
184. Frackelton, A.R., Jr., et al., *p66 Shc and tyrosine-phosphorylated Shc in primary breast tumors identify patients likely to relapse despite tamoxifen therapy.* Breast Cancer Res, 2006. **8**(6): p. R73.
185. Dankort, D., et al., *Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: implications for human breast cancer.* Mol Cell Biol, 2001. **21**(5): p. 1540-51.
186. Siegel, P.M., et al., *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.* EMBO J, 1999. **18**(8): p. 2149-2164.
187. Dankort, D., et al., *Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins.* Journal of Biological Chemistry, 2001. **276**(42): p. 38921-38928.
188. Guy, C.T., R.D. Cardiff, and W.J. Muller, *Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease.* Molecular and Cellular Biology, 1992. **12**(3): p. 954-961.
189. Ursini-Siegel, J., et al., *Receptor Tyrosine Kinase Signaling Favors a Protumorigenic State in Breast Cancer Cells by Inhibiting the Adaptive Immune Response.* Cancer Research, 2010. **70**(20): p. 7776-7787.
190. Ursini-Siegel, J., et al., *The ShcA SH2 domain engages a 14-3-3/PI3[prime]K signaling complex and promotes breast cancer cell survival.* Oncogene, 2012. **31**(48): p. 5038-5044.
191. Saucier, C., et al., *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 of the United States of America, 2004. **101**(8): p. 2345-2350.
192. Morris, P.N., B.J. Dunmore, and N.P.J. Brindle, *Mutant Tie2 causing venous malformation signals through Shc.* Biochemical and Biophysical Research Communications, 2006. **346**(1): p. 335-338.
193. Northey, J.J., et al., *Signaling through ShcA Is Required for Transforming Growth Factor β- and Neu/ErbB-2-Induced Breast Cancer Cell Motility and Invasion.* Molecular and Cellular Biology, 2008. **28**(10): p. 3162-3176.
194. Pellegrini, M., S. Pacini, and C.T. Baldari, *p66SHC: The apoptotic side of Shc proteins.* Apoptosis, 2005. **10**(1): p. 13-18.
195. Xie, Y. and M.-C. Hung, *p66ShcIsoform Down-Regulated and Not Required for HER-2/neu Signaling Pathway in Human Breast Cancer Cell Lines withHER-2/neuOverexpression.* Biochemical and Biophysical Research Communications, 1996. **221**(1): p. 140-145.
196. Stevenson, L.E. and A.R. Frackelton, Jr., *Constitutively tyrosine phosphorylated p52 Shc in breast cancer cells: correlation with ErbB2 and p66 Shc expression.* Breast Cancer Res Treat, 1998. **49**(2): p. 119-28.

197. Jackson, J.G., et al., *Elevated Levels of p66 Shc Are Found in Breast Cancer Cell Lines and Primary Tumors with High Metastatic Potential.* Clinical Cancer Research, 2000. **6**(3): p. 1135-1139.
198. Ventura, A., et al., *The p66Shc Longevity Gene Is Silenced through Epigenetic Modifications of an Alternative Promoter.* Journal of Biological Chemistry, 2002. **277**(25): p. 22370-22376.
199. Lai, K.-M.V. and T. Pawson, *The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo.* Genes & Development, 2000. **14**(9): p. 1132-1145.
200. Migliaccio, E., et al., *The p66shc adaptor protein controls oxidative stress response and life span in mammals.* Nature, 1999. **402**(6759): p. 309-313.
201. Giorgio, M., et al., *Electron Transfer between Cytochrome c and p66Shc Generates Reactive Oxygen Species that Trigger Mitochondrial Apoptosis.* Cell, 2005. **122**(2): p. 221-233.
202. Pacini, S., et al., *p66SHC Promotes Apoptosis and Antagonizes Mitogenic Signaling in T Cells.* Molecular and Cellular Biology, 2004. **24**(4): p. 1747-1757.
203. Gertz, M., et al., *Identification of Peroxiredoxin 1 as a novel interaction partner for the lifespan regulator protein p66Shc.* Aging-Us, 2009. **1**(2): p. 254-265.
204. Pinton, P., et al., *Protein Kinase C ß and Prolyl Isomerase 1 Regulate Mitochondrial Effects of the Life-Span Determinant p66Shc.* Science, 2007. **315**(5812): p. 659-663.
205. Giorgio, M., et al., *Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals?* Nat Rev Mol Cell Biol, 2007. **8**(9): p. 722-728.
206. Polyak, K., et al., *A model for p53-induced apoptosis.* Nature, 1997. **389**(6648): p. 300-305.
207. Li, P.-F., R. Dietz, and R. von Harsdorf, *p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2.* EMBO J, 1999. **18**(21): p. 6027-6036.
208. Schuler, M., et al., *p53 Induces Apoptosis by Caspase Activation through Mitochondrial Cytochrome c Release.* Journal of Biological Chemistry, 2000. **275**(10): p. 7337-7342.
209. Orsini, F., et al., *The Life Span Determinant p66Shc Localizes to Mitochondria Where It Associates with Mitochondrial Heat Shock Protein 70 and Regulates Trans-membrane Potential.* Journal of Biological Chemistry, 2004. **279**(24): p. 25689-25695.
210. Trinei, M., et al., *A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis.* Oncogene, 2002. **21**(24): p. 3872-8.
211. Okada, S., et al., *The 66-kDa Shc Isoform Is a Negative Regulator of the Epidermal Growth Factor-stimulated Mitogen-activated Protein Kinase Pathway.* Journal of Biological Chemistry, 1997. **272**(44): p. 28042-28049.
212. Kamp, D.W., E. Shacter, and S.A. Weitzman, *Chronic inflammation and cancer: the role of the mitochondria.* Oncology (Williston Park), 2011. **25**(5): p. 400-10, 413.

213. Veeramani, S., et al., *Mitochondrial redox signaling by p66Shc is involved in regulating androgenic growth stimulation of human prostate cancer cells.* Oncogene, 2008. **27**(37): p. 5057-5068.
214. Ma, Z., et al., *p66Shc mediates anoikis through RhoA.* The Journal of Cell Biology, 2007. **179**(1): p. 23-31.
215. Grossman, S.R., et al., *p66 Shc tumor levels show a strong prognostic correlation with disease outcome in stage IIA colon cancer.* Clinical Cancer Research, 2007. **13**(19): p. 5798-5804.
216. Lee, M.-S., et al., *p66Shc protein is upregulated by steroid hormones in hormone-sensitive cancer cells and in primary prostate carcinomas.* International Journal of Cancer, 2004. **108**(5): p. 672-678.
217. Capitani, N., et al., *Impaired expression of p66Shc, a novel regulator of B-cell survival, in chronic lymphocytic leukemia.* Blood, 2010. **115**(18): p. 3726-3736.
218. Subik, K., et al., *The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines.* Breast Cancer (Auckl), 2010. **4**: p. 35-41.
219. Owusu-Ansah, E., A. Yavari, and U. Banerjee, *A protocol for _in vivo_ detection of reactive oxygen species.* 2008.
220. Fidler, I.J., *The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited.* Nat Rev Cancer, 2003. **3**(6): p. 453-8.
221. Hubbard, S.R., *Juxtamembrane autoinhibition in receptor tyrosine kinases.* Nat Rev Mol Cell Biol, 2004. **5**(6): p. 464-471.
222. Hooper, C. *Epidermal growth factors and cancer*. 2013; Available from: http://www.abcam.com/index.html?pageconfig=resource&rid=10723.
223. Abe, J.-i. and B.C. Berk, *Reactive Oxygen Species as Mediators of Signal Transduction in Cardiovascular Disease.* Trends in Cardiovascular Medicine, 1998. **8**(2): p. 59-64.
224. Cairns, R.A., I.S. Harris, and T.W. Mak, *Regulation of cancer cell metabolism.* Nat Rev Cancer, 2011. **11**(2): p. 85-95.
225. Albini, A. and M.B. Sporn, *The tumour microenvironment as a target for chemoprevention.* Nat Rev Cancer, 2007. **7**(2): p. 139-147.