Investigating the roles of 14-3-3ζ in the mammary tumour microenvironment

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Abbreviations

ADH	atypical ductal hyperplasia
ANOVA	analysis of variance
APS	ammonium persulfate
AT	adipose tissue
BAX	bcl-2 associated X protein
BC	breast cancer
BCA	bicinchoninic acid
BCL	B-cell lymphoma unspecified
BEP	basal enduring progenitors
bFGF	basic fibroblast growth factor
BIM	bcl-2-like 4
BrdU	bromodeoxyuridine
BSA	bovine rerum albumin
BUV	Brilliant Ultraviolet
BV	Brilliant Violet
CAA	cancer-associated adipocyte
CAF	cancer-associated fibroblast
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CK	cytokeratin
CO_2	carbon dioxide
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DAB	3,3’ diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
DCIS	ductal carcinoma in situ
ddH2O	double distilled water
dLN	draining lymph node
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
ECL	electrochemiluminescence
ECM	extracellular cell matrix
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial–mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal-regulated kinases

ES	embryonic stem cell
EV	extracellular vesicle
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FAP	fibroblast activation protein
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FOXO3	forkhead homeobox type O 3
FOXP3	forkhead box P3
FSC	forward scatter
FVB	Friend Virus B Type
FVS	Fixable Viability Stain
GAS	gamma activated site
GFP	green fluorescent protein
GH	growth hormone
GPCR	G-protein coupled receptor
GTP	guanosine-5' triphosphate
H_20_2	hydrogen peroxide
HCC	hepatocellular carcinoma
HEK293T	human embryonic kidney cell line
HET	heterozygous
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
IDC	invasive ductal carcinoma
IF	immunofluorescence
IFN	interferon
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
ILC	invasive lobular carcinoma
IMDM	Iscove's Modified Dulbecco's Medium
IQR	interquartile range
IRF1	interferon-regulatory factor 1
ISRE	interferon-stimulated response element
JAK	Janus activated kinase
KD	knock down
KO	knockout
LAG-3	lymphocyte activation gene 3
LAMP	lysosomal associated membrane glycoproteins
LCIS	lobular carcinoma in situ
LEP	luminal enduring progenitors
LIF	leukemia inhibitory factor
LN	lymph node
LOX	lysyl oxidase
MAPK	mitogen-activated protein kinase
MaSC	mammary stem cell
MDSC	myeloid-derived suppressor cell
MEC	mammary epithelial cell
MEK	mitogen activated protein kinase

METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
MFI	mean fluorescence intensity
MFP	mammary fat pad
MHC	major histocompatibility complex
miRNA	micro ribonucleic acid
MLC	myosin light chain
MMP	matrix metalloproteinase
MMTV	mouse mammary tumour virus
MRCLB	mouse red cell lysis buffer
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
MYPT	myosin phosphatase target subunit
NCBI	National Centre for Biotechnology Information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NK	Natural Killer cell
NKT	natural killer T
NO	nitric oxide
NSCLC	non-small cell lung carcinoma
OSCC	oral squamous cell carcinoma
PAM50	prediction analysis of microarray 50
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet derived growth factor
PDK	3-phosphoinositide-dependent kinase
PD-L1	programmed death-ligand 1
PE	Phycoerythrin
PerCP-Cy5.5	PerCP Cyanine 5.5
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonyl fluoride
PR	progesterone receptor
PyMT	Polyoma middle T antigen
qPCR	quantitative real-time polymerase chain reaction
RANTES	regulated on activation normal T cell expressed and secreted
RBC	red blood cell
RFI	relative fluorescent intensity
RIPA	radioimmunoprecipitation assay
ROCK	Rho-associated, coiled-coil containing protein kinase
ROI	region of interest
ROS	reactive oxygen species
RPPA	Reverse Phase Protein Array
RT	room temperature
SCC	squamous cell carcinoma
SD	standard deviation
SDF-1	stroma-derived factor -1
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	standard error of the mean
SHG	second harmonic generation
siRNA	small interfering RNA
SMA	smooth muscle actin
SSC	side scatter
STAT	signal transducer and activator of transcription
T reg	regulatory T cell
TAM	tumour-associated macrophage
TAN	tumour associated neutrophil
TBS	tris buffer saline
TBS-T	tris buffer saline+Tween20
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TEB	terminal end bud
TEMED	tetramethyl ethylenediamine
TGF	transforming growth factor
Th	helper T cell
TIL	tumour infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin-domain containing 3
TMA	alkylated trimethyl ammonium
TME	tumour microenvironment
TNBC	triple negative breast cancer
TNF	tumour necrosis factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UTR	untranslated region
UV	ultraviolet
VEGF	vascular endothelial growth factor
WB	Western Blot
WHO	World Health Organisation
WT	wildtype
YAP	yes associated protein

Publications arising from this thesis

1. **Poltavets, V**., Kochetkova, M., Pitson, S. M., & Samuel, M. S. (2018). The Role of the Extracellular Matrix and Its Molecular and Cellular Regulators in Cancer Cell Plasticity. *Front Oncol*, 8, 431. doi:10.3389/fonc.2018.00431

2. **Poltavets, V**., Esmaeili, Z., Boyle, S. T., Ramshaw, H. S., Lopez, A. F., Kochetkova, M., & Samuel, M. S. (2021). 14-3-3ζ-depletion impairs mammary gland development in the mouse. *bioRxiv*, 2021.2002.2003.425513. doi:10.1101/2021.02.03.425513

3. Boyle, ST*; **Poltavets, V***; Samuel, MS. Mechanical Signalling in the Mammary Microenvironment - From Homeostasis to Cancer. Chapter in Springer Nature *Advances in Experimental Medicine and Biology Book Series*, Tumor Microenvironment – Novel Concepts volume. Submitted *Joint first authors

4. **Poltavets, V**., Esmaeili, Z., Woodcock J.M, Pitson, S. M., Lopez, A. F., Kochetkova, M., & Samuel, M. S. Pharmacological inhibition of 14-3-3 reduces mammary cancer growth and is associated with enhanced anti-tumour immunity. *Clinical Sciences* Manuscript in preparation.

Summary

Breast cancer is the most commonly diagnosed cancer among women. With the introduction of global early detection and screening programs, awareness campaigns and targeted therapies, the mortality rates for breast cancers have been declining over the last two decades. However, later stage breast cancers, which are predominantly metastatic, remain incurable. Therefore, it is paramount to continue to dissect mechanisms of breast cancer progression in order to identify putative therapeutic targets for late-stage breast cancers.

14-3-3ζ is a member of the highly evolutionary conserved 14-3-3 protein family and is a phospho-serine binding protein. 14-3-3 proteins are known to interact with over 200 protein clients and regulate majority of cellular functions such as control of the cell cycle, survival, apoptosis, intracellular trafficking, cytoskeletal arrangement, transcription, and metabolism. Their roles are highly diverse and are context and tissue specific. 14-3-3ζ is abundantly expressed in breast cancers and this often correlates with disease recurrence and poor patient survival. Yet, when I started this project, the role of 14-3-3ζ in mammary cancers in vivo remained largely unexplored.

In order to establish the role for 14-3-3ζ in mammary cancer progression, I have intercrossed MMTV-PyMT tumour mouse line with the 14-3-3ζ genetic knock out model. Since the mammary cancers arise in the epithelial compartment of the mammary gland, it was critical to establish the role for 14-3-3ζ in normal mammary development prior to characterisation of the mammary tumorigenesis in vivo. It is also important to note, that oftentimes in cancers developmental mechanisms are corrupted by cancer cells, therefore this investigation could also provide clues to the roles of 14-3-3ζ in cancer progression.

Hence, I have extensively analysed mammary gland development in 14-3-3ζ-deficient mice. Interestingly, 14-3-3ζ was crucial for normal mammary gland development, with knock out animals showing significant delay in epithelial branching. This delay was associated with reduced proliferation rate of the mammary epithelial cells and the reduction in the basal epithelial compartment. Together, these results indicated that 14-3-3ζ is important for normal mammary gland development, cellular proliferation and potentially stem cell phenotype. Surprisingly, the 14-3-3ζ heterozygous animals had comparable development phenotype as

wildtype. This was important for my further tumorigenesis studies, and I have established MMTV-PyMT tumour model with 14-3-3ζ heterozygous genotype.

Genetic reduction of 14-3-3ζ led to delayed tumour initiation, reduced tumour burden in mice, reduced cancer cell proliferation and overall affected proliferative and apoptotic pathways.

I have also tested a novel pharmacological inhibitor of 14-3-3, RB-012 in a spontaneous mammary tumorigenesis model. The treatment of established tumours was significantly reduced upon three-week treatment with the drug, suggesting that pharmacological inhibition of 14-3-3 could be further analysed in order to find specific pathways that are affected and potentially serve as a combination treatment with other inhibitors.

Furthermore, I have also demonstrated that 14-3-3ζ could be important in the mammary cancer microenvironment. My preliminary experimental data suggests that 14-3-3ζ reduction in the microenvironment could influence the recruitment of cytotoxic T cells and their IFN-γ mediated responses at the tumour site.

Collectively, I have demonstrated that 14-3-3ζ has a role in mammary cancer progression via cellular proliferation mechanisms and that pharmacological inhibition of 14-3-3 reduces tumour burden in already established mammary tumours in vivo. I have also demonstrated that this protein has a putative role in the tumour microenvironment, suggesting that 14-3-3ζ could be important in T cell mediated immune responses in mammary cancers. This study could be of benefit to the ongoing efforts for enhancing tumour-microenvironment targeted therapies in advanced breast cancers.

Declaration

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge, it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.



13th October 2021

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Dedication



Dedicated to my beloved grandmother Valentina Kusheva (1921-2009): the Second World War survivor, the battlefield surgeon, the most inspiring person in my life, the woman of great stamina and endurance.

Finally, granny, I fulfilled my promise, I will be a doctor.

Chapter 1. Introduction

1.1. Mammary Gland Development

The mammary gland is a unique organ with the specific function of milk production and secretion. This complex organ harbours multiple cell types that support the development and maintenance of the ductal epithelial structures important for milk production, namely adipocytes that comprise a large proportion of the stromal area of the fat pad; fibroblasts that are found in close proximity to the epithelial cells; vascular endothelial and lymphatic cells that provide networks for the blood vessel and lymphatic vessel formation; and a range of immune cells that have diverse functions at all stages of mammary gland development (Inman et al. 2015).

1.1.1. ***Stages of the Mouse Mammary Gland Development***

Throughout the lifetime of a female, the mammary gland undergoes multiple rounds of dramatic changes that affect its structure and function. These changes occur during physiologically important stages of the lifespan of the animal, namely onset of puberty, throughout the menstrual cycle, pregnancy, lactation, and involution (Inman et al. 2015; Macias & Hinck 2012).

The development of the mammary gland occurs in three defined stages: embryonic, pubertal, and reproductive. Each stage is closely associated with the specific hormonal cues, growth factors and mechanical signals that initiate and sustain the required morphological and cellular changes of development (Macias & Hinck 2012).

1.1.1.1. Embryonic Stage

Embryonic development of the mammary gland in mice occurs around E10 with an establishment of milk lines (multilayered ectoderm) at the ventral surface of the embryo. During the embryogenesis around E11.5, ectoderm-derived structures termed placodes appear from the milk lines. Further on, placodes become epithelial mammary buds and descend into the underlying dermis. The surrounding mesenchymal cells around the bud contract and emerge as a mammary mesenchyme. The fat pad precursor (or a secondary mesenchyme) develops around E15.5. Later, the epithelial mammary bud proliferation and elongation leads to sprout formation that invades the fat pad precursor. The rudimentary ductal tree is formed by E18.5 and remains quiescent until the onset of puberty (Hens & Wysolmerski 2005; Inman et al. 2015). Schematic representation of embryonic mammary fat pad development is depicted in **Figure 1.1**.

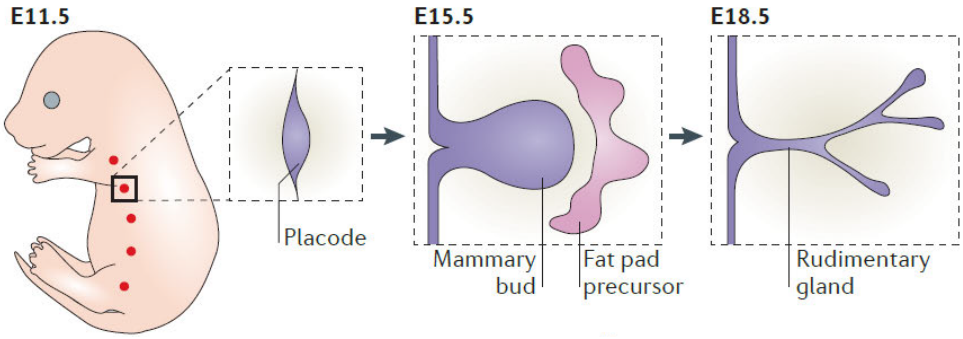


Figure 1.1: Schematic representation of embryonic mouse mammary gland development.

The embryonic development of the mammary gland occurs ~E10 the with an establishment of milk lines (multilayered ectoderm). Around E11.5 ectoderm-derived structures termed placodes appear from the milk lines. The placodes become epithelial mammary buds and descend into the underlying dermis. At E15.5 the fat pad precursor develops, and epithelial mammary bud proliferation and elongation leads to sprout formation that invades the fat pad precursor. By E18.5 the rudimentary gland is formed and remains quiescent until the onset of puberty. Adapted from (Gjorevski & Nelson 2011).

1.1.1.2. Pubertal Stage

Substantial development of the mammary gland occurs at the onset of puberty under the influence of sex hormones as well as growth factors. Hormone-driven activation of the mammary morphogenesis occurs during puberty when endocrine signals from the ovary (estrogen and progesterone) and pituitary gland (growth hormone) activate downstream paracrine signalling from both epithelial and stromal cells to drive the formation of the terminal end buds (TEBs) and the process of epithelial branching. The elongation of the epithelial ducts is activated and controlled by estrogen and the estrogen receptor (ER)α. Mammary epithelial cells exhibit proliferative responses to estrogen and progesterone with cyclical regularity thorough the lifespan of the animal (Fendrick, Raafat & Haslam 1998; Mueller, SO et al. 2002). Cellular proliferation, survival and branching are mainly driven by estrogen insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Gjorevski & Nelson 2011).

layer of highly proliferative cap epithelial cells and underlying body cells that drive the process by elongating and branching as shown in **Figure 1.2**. Subsequently cap cells give rise to the differentiated myoepithelium and body cells to luminal epithelium (Fu et al. 2020). The schematic of the structure of the TEB is represented in **Figure 1.3**.

The main drivers of proliferation during the process of branching morphogenesis are the TEBs. The highly proliferative cap epithelial cells form the outer layer whereas the inner multi-layer is comprised of body cells. EGFR and TGF-α signalling are important for proliferative events in both cell types of the TEBs (Snedeker, Brown & DiAugustine 1991), whereas TGF-β signalling restricts ductal growth by inhibition of proliferation of the cap cells (Daniel et al. 1989). Furthermore, the continuation of ductal elongation is dependent on the ErbB2 and ErbB3 signalling (Andrechek, White & Muller 2005; Qu et al. 2006).

Secondary branching occurs laterally from the primary ducts and the tree-like pattern of the mammary epithelium emerges. The lateral branching is regulated by progesterone receptor (PR) signalling and side-branching of the mammary ducts is dependent on Wnt1 and Wnt4 signalling (Brisken et al. 2000).

Pubertal stage of mammary gland development is a critical stage of the development. It is characterised by spatially and temporally coordinated burst of cellular proliferation and mammary tree elongation which are orchestrated by hormonal, growth factor and developmental cues. Pubertal stage prepares an adult mammary gland for its main function of milk production in the next, reproductive stage of the development.

1.1.1.3. Reproductive Stage

The process of gland maturation and alveolar differentiation occurs throughout the reproductive phase of mammary gland development. During pregnancy, under the influence of estrogen, progesterone and prolactin, secondary branching increases and tertiary branching of the mammary epithelial tree commences. The cells of the luminal epithelial layer proliferate and differentiate to form structures termed alveoli, which are milk-producing and secreting units of the mammary gland. Alveoli become fully mature when lactation commences (Macias & Hinck 2012).

During lactation, luminal cells produce and secrete milk, whilst basal cells (myoepithelial cells) contract to propel the milk out of the ducts. Thus, both cell types experience compressive force due to accumulated milk and ductal distension (Inman et al. 2015). Myoepithelial cells express high levels of smooth muscle actin and cytoskeletal proteins that allow them to contract upon oxytocin stimulus (Haaksma, Schwartz & Tomasek 2011).

Upon weaning, the process of involution ensues, when milk-producing epithelial cells undergo extensive apoptosis and widespread gland tissue remodelling occurs (Watson 2006). During involution, accumulation of residual milk leads to intraluminal pressure build up and it is suggested that the resulting epithelial cell stretching may contribute to Leukemia inhibitory factor (LIF) expression and STAT3 activation that define the initiation of involution (Quaglino et al. 2009).

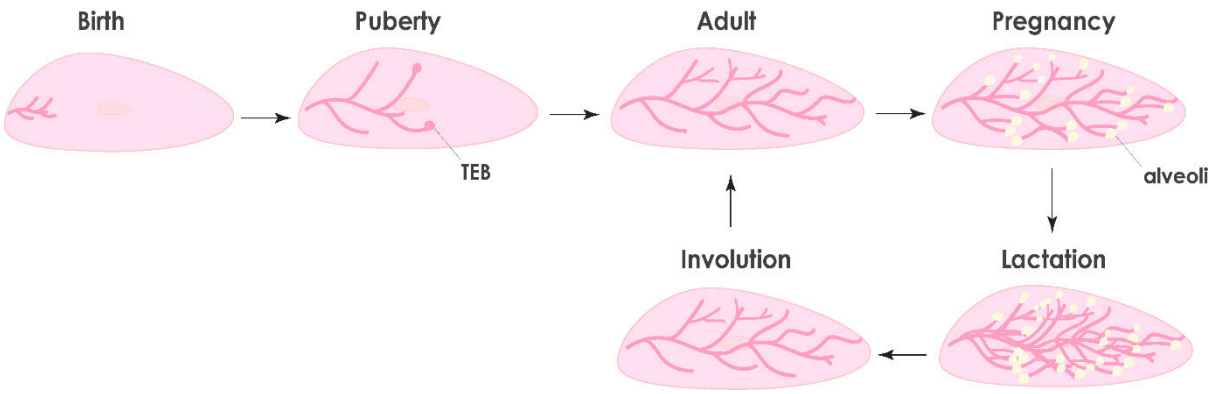


Figure 1.2: Overview of postnatal mouse mammary gland development.

The rudimentary gland remains quiescent until puberty. Endocrine signalling drives the formation of the terminal end buds (TEBs), the main centres of proliferation in the mammary gland. Ductal elongation then continues throughout the adult stage, until the physical limits of the fat pad are reached. During pregnancy, under the influence of steroid hormones secondary branching increases and tertiary branching of the mammary epithelial tree occurs. The luminal epithelial layer proliferates and differentiates into structures termed alveoli, the milk-producing and secreting units of the mammary gland. During lactation, luminal cells produce and secrete milk, whilst basal cells (myoepithelial cells) contract to propel the milk out of the ducts. Following lactation, the process of involution commences, extensive epithelial remodelling occurs, and the mammary fat pad returns to its resting state. Adapted from (Fu et al. 2020; Macias & Hinck 2012).

1.1.2. *Stem/Progenitor Cell Hierarchy in the Mammary Ductal Morphogenesis*

The field of mammary stem cell (MaSC) research is still evolving and efforts to clear up the uncertainties surrounding the identity and differentiation potential of MaSCs are ongoing.

One of the hypotheses, based on lineage tracing data, is that the multipotent MaSCs are only active at the embryonic stages of development. After birth, however, the basal enduring progenitors (BEP) and luminal enduring progenitors (LEP) are unipotent and fully lineage

committed. BEPs give rise to the ductal myoepithelial cells in developing and mature ducts and alveolar myoepithelial cells in pregnancy; whereas LEPs give rise to ductal and luminal progenitors and luminal epithelial cells (Watson & Khaled 2020).

An alternative hypothesis is that the adult mammary gland harbours a population of quiescent stem cells that are bi-potent and can give rise to both luminal and basal progenitors, which are then lineage-committed and subsequently give rise to luminal and basal mammary epithelial cell populations (Fu et al. 2020).

Based on the combination of transplantation studies and immunophenotyping, the MaSCs with repopulation potential were found to be in the basal epithelial compartment and defined as $Lin^-CD24^+CD29^{high}CD49f^{high}Sca1^{low}$ (Shackleton et al. 2006; Stingl et al. 2006). The luminal compartment of the mammary epithelium is defined by $Lin^-CD24^+CD29^{low}CD49f^{low}$ and does not have repopulation capacity. The luminal mouse mammary epithelium could be further broadly classified into the progenitor cells and are defined based on the surface markers as $Lin^-CD24^+CD29^{low}CD61^+$ and differentiated luminal cells $Lin^-CD24^+CD29^{low}CD61^-$ (Asselin-Labat et al. 2007a).

Below, I briefly review the major molecular pathways that are crucial in the regulation and differentiation of MaSC/basal progenitor and luminal progenitor populations in the developing mammary gland.

The Wnt pathway is the major regulator of basal stem/progenitor cells. Canonical Wnt signalling and its Frizzled receptors play complex roles in MaSC regulation (Slepicka, Somasundara & Dos Santos 2020). Wnt pathway co-receptors Lrp5 and Lrp6 are highly expressed in basal cells that have high repopulation ability (Lindvall et al. 2006). Lgr5, a target gene of Wnt pathway, is expressed in the basal compartment of adult glands and its deletion impairs ductal development (Plaks et al. 2013). *Axin2*, another target gene and a negative regulator of Wnt pathway, is also highly expressed in basal cells and its deletion potentiates the in vivo mammary reconstitution capacity of MaSCs (Zeng & Nusse 2010).

The Notch signalling pathway is the key regulator of the luminal cell fate specification, and its activation promotes MaSC commitment to the luminal lineage (Bouras et al. 2008). The transcription factor Gata-3 is critical for luminal progenitor cell differentiation downstream of Notch, with Gata-3 protein restricted to the luminal cells essential for the differentiation of luminal progenitors into the alveolar subset (Asselin-Labat et al. 2007a). Interestingly, *Gata-3* gene expression is observed in both luminal and basal cells and is crucial for TEB formation

in the developing glands (Kouros-Mehr et al. 2006). Lower levels of *Gata-3* expression are found in stem cell-enriched populations (Asselin-Labat et al. 2007b).

Another level of regulation of the cell fate of the basal/MaSC, luminal progenitors and mature cells is epigenetic. For example, H3K27me3-mediated gene silencing is prominent in the luminal progenitors and thus, in the context of the mammary gland, could be indicative of the luminal identity of the mammary epithelial population (Dravis et al. 2018). An important regulator of the histone methylation is Pygo2, which is found to supress luminal differentiation in the MaSCs. Pygo2-mediated chromatin regulation connects Wnt and Notch signalling and restricts luminal differentiation of the MaSCs (Gu, B et al. 2013).

1.1.3. *Mammary Gland Microenvironment in Pubertal Development*

Mammary gland stroma is comprised of specific cell types that guide and support the process of TEB formation and of the branching morphogenesis. Adipocytes comprise a large proportion of these cells and regulate mammary epithelial growth and function (Hovey & Aimo 2010). Ablation of adipocytes in the mammary gland triggers changes in proliferation and apoptosis in the epithelium associated with the TEBs (Landskroner-Eiger et al. 2010). Adipocytes are also important for regulation of the angiogenesis via secretion of the vascular endothelial growth factor (VEGF) (Inman et al. 2015). Schematic representation of the cellular composition of the TEB in developing mouse mammary gland and its surrounding microenvironment is illustrated in **Figure 1.3**.

Stromal fibroblasts provide growth factor and survival signalling to the epithelial cells during the branching morphogenesis (Howard & Lu 2014). Fibroblasts are also the key cells that synthesise the extracellular cell matrix (ECM) components such as collagens, proteoglycans and fibronectin that provide structural support for the epithelial tree. Fibroblasts are also the sources of the metalloproteinases (MMPs) that are crucial regulators of the ECM during for the process of epithelial invasion (Wiseman & Werb 2002).

Lymphatic and vascular networks are essential for the development of the mammary epithelium, however not much is known about their function in TEB formation and ductal elongation. The vascular and lymphatic networks are closely associated with the mammary epithelial tree, which suggests that this proximity plays a role on how these vascular networks regulate development of the mammary epithelial tree. However, the exact molecular interactions are still under investigation (Betterman et al. 2012).

The immune cells, specifically mast cells, macrophages, and eosinophils, are crucial regulators of the TEB formation and branching morphogenesis in the mammary gland. The proximity of mast cells to the TEB structures and their active degranulation is essential for the mammary branching morphogenesis and epithelial cell proliferation (Lilla & Werb 2010). Macrophages and eosinophils are recruited to the distal end of the epithelial duct to maintain the mammary fat pad invasion, maintenance of structured collagen organisation and mammary tree elongation (Dawson et al. 2020; Gouon-Evans, Rothenberg & Pollard 2000; Ingman et al. 2006).

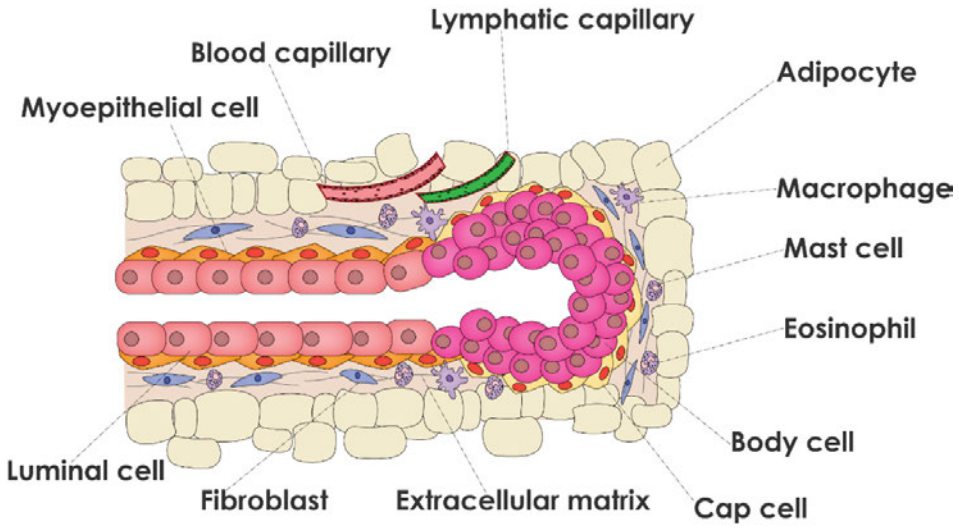


Figure 1.3: Schematic representation of the terminal end bud (TEB) in a pubertal mouse mammary gland.

TEB structures are the driving elements of the branching morphogenesis in pubertal mouse. TEBs consist of a single layer of highly proliferative cap epithelial cells and underlying body cells. Mature ducts are comprised of the outer layer of the myoepithelial cells and the inner layer of luminal cells. Mammary gland stroma is comprised of specific cell types that guide and support the process of TEB formation and of the branching morphogenesis including fibroblasts, adipocytes, macrophages, mast cells and eosinophils. The blood and the lymphatic networks play important roles in the support of branching morphogenesis. Adapted from (Fu et al. 2020).

1.2. Breast Cancer

1.2.1. *The Burden of the Disease in the World and Australia*

In 2020, 2.3 million women were diagnosed with breast cancer worldwide, and 685 000 deaths were attributed to this disease. In the majority of countries, breast cancer is the most commonly

diagnosed cancer among women, with ~14% of all cancer-related deaths among women attributed to this disease (Sung et al. 2021; WHO, 2021).

In 2021, it is estimated that 20,825 Australians will be diagnosed with breast cancer (AIHW, 2020). Breast cancer primarily affects older women, with an average diagnosis age of 61 years. The probability of surviving for at least five years following a diagnosis of breast cancer is ~91% and that for ten years is 83%; in addition, the aging population in Australia means that the incidence of breast cancer is increasing each year (AIHW, 2020).

Even though with the introduction of global screening programs, early detection, awareness campaigns and targeted therapies, the mortality rates for breast cancer have been declining over the last two decades, later stage breast cancers, which are predominantly metastatic, are currently incurable (Sung et al. 2021).

Specifically, in Australia, pre-menopausal women and Aboriginal and the Torres Strait Islander women generally have a poorer survival outcome due to metastatic disease, compared to older women. Therefore, further research into mechanisms of breast cancer progression and targeted therapeutic treatments for breast cancer patients is paramount (AIHW, 2020).

1.2.2. ***Breast Cancer Classification***

1.2.2.1. Histopathological Features of Breast Cancer

Breast cancers are categorised into non-invasive (in situ) or invasive carcinomas depending on the levels of expansion of the cancer cells from their anatomical location (lobule or duct). Broadly, histological subtypes are subdivided to ***preinvasive*** ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS); and ***invasive*** ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Malhotra et al. 2010). Key stages of breast cancer progression are shown in **Figure 1.4**.

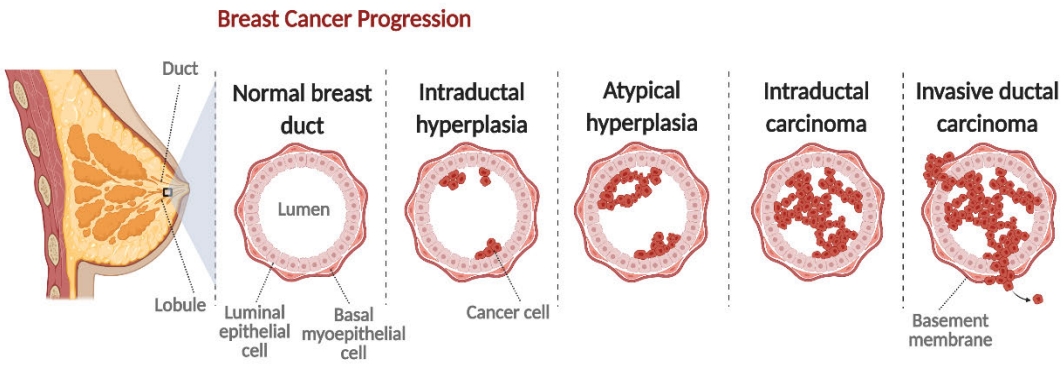


Figure 1.4: Key stages of breast cancer progression.

The breast cancer initiates via proliferation of the tumour cells within the mammary duct. Intraductal and the atypical ductal hyperplasia (ADH) are pre-malignant lesions and are characterised by several layers of cancer cells within the ducts. Next stage of the progression is the intraductal carcinoma in situ (DCIS) where cancer cells occlude the lumen; finally, invasive ductal carcinoma (IDC) is characterised by invasion of cancer cells beyond the myoepithelial layer and basement membrane and growth outside the duct (Harris 2014).

Important pathological features of breast cancer progression are classified via grade and stage. Grade (G1-3) is indicative of the growth rate and differentiation status of cancer cells. Grade indicates the potential aggressiveness and is a strong prognostic factor in breast cancer. Stage of the disease is assigned from O-IV. The stage is the measurement of the advancement of the disease which takes into consideration the primary tumour size (T), regional lymph node status (L) and the spread to metastatic sites (M) (Brierley, Gospodarowicz & Wittekind 2017).

1.2.2.2. Molecular subtypes

It is now well established that breast cancers with the same histopathological features can exhibit very different specific molecular characteristics. The framework for molecular subtyping of breast cancer is always evolving. Nevertheless, the molecular subtype framework revolutionised the therapeutic decision-making for clinicians and assists with specific course of treatment for individual breast cancer patients, it is also a useful tool in breast cancer research.

Molecular characteristics of the breast cancers were first described as **intrinsic subtypes** (Perou et al. 2000) from profiling 84 breast cancer samples for 1,753 genes. Based on specific gene signature clustering of bulk tumour sequencing, four major clusters emerged: *Luminal A and B* (expressing estrogen receptor (ER) and progesterone receptor (PR)), *HER2* (typically ER negative) and *Basal-like* (typically negative for HER2, ER and PR, also referred as 'triple

negative') (Foulkes, Smith & Reis-Filho 2010). *Claudin-low* subtype was later defined using human breast and mouse mammary tissue gene profiling and is characterised by low expression of tight junction and cell-cell adhesion genes (Claudins 3, 4, 7, Occludin, and E-cadherin) (Herschkowitz et al. 2007). This subtype classification is widely used in the breast cancer research field and in the clinic (Harbeck et al. 2019).

In current clinical settings the **surrogate intrinsic subtype** classification is used (Cheang et al. 2015). The surrogate intrinsic subtypes emerged by immunohistochemical examination of key proteins in breast cancer: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5/6, epidermal growth factor receptor (EGFR) and proliferation marker Ki-67. Breast cancer tumours that express ER and/or PR are defined as 'hormone receptor-positive'. These include HER2-enriched, Luminal B-like HER2+, Luminal B-like HER2- and Luminal A-like. Tumours that do not express ER, PR and HER2 are termed Triple-negative (Harbeck et al. 2019). **Figure 1.5** summarises intrinsic and surrogate subtypes of breast cancers.

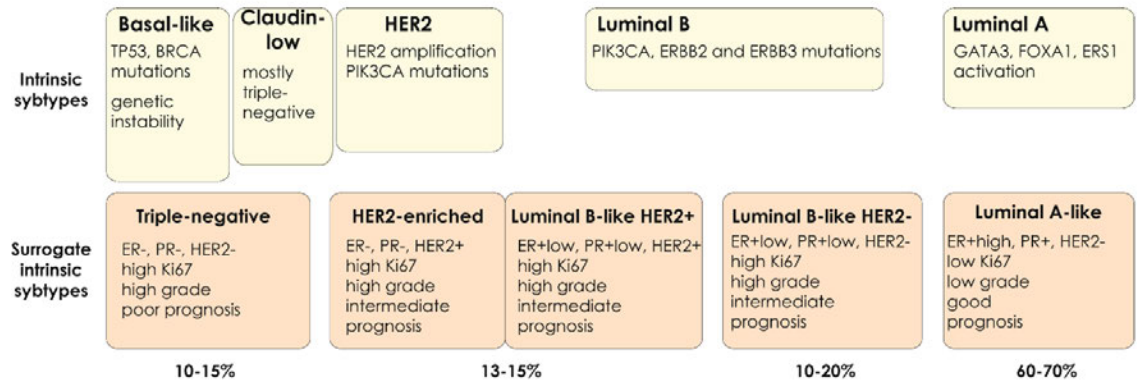


Figure 1.5: Intrinsic and surrogate intrinsic subtypes of breast cancers.

Two separate classification systems of molecular subtypes of the breast cancers. Intrinsic subtype considers gene expression prolife breast cancers, mutations, and hormone receptor status (ER-estrogen receptor; PR -progesterone) and human epidermal growth factor receptor 2 (HER2) status. The surrogate intrinsic classification takes into consideration immunohistochemical examination of the breast samples, including hormone receptor status, HER2 status, and cancer cells proliferation measured by Ki-67 as well as gross tissue morphology. Percentages represent the proportion of breast cancer diagnosed according to subtypes. Adapted from (Harbeck et al. 2019).

1.2.3. ***Genetic and Molecular Drivers of Breast Cancer***

1.2.3.1. Gene Alternations

The precise mechanism of breast cancer initiation is unknown, however some major insights into genetic and molecular alterations leading to significantly increased risk of breast cancer have been identified.

Familial breast cancers are strongly associated with inherited mutations of tumour suppressor genes BRCA1 and BRCA2 (both important for DNA repair of double-stranded breaks). BRCA1/2 increase the risk of developing breast cancer to age 80 by 72% and 69% respectively (Kuchenbaecker et al. 2017). Among other genes to determine the inherited breast cancer risk are ATM, PALB2, PTEN, STK11, TP53 (Easton et al. 2015).

Most frequent gene alterations in breast cancer cells are TP53, PIK3CA, MYC, PTEN CCND1, ERBB2, FGFR1 and GATA3. These genes encode the components of cell cycle regulation, proliferation, inhibition of apoptosis or inhibitors of oncogenic signalling (Nik-Zainal et al. 2016).

1.2.3.2. Key Signalling Pathways in Breast Cancer

Over the years, cancer 'hallmarks' have been described in a conceptual framework as key characteristics of cancers. These characteristics include sustained proliferative signalling, evasion of growth suppression, invasion and metastasis, replicative immortality, angiogenesis, and resistance to cell death, as well as emerging hallmarks of immune destruction avoidance, tumour-promoting inflammation, genome instability and mutation, and deregulation of cellular energetics. These characteristics are acquired via genetic mutations or altered signal transduction (Hanahan & Weinberg 2000, 2011).

In breast cancers, the major contributors to the disease progression are pathways mediated by the estrogen receptor (ER) and human epidermal growth factor type-2 (HER2/ ERBB2) (Feng, Y et al. 2018). Estrogen exposure is a major risk for development of sporadic breast cancers since the breast epithelial cells are highly responsive to the effects of the estrogen (Dall & Britt 2017).

Hormonal stimulation of the breast tissue occurs during puberty, menstrual cycles, and pregnancy. Estrogen has a direct effect on the mammary epithelial cell proliferation. Animal studies confirm that local estrogen stimulates mammary ductal growth (Daniel, Silberstein &

Strickland 1987; Silberstein et al. 1994). Furthermore, progesterone causes increased division of the epithelial mammary cells during luteal phase of the menstrual cycle (Navarrete et al. 2005). Interestingly, it has also been suggested that synergistic action between progesterone and estrogen during menstrual cycles increases cellular proliferation in the breast (Nazario et al. 1995; Pike et al. 1993). Thus, hormonal stimulation of the mammary tissue throughout the lifecycle of the female creates more potential for DNA damage and tumour-promoting mutations in the epithelial cells.

Estrogen receptors (ERs) are divided into the membrane and nuclear receptors (ERα, β) that are transcriptional factors and mediate downstream target gene expression upon ligand binding (Feng, Y et al. 2018). Estrogen binds to its receptor ERα in the nucleus, causing receptor dimerisation and binding to estrogen response elements (EREs) that are found at promoter regions of target genes. (Arnal et al. 2017; Bjornstrom & Sjoberg 2005). In ER+ve primary and recurrent breast cancers the most mutated pathways are PI3K/AKT/mTOR which regulate cellular growth, proliferation, survival, and metabolism (Ciruelos Gil 2014).

HER2 is a member of the epidermal growth factor receptors (EGFRs), a family of tyrosine kinase receptors that consists of an extracellular ligand-binding domain, a transmembrane domain, and intracellular domain (Feng, Y et al. 2018). Ligand binding activates receptor dimerisation and stimulates tyrosine phosphorylation in the intracellular domains and subsequent activation of Ras, MAPK and PI3K/AKT/mTOR signalling pathways that govern proliferation, cell survival and metastasis (Ortega et al. 2020). A schematic representation of the ERα and HER2 receptor signalling in breast cancer is shown in **Figure 1.6**.

The PI3K/AKT/mTOR pathway controls major cellular functions including proliferation, growth, survival, and metabolism. PI3K (phosphatidylinositol 3-kinase) is a kinase that is activated by receptor tyrosine kinases and by G-protein coupled receptors (GPCRs) resulting in phosphorylation of phosphatidylinositol at the 3' position hydroxyl of the inositol ring. A downstream target of PI3K action is the serine/threonine protein kinase AKT (Protein kinase B, PKB). Downstream of AKT, a serine/threonine kinase mTOR (mammalian target of rapamycin) is a crucial mediator of cellular metabolic activity (Nunnery & Mayer 2020). In breast cancers, hyperactivation of PI3K signalling leads to cellular transformation, tumour growth, resistance to apoptosis and metabolic adaptation of epithelial cells to the hostile microenvironment. One of the most common genetic mutations associated with breast cancer occur in *PIK3CA* which encodes p110α subunit of the PI3K, leading to enhanced activity of this enzyme (Miricescu et al. 2020).

Raf/MEK/ERK pathway (MAPK mitogen-activated protein kinase pathway) is a signalling pathway that mediates cellular proliferation, cell survival and apoptosis. This pathway consists of three kinase cascades including Raf (rapidly accelerated fibrosarcoma), a serine-threonine kinase, MEK1/2 (mitogen-activated protein kinases 1 and 2) and ERK1/2 (extracellular signal-regulated kinases 1 and 2). MEK1/2 and ERK1/2 pathways are often upregulated in breast cancer cells (Santen et al. 2002). Ras is a small GTPase that transmits external stimuli into the cell. Ras is an oncoprotein and is the most frequently mutated gene in human cancers. Ras is a key upstream activator of Raf and initiates signalling via both the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in breast cancers (Roberts & Der 2007; Saini et al. 2013).

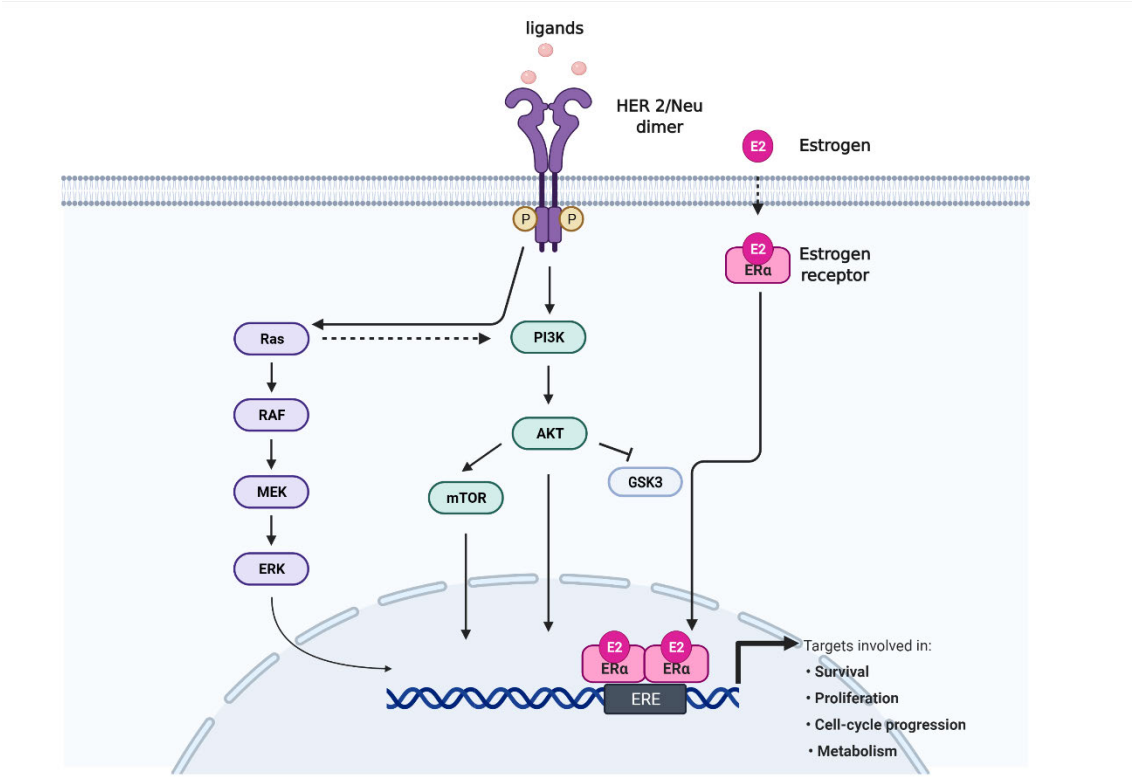


Figure 1.6: Key signalling pathways in breast cancer.

In breast cancers, the major contributors to disease progression are signalling pathways mediated by the estrogen receptor (ERα) and human epidermal growth factor type-2 (HER2/ERBB2). Estrogen binding activates the ERα receptor predominantly found in the nucleus, leads to receptor dimerisation and binding to estrogen response elements (EREs) that are localised in the promoter regions of target genes. Ligand binding to HER2/Neu leads to activation of downstream Ras, MAPK and PI3K/AKT/mTOR signalling pathways. Together these pathways govern breast cancer cell proliferation, survival, metabolism, and metastasis. Adapted from (Feng, Y et al. 2018; Ortega et al. 2020).

1.2.4. *Components of Breast Cancer Microenvironment*

It is becoming increasingly evident that the study of tumour biology should encompass not only the various individual cancer cell types that the cancer is comprised of but also the tumour microenvironment that develops during process of tumourigenesis. Over the past decade, considerable evidence has accumulated regarding the tumour microenvironment and its direct and complex role in tumour promotion suggesting that signals from the microenvironment may not only act as mutagens but could in some cases drive the tumour phenotype (McAllister & Weinberg 2014; Mueller, MM & Fusenig 2004; Quail & Joyce 2013).

The breast cancer microenvironment consists of several key cellular components including fibroblasts, adipocytes, immune cells, and endothelial cells. Non-cellular components such as the extracellular matrix (ECM) and secreted cytokines, chemokines, and growth factors are also essential to the breast cancer microenvironment (Soysal, Tzankov & Muenst 2015). Schematic representation of breast cancer microenvironment is depicted in **Figure 1.7**.

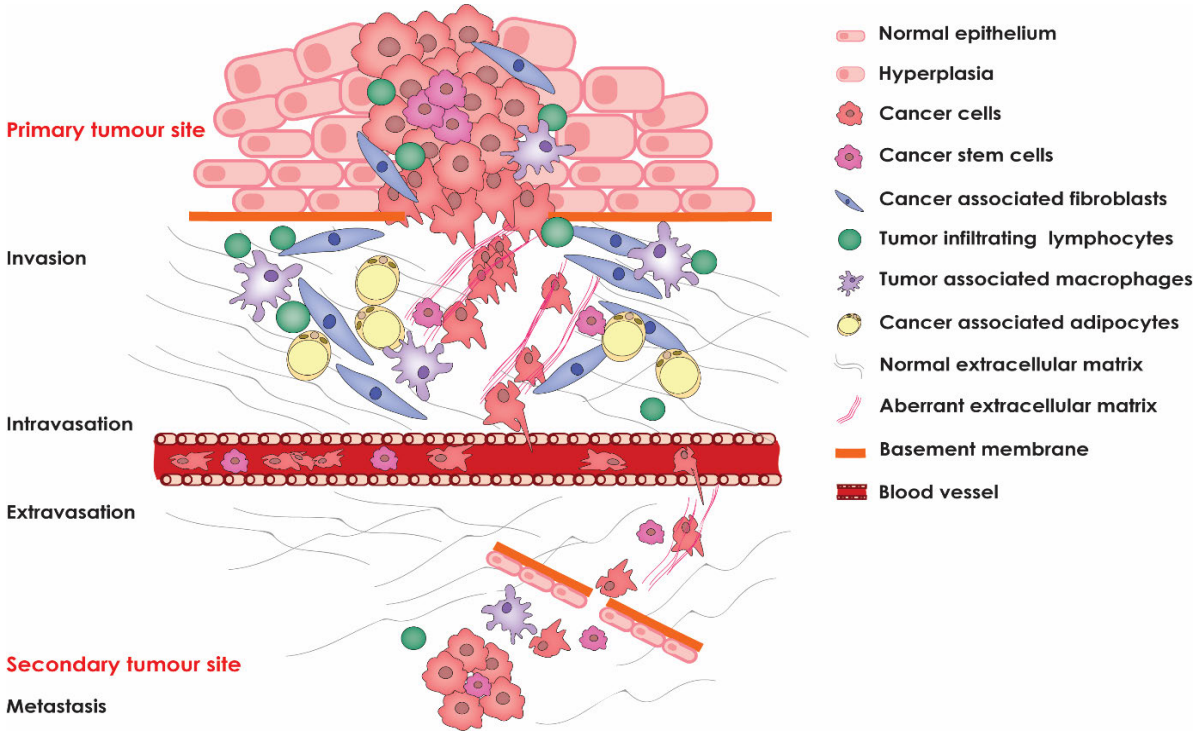


Figure 1.7: Components of the breast cancer microenvironment.

Breast cancer microenvironment consists of cellular components including cancer associated fibroblasts, adipocytes, immune cells, and endothelial cells. Non-cellular components such as extracellular matrix (ECM), cytokines, chemokines, and growth factors are also essential to the breast cancer microenvironment. The tumour microenvironment may support or inhibit tumour progression at any stage of tumourigenesis. Adapted from (Poltavets et al. 2018).

1.2.4.1. Cancer Associated Fibroblasts (CAFs)

Activated fibroblasts in the tumour microenvironment are termed cancer-associated fibroblasts (CAFs). CAFs can become activated via a number of paracrine and/or autocrine signals from cancer cells and other cells in the tumour microenvironment including adipocytes, pericytes, immune cells and resident fibroblasts (Ping et al. 2021). Pro-inflammatory factors such as TGF-β, IL-6, platelet-derived growth factor (PDGF), hypoxia and reactive oxygen species (ROS) have been demonstrated to activate quiescent fibroblasts (Kalluri 2016). Work from our laboratory has recently demonstrated that in breast cancers, fibroblasts can be actively reprogrammed to a tumour-promoting CAF phenotype by tumour-cell secreted protein Creld-2 (Boyle et al. 2020).

CAFs constitute a highly plastic and heterogeneous population and may arise from normal fibroblasts, from the mesenchymal cellular lineage or elsewhere, meaning that the origin of CAFs still remains an unsettled question (Sahai et al. 2020). Identification of CAFs is based on the combination of morphology, protein expression, and specific gene expression profiles. The defining markers such as vimentin, alpha-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), and platelet-derived growth factor alpha (PDGF-α) could be used to define CAFs (Ping et al. 2021).

CAFs can contribute to tumour progression in two major ways. One is by secreting factors that promote the proliferation of tumour cells or enhance the tumour-promoting functions of other stromal cells (immune cells, adipocytes, endothelial cells etc.). CAFs produce an array of growth factors (TGF-β, FGF, VEGF and EGF), cytokines (IL-6) and chemokines (CXCL8, CXCL12) that can contribute to tumour cell growth, motility, invasion and epithelial-mesenchymal transition (EMT) (Augsten 2014). In breast cancers, CAFs secrete SDF-1/CXCL12 to recruit endothelial progenitor cells (EPCs) that enhance angiogenesis and contribute to tumour growth (Orimo et al. 2005). The other is by remodelling the ECM into a tumour-promoting form by producing ECM proteins or modifying existing ECM. CAFs determine the nature of the tumour extracellular matrix by deploying secreted or membrane-bound enzymes and ECM components to direct changes in ECM composition and architecture, and therefore its mechanical properties. Various studies confirmed that CAFs produce the ECM components collagen, laminin, periostin and tenascin C, as well as ECM remodelling matrix metalloproteases (MMPs) and the collagen cross-linking enzymes lysyl oxidases (LOXs) (Ishii, Ochiai & Neri 2016).

In human breast cancers, immunophenotyping of CAFs identified four major subpopulations (S1-S4) based on the distinct markers and activation status. Interestingly, different subtypes of CAFs had a separate molecular prolife, were associated with specific locations within the tumour tissue and correlated with distinct breast cancer subtypes. CAF-S3 were found to be mostly adjacent to tumours in multiple BC subsets, and CAF-S2 were located in both tumour and juxta-tumour tissues and were enriched in LumA tumours. CAF-S1 and CAF-S4 were preferentially associated with TNBCs, where CAF-S1 were found to regulate ECM organisation and contributed to the immunosuppressive microenvironment by recruiting $CD4^+CD25^+$ T regulatory cells and accumulated mostly in close proximity to tumour cells. CAF-S4 were characterised by myofibroblastic protein expression and were also highly detected in HER2 positive tumours. Importantly, the link between the immune response and CAF populations was established since the presence of specific subsets of CAFs was also associated with the presence of specific T cell populations. Tumours with higher CAF-S1 population had a higher T-regs to $CD8^+$ T cells ratio, where inverse correlation was observed in CAF-S4 enriched tumours (Costa et al. 2018).

Evidence is progressively accumulating that suggests a significant role for CAFs in regulating immune response in the tumour microenvironment (An et al. 2020). CAFs can secrete pro-inflammatory mediators such as IL1-β, IL-8, IL-10, CCL-2 and CXCL12 contributing to recruitment of tumour-promoting immune cells (Tower, Ruppert & Britt 2019). A recent single-cell analysis of the TNBC patient cohort suggests that there is strong correlation between the inflammatory CAFs and cytotoxic T cell exclusion, thus suggesting that there is a potential for the anti-CAF therapy to complement and advance current immunotherapeutic strategies in breast cancer (Wu, SZ et al. 2020).

Tumour-promoting properties of CAF populations in cancers progression have been extensively confirmed. However, their role as molecular coordinators of the tumour microenvironment is poorly understood, especially in BCs. The emerging roles of CAFs as critical regulators of the tumour immune microenvironment remains poorly understood and is an important area of investigation since it harbours great potential for improving immune therapy treatments of cancers (Kochetkova & Samuel 2021).

1.2.4.2. Cancer Associated Adipocytes

Adipocytes are the lipid-storing cells of adipose tissues (AT) that regulate energy storage and metabolism within the body. Adipocytes mainly arise from mesenchymal stem cells (MSCs)

or undifferentiated adipocyte precursors within adipose tissue stroma (Rodeheffer, Birsoy & Friedman 2008; Tang & Lane 2012). A small proportion of adipocytes can also be derived from hematopoietic stem cells (HSCs) (Sera et al. 2009).

Tumour cells can 'activate' adipocytes and subvert their cellular programs to facilitate tumour-promotion. Such activated cancer-associated adipocytes (CAAs) are distinct from normal adipocytes in morphology and function. Adipocytes co-cultured with cancer cells exhibit de-lipidation, decreased expression of adipocyte markers such as Ap2 and FABP4, increased expression of MMP11, and enhanced release of inflammation-promoting cytokines IL-6 and IL-1β. Importantly, the presence of CAAs expressing IL-6 was confirmed ex vivo using primary breast cancer samples (Dirat et al. 2011).

CAAs secrete hormones and other molecules, collectively termed adipokines, including leptin, adiponectin, IL-6, CCL-2 and CCL-5 that exert paracrine and endocrine regulatory roles in inflammation, tumourigenesis and cancer metabolism, and promote tumour cell proliferation, invasion and metastasis (Zhao, C et al. 2020).

CAAs contribute to cancer in a number of ways. Mature human breast adipocytes increase cell motility of both pre-malignant and malignant breast cancer cell lines (Carter & Church 2012). In vitro studies show that paracrine signalling from cancer cells induces the release of free fatty acids from CAAs resulting in CAA de-lipidation and increased secretion of inflammatory cytokines and proteases that promote tumour cell invasiveness (Park & Scherer 2012). When co-cultured with breast cancer cells, primary adipocytes derived from breast cancer patients reverted to a more immature phenotype, secreting IL-6 and CCL-2 and promoting cancer cell migration (Fujisaki et al. 2015).

Intriguingly, adipocyte-immune interaction is now also emerging in the context of immune checkpoint inhibition in breast cancer. A recent study shows that adipocyte-specific PD-L1 (programmed death-ligand 1) can cause tumour immunosuppression, enhanced mammary tumour growth and inhibition of anti-tumour immunity in mammary cancers (Wu, B et al. 2020).

The tumour-promoting roles of CAA and their interaction with cancer cells have been extensively described. Yet, the contribution of the CAA in regulating immune components or other cell types in the tumour microenvironment, remains currently under investigated (Wu et al. 2021). Given CAA strongly contribute to the metabolic characteristics of the tumour cells (Nieman et al. 2011), the roles of CAA in regulating immune responses via metabolic control of the immune microenvironment should be carefully investigated.

1.2.4.3. Endothelial cells and tumour angiogenesis

Endothelial cells are tightly adherent cells that line the inner blood vessel walls. Specific signalling molecules (e.g., vascular endothelial growth factor (VEGF), angiopoietin, and fibroblast growth factor (FGF)) that engage receptors displayed on the surface of the vascular endothelial cells help to promote their growth (Hanahan & Weinberg 2011; Quail & Joyce 2013).

In breast cancers, tumour vascularisation is largely caused by a hypoxic microenvironment and mediated by hypoxia-inducible factors (HIF-1 and HIF-2). HIF pathway activation in the cancer cells induces the production of pro-angiogenic factors including VEGF, angiopoietins and basic fibroblast growth factor (bFGF) that mediate angiogenesis (Madu et al. 2020). Other components of the microenvironment can contribute to the pro-angiogenic milieu. Tumour-associated macrophages (TAMs) secrete CCL-18, and CAFs can contribute to vascularisation by releasing pro-angiogenic factors such as VEGF, SDF-1 and TGF-β into the tumour stroma thereby enhancing tumour angiogenesis (Lin, L et al. 2015; Wang et al. 2019). Higher microvascular density in breast cancers is associated with larger tumour size, high histological grade and overall poor prognosis (Madu et al. 2020).

1.2.4.4. Extracellular matrix (ECM)

The extracellular matrix provides structural and biochemical support for the cells, and a tightly regulated balance exists between cells and the surrounding matrix. Based on biochemical and structural properties, the ECM is subdivided into two distinct components, the basement membrane (at the basal surface of epithelial or endothelial cells) and the interstitial (stromal) matrix. The basement membrane consists of collagen IV, laminin, fibronectin, and several types of proteoglycans. The main role of the basement membrane is to provide a physical barrier between the epithelial cells and the mesenchyme. The interstitial matrix is mainly deposited by mesenchymal cells and consists of collagens I and III and fibronectin. In solid cancers, the basement membrane is often degraded, and activated epithelial cells can undergo an epithelial to mesenchymal transition (EMT) and migrate into the surrounding stroma. Such activated epithelial cells can then provide signals for the activation of the stromal cells thus creating a tumour-promoting microenvironment (Malik, Lelkes & Cukierman 2015).

In normal ECM, filaments are arranged at random (homeostatic parenchyma), however organised straight fibres are characteristic of pathological conditions (desmoplasia) and it has been observed that the presence of organised fibres of the ECM correlates with poor patient

outcome. It has been shown that such organised fibrils provide 'trails', which migratory cancer cells can use to their advantage. The main regulators of ECM remodelling during tumourigenesis are CAFs of myofibroblastic nature, which deposit high levels of collagen I, fibronectin and periostin (Malik, Lelkes & Cukierman 2015).

Cell-ECM interactions are mainly mediated via integrins, which constitute a large family of cell-surface receptors. Integrins also mediate cytoskeleton organisation and can activate intracellular signalling pathways, conveying both mechanical and chemical signalling. Besides their roles in cell adhesion and migration they also transmit signals for cell proliferation and survival. The majority of integrins activate focal adhesion kinase (FAK). This, in turn can promote directional cell motility of both tumour and stromal cells, and further modify ECM organisation altering mechanical properties of the tumour microenvironment (Guo & Giancotti 2004; Malik, Lelkes & Cukierman 2015).

1.2.5. *The Immune Microenvironment of Breast Cancer*

The nature of the immune microenvironment and its interaction with tumour cells in breast cancer progression is complex. Tumour cells and other components of the tumour microenvironment influence the recruitment and the composition of the immune infiltrating cells within the breast tumour (Gatti-Mays et al. 2019).

Our current understanding of breast cancer immunology suggests that there are broadly two categories of immune cells that induce either tumour-promoting or tumour-supressing effects. Tumour suppression is supported by natural killer (NK) cells, CD8+ T cells, $CD4^+$ Th1 cells, B cells, M1 type macrophages and dendritic cells (DCs) and mediated via tumour inhibitory cytokines such as IFN-γ, TNF-α, IL1-β, IL-2 and IL-12, as well as cytotoxic granules (granzymes and perforins) (Nagarajan & McArdle 2018; Thorsson et al. 2018).

The tumour-promoting immune cells include regulatory $CD4^+$ Th2 cells, T regulatory cells (Tregs), M2 type macrophages and myeloid-derived suppressor cells (MDSCs) and create pro-tumourigenic microenvironment via release of cytokines such as TGF-β, VEGF, IL-6, IL-8, and IL-10. Such influx of the immune cells in turn, may lead to immunosuppression, immune evasion, or potentiation of tumour-promoting inflammatory responses that drive cancer progression (Tower, Ruppert & Britt 2019).

To understand the nature of immune cell responses in breast cancers, the discussion of the cancer immunoediting hypothesis is necessary. The cancer immunoediting hypothesis

postulates that there are three major stages in immune system-cancer cell interactions: elimination, equilibrium, and escape (Dunn, Old & Schreiber 2004).

In the early stages of tumour development, as a result of oncogenic transformation, cancer cells express tumour-specific antigens. Immune cells recognise these antigens and initiate a potent anti-tumour response. If the elimination phase is successful, tumour suppression occurs. If, however tumour cells persist, they mutate under selective pressure of the elimination phase, producing tumour cell clones that are less immunogenic and entering an equilibrium phase where tumour dormancy may occur. Finally, tumour cell modifications lead to an immunosuppressive microenvironment, which leads to the proliferation of tumour cells that evade the immune response, leading to tumour growth (Dunn, Old & Schreiber 2004; Tower, Ruppert & Britt 2019).

In breast carcinomas, the most prominent immune cell types identified are tumour infiltrating lymphocytes (TILs) and macrophages (Hussein & Hassan 2006; Ruffell et al. 2012). These two classes of tumour infiltrating immune cells have prognostic value in breast cancers and are important indicators of the responses to the therapy and the progression of the disease (Gatti-Mays et al. 2019). **Figure 1.8** illustrates a broad overview of the breast cancer immune microenvironment.

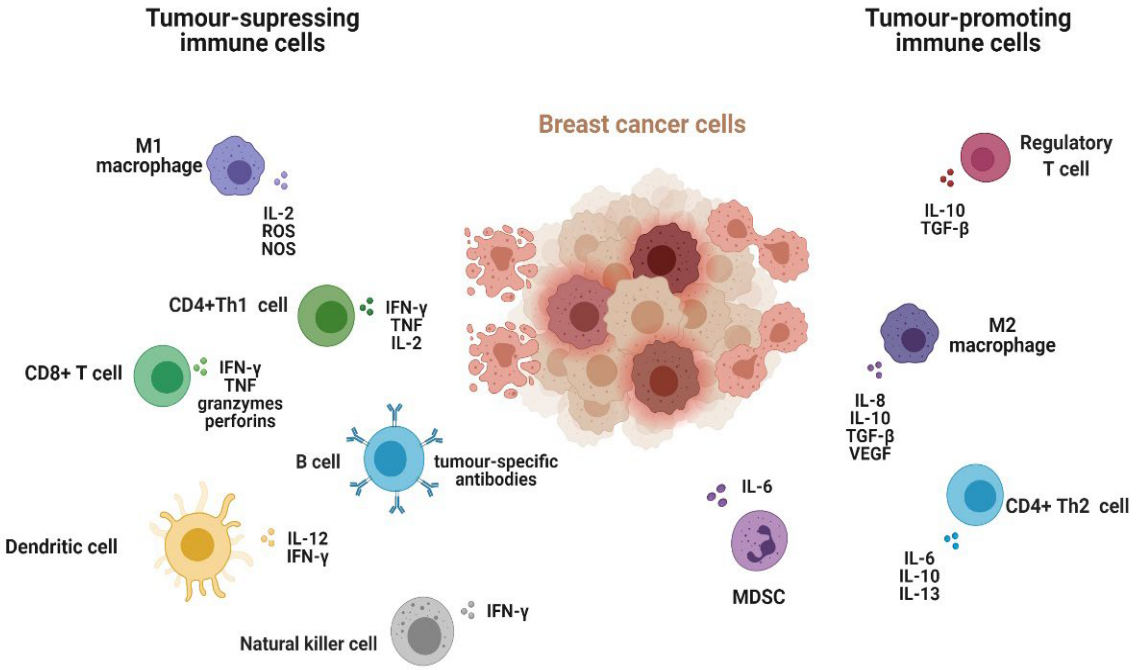


Figure 1.8: The immune microenvironment of breast cancer.

The breast cancer immune microenvironment consists of cells that either supress or promote tumourigenesis. Tumour suppression is supported by natural killer (NK) cells, CD8+ T cells, $CD4^+$ Th1 cells, B cells, M1 type macrophages and dendritic cells (DCs) and is mediated via tumour inhibitory cytokines such as IFN-γ, TNF-α, IL-2, and IL-12, cytotoxic granzymes, perforins, reactive oxygen, and nitrogen species (ROS, NOS). Tumour-promoting immune cells include regulatory $CD4^+$ Th2 cells, T regulatory cells (Tregs), M2 type macrophages and myeloid-derived suppressor cells (MDSCs) and create pro-tumourigenic microenvironment via release of tumour-promoting cytokines such as TGF-β, VEGF, IL-6, IL-8, and IL-10. Adapted from (Gatti-Mays et al. 2019; Tower, Ruppert & Britt 2019).

1.2.5.1. Tumour infiltrating lymphocytes (TILs)

Tumour infiltrating lymphocytes (TILs) consist of the lymphocytic cell populations that infiltrate the tumour tissue. In breast cancers, the major components of TILs are cytotoxic $CD8^+$ T cells, $CD4^+$ T cells, T regulatory cells and B cells (Basu, A et al. 2019).

T cells are the component of the adaptive immune responses that have the high specificity and capacity for immunological memory. Both CD4+ and $CD8^+$ T cells require MHC-mediated antigen presentation to be activated and mature into the effector cells that can exert their functional roles.

$CD8^+$ T cells are the main anti-tumour effectors in breast cancers. $CD8^+$ T cell maturation depends on the direct engagement of the TCR with MHC class I receptors that are expressed on the target cells and leads to activation of the cytotoxic effector responses. This activation leads to the release of granzymes and perforins that directly kill target cells, and other cytokines such as IFN-γ and TNF-α that create tumour-suppressive microenvironment (Basu, A et al. 2019; Edechi et al. 2019). Invasive breast cancer tissues have elevated levels of $CD8^+$ cytotoxic T lymphocytes (CTLs) and their number positively correlates with the increased survival in HER2+ and triple negative breast cancer patients (Mahmoud et al. 2011; Salgado et al. 2015). Compared to the invasive BCs, the pre-invasive tissues harbour significantly higher number of activated effector cytotoxic T cells expressing IFN-γ (Gil Del Alcazar et al. 2017).

$CD4^+$ T cells maturation occurs via co-stimulatory signalling between T cell receptor (TCR) and MHC class II (expressed on antigen-presenting cells such as dendritic cells (DCs), B cells and macrophages). $CD4^+$ cells mainly secrete a plethora of diverse immunomodulatory cytokines that create either tumour-promoting or tumour supressing microenvironment. $CD4^+$ T cells can differentiate into effector cells, including T helper 1 (Th1) that secrete IL-2, INF-γ, TNF-α and engage macrophages and have antitumour effects. T helper 2 (Th2) cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 that block the activity of antitumour effector T cells and recruit tumour promoting macrophages (Basu, A et al. 2019; Edechi et al. 2019). Increased Th2 to Th1 cell ratios in the primary breast tumours correlate with reduced overall survival of breast cancer patients (Ali et al. 2016).

Regulatory T cells (Tregs) are $CD4^+$ T cells that express Forkhead box P3 (FOXP3) protein. The conventional role of Tregs is to regulate self-tolerance in exhibiting immunosuppressive functions. However, in the context of breast cancers, Tregs contribute to pro-tumour responses, secreting IL-10, supressing activities of cytotoxic $CD8^+$ T cells and $CD4^+$ Th1 cells (Gupta et al. 2007). Ablation of Tregs in advanced primary tumours in mice promotes anti-tumour responses by $CD4^+$ Th1 cells (Bos et al. 2013). High Treg infiltration in the breast tumour is associated with shorter overall survival of breast cancer patients (Bates et al. 2006). The high ratio of $FOXP3^+/CD8^+$ T cells in the luminal breast cancers is associated with poor prognosis and reduced disease-free survival (Fukui et al. 2020), consistent with the immune suppressive functions of these cells.

B cells, components of the adaptive immune system, have the capacity to create antibodies that are highly specific for tumour antigens. Antibodies released by the B cells act via neutralising cancer cells or activating other immune responses against the cancer cells (Basu, A et al. 2019; Edechi et al. 2019). The prognostic value of B cell infiltrates and the roles of B cells in breast

cancer are still unresolved since evidence supports both pro-tumour and anti-tumour roles of B cells. Some reports indicate that B cell infiltration correlates with improved clinical outcomes for breast cancer patients, however there is also evidence for pro-tumourigenic effects of B cells secreting IL-10, and higher density of B cells infiltrating tumours is associated with poor prognosis (Kuroda et al. 2021; Shen, Wang & Ren 2018), suggesting that a careful examination of B-cells in situ mammary tumours and the confirmation of their functional responses in vivo in relation to their anti- or pro-tumourigenic properties is further required.

1.2.5.2. Tumour associated macrophages

Macrophages belong to the innate component of the immune system of myeloid lineage, the phagocytic cells that can engulf cells that express abnormal antigens. They are highly adaptable cells and exhibit a high degree of plasticity depending on the signals in their immediate environment (Qian & Pollard 2010). In breast cancers, tumour associated macrophages (TAMs) can differentiate from peripheral blood circulating monocytes originating in either the bone marrow or the spleen (Franklin et al. 2014), or the tissue resident macrophages of breast stroma can also be converted to TAMs (Tower, Ruppert & Britt 2019).

Macrophages are largely influenced by the signals produced by the cancer cells, hypoxia-induced factors and the cytokines from other immune cells recruited at the tumour site. Tumour-associated macrophages are sometimes referred to as existing in a continuum of functional states characterised by extremes designated M1 and M2. Macrophages polarised towards the 'M1' state are 'classically' activated and are tumour suppressive. This population is activated by IFN-γ, TNF-α and tumour antigens and has antitumour properties by producing pro-inflammatory agents such as IL-2, reactive nitrogen, and oxygen species (Gabrilovich, Ostrand-Rosenberg & Bronte 2012; Qiu et al. 2018). In breast cancer tissues, a higher proportion of M1 population has been associated with better patient prognosis (Bense et al. 2017).

Macrophages polarized by IL-10 and TGF-β signals towards the 'M2' state are 'alternatively' activated. They secrete anti-inflammatory cytokines such as IL-8, IL-10, TGF- β, and VEGF and EGF that largely suppress inflammatory responses, contribute to the angiogenesis in tumour tissues and promote tumour proliferation and fibroblast activation (Goswami et al. 2005; Wyckoff et al. 2004). In breast cancers, M2 macrophages can recruit Tregs and can hinder CD8+ T cell mediated anti-tumour responses (Gabrilovich, Ostrand-Rosenberg & Bronte 2012; Peranzoni et al. 2018). In breast biopsies, the M2 macrophage presence in the

stroma confirms the presence of the breast lesion (Sousa et al. 2015) and correlates with poor prognosis of breast cancer patients (Ali et al. 2016).

1.2.5.3. Immune Landscape of Breast Cancers and IFN-γ-mediated Responses

Given the complexity of the interaction between tumour cells and the immune components during the breast cancer progression, it is important to point out that the immune landscape of breast cancer is highly diverse and heterogenous across patients and cancer subtypes. There are several attempts to classify the breast cancer immune landscape based on the subtypes and disease stage which can be then used in patient treatment and prognostic applications.

A recent study based on gene signatures from The Cancer Genome Atlas database, identified six separate immune subtypes that are common to cancers based on gene signatures of macrophages, lymphocytes, Th1/Th2 cell ratios and several tumour cell characteristics. There were designated wound healing, IFN-γ dominant, inflammatory, lymphocyte depleted, immunologically quiet and TGF-β dominant (Gatti-Mays et al. 2019; Thorsson et al. 2018).

The most immunogenic subtypes in breast cancers are IFN-γ dominant, wound healing and inflammatory. IFN-γ dominant subtype is classified having the highest M1/M2 macrophage ratio and strong CD8+ T cell signal. Wound healing had elevated expression of angiogenic genes and Th2 cell signatures. The inflammatory subtype of breast cancers had elevated Th17 and Th1 genes and lower tumour cell proliferation signatures (Thorsson et al. 2018).

IFN-γ is a pleiotropic cytokine, the only member of the type II interferon family, and has a variety of immunomodulatory functions that act on both innate and adaptive immune systems (Jorgovanovic et al. 2020). **Figure 1.9** represents IFN-γ signalling pathway. IFN-γ signalling is mediated via receptors IFNγR1 and IFNγR2 and downstream engagement of Janus activated kinase (JAK) 1 and 2 and subsequent phosphorylation of signal transducer and activator of transcription STAT1. STAT1 oligomers translocate to the nucleus and bind gamma-activated site (GAS) of primary IFN-γ response genes. Further secondary response genes are activated via STAT1-mediated transcription of interferon-regulatory factor 1 (IRF1) which binds interferon-stimulated response element (ISRE). These secondary response genes initiate transcription of a plethora of genes mediating apoptosis, DNA damage and of molecules involved in direct tumouricidal effects. IFN-γ signalling also orchestrates upregulation of cytokine and chemokine receptors, membrane receptors, cellular adhesion proteins, and cellular signalling that have proinflammatory and anti-tumour properties (Castro et al. 2018).

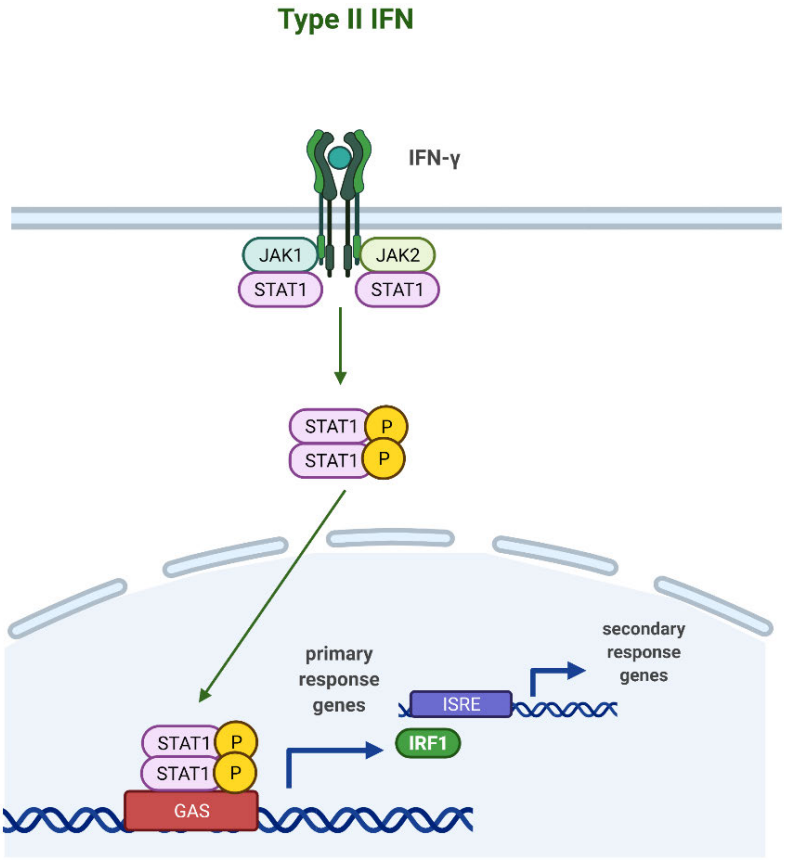


Figure 1.9: Schematic representation of IFN-γ signalling pathway

IFN-γ is a pleiotropic cytokine that activates IFN-γ pathway signalling via IFNγR1 and IFNγR2. IFNγR oligomerisation activates downstream targets Janus activated kinase 1 and 2 (JAK 1 and JAK2). Further, phosphorylation of IFNγR by JAKs creates binding site for the signal transducer and activator of transcription 1 (STAT1). Phosphorylated STAT1 homodimerizes and translocate to the nucleus and binds to gamma-activated site (GAS) activating primary response genes. Upon induction, transcription factor interferon-regulatory factor 1 (IRF1) binds to interferon-stimulated response element (ISRE) and enhances the transcription of secondary response genes that mediate a plethora of downstream immunomodulatory and anti-tumour functions. Adapted from (Castro et al. 2018; Jorgovanovic et al. 2020).

In breast cancers, IFN-γ plays a critical role in regulating anti-tumour immune responses. It is secreted predominantly by NK T cells and activated lymphocytes, both $CD8^+$ cytotoxic T cells and Th1 $CD4^+$ T cells (Heimes et al. 2020).

The direct tumouricidal effect of IFN-γ on the cancer cells has been described in prostate cancer and in non-small-cell lung carcinomas (NSCLC) via activation of apoptosis-associated genes including caspases 3,7, 8, 9, as well as BAD and BID (Guinn, Brown & Petro 2017; Kundu,

Roy & Pahan 2017). However, the major anti-tumour effects of IFN-γ are associated with the tumour microenvironment and immune cell recruitment and regulation.

IFN-γ has anti-angiogenic effects in solid tumours leading to blood vessel destruction and tumour necrosis (Briesemeister et al. 2011). In a fibrosarcoma engraftment model, IFN-γ acted on cancer-associated fibroblasts inhibiting VEGF-A production leading to suppression of tumour angiogenesis (Lu, Y et al. 2009).

IFN-γ has an important role in polarisation of M1 macrophages and subsequent sustained production of IFN-γ by cytotoxic $CD8^+$ lymphocytes (CTLs) further enhance M1-mediated anti-tumour responses (Baer et al. 2016). Conversely, IFN-γ secreted by CTLs indirectly affects the metabolic M2 macrophage suppression in melanoma model (Liu, C et al. 2019). Interestingly, in a melanoma model, Tregs reprogrammed to secrete IFN-γ had an inhibitory effect on surrounding Treg populations and led to anti-tumour immunity (Overacre-Delgoffe et al. 2017).

While there is a paucity of information regarding molecular roles for IFN-γ specifically in the breast cancer microenvironment, the prognostic and predictive effects of IFN-γ signatures in breast cancer patient samples have been described and are under current investigation in relation to checkpoint inhibition in breast cancers (Heimes et al. 2020).

More than two decades ago, the IFN-related gene signature was initially outlined in breast cancers (Perou et al. 2000). However, only several years later the research relating IFN-related gene signature from breast cancer biopsies to the favourable response to chemotherapy and radiotherapy in breast cancer patients emerged (Weichselbaum et al. 2008). Importantly, the IFN-γ related gene signature predicts clinical response to immune therapy with inhibitors of programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand (PD-L1) in breast cancers (Nakayama et al. 2019).

Moreover, in breast cancers, it appears that IFN-γ is an important player in subtype specific disease. This is particularly evident in HER2 positive breast cancers that are an aggressive type of breast cancer and are associated with high relapse and mortality rates (Harbeck et al. 2019). The combination therapy of IFN-γ and anti-ErbB2 antibody on inhibition of mammary tumour growth in vivo was associated with increased M1 accumulation and increased CTLs cytotoxicity in the tumours (Nagai et al. 2017). An ongoing clinical trial investigating combination therapy of anti-HER2 inhibitors transtuzumab and petuzumab with IFN-γ in HER2+ breast cancer patients is currently in Phase II (NCT03112590). A recent study also found that combination of anti-HER2 therapy and IFN-γ reduces growth of therapy resistant

HER2 expressing breast cancer cells, suggesting that the combination therapy may help to overcome the resistance to HER2-targeted therapies (Jia et al. 2021).

All of the above information shows that understanding the molecular roles of IFN-γ in the tumour microenvironment and the regulators of IFN-γ signalling are crucial in further expanding therapeutic options for subtype-specific breast cancers.

1.3. The 14-3-3 Protein Family

1.3.1. *Introduction*

In mammals, the 14-3-3 protein family consists of seven highly evolutionary conserved, small (28–33 kDa) phospho-serine binding members (β, γ, ε, σ, ζ, θ/τ, η). 14-3-3 proteins form obligate homo- or heterodimers and the crystal structures of all seven family members confirm their dimeric arrangement (Aitken 2006; Obsil & Obsilova 2011).

Each monomer has nine antiparallel helices. The two monomers combined in a dimer arrangement form a wide channel **Figure 1.10**.

This wide channel has a conserved sequence region inside with a net negative charge, while the variable regions are located on the outside. The conserved region is the location of the phospho-serine binding with the target protein.

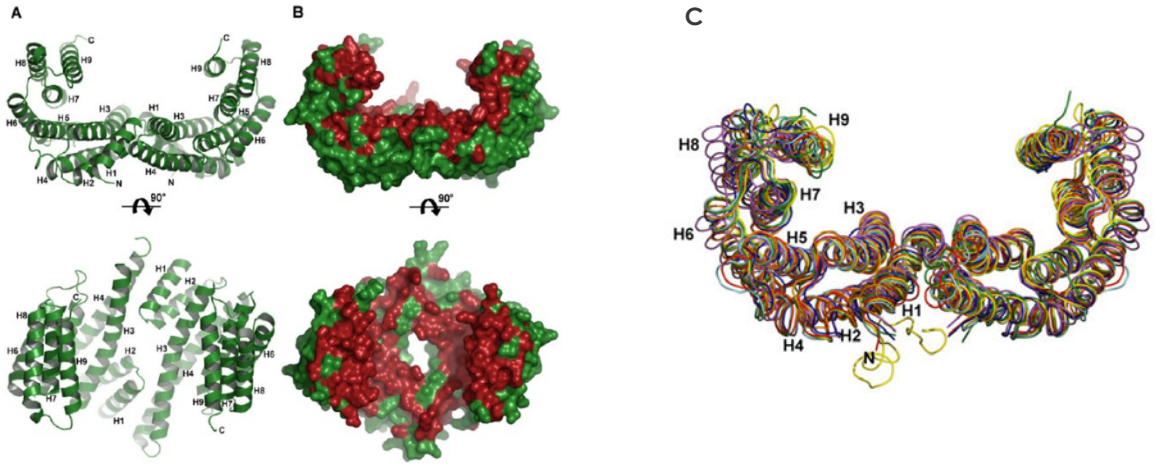


Figure 1.10: Crystal structure of the 14-3-3ζ protein and the superimposed structures of all 14-3-3 family members.

The ribbon **(A)** and surface **(B)** representations. Conserved regions in all family members in red. **(C)** Superimposed seven human 14-3-3 isoforms. Adapted from (Obsil & Obsilova 2011).

This dimeric arrangement permits 14-3-3 dimers to have a defined architecture and promote conformational changes in binding partners. 14-3-3 proteins bind phosphorylated serine or threonine on their clients within the consensus motif RXXpS/TXP (Yaffe et al. 1997). 14-3-3's interaction with a client protein can alter this protein's catalytic activity, prevent protein degradation, destabilise its conformation or alter its subcellular localisation (Morrison 2009). Another mode of operation can include steric hindrance, when 14-3-3 binding can obscure intrinsic localisation, phosphorylation, or other motifs on the target proteins. Since each monomer has independent ligand-binding channels, the dimer can simultaneously bind two sites, either on single or separate targets (Aitken 2006).

1.3.2. *14-3-3 Proteins and the Regulation of Major Cellular Functions in Homeostasis and Cancer*

14-3-3 proteins are known to interact with over 200 protein clients and regulate the majority of cellular functions such as control of the cell cycle, survival, apoptosis, intracellular trafficking, cytoskeletal arrangement, transcription, and metabolism (Aghazadeh & Papadopoulos 2016).

14-3-3 proteins increase the activity of proliferative and pro-survival proteins via interaction with Raf-MAPK, Hippo and PI3K-PDK-AKT signalling networks (Morrison 2009). 14-3-3 interacts with Cdc25C to regulate cell cycle progression (Peng et al. 1997). It has also been shown that 14-3-3 can antagonise the apoptosis-promoting proteins (BAD, BIM, BAX) and thus sustain cell survival (Zha et al. 1996). 14-3-3s are also involved in regulation of cell growth signalling via the mTOR pathway. The 14-3-3 proteins bind four regulators of the mTOR complex 1 (TORC1), thus promoting cell growth (Kleppe et al. 2011).

14-3-3 is a negative regulator of the cofilin regulator slingshot1-like (SSH1L), cortactin (an F-actin-binding protein that is enriched in membrane ruffles, lamellipodia and invadopodium) and IRSp53, which are implicated in actin remodelling (Freeman & Morrison 2011).

Another essential process that involves 14-3-3 is an epithelial to mesenchymal transition), during which epithelial cells acquire mesenchymal characteristics. 14-3-3 binds Snail, a key transcriptional regulator of EMT, eliciting positive or negative effects depending on the phosphorylation sites it binds (Hou et al. 2010) .

14-3-3s play key roles in the regulation of major cellular processes and signalling networks in homeostasis. Thus, it is not surprising that over the last decade, 14-3-3 proteins have been

found to have roles in cancer progression in connection with dysregulation of proliferation, cell cycle control, apoptosis, signal transduction, intracellular trafficking, cytoskeletal arrangement, transcription, and metabolism (Pennington et al. 2018).

Most of the 14-3-3 isoforms are upregulated in a variety of cancers including breast, lung, pancreatic, head and neck, colon, and ovarian (Fan, X et al. 2019). In this section, I will briefly discuss the roles of 14-3-3 isoforms in breast cancer, and in the following section will mainly focus on roles of 14-3-3ζ in cancers. The majority of studies on 14-3-3 isoforms in breast cancer have been undertaken in cell lines in vitro, and only a few studies combine in vivo and in vitro approaches. Therefore, their conclusions must be carefully examined in the context of the specific system employed.

Ectopic expression of 14-3-3β in MCF-7 breast cancer cells leads to enhanced direct interaction between 14-3-3β and ERα which leads to increased cellular proliferation (Kim et al. 2011). 14-3-3γ was located in the pseudopodia in MDA-MB-231 breast cancer cell line and its level was associated with increased invasion (Hiraoka et al. 2019). Ectopic expression of 14-3-3γ promotes invasion of the MDA-MB-231 cell line in an experimental model of metastasis in mice (Yoo et al. 2016). Knock-down of 14-3-3ε in MDA-MB-231 cell line inhibited invasion and migration and reduced the expression of promoters of EMT Snail and Twist (Yang, YF et al. 2019). The molecular roles of 14-3-3η in breast cancer are poorly understood. One study identified significantly higher expression of 14-3-3η in the ducal carcinomas in breast cancer patients compared to normal tissues (Liang, Singh & Gam 2010). However, there is no evidence of any prognostic value for this isoform in breast cancers (Mei et al. 2020). Overexpression of 14-3-3θ is associated with reduced survival in breast cancer patients and the knockdown of 14-3-3θ in MDA-MB-231 cells inhibited tumour growth and metastatic burden in mouse xenograft models (Li, N et al. 2014). The studies highlighted provide initial evidence that specific 14-3-3 isoforms may have defined functions in a tissue-specific context. Thus, further investigation in the roles of specific 14-3-3 isoforms is paramount and may reveal unique vulnerabilities of cancer cell types that could be further targeted in cancer treatments.

The analysis of tissue samples from cancer patients has revealed peculiar patterns in expression of 14-3-3σ and ζ. 14-3-3σ is expressed in normal epithelial cells and almost exclusively forms homodimers. For a very long time it was considered a tumour suppressor because of its known role in regulating p53 during DNA damage (Yang, HY et al. 2003). The downregulation of the 14-3-3σ protein has been observed in a variety of epithelial cancers, and recently it has been shown that in ErbB2-overexpressing breast cancers, the loss of 14-3-3σ contributes to disease progression. At the same time, various reports show that overexpression of 14-3-3σ correlates

with worse patient outcome in prostate and colorectal as well as pancreatic cancers. Thus, the previously well-accepted role of the σ isoform as a tumour suppressor is now less clear (Freeman & Morrison 2011). Therefore, such tissue-specific roles for 14-3-3 isoforms call for more careful examination in the context-specific, tissue-specific, and cell-specific manners.

1.3.3. *The Roles of 14-3-3ζ in Cancer*

14-3-3ζ is abundantly expressed in a number of tumours and this often correlates with disease recurrence and poor patient survival (Neal et al. 2009). Several reports confirm the tumour-promoting roles for 14-3-3ζ in multiple cancers including hepatocellular carcinoma, and colorectal, lung, and prostate cancers (Gan, Ye & He 2020; Mei et al. 2020). The roles of 14-3-3ζ has been investigated in vivo in an overexpression model in context of breast cancer (Rehman et al. 2014), as well as in 14-3-3ζ-deficient mouse models in the context of skin cancer (Kular et al. 2015), breast cancer (Joshi et al. 2017) and also in pathology such as impaired adipocyte differentiation (Lim et al. 2015) and schizophrenia (Ramshaw et al. 2013). **Table 1.1** summarises major roles for the 14-3-3ζ identified in different cancer types.

Table 1.1: 14-3-3ζ functions and molecular interactors by cancer type

Cancer type	Function	Target pathway/molecule	References
Hepatocellular carcinoma	Increased cell proliferation, increased invasion, inhibition of apoptosis	AKT, ERK1/2, Caspase-3, TGF-β	(Reichl et al. 2015; Zhao, JF et al. 2018)
Colorectal cancer	Increased cell proliferation and invasion, EMT	Foxo3, β-catenin, Snail, E-cadherin	(Li, YY et al. 2015; Mulvey et al. 2015)
Lung cancer	Increased cell proliferation and invasion, EMT	PKC/NF-κB, Snail, β-catenin	(Chen, CH et al. 2012; Tong et al. 2016)
Prostate cancer	Increased cell proliferation and survival, enhanced cell motility	Androgen receptor, Rac1	(Goc et al. 2012; Murata et al. 2012)

High levels of 14-3-3ζ expression has a dramatic effect on similar cellular processes and client proteins in multiple cancers. There are common cellular functions that are affected, namely enhanced cell proliferation, increased migration, invasive phenotype, EMT and inhibition of apoptosis. These major functions become corrupted via common signalling pathways and their effectors such as PI3K/Akt; TFG-β; ERK1/2; Wnt/β-catenin Snail; Caspase-3 and Bax (Gan, Ye & He 2020).

Below I summarised in detail major findings regarding the role of 14-3-3ζ in breast cancer from the published literature available to date.

14-3-3ζ overexpression promotes breast cancer cell proliferation via upregulation of MAPK/c-Jun signalling and increase in miR-221 transcription (Rehman et al. 2014). Overexpression of 14-3-3ζ activates PI3K via p85, leading to breast cancer cell survival and proliferation (Neal et al. 2012). Tamoxifen chemotherapy leads to miR-451 downregulation, which in turn results in 14-3-3ζ overexpression and promotion of survival of breast cancer cells (Bergamaschi & Katzenellenbogen 2012). In mammary epithelial cells, 14-3-3ζ destabilisation of p53 leads to 14-3-3σ downregulation and disabling of TGF-β tumour suppression (Xu et al. 2015). These studies highlight that the role of 14-3-3ζ have been mainly addressed in the context of the cancer cell function and have relied on cell lines and siRNA knock down approaches (Bergamaschi & Katzenellenbogen 2012; Jin et al. 2016; Lu, J et al. 2009; Neal et al. 2012; Neal et al. 2009; Rehman et al. 2014; Xu et al. 2015). Yet, to date there is only a handful of studies that have addressed the roles of 14-3-3ζ in the context of the tumour microenvironment, and they have revealed surprising results. Although the majority of studies link 14-3-3ζ overexpression in tumour promotion, a study from our laboratory identified 14-3-3ζ as a negative regulator of signalling through Rho-associated kinase (ROCK) and strongly implicates the absence of 14-3-3ζ in tumour progression in skin cancer. In 14-3-3ζ-deficient dermal fibroblasts, signalling via the ROCK pathway is enhanced. This causes ECM stiffening, enhanced cell proliferation and leads to increased tumour size of cutaneous squamous cell carcinoma (SCC) (Kular et al. 2015). A study in hepatocellular carcinoma revealed that high 14-3-3ζ levels impair T cell mediated anti-tumour responses (Wang, X et al. 2018). Yet to date, no studies carefully examined the role of 14-3-3ζ in breast cancer microenvironment. Thus, a careful examination of the specific role of 14-3-3ζ is required in the tissue- and cell-specific contexts in vivo to dissect its contribution to the tumour-promoting or inhibiting roles in the tumour microenvironment.

Since it has been found that in several cancers, including breast, 14-3-3ζ overexpression correlates with disease relapse in patients, 14-3-3ζ has been proposed as both a potential biomarker of cancer progression (Neal et al. 2009) and as therapeutic target in multiple cancers (Woodcock et al. 2015; Yang, X et al. 2012). These tumour-promoting roles have further propelled an interested in the development of the pharmacological 14-3-3 inhibitors.

1.3.4. *Inhibitors of 14-3-3*

Broadly, modulators of 14-3-3 function can be classified into two categories, inhibitors, or stabilisers. A recent review describes ~30 specific small molecular inhibitors of 14-3-3. None of the described modulators are isoform-specific, however some show higher affinity for specific isoforms over others. Most of these inhibitors bind in the phospho-client binding groove of 14-3-3, and so inhibit by competing with client binding (Stevers et al. 2018).

Such compounds as xanthine and lacosamide derivatives are found to bind the ζ isoform with a higher degree of specificity. Even though 14-3-3 isoforms are encoded by different genes, they share a high degree of sequence similarity, making it difficult to create isoform-specific modulators (Hartman & Hirsch 2017).

Recently, our laboratories have identified a radically different approach to targeting 14-3-3 proteins: disruption of the 14-3-3 dimer using derivatives of FTY720 (Fingolimod). This approach was based on previous observations that phosphorylation of the 14-3-3 dimer interface was able to disrupt 14-3-3 dimerisation and function and that sphingosine, a lipid long associated with the induction of cell death, was a physiological regulator of 14-3-3 (Ma, Y et al. 2005; Woodcock et al. 2010). The synthetic sphingosine analogue FTY720 and other sphingo-mimetic molecules (alkylated trimethyl ammonium or TMA) were able to enhance 14-3-3 phosphorylation by PKA and PKCδ. This led to the development of a new type of sphingo-mimetic molecules that disable 14-3-3 dimerisation. Indeed, compounds RB-011, RB-012, RB-066, RB-067 and RB-068 were able to induce apoptosis in vitro, with RB-011 and RB-012 exhibiting greatest potency. Furthermore, RB-012 significantly reduced tumour burden in a mouse model of non-small cell lung cancer (Woodcock et al. 2015).

1.4. Summary

Breast cancer is the most diagnosed cancer in females and accounts for ~14% of all cancer-related deaths among women worldwide. Intense focus on the breast cancer research has furthered our understanding about the molecular characteristics of this malignancy and spurred great achievements in terms of therapeutic options for breast cancer patients and their

outcomes. Yet, currently, the majority of therapies still mainly target cancer cells, and it is only recently, as novel research into the tumour-promoting roles of the crucial components of the tumour microenvironment is emerging, the opportunities for combination therapies that targeted both cancer cells and their immediate microenvironment become a possibility. Still, since the complexity of the tumour cell and microenvironment co-evolution along the continuum of the disease is becoming more apparent, it also makes further investigation into the contribution of the key components of the tumour microenvironment a priority. Such knowledge will be informative of improving current therapies for metastatic breast cancer disease that currently remains incurable.

In my thesis, I examined the role of 14-3-3ζ in normal mammary development with a view that this could provide molecular clues to tumour progression. Furthermore, I investigated the contribution of 14-3-3ζ to the tumour-promoting properties of cancer cells in vivo and addressed the possibility of pharmacological inhibition of 14-3-3 proteins in mammary cancers. Finally, I examined the role of 14-3-3ζ and its impact on the immune tumour microenvironment in the context of breast cancer progression with the aim of finding approaches to enhance therapeutic outcomes for breast cancer patients.

1.5. The Research Project

The overall hypothesis of this thesis is that 14-3-3ζ has a tumour-promoting role in mammary tumour progression.

In order to investigate the validity of this hypothesis, the following aims are addressed in this work:

1. To compare mammary gland development in 14-3-3ζ-deficient mice relative to wild-type mice that express normal levels of 14-3-3ζ and to investigate the causes of the differences.

2. To characterise tumour progression in a mouse model of mammary cancer on a background of 14-3-3ζ deficiency.

3. To examine the translational potential of pharmacological inhibition of 14-3-3 in mammary cancers.

4. To investigate the roles of 14-3-3ζ in the tumour microenvironment and its role in the modulation of the immune cell compartment.

Chapter 2. Materials and Methods

2.1. Models and Materials

2.1.1. *Mouse models*

All animal procedures were approved by the institutional animal ethics committees of the Central Adelaide Local Health Network/SA Pathology and the University of South Australia. To enhance fertility, all breeders were maintained on a 22% fat diet (Cat. #SF01-025, Specialty Feeds Pty. Ltd).

2.1.1.1. 14-3-3ζ-deficient FVB/NJ Mice

Generation of the 14-3-3ζ-deficient strain of mice has been previously described (Cheah et al. 2012). Briefly, 14-3-3ζGt (OST390) Lex mutant mice containing gene trap constructs were derived from Lexicon Genetics ES cell line. The gene trap vector was inserted into the second intron of 14-3-3ζ. Mice were originally generated on a mixed C57BL/6 x 129/SvJ strain background and subsequently backcrossed onto the FVB/NJ strain background for at least 10 generations to obtain 14-3-3ζ-deficient FVB/NJ mice.

2.1.1.2. 14-3-3ζ-deficient MMTV-PyMT-FVB/NJ Mice

FVB/NJ-Tg(MMTV-PyMT)634Mul/J (MMTV-PyMT) mice (Guy, Cardiff & Muller 1992) were obtained from the Jackson Laboratory (Stock No: 002374). The MMTV-PyMT transgene expresses polyoma virus middle T antigen under the control of the mouse mammary tumour virus long terminal repeats. 14-3-3ζ-deficient MMTV-PyMT-FVB/NJ mice were generated by inter-crossing MMTV-PyMT positive males with 14-3-3$\zeta^{-/+}$ females.

All tumourigenesis studies were performed using MMTV-PyMT FVB/NJ (WT) and 14-3-3$\zeta^{-/+}$ MMTV-PyMT FVB/NJ (HET) mice. Tumour analysis at 8 and 11 weeks of age from WT and HET mice confirmed the presence of heterogenous tissue regions in both genotypes corresponding to the progressive tumour development **(Figure 2.1)**.

Furthermore, analysis of the mammary tumour tissues from 11-week-old WT and HET mice has revealed that both gene *(Ywhaz)* and 14-3-3ζ protein levels were significantly reduced in the HET animals **(Figure 2.2)**. These observations are consistent with the genotype of the experimental animals and are therefore validated for further studies.

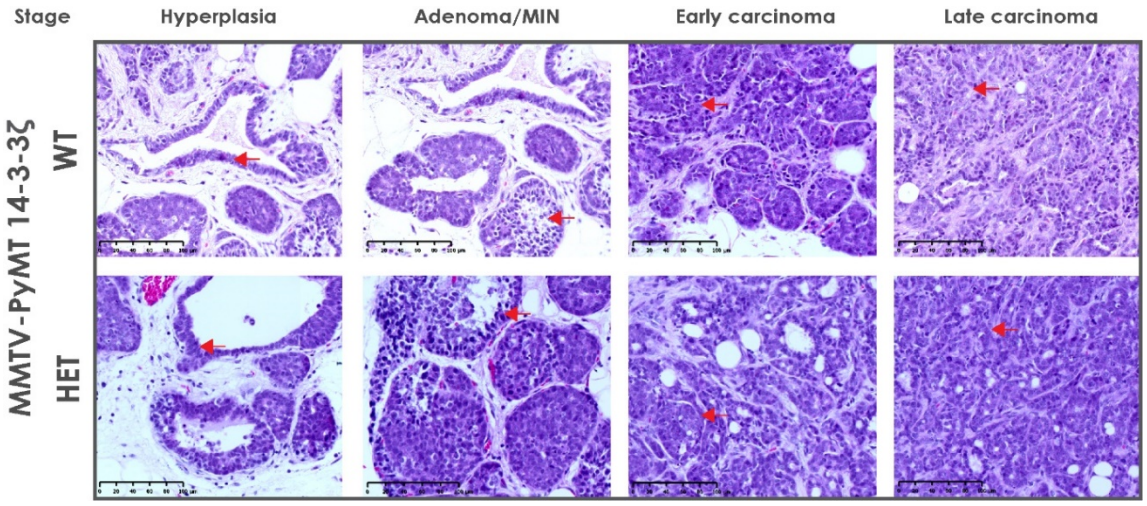


Figure 2.1: Tumour heterogeneity and progression is a feature of both MMTV-PyMT FVB/NJ (WT) and 14-3-3ζ$^{-/+}$ MMTV-PyMT FVB/NJ (HET) mice.

Comparable H&E images of the tumour regions from WT and HET mice at 8 weeks (hyperplasia and adenoma/MIN) and 11 weeks (early carcinoma, late carcinoma). Red arrows indicate the corresponding regions.

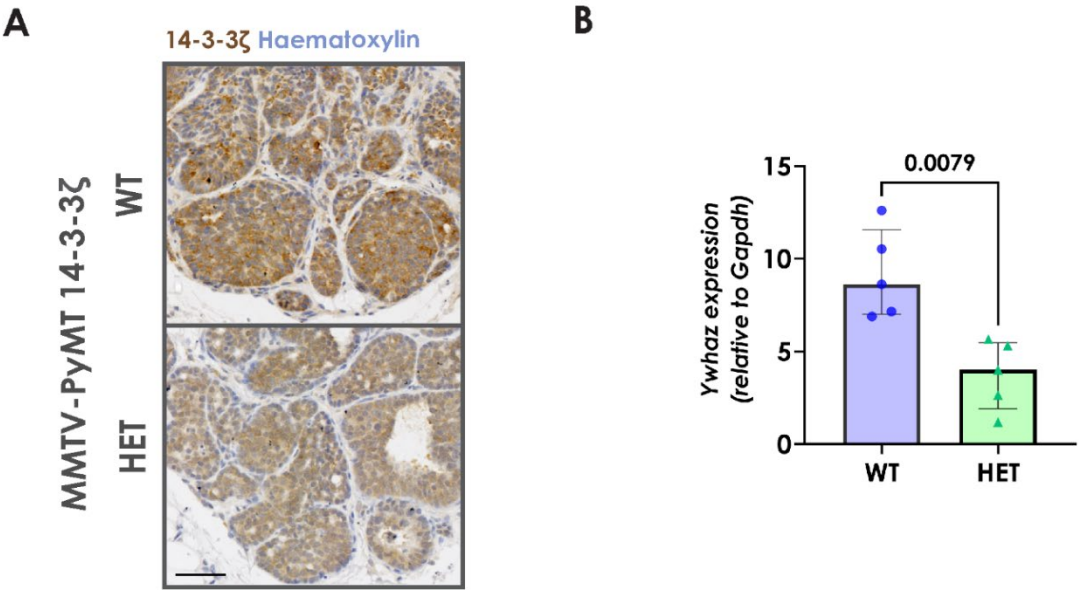


Figure 2.2: Protein expression and 14-3-3ζ gene (*Ywhaz*) in MMTV-PyMT FVB/NJ (WT) and 14-3-3ζ-/+ MMTV-PyMT FVB/NJ (HET) mice at 11 weeks of age are consistent with the genotype.

(A) Representative images of immunohistochemistry analysis of 14-3-3ζ protein expression in WT and HET mice. Scale bar=50um **(B)** *Ywhaz* (14-3-3ζ gene) qRT-PCR of the whole tumour mRNA from WT and HET relative to *Gapdh*, n=5 per genotype. Data is Mean±SEM; Student t-test.

2.1.2. *Genotyping*

Mouse genotypes from tail biopsies were determined using real time PCR with proprietary probes designed for each gene (Transnetyx, Cordova, TN). qPCR genotyping was validated by in-house PCR assays.

2.1.3. *Antibodies*

Antibodies and their dilutions used in immunohistochemistry, immunofluorescence and Western blot analyses are summarized in **Table 2.1** and antibodies used in flow cytometry analyses summarised in **Table 2.2.**

Table 2.1: Primary and secondary antibodies for immunohistochemistry, immunofluorescence and Western blot analyses

Antibody	Species	Supplier	Dilution *(method)*	Incubation *Temp (°C) [Time (hrs)]*
14-3-3ζ (C16)	Rabbit	Santa Cruz	1:1000 (IF)	4[18]
Akt Phospho-(Thr308) #9275	Rabbit	Cell Signalling Technology	1:1000 (WB)	4[18]
Akt #9272	Rabbit	Cell Signalling Technology	1:1000 (WB)	4[18]
AlexaFluor 2° Abs	Goat	ThermoFisher Scientific	1:400(IF)	25[1]
BrdU (B44)	Mouse	BD Biosciences	1:500 (IHC)	4[18]
CD45	Rat	BD Biosciences	1:100 (IHC)	4[18]
Cytokeratin 8	Rabbit	Abcam	1:200 (IF)	4[18]
Cytokeratin 14	Mouse	Leica Microsystems	1:100 (IF)	4[18]
E-Cadherin AlexaFluor-555	Mouse	BD Biosciences	1:200(IF)	25[1]
Horseradish Peroxidase 2° Abs	Goat/ Donkey	Rockland Inc./Abcam	1:10,000 (WB)	25[1]
Ki-67	Mouse	Leica Microsystems	1:50 (IHC)	4[18]
Mlc2-Phospho (Ser19)	Rabbit	Cell Signalling Technology	1:50 (IF)	4[18]
Mypt1-Phospho (Thr696)	Rabbit	Merck	1:100(IF)	4[18]
Periostin (Clone 47)	Mouse	Sigma-Aldrich	1:500(IF)	4[18]
Vimentin (LN-6)	Mouse	Sigma-Aldrich	1:250(IF)	4[18]

Table 2.2: Antibodies used for flow cytometry analyses

Antibody	Species	Clone	Supplier	Dilution
CD107a BV510	Rat IgG2a, κ	1D4B	Biolegend	1:50
CD3e BUV395	Armenian Hamster IgG1, κ	45-2C11	BD Biosciences	1:50
CD4 BV786	Rat IgG2α, κ	RM4-5	BD Biosciences	1:50
CD8α PerCP-Cy5.5	Rat IgG2a, κ	53-6.7	Biolegend	1:50
CD8α PE-Cy7	Rat IgG2a, κ	53-6.7	BD Biosciences	1:50
CD44 PE	Rat IgG2b, κ	Rat IgG2b, κ	Biolegend	1:50
CD45 FITC	Rat IgG2b, κ	30-F11	Biolegend	1:200
CD45 BUV737	Rat IgG2b, κ	30-F11	BD Biosciences	1:100
CD69 BV421	Armenian Hamster IgG1, κ	H1.2F3	Biolegend	1:50
IFN-γ BV421	Rat IgG1, κ	XMG1.2	BD Biosciences	1:20
TNF-α AF647	Rat IgG1, κ	MP6-XT22	Biolegend	1:40

2.1.4. *Inhibitor of 14-3-3*

RB-012 compound (14-3-3 inhibitor) was generously provided by Joanna Woodcock (Molecular Signalling Laboratory, Centre for Cancer Biology). Briefly, the inhibitors were generated using derivatives of FTY720 (Fingolimod) and combination of sphingo-mimetic molecules (alkylated trimethylammonium compounds). These compounds act by inhibiting the dimerisation of 14-3-3 monomers (Woodcock et al. 2015).

2.1.5. *Buffers and Solutions*

All buffers and solutions used in the experimental work are summarized in **Table 2.3**.

Table 2.3: Buffers and solutions

Buffer/Solution	Components
Blocking and antibody dilution solution (IF, IHC)	5% normal goat serum in CAS-Block™ (Life Technologies)
Carnoy fixative	60% ethanol, 30% chloroform, 10% glacial acetic acid
Carmine Alum	Boil 1 g carmine (Cat. #C-1022, Sigma), 2.5 g aluminium potassium sulphate in 500 mL H_2O for 20 min, filter sterilize and add thymol as a preservative. Store at 4 °C.
Citrate buffer	10 mM Sodium Citrate pH=6
Formalin buffer	3.7% formaldehyde in PBS
Flow buffer	2% heat inactivated FBS in PBS
IMDM medium (complete)	IMDM (Gibco), 10% heat inactivated FBS, 54nM 2-mercaptoethanol (Gibco), Pen/Strep (Gibco)
Re-stimulation buffer	Complete IMDM supplemented with 20 ng/mL PMA, 1 µM ionomycin (both Sigma-Aldrich) and 1:1500 dilution GolgiStop (BD Biosciences)
Mouse Red Blood Cell Removal Buffer (MRBCRB)	Solution A: 8.3 g NH_4Cl in 1 L H_2O; Solution B: 20.594 g Tris base in 1 L H_2O pH=7.65; Mix 900 mL solution A with 100 mL solution B to make 1 L adjust pH to 7.2; filter sterilise using 0.2 mm; Store at 4 °C.
PBS	Cell culture grade PBS was prepared from 10xPBS stock (Gibco) diluted in ddH_2O
Protein electrophoresis buffer	25 mM Tris, 190 mM glycine, 0.1% SDS
Protein transfer buffer (Western blot)	25 mM Tris, 190 mM glycine, 20% methanol, for proteins larger than 80 kDa add 0.1% SDS
Resolving gel (Western blot)	40% acrylamide/Bis solution (Bio-Rad), 1.5 M Tris-HCl pH=8.8, 0.01% SDS, 0.005% APS, 0.005% TEMED
Sample loading buffer (Laemmli)	x4 buffer: 62.5 mM Tris-HCl, pH=6.8, 10% glycerol, 1% SDS 0.005% Bromophenol Blue
RIPA buffer	10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 40 mM sodium tetrapyrophosphate, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM PMSF, 0.025% w/v SDS, 150 mM

	sodium chloride) containing 1xPhosSTOP™ and 1x cOmplete™ Protease Inhibitor Cocktail (Roche).
Stacking gel (Western blot)	40% acrylamide/Bis solution (Bio-Rad), 1 M Tris-HCl pH= 6.8, 0.01% SDS, 0.005% APS, 0.005% TEMED
TBS and TBS/T	20 mM Tris pH=7.5, 150 mM NaCl/0.1% Tween-20
Tris-EDTA buffer	10 mM Tris-Cl pH=9, 1 mM EDTA
TUNEL blocking buffer	0.1 M pH=7.5 Tris-HCl, containing 3% BSA and 20% FBS

2.2. Methods

2.2.1. *In vivo Methods*

2.2.1.1. Studies of Spontaneous Tumourigenesis

Onset of mammary tumourigenesis was monitored by manual palpation. Upon tumour onset, digital calliper measurements were taken twice weekly under anaesthesia up to 11 weeks of age or until the cumulative tumour burden reached 2 cm^3 (humane end point). Tumour volume was calculated as length X width X width in mm^3. Two hrs prior to sacrifice a single dose of 1mg of BrdU cell proliferation labelling reagent (GE Healthcare) was administered intraperitoneally.

2.2.1.2. Primary Tumour Cell Engraftment Studies

Primary tumour epithelial cells from 8-9-week-old MMTV-PyMT or MMTV-PyMT;14-3-3 $\zeta^{+/-}$ mice were isolated as described in Section 2.2.2.2. $3x10^4$ donor cells were suspended in 20% Matrigel (Corning)/DMEM mixture. 20 µL of cell suspension was engrafted into the fourth inguinal mammary fat pad of 6-8 weeks old anaesthetised recipient mice using a Hamilton syringe fitted to a 26-gauge needle. Mice were monitored for the onset of tumour development by manual palpation and digital calliper measurements were taken twice weekly.

2.2.1.3. Tumour Cell Line Engraftment Studies

The FVB/NJ PyMT+ve cell line was established by K. Blyth (Blyth et al. 2016) and previously used for the mammary tumour engraftment experiments (Boyle et al. 2020). Cells were cultured in DMEM supplemented with 10% FBS and Antibiotic-Antimycotic (Gibco) under standard cell culture conditions) at 37 °C and 5% CO_2.

$5x10^4$ cells were suspended in 20% Matrigel (Corning)/DMEM mixture. 20 µL of cell suspension was engrafted into the fourth inguinal mammary fat pad of a 6-week old anaesthetised recipient mice using a Hamilton syringe fitted to a 26-gauge needle. Mice were monitored for the onset of tumour development by manual palpation. 21 days post engraftment mammary fat pads with tumour lesions were harvested and processed for flow cytometry analysis.

2.2.1.4. In vivo Inhibitor Studies

14-3-3 inhibitor (RB-012 compound) was diluted in 0.9% sodium chloride (Baxter) administered by intraperitoneal injection at 7.5 mg/kg body weight, daily for 23 days from eight weeks of age in the spontaneous MMTV-PyMT model. Prior to injection tumour burden was assessed by digital calliper measurements and subsequent growth monitored twice weekly under anaesthesia until the end of treatment. Tumour volume was calculated as length x width x width in mm^3. Two hours prior to sacrifice a single dose of 1mg of BrdU cell proliferation labelling reagent (GE Healthcare) was administered intraperitoneally.

2.2.2. ***Ex vivo Methods***

2.2.2.1. Mammary Gland Whole Mount Preparations, Imaging, and Analysis

Whole 4th mammary fad pads were dissected, spread onto glass slides, and fixed using Carnoy's fixative for 3-4 hours at room temperature. Slides were washed in 70% ethanol (~10 min) and slowly diluted with ddH2O. Slides were stained with carmine alum solution overnight at room temperature, dehydrated through serial ethanol washes (70%, 90% and 100%), cleared in xylene and mounted in Permount (ProSciTech Pty. Ltd). Tissues were visualised using a dissecting microscope (Model MVX10, Olympus) connected to a CCL12 camera. Image stitching and processing was performed using MosaicJ plugin (http://bigwww.epfl.ch/thevenaz/mosaicj/) for ImageJ (NIH). Quantification of epithelial branching, length of main duct and enumeration of terminal end buds was performed according to the Mammary Gland Whole Mount Robin Summary (Fenton, SE 2009). Tumour initiation analysis was performed using area measurement and object counting tools (ImageJ).

2.2.2.2. Isolation of Primary Mammary Epithelial Cells, and Total Mammary Cells Isolation from Normal and Tumour Tissues

All mouse mammary fat pads or tumours were extracted with the removal of lymph node, manually dissociated using scissors and then digested in DMEM (Gibco) supplemented with 1 mg/ml collagenase IA, 100 U/ml hyaluronidase (Worthington Biochemical Corporation), and 12 U/mL DNAse (Gibco) and 2% foetal calf serum for 2-3 hrs at 37 °C with gentle rotation using HulaMixer (Invitrogen). Tissue digests were washed twice in DMEM+2%FBS. Resultant total mammary cell isolation preps were processed in TRIzol® using The FastPrep-24® (MP Biomedicals) tissue homogeniser.

To obtain single epithelial cells, a second digest was performed for further 30 min with 6 U/ml dispase (BD) and 12 U/ml DNase (Gibco) at 37 °C. Freshly isolated total cell preparations were filtered through 70 µm filter (Falcon) and cultured overnight in non-adherent conditions (1:1 volumes of DMEM) and Ham's F12 nutrient mix (Thermo Fisher Scientific), supplemented with 10 ng/ml EGF, 20 ng/ml bFGF (both PeproTech®) and 0.5xB27 (Gibco) to obtain a pure epithelial cell culture, which was then cultured adherently for 24 hours in DMEM supplemented with 10% FBS and Antibiotic-Antimycotic (Gibco). After 48 hours in culture, cells were either dissociated using trypsin, washed, and used for engraftment studies or lysed in RIPA buffer for protein analysis or TRIzol® for RNA isolation.

2.2.2.3. Isolation of Single Cells for Flow Cytometry Analysis From Tumour Tissues and Lymph Nodes

Tumour lesions or tumour tissues were dissected, manually dissociated using scissors in DMEM (Gibco) supplemented with 1 mg/ml collagenase IA, 100 U/ml hyaluronidase (both Worthington Biochemical Corporation) and 2% foetal calf serum for 2 hrs at 37 °C with gentle rotation using HulaMixer (Invitrogen). Freshly isolated total cell preparations were filtered through 70 µm filter (Falcon) and lysed of red blood cells. Draining lymph nodes were excised, and single cell suspensions derived by pressing through 70 µm filter (Falcon).

2.2.3. ***Flow Cytometry***

Cell counts were performed using Countess™ II FL automated cell counter (ThermoFisher). Single-cell suspension were plated into V-bottom 96-well trays (Corning) at $1.5x10^6$ cells/well, blocked with 10 mg/mL mouse γ-globulin (ThermoFisher) and stained in flow buffer (2%FBS

in PBS) for 30-40 min on ice. Dead cells were excluded by staining with Fixable Viability Stain 780 (BD Biosciences) according to manufacturer's protocol.

For intracellular cytokine staining single-cell suspension were plated into U-bottom 96-well trays at $2x10^6$ cells/well washed in re-stimulation buffer (pre-warmed complete IMDM and incubated with 20 ng/mL PMA, 1 uM ionomycin (both Sigma-Aldrich) and 1:1500 dilution GolgiStop (BD Biosciences)) for 4 hrs at 37 °C. For intracellular staining BD Cytofix/Cytoperm™ was used according to manufacturer's protocol.

Data acquisition was performed using BD LSR Fortessa™ Flow Cytometer and analysed using FlowJo software (BD Biosciences). Gating strategies for flow cytometry analyses represented in **Figure 2.3** and **Figure 2.4.**

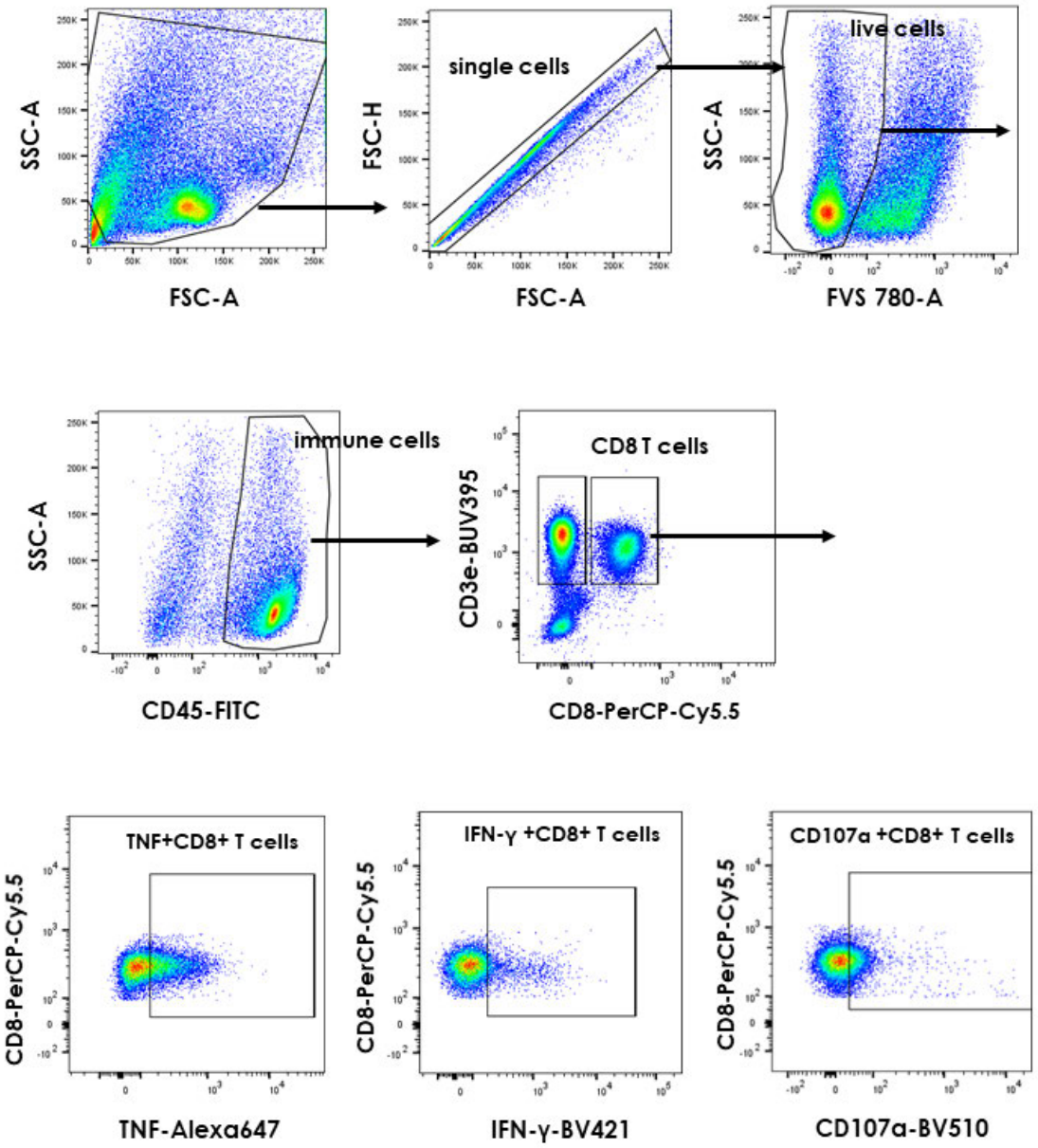


Figure 2.3: Gating strategy for flow cytometry analysis of $CD8^+$ intracellular cytokine profile.

Representative images for flow cytometry gating strategy with gates and populations as indicated.

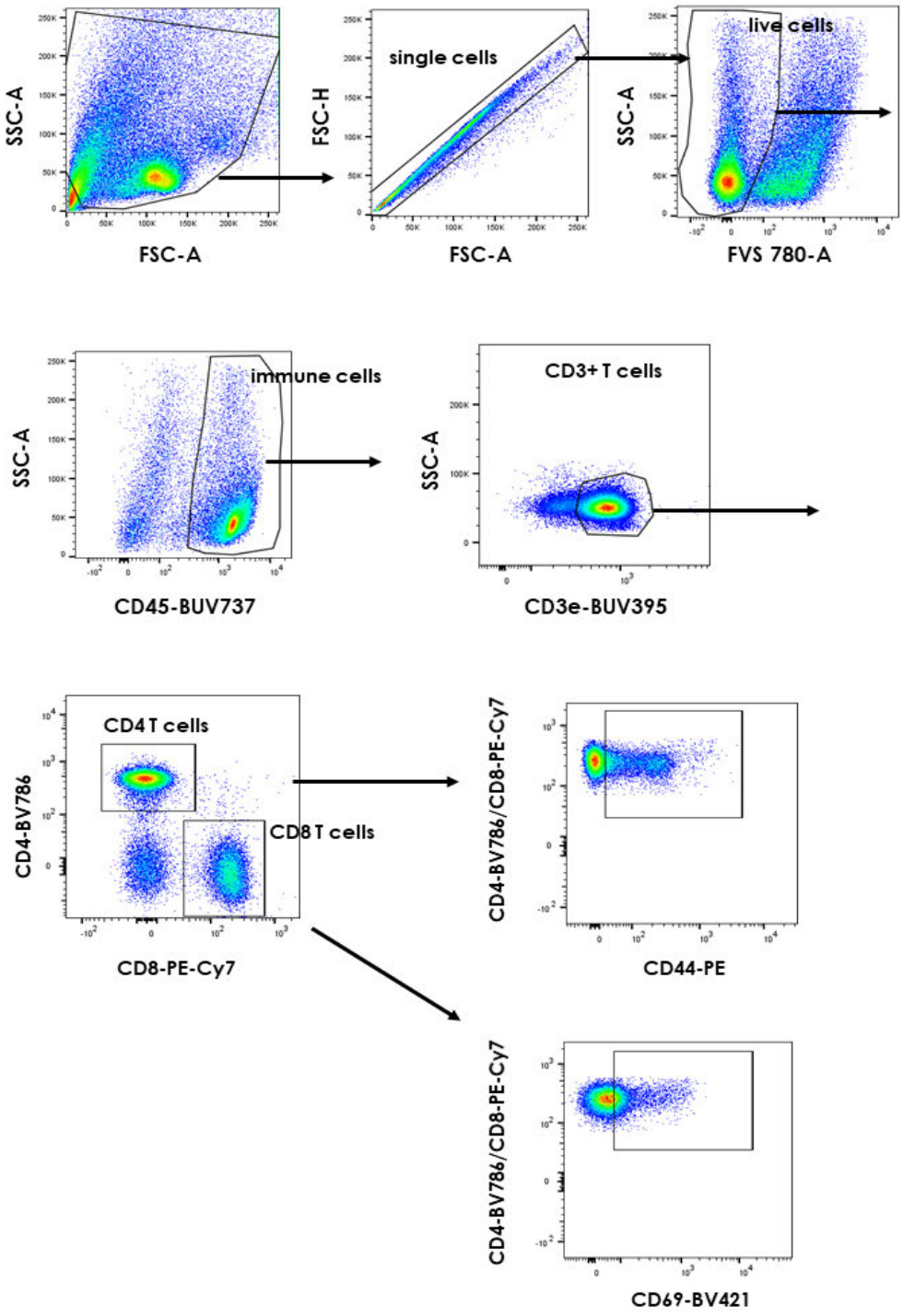


Figure 2.4: Gating strategy for flow cytometry analysis of T cell populations in non—tumour spleens and lymph nodes.

Representative images for flow cytometry gating strategy with gates and populations as indicated.

2.2.4. ***Histology Methods***

2.2.4.1. Tissue Processing and Fixation

Dissected tissues were fixed in 10% neutral formalin buffer overnight at 4 °C, washed in 70% ethanol and processed by University of Adelaide Medical School Histology services. Paraffin embedding was performed using Tissue Tek (Sakura) equipment. Tissues were sectioned at 4 μm unless otherwise specified using a RM 2235 Leica Biosystems microtome and mounted on SuperFrost plus (Thermo Fisher Scientific) slides.

2.2.4.2. Immunohistochemistry, Immunofluorescence Analysis and SHG

Prior to staining, cut tissue sections on slides were baked at 37 °C overnight, dewaxed by 2x5min xylene washes and rehydrated using sequential ethanol washes (2x100%, 70% and 50%). Antigen retrieval was performed by boiling slides in a pressure cooker in citrate buffer (10mM Citric acid pH 6.0) for all antigens, except for phospho-Mlc2, where the Tris-EDTA buffer (10 mM Tris-Cl and 1 mM EDTA pH 9.0) was used.

2.2.4.2.1. Immunohistochemistry

Following antigen retrieval sections were pre-treated with 3% hydrogen peroxide in methanol for 20 min and blocked in 5% normal goat serum in CAS-Block (Thermo Fisher Scientific) for 30-45 min. After the primary antibody incubation tissues were further processed with EnVision+Dual Link System-HRP (Dako) for 120 min and visualised using Liquid DAB+Substrate Chromogen System (Dako) according to the manufacturer's instructions.

Sections were counter-stained with hematoxylin (Sigma), serial washed in 1% acid alcohol, Scott's tap water substitute and deionised water and further dehydrated using sequential ethanol washes (5 minX50%, 5 minX70%, 1 minX100%, 5 minX100%) and 2X 5 min xylene washes and mounted with DPX mounting medium (Southern Biological).

Sections were scanned using a Hamamatsu Nanozoomer NDP (Hamamatsu Photonics) slide scanner. Images were visualised using NDP Virtual Slide Viewer software. Positive pixel analysis was performed using Aperio Positive Pixel Count Algorithm (Leica Biosystems) or QuPath Digital Pathology Image Analysis software using Positive Cell Detection algorithm (Bankhead et al. 2017).

2.2.4.2.2. Immunofluorescence

Following antigen retrieval, tissues were blocked in 5% normal goat serum in CAS-Block (Thermo Fisher Scientific) for ~30-45 min. Primary and secondary antibody dilutions are summarised in **Table 2.1**. Fluorescently immunolabelled sections were mounted in a hard set mounting medium supplemented with DAPI (Vector Laboratories). Imaging was performed using an LSM 700 confocal microscope (Zeiss). ImageJ was used to calculate integrated density (intensity) and/or area coverage by signal per ROI (duct) image, after conversion to a binary image based upon a single manually determined threshold value applied across all images as previously described (Kular et al. 2015).

2.2.4.3. Terminal Deoxynucleotidyl Transferase dUTP Nick end Labelling (TUNEL)

Apoptotic cell analysis was performed using In Situ Cell death detection Fluorescein kit (Roche). Formalin-fixed, paraffin-embedded sections were rehydrated and treated according to manufacturer's instructions for difficult tissues. Briefly, slides were microwave treated at 750 W for 1 min in 10 mM Citrate buffer (pH=6.0), blocked for 30 min in TUNEL blocking buffer (Tris-HCl, 0.1 M pH=7.5, containing 3% BSA and 20% FBS), stained and mounted in a hard set mounting medium supplemented with DAPI (Vector Laboratories). ImageJ particle analysis algorithm was used to calculate TUNEL positive nuclei.

2.2.4.4. 2-Photon Second Harmonic Generation (SHG) Microscopy

Collagen imaging was performed using SHG microscopy. Briefly mammary tumour tissue sections were cut at 8 μm, rehydrated and counter-stained with hematoxylin (Sigma), serial washed in 1% acid alcohol, Scott's tap water substitute, deionised water and further dehydrated using sequential ethanol washes (5minX50%, 5minx70%, 1minX100%, 5minX100%) and 2X 5min xylene washes and mounted with DPX mounting medium (Southern Biological). Images were acquired using a 20x water immersion objective on an upright fixed-stage two-photon LSM 710 laser scanning microscope system (Zeiss). The excitation source was a Ti:Sapphire femto-second laser cavity (Newport Mai Tai), coupled into a LSM710 scan module. An excitation wavelength of 890 nm was used to collect SHG signal (435±20 nm) from collagen. Transmission images were co-acquired with SHG data. SHG signal was analysed using ImageJ.

2.2.5. *RNA Analysis*

2.2.5.1. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Snap-frozen normal or tumour tissues were pulverised while frozen using a pestle and mortar and liquid nitrogen extraction. RNA was extracted using TRIzol® according to the manufacturer's instructions. RNA concentration was determined by NanoDrop spectrometer (Thermofisher).

First strand cDNA synthesis and qPCR amplification carried out using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Validated qPCR primers (QuantiTect Primer Assays) were used together with QuantiTect SYBR Green PCR kit (QIAGEN) for gene expression analysis on Rotor-Gene Q(CA) thermocycler (QIAGEN). Relative quantification was performed using Rotor-Gene Q (CA) software (QIAGEN).

Qiagen catalogue numbers and primer sequences for the primer assays used are:

mRNA Species	**Primer Assay/Sequence**
Dach1	F 5'-GGCTTCCTTTACGGTGGAGG-3' R 5'-CAGCTTGGTGTAGACGGTGTG-3'
Gapdh	F 5'-GTTCCAGTATGACTCCACTCACG-3' R 5'-AGATCCACGACGGACACATTG-3'
Gata3	F 5'-CTCGGCCATTCGTACATGGAA-3' R 5'-GGATACCTCTGCACCGTAGC-3'
Notch1	F 5'-GATGGCCTCAATGGGTACAAG-3' R 5'-TCGTTGTTGTTGATGTCACAGT-3'
Pygo2	F 5'-AGCGAAGAAAGTCCAATACTCAG-3' R 5'-GTTAGAAGCGACCAGATGATCC-3'
Ywhab	QT00126133
Ywhaz	QT00105350
Ywhah	QT01776012
Ywhag	QT00288575

Ywhae	QT00150738
Ywhaq	QT01061165

2.2.6. ***Protein Analysis***

2.2.6.1. Sample Preparation and Western Blot Analysis

Cells were lysed with RIPA buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 40 mM sodium tetrapyrophosphate, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM PMSF, 0.025% w/v SDS, 150 mM sodium chloride) containing protease and phosphatase inhibitors (Roche).

Protein concentrations were determined using Pierce™ BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's protocol. The protein concentration was measured at 540nm using the Epoch Microplate Spectrophotometer (Biotek) and Gen5™ (Biotek) analysis software.

Proteins were separated by 10% or 12% SDS-PAGE and immobilized onto nitrocellulose membrane. Western analysis was performed by blocking the membrane in 5% skim milk in Tris buffered saline (TBS)/Tween-20 (TBS-T) for 1 h at room temperature followed by addition of primary antibodies overnight at 4°C. Immunoblotting was carried out using antibodies detailed in **Table 2.1**. Bands were visualized using a ChemiDocTM Imaging System (Bio-Rad Laboratories Pty. Ltd.) using Pierce® ECL (Thermo Fisher Scientific) according to manufacturer's protocol.

2.2.6.2. ***Reverse Phase Protein Array (RPPA)***

Tumour tissues from eleven weeks old MMTV-PyMT FVB/NJ (WT) and 14-3-3ζ-/+ MMTV-PyMT FVB/NJ (HET) mice were processed using The FastPrep-24® (MP Biomedicals) tissue homogeniser and lysed in proprietary LCB1 buffer. RPPA analysis was performed in Victorian Centre for Functional Genomics at Peter MacCallum Cancer Centre. Data output for RPPA is returned as Relative Fluorescence Intensity (RFI) values (calculated by the Zeptoview software). The fluorescence intensity value is background corrected to both the local background and to the secondary alone control to give us the RFI value for each sample/antibody combination.

2.2.7. *Gene Expression Analysis Using METABRIC Dataset*

METABRIC dataset was obtained from the The Oncomine Platform (http://www.oncomine.org/resource/login.html) and contains microarray gene expression (Log2-Median Centred Intensity) from Illumina HumanHT-12 V3.0 R2 (Curtis et al. 2012). Probability of survival was determined by Log-Rank (Mantel-Cox) Test. High and low expression of *YWHAZ* was assigned above or below the median expression level.

2.2.8. *Statistical Analysis and Figures*

Statistical analysis was performed using GraphPad Prism v.7 &8 (La Jolla, CA). For in vivo data analysis normality Shapiro-Wilk test was performed. For variable that followed Gaussian's distribution, a student's t-test was used for two variables and One-way ANOVA for multiple comparisons. In all other cases, a Mann-Whitney test was performed for two variables and Kruskal-Wallis test performed for multiple comparisons. All figure legends indicate the statistical analysis undertaken. P-values used to indicate statistical significance. Levels of significance: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$. Figures and illustrations were prepared using Adobe Illustrator and BioRender (https://biorender.com/).

Chapter 3. The Role of 14-3-3ζ in Mammary Gland Development

3.1. Introduction

The mammary gland is an organ unique to mammals, with the specific function of milk production and delivery. In both humans and rodents, the development of the mammary gland occurs in three defined stages: embryonic, pubertal, and reproductive. Each stage is controlled by specific hormonal cues and growth factors (Inman et al. 2015; Macias & Hinck 2012).

The pubertal stage of mammary gland development is of particular interest, since it is an ideal model to study dynamic changes in mammary gland morphogenesis, which are characterised by integration of proliferative, differentiation and apoptotic signalling (McNally & Stein 2017). These same signalling pathways can become corrupted in the process of breast cancer development, and the study of mammary gland development is therefore essential for dissecting potential mechanisms of breast cancer progression (Butcher, Alliston & Weaver 2009; Watson & Khaled 2020).

In mice, onset of puberty is characterised by elevation of ovarian hormone levels, including estrogen. Puberty onset variability has been observed in different mouse strains, with vaginal opening in FVB mice detected at ~3.9 weeks of age and in the C57Bl/6 strain background at ~4.7 weeks of age (MacLennan, Anderson & Ma 2011). Upon hormonal stimulation the rudimentary mammary epithelium commences invasion into the mammary fat pad and continues until the physical limits of the mammary fat pad are reached (Sternlicht 2006). This process is termed branching morphogenesis. The main drivers of primary branching morphogenesis are the terminal end buds (TEBs) located at the invading front of the branch **Figure 3.1.**

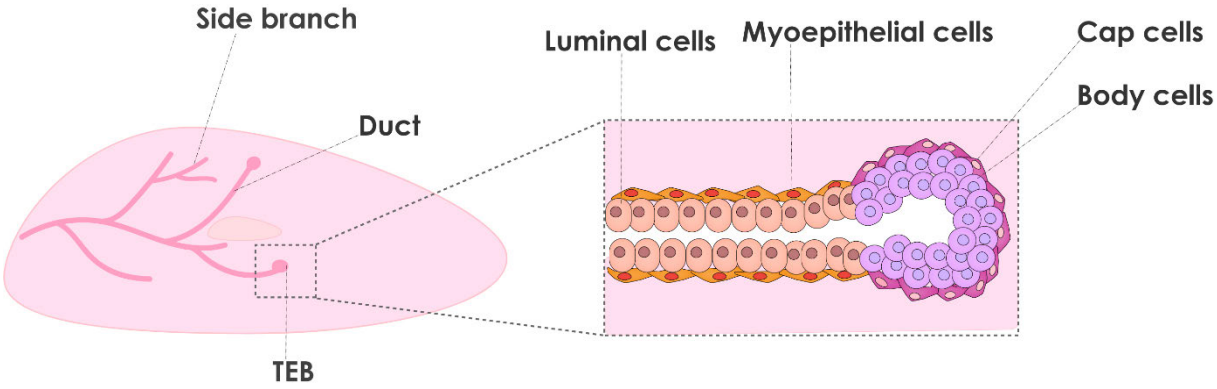


Figure 3.1: Schematic representation of the developing mouse mammary gland and the terminal end bud (TEB) structure.

TEB structures consist of a single layer of highly proliferative cap epithelial cells and underlying body cells that drive the process by elongating and branching. Cap cells are thought to be the reservoir of the mammary stem cells and primarily give rise to myoepithelial cells that form the basal cell population. Some markers of the basal lineage include keratins 5 and 14. Body cells give rise to the luminal cells of the duct, and the alveolar subset, which give rise to alveoli during pregnancy and lactation. These cells express keratin 6, 8 and 18 (Paine & Lewis 2017). Secondary branching occurs laterally from the primary ducts and the tree-like pattern of the mammary epithelium emerges (Hinck & Silberstein 2005).

The Notch and Wnt/β-catenin signalling pathways are the main regulators of lineage specification and differentiation of the basal and myoepithelial cells of the mammary epithelium, and as a result influence the process of primary branching morphogenesis. Importantly, these pathways also become corrupted during breast cancer progression (Rangel et al. 2016). Branching morphogenesis is also governed by several key genes including *Dach1* (Jiao et al. 2019), *Gata3* (Asselin-Labat et al. 2007a; Kouros-Mehr et al. 2006), *Notch1* (Bouras et al. 2008) and *Pygo2* (Gu, B et al. 2013) that regulate mammary epithelial cell fate specification at the early stages of the differentiation cascade.

14-3-3 proteins are a family of proteins with roles in many signalling pathways, that regulate important processes including cellular proliferation, cell cycle control, apoptosis, signal transduction, intracellular trafficking, cytoskeletal arrangement, transcription, and metabolism (Morrison 2009). Little is known about the functions of these important regulators of signalling in normal mammary gland development. 14-3-3σ is however known to be important in maintaining epithelial polarity (Ling et al. 2010) and 14-3-3ε in regulating cell proliferation and casein synthesis via PI3K-mTOR signalling in bovine mammary epithelial cells (Wang, B et al. 2018). 14-3-3γ regulates protein synthesis and cell proliferation at both translational and transcriptional levels via mTOR and Stat5a signalling (Yu, C et al. 2014).

While no investigations into whether 14-3-3ζ is involved in mammary gland development in vivo appear in the published literature, a few studies have showed that 14-3-3ζ has important functions in breast cancer (Lu, J et al. 2009; Xu et al. 2015). Given the significant roles of 14-3-3ζ in mammary cancer pathobiology and its potential as a therapeutic target for breast cancer treatment (Yang, X et al. 2012) it is important to understand its role in mammary development and homeostasis since these could be impaired by therapies targeting 14-3-3ζ. Therefore, I sought to investigate roles for 14-3-3ζ in normal mammary gland development using mice as a model.

3.2. Hypothesis and Aims

I hypothesised that 14-3-3ζ is required for normal mammary gland development.

The aims of the work presented in this chapter were to compare mammary gland development in 14-3-3ζ-deficient mice relative to wild-type mice that express normal levels of 14-3-3ζ and to identify potential mechanisms that account for the developmental differences.

3.3. Results

3.3.1. *Mammary Gland-Specific 14-3-3ζ Gene Expression and Protein Levels are Consistent with the Animal Genotype.*

To assess the levels of 14-3-3ζ gene expression in the mammary gland tissue derived from 14-3-3ζ wildtype (WT), 14-3-3ζ heterozygous (HET) and 14-3-3ζ-deficient (KO) mice I performed qPCR analysis on whole mammary glands isolated from mice of these genotypes. To assess the protein levels, I performed immunofluorescence analysis of the mammary ducts of mice of the same genotypes using commercially available antibodies against 14-3-3ζ.

These analyses revealed that the *Ywhaz* mRNA **(Figure 3.2A)** and 14-3-3ζ protein **(Figure 3.2B)** were absent in mammary tissue from KO mice and that tissue from HET mice exhibited around half the levels of *Ywhaz* mRNA and 14-3-3ζ protein compared to tissues from WT mice.

These data show that mammary gland-specific 14-3-3ζ gene expression and protein levels are consistent with the animal genotype and also validate the 14-3-3ζ antibody.

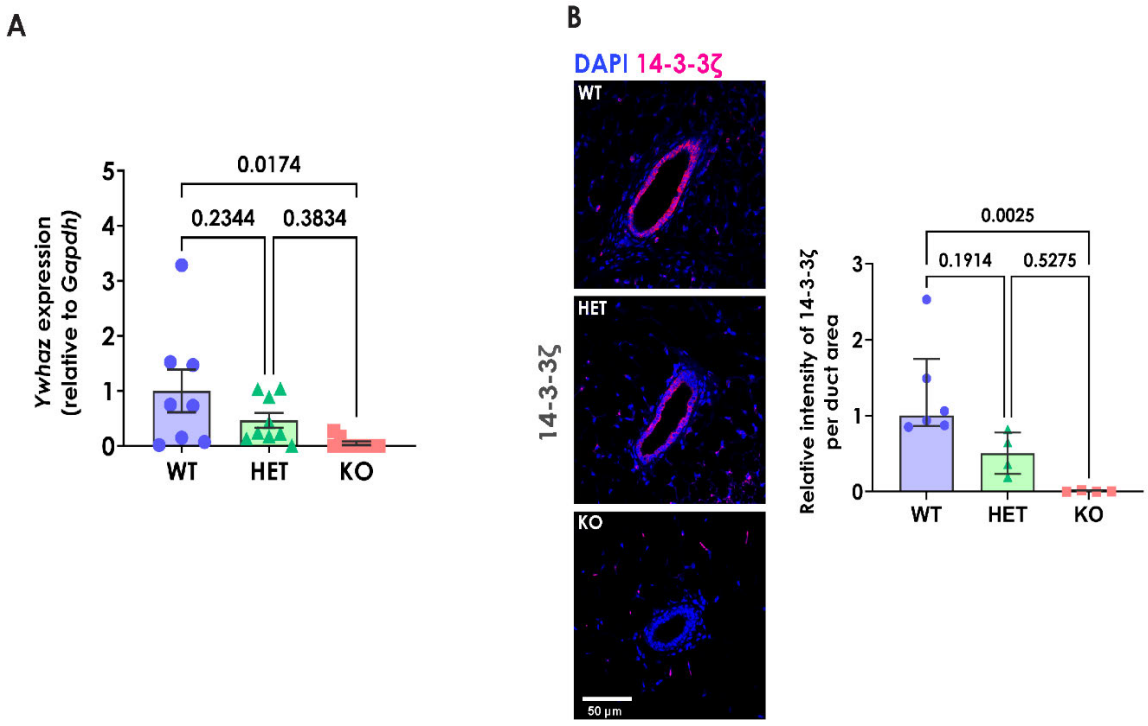


Figure 3.2: Mammary gland-specific 14-3-3ζ gene expression and protein levels are consistent with the animal genotype.

(A) qRT-PCR of the whole mammary gland mRNA in 14-3-3ζ WT, HET and KO mice relative to *Gapdh* and expressed as fold change relative to WT. n=8 (WT), n=9 (HET, KO). **(B)** Representative images of immunofluorescence analysis of 14-3-3ζ in WT, HET and KO mammary glands of 6-week-old mice. Relative intensity of signal was quantified per area of

ductal epithelium. Data is Mean±SEM; P values were determined using one-way ANOVA; (n=6 (WT), n=4 (HET and KO) individual mammary fat pads).

3.3.2. *Gene Expression of Other 14-3-3 Family Members is Unchanged in the Mammary Glands of 14-3-3ζ-Deficient Mice*

14-3-3 proteins share considerable sequence similarity at the protein level and some context-dependent functional redundancy between family members is reported (Aghazadeh & Papadopoulos 2016). However, there is also evidence to suggest non-redundant and tissue specific functions of 14-3-3 family members (Cheah et al. 2012; Kular et al. 2015).

I therefore sought to establish whether other 14-3-3 family members may be differentially regulated to perform compensatory roles in mammary gland development when 14-3-3ζ is not available. qRT-PCR analysis revealed that none of the other family members were significantly changed at the mRNA level in the mammary gland of HET or KO animals **(Figure 3.3A-F)**.

It is also important to point out that there is a trend toward reduction in 14-3-3η **(Figure 3.3D)** and 14-3-3σ gene **(Figure 3.3F)** expression in mammary epithelium from 14-3-3ζ KO mice, indicating that these isoforms may be affected by 14-3-3ζ deletion. However, overall, none of the other 14-3-3 family members were consistently differentially regulated in KO and HET mice in a statistically significant manner, suggesting that it is unlikely that the phenotype observed arises from the consistent differential regulation of 14-3-3 family members as a result of 14-3-3ζ depletion.

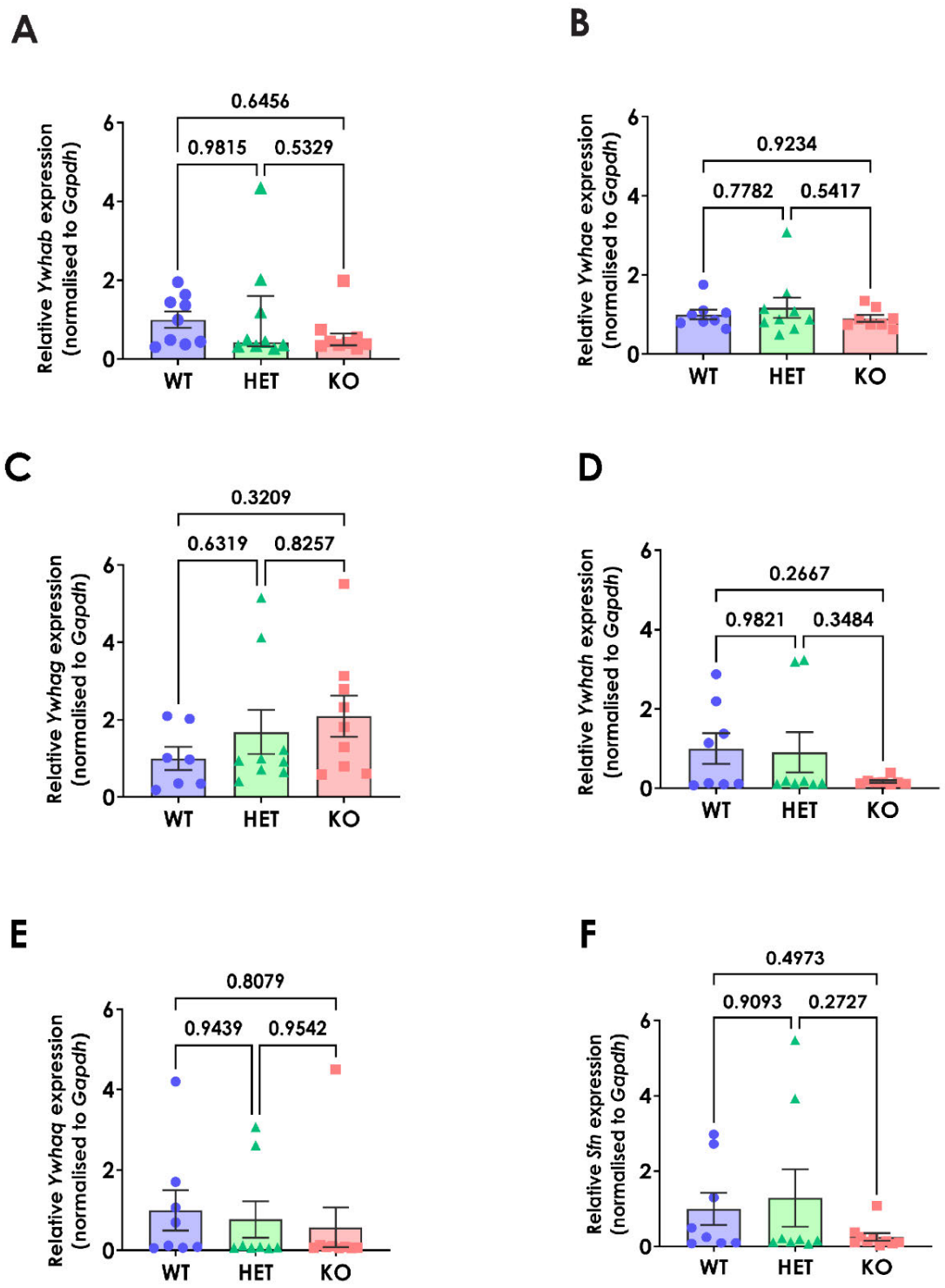


Figure 3.3: Gene expression of other 14-3-3 family members is unchanged in the mammary glands of 14-3-3ζ-deficient mice

(A)*Ywhab* (14-3-3β), **(B)** *Ywhae* (14-3-3ε), **(C)** *Ywhag* (14-3-3γ), **(D)** *Ywhah* (14-3-3η), **(E)** *Ywhaq* (14-3-3θ), **(F)** *Sfn* (14-3-3σ) qRT-PC analysis of mRNA levels of 14-3-3 family members in the mammary glands of WT, HET and KO mice at 6-11 weeks of age relative to *Gapdh* and expressed as fold change relative to WT. Data is Mean±SEM; P values are shown on the graphs and were determined by one-way ANOVA (n=8-9 mice per genotype).

3.3.3. *Mammary Gland Development is Transiently Delayed in 14-3-3ζ-Deficient Mice*

To investigate whether 14-3-3ζ is necessary for normal mammary gland development, I compared the epithelial structures within the fourth mammary fat pads of WT, HET and KO female mice at 4, 6, 9 and 11 weeks of age. These timepoints were chosen because they represent key stages of mammary development in mice on the FVB/NJ strain background, namely onset and early puberty (3-6 weeks), mid-puberty (7-10 weeks) and maturity (~10-12 weeks) (MacLennan, Anderson & Ma 2011; Plante, Stewart & Laird 2011).

The rudimentary epithelial structures of the murine mammary gland remain quiescent until ~3 weeks of age when the onset of puberty, triggered by sex hormones, leads to the appearance of terminal end buds (TEBs) - the main proliferative centres of the mammary tree. The process of ductal elongation ensues and continues till ~8-9 weeks of age. At ~10-12 weeks the epithelial ductal tree reaches the physical limits of the fat pad and the process of mammary epithelial elaboration is then complete (Richert et al. 2000).

Development of the mammary gland is assessed by gross morphological examination of the fourth mammary gland using key parameters outlined in Mammary Gland Whole Mount Round Robin Summary (Fenton, S et al. 2009). The length of the main duct is defined as the distance of the longest duct extending from the nipple to the opposite end of the fat pad; TEBs are defined as teardrop-shaped structures at the end of the duct with area larger than 0.03 mm^2; the secondary branching is determined by enumerating branch points per mm along three separate ducts in three different areas of the mammary gland (Boyle et al. 2016; Fenton, S et al. 2009).

Gross morphological observation of the mammary fat pads revealed that although the mammary glands of KO mice appeared to have developed to the same stage as those of WT mice at 4 weeks of age, a pronounced developmental delay in epithelial branching occurred by 6 and 9 weeks in KO animals and persisted till 11 weeks of age. Interestingly however, the mammary glands of the WT and HET animals were developmentally indistinguishable throughout this age range **(Figure 3.4)**.

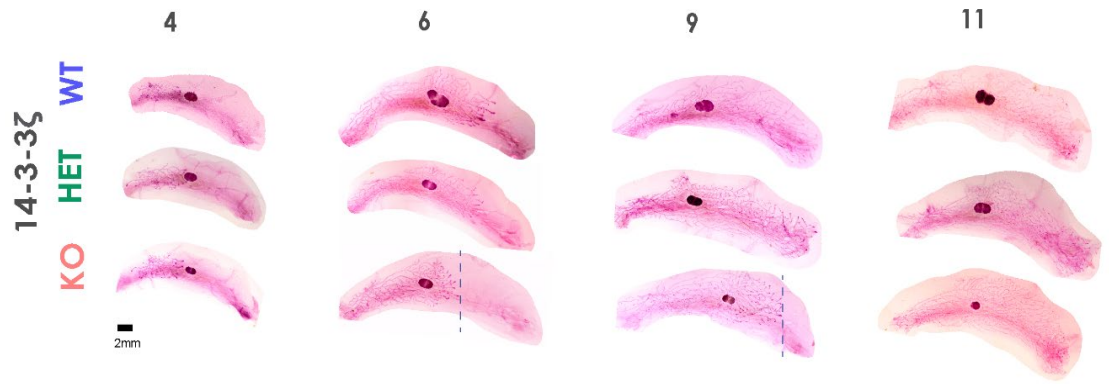


Figure 3.4: Mammary gland development is transiently delayed in 14-3-3ζ-deficient mice

Representative whole mount images of the fourth mammary fat pads taken from 14-3-3ζ WT, HET and KO mice at indicated ages. The extent of mammary tree elaboration of KO animals is indicated by the dotted line.

The most striking difference was apparent in a 1.5-fold reduction in the length of the main duct in KO animals at both 6 and 9 weeks of age compared to WT counterparts. This indicates that the rate of branching morphogenesis is significantly lower in KO animals. Interestingly, by 11 weeks of age the lengths of the main ducts were identical between these groups suggesting a transient developmental defect **(Figure 3.5A)**. Importantly, there was no difference in main duct length between WT and HET animals at any of the ages analysed.

To further evaluate the process of branching morphogenesis, I counted the number of TEBs. I observed a 3.5-fold more TEBs in KO mammary fat pads at both 9 and 11 weeks of age compared to those in WT mice of the corresponding ages, suggesting that the process of epithelial outgrowth is slower in the KO mice compared to the WT. It is important to note that even though at 4 weeks of age there is no significant difference in the number TEBs between WT and KO animals, there is a trend towards fewer TEBs in the KO animals compared to WT animals, potentially indicative of delayed puberty **(Figure 3.5B)**.

I next analysed the process of secondary branching in developing mouse mammary glands. Interestingly, even though by 11 weeks of age the length of the main duct is similar in WT and KO animals **(Figure 3.5A)** a trend towards a reduction in secondary branching was observed in KO animals at 6 and 11 weeks of age **(Figure 3.5C)**, suggesting that 14-3-3ζ may play a role in the secondary branching process. Similar to my observations with the length of the main duct, the extent of secondary branching was indistinguishable in WT and HET mammary glands at each time point.

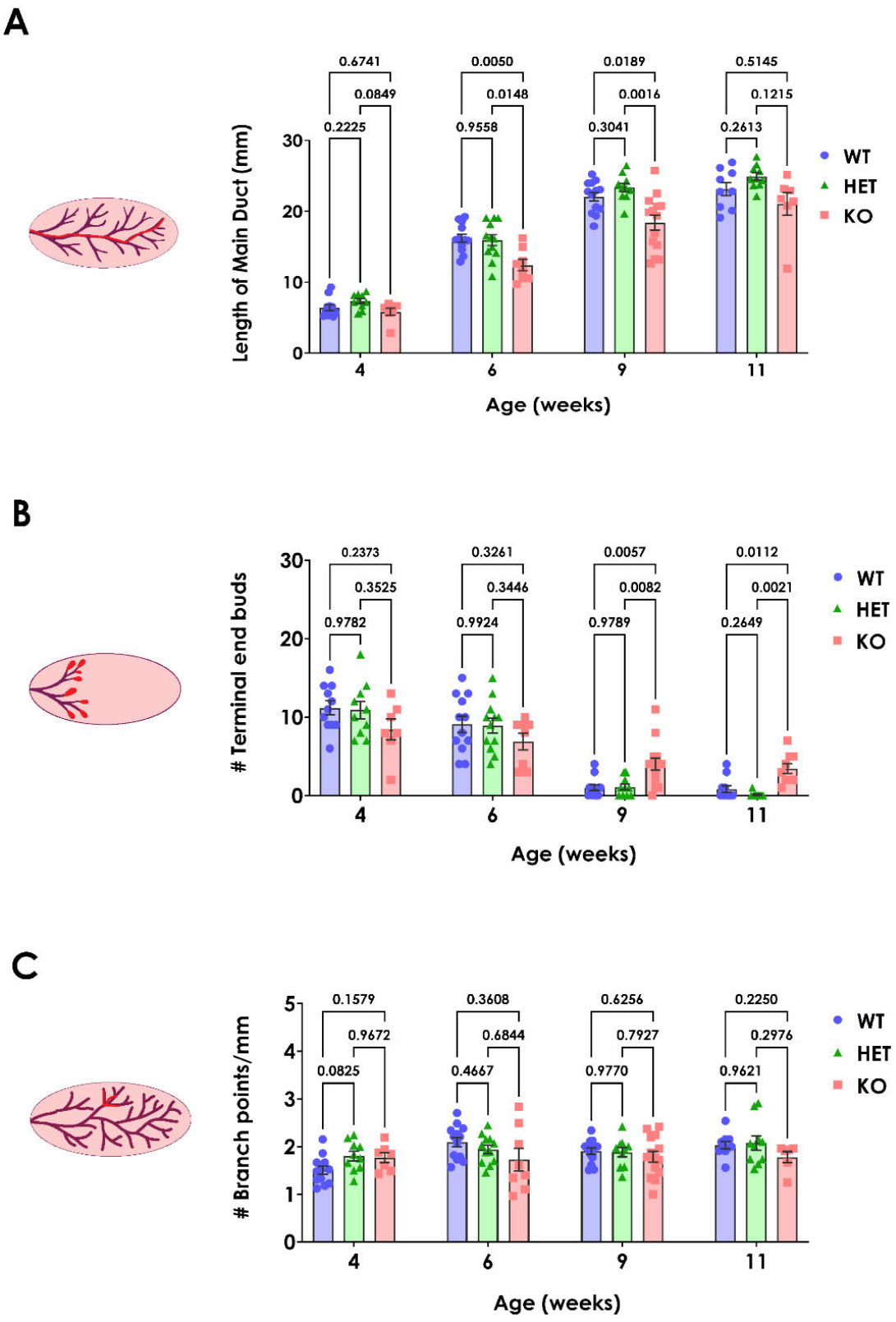


Figure 3.5: Length of the main duct and terminal end buds but not the number of secondary branches is reduced in the mammary fat pads of 14-3-3ζ-deficient mice

Quantification of the length of the main duct **(A),** terminal end buds (TEBs) **(B)** and secondary branching **(C)** within mammary epithelium. Data is Mean±SEM; P values are shown on the graphs and were determined by two-way ANOVA (n=5-6 mice per genotype). Each dot represents a separate mammary fat pad.

Since the body weight of the animal could be a potential co-variate in the evaluation of the mammary gland development (Fenton, S et al. 2009), I measured the weights of each animal prior to mammary fat pad removal. However, I found no significant differences between the weights of the experimental animals of all 14-3-3ζ genotypes **(Figure 3.6)**. These data suggest that between 4 and 11 weeks of age, differences in overall body weight cannot explain the delayed mammary development observed in KO mice relative to their WT littermates.

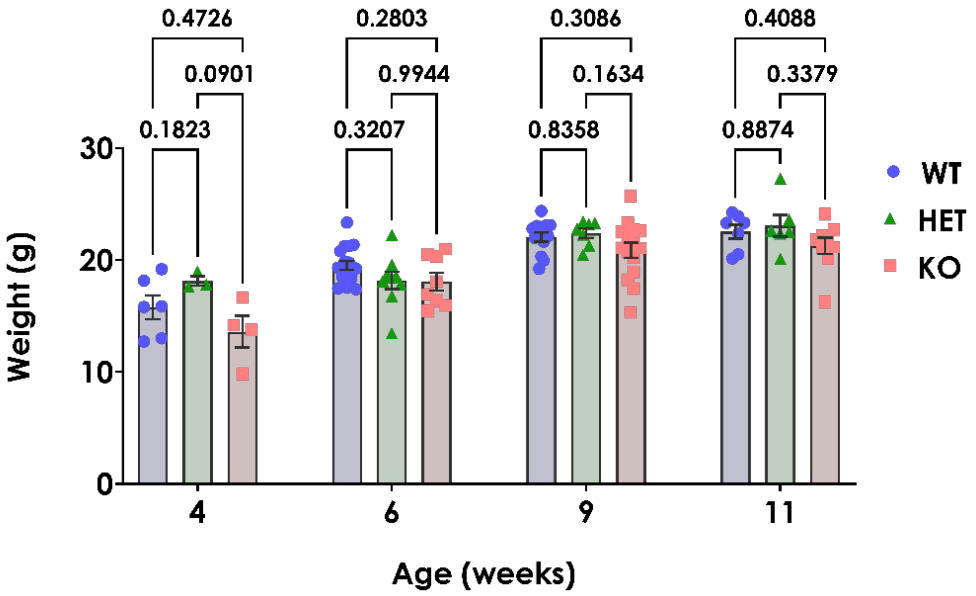


Figure 3.6: The body weight is unchanged between the genotypes

Body weight comparison between WT, HET and KO mice between 4 and 11 weeks of age. Data is Mean±SEM; P values are shown on the graphs and were determined by two-way ANOVA (n=3-9 mice per genotype). Each dot indicates a separate mouse.

3.3.4. *14-3-3ζ Deletion Inhibits Cell Proliferation but Does not Influence Apoptosis Rates in the Developing Mammary Gland*

14-3-3 protein family members have been strongly implicated in cell growth, survival, and differentiation (Mhawech 2005). I therefore investigated whether 14-3-3ζ deletion had an effect on either cell proliferation or apoptosis within the mammary epithelium in this model of mammary gland development.

To assess whether 14-3-3ζ deficiency influences the proliferation of mammary epithelial cells, I utilised Ki-67 immunohistochemistry to identify proliferating cells at 6 weeks of age in mice of all three genotypes, since it is at this timepoint that I observed the most pronounced differences in mammary gland development between the genotypes. This analysis revealed a 15% reduction in Ki-67 positive cells in KO mammary epithelium relative to WT mammary epithelium, whereas the proliferation rate within the mammary epithelium in HET animals was similar to that observed in WT mice **(Figure 3.7A).**

Similarly, to assess whether 14-3-3ζ deficiency influences apoptosis of mammary epithelial cells, I undertook terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) analyses on tissue sections of mammary glands isolated from 6-week-old mice of all three genotypes. TUNEL was not significantly different between the three genotypes **(Figure 3.7B)**.

Taken together, these data suggest that at the delay in the development of mammary glands of KO mice is likely explained by reduced proliferation rather than enhanced apoptosis and that 14-3-3ζ is required for normal proliferation within the developing mammary epithelium.

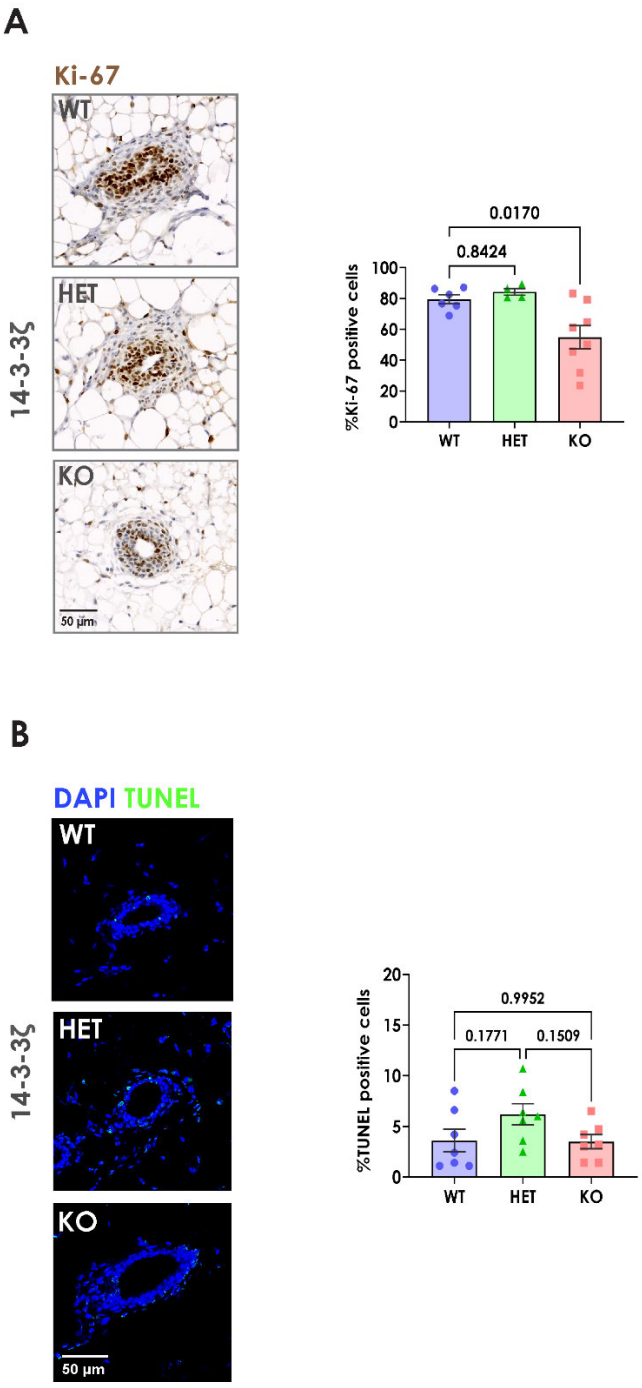


Figure 3.7: 14-3-3ζ deletion reduces cell proliferation but does not affect apoptosis in the developing mammary gland

(A) Representative images and quantification of Ki-67 immunohistochemistry in the mammary glands of 14-3-3ζ WT, HET and KO mice at 6 weeks of age. Data are shown as median±IQR, P values were determined using one-way ANOVA, (n=2-4 mice per genotype). Each dot indicates a separate mammary fat pad. **(B)** Representative images and quantification of cell death (TUNEL) in the mammary glands in 14-3-3ζ WT, HET and KO mice at 6 weeks of age. Data shown as median±IQR, P values are shown on the graph and were determined by one-way ANOVA, (n=3-4 mice per genotype). Each dot indicates a separate mammary fat pad.

3.3.5. *14-3-3ζ deficiency limits the basal but not the luminal epithelial cell population in the developing mammary gland*

The mammary epithelium comprises of two key cell populations: basal and luminal mammary epithelial cells. To investigate whether 14-3-3ζ deficiency influences the differentiation of these two cell lineages, I performed immunofluorescence analysis of the mammary glands of 6-week-old mice using well-established cytokeratin lineage markers: cytokeratin 14 (CK14) to label basal epithelial and cytokeratin 8 (CK8) to label luminal epithelial cells (Mikaelian et al. 2006; Sun et al. 2010).

Strikingly, basal cell populations were greatly reduced in KO mammary ducts compared to those in WT mice **(Figure 3.8A**, **C)**, whereas there was no impact on luminal populations **(Figure 3.8A, B)**. I also observed a trend towards a reduction of the basal population in HET animals compared to WT mice **(Figure 3.8C).**

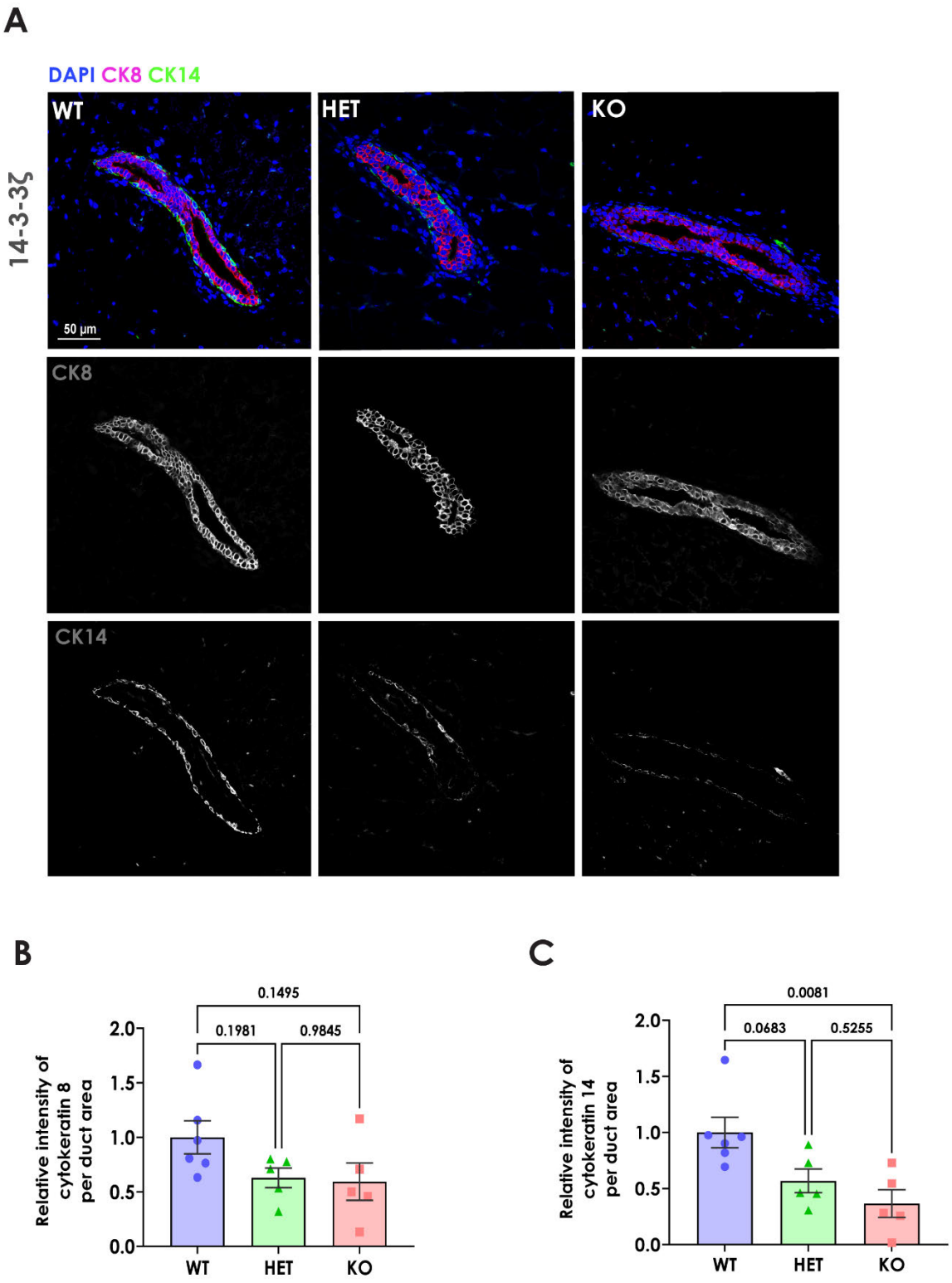


Figure 3.8: 14-3-3ζ deletion limits the basal but not the luminal epithelial cell population in the developing mammary gland

(A) Representative images of cytokeratin 8 (luminal) and cytokeratin 14 (basal) immunofluorescence analysis of mammary ducts at 6-week-old mice. Relative intensity of CK8 **(B)** and CK14 **(C)** signal quantified per area of ductal epithelium and normalised to WT. Data is Mean±SEM; P values are shown on the graphs and were determined by the unpaired t-test; (n=6 (WT), n=4 (HET and KO) individual mammary glands).

3.3.6. *14-3-3ζ deficiency represses the expression of key genes governing ductal branching morphogenesis*

Since 14-3-3ζ deletion suppressed mammary ductal elaboration, I decided to investigate whether it also influenced the expression of genes known to govern developmental processes in the murine mammary gland **(Figure 3.9)**. *Dach1* (Jiao et al. 2019), *Gata3* (Asselin-Labat et al. 2007a; Kouros-Mehr et al. 2006), *Notch1* (Bouras et al. 2008) and *Pygo2* (Gu, B et al. 2013) have been shown to regulate mammary epithelial cell fate specification at the early stages of the differentiation cascade. qRT-PCR analysis of 6-week-old animals revealed five-fold lower *Notch1* and *Pygo2* mRNA in 14-3-3ζ-deficient mammary glands compared to WT mammary glands. A trend towards lower gene expression of *Dach1* and *Gata3* was also observed in KO mammary glands, but these were not statistically significant.

These data suggest that that impaired branching morphogenesis observed in 14-3-3ζ KO mammary glands may result from the repression of *Notch1* and *Pygo2* gene expression.

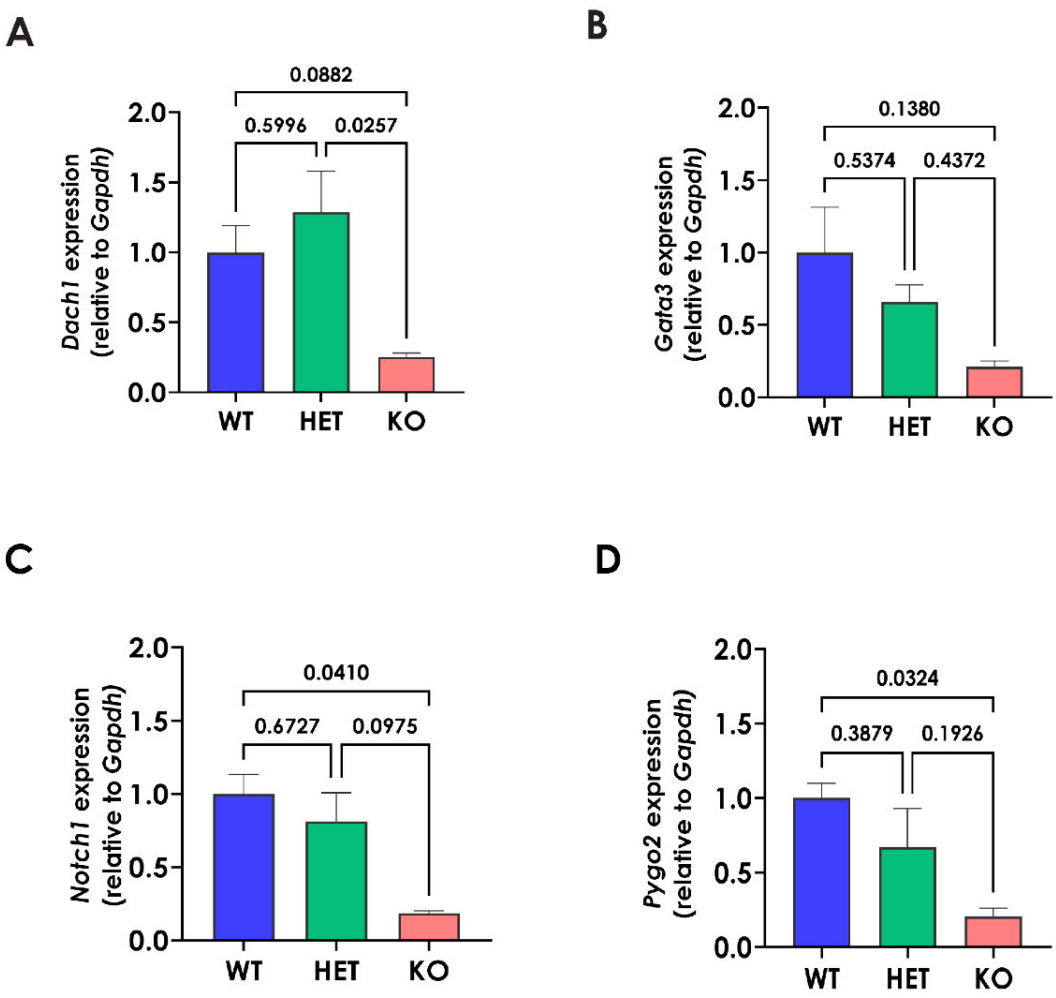


Figure 3.9: 14-3-3ζ deletion reduces expression of key genes governing ductal branching morphogenesis.

qRT-PCR to quantify the mRNA expression of genes regulating branching morphogenesis, in the mammary glands of 6-week-old WT, HET and KO mice. to quantify the mRNA expression of genes regulating branching morphogenesis, in the mammary glands of 6-week-old WT, HET and KO mice. Expression levels of *Dach1* **(A),** *Gata3* **(B),** *Notch1* **(C)** and *Pygo2* **(D)** relative to *Gapdh* and expressed as fold change relative to WT. Data are Mean ± SEM; P values are shown on the graphs and were determined by one-way ANOVA; n=3 mice per genotype.

3.4. Discussion

In this chapter, I sought to determine whether 14-3-3ζ has a role in post-natal mammary gland development. The rationale for these series of investigations was to understand whether 14-3-3 inhibitor therapies proposed for select cancers and to accelerate the healing of chronic wounds may impair this normal developmental process. Overall, my observations suggest that 14-3-3ζ plays an important role in process of branching morphogenesis of the mammary gland, since its deficiency impairs elaboration of the mammary epithelial tree. I have established here, that 14-3-3ζ is required to maintain normal levels of cell proliferation for development and may also play a role in the differentiation of mammary epithelial cell lineages.

My observations are similar but not identical to those of a report (Joshi et al. 2017) published while I was conducting my analyses, also showing a transient delay in mammary gland development in 14-3-3ζ KO animals on the FVB/NJ strain background. However, this paper reported that the developmental delay only persisted until 8 weeks of age, on the basis of a relatively cursory analysis. My careful analysis demonstrates that mammary gland development is in fact delayed till at least 11 weeks of age in 14-3-3ζ-deficient mice.

An important point to be made here is that 14-3-3ζ KO mice lack 14-3-3ζ throughout the whole body and throughout their development, and therefore systemic effects of 14-3-3ζ deficiency likely strongly influence mammary development in these mice. Pituitary Growth Hormone (GH) and ovarian estrogen promote cell proliferation and formation of TEBs (Brisken & Ataca 2015). It would therefore be important to establish, for instance, whether 14-3-3ζ deficiency in these organs impairs hormone production and therefore whether the observed mammary developmental defect may be independent of 14-3-3ζ expression within the mammary gland itself. I have also observed impaired fertility in both female and male KO animals, which could be an indication that the effect of 14-3-3ζ deletion may be systemic. Thus, deletion of 14-3-3ζ could be influencing the levels of sex hormones in this animal model, potentially giving rise to the transient defect in the mammary gland development. These issues could be dissected out using an inducible model of 14-3-3ζ-deficient mice when the deletion can be temporally regulated. Such a model is currently in development in our laboratory. Another way to assess the contribution of the endocrine system is to assess the circulating levels of GH and estrogen, and if a reduction is observed, evaluate the effect of supplementation on mammary development in 14-3-3ζ-deficient mice. However, given the transient nature of the

developmental impairment observed, it is unlikely that such crucial regulator of branching morphogenesis as estrogen is significantly affected at a systemic level in this model.

Furthermore, at the level of mammary tissue, there are two interrelated ways in which the developmental process in the mammary gland could be influenced by 14-3-3ζ, the epithelial cell specific effects and the stromal-generated influences. It is now well-established that stromal components such as fibroblasts (Hammer et al. 2017), adipocytes (Hovey & Aimo 2010), macrophages (Dawson et al. 2020; Ingman et al. 2006), mast cells (Lilla & Werb 2010) and extracellular matrix (Ghajar & Bissell 2008) play essential roles in mammary gland morphogenesis. It therefore remains to be established whether 14-3-3ζ expressed within the epithelium, the cells of the stroma or indeed both may influence mammary gland development in this model. This could also be addressed using floxed 14-3-3ζ mice inter-crossed onto the appropriate strains expressing cre-recombinase in the specific cells of interest, such as for instance, MMTV-Cre (mammary epithelium-specific) and Col1a2-Cre (normal tissue fibroblasts-specific), which would permit the ablation of 14-3-3ζ in those specific cells.

14-3-3 potentiates proliferative and pro-survival signalling pathways such as the Raf-MAPK, Hippo and PI3K-PDK-AKT pathways (Morrison 2009), and it has been shown that PI3K signalling is required for both branching initiation and elongation (Zhu & Nelson 2013). A potential role for 14-3-3ζ deficiency in mammary epithelial cells on mammary gland development could therefore be investigated by assessing its influence on PI3K signalling within the mammary epithelium by assessing AKT phosphorylation levels. Another potential avenue for investigation is the insulin-like growth factor 1 receptor (IGF-1R), which has been shown to interact with the 14-3-3 protein family (Parvaresch et al. 2002) and is known to regulate the proliferation of cells comprising TEBs (Bonnette & Hadsell 2001).

I have shown that the expression of *Notch-1, Pygo-2, Gata-3* and *Dach1* genes are reduced upon 14-3-3ζ deletion. *Notch-1* is important in stem cell maintenance, cell-fate specification, and differentiation (Bouras et al. 2008). *Dach1* is crucial for the expansion of mammary gland basal cells (Jiao et al. 2019). *Gata-3* is important for the formation of the TEBs (Kouros-Mehr et al. 2006) and low levels of *Gata-3* expression are found in stem cell-enriched populations (Asselin-Labat et al. 2007a). These observations could indicate that 14-3-3ζ deletion impairs mammary epithelial lineage specification.

Previously, deletion of 14-3-3ζ has been implicated in a reduction in interneurons during embryonic development (Greenberg 2017), which could be associated with the stem cell defects. Since we have observed the reduction in basal epithelial cell populations, another

possibility is that 14-3-3ζ functions in stem cell development and differentiation of progenitor populations in the mammary gland (Lloyd-Lewis et al. 2017; Van Keymeulen et al. 2011). This can be investigated further using flow cytometry analysis of the lineage depleted epithelial cells from the mammary glands for proportions of basal and luminal epithelial compartments and performing downstream gene expression analysis on the sorted cellular population (Fu et al. 2020) to determine how progenitor populations are affected by 14-3-3ζ deficiency.

It would be essential to conduct a closer analysis of the morphology of the TEB structures since there was such a striking difference in the TEB numbers between WT and KO animals. It would be interesting to examine the proliferation rates in the intact TEB structures using current tissue clearing techniques and confocal microscopy (Lloyd-Lewis et al. 2016; Rios et al. 2019) which could reveal the spatial distribution of the proliferating cells and the potential implications of 14-3-3ζ in directing apical-basal polarity, which is also crucial for the process of branching morphogenesis in the mammary gland (Huebner, Lechler & Ewald 2014).

The stromal effects of 14-3-3ζ deletion on the mammary gland development could be further investigated in relation to specific cell types. The adipose microenvironment is the source of important factors that influence epithelial cell proliferation and survival which affects TEB formation and branching morphogenesis (Landskroner-Eiger et al. 2010). Since adipocytes are significantly smaller and less differentiated in 14-3-3ζ-deficient animals this should be further investigated (Lim et al. 2015). Interestingly a similar developmental phenotype (reduction in both the number of TEBs and epithelial cells proliferation rate) is observed when mast cells are deleted from the mammary gland (Lilla & Werb 2010).

Furthermore, at specific developmental time separating the TEBs, mature duct cells and stroma by microdissection and conducting a thorough analysis of the gene regulation could be performed (Kouros-Mehr & Werb 2006).

3.5. Conclusion

In conclusion, I have established that 14-3-3ζ plays an important role in the process of branching morphogenesis of the mammary gland by maintaining normal levels of cell proliferation and potentially regulating normal basal cell differentiation. However, my investigations have revealed that the impairment of mammary gland development in 14-3-3ζ KO mice renders them less than ideal for the study of 14-3-3ζ in mammary cancer development.

This is because any differences observed in tumourigenesis between WT and 14-3-3ζ deficient mice cannot be unambiguously attributed to a function of 14-3-3ζ in tumours as distinct from the pre-existing developmental impairment of the 14-3-3ζ-deficient mammary gland. While this is somewhat disappointing, this study also clearly demonstrates that since 14-3-3ζ heterozygous mice exhibit overall normal mammary gland development, they can be used to investigate whether 14-3-3ζ haploinsufficiency modulates tumour growth. This investigation is the subject of the next chapter of my thesis.

Chapter 4. Investigating the Roles of 14-3-3ζ in Mammary Cancer

4.1. Introduction

Over the past 15 years, systematic approaches to diagnostics and treatments of early breast cancers have dramatically improved the overall patient survival rates, leading to a 70-80% cure rate. In contrast, advanced invasive and metastatic disease remains the main cause of breast cancer-related deaths and is currently incurable (Harbeck et al. 2019). Therefore, there is a pressing need to investigate the molecular mechanisms that drive invasive disease progression and to identify novel therapeutic approaches in breast cancer treatment.

14-3-3ζ is proposed as a therapeutic target in breast cancers since it upregulated at the mRNA level in both ductal breast carcinomas in situ (DCIS) and invasive ductal carcinomas (IDC) (Mei et al. 2020). High levels of 14-3-3ζ in breast cancer patients correlates with disease recurrence and overall poor survival (Neal et al. 2009). However, there is as yet no published studies that investigate therapeutic 14-3-3ζ inhibition in in vivo mammary cancer models.

Prior to commencement of my experimental work, the role of 14-3-3ζ in mammary cancers in vivo had only been investigated in an overexpression mouse model (Rehman et al. 2014) and the majority of in vitro studies relied on cell lines and siRNA knock down approaches (Bergamaschi & Katzenellenbogen 2012; Jin et al. 2016; Lu, J et al. 2009; Neal et al. 2012; Neal et al. 2009; Rehman et al. 2014; Xu et al. 2015). Whilst in vitro approaches are indispensable for dissecting signalling pathways in tumourigenesis, such approaches must be complemented by investigations in physiologically relevant pre-clinical in vivo models. Given the importance of the tumour microenvironment in the breast cancer progression, in vivo models are essential for accurately modelling the effects of the microenvironment on the process of tumourigenesis (Soysal, Tzankov & Muenst 2015).

Our laboratories identified a unique approach to inhibit 14-3-3 protein function, using a family of sphingo-mimetic compounds designated RB-011, RB-012, RB-066, RB-067 and RB-068, which I discussed in more detail in Section 1.3.4 (page 33). RB-011 significantly reduced tumour burden in vivo in a xenograft mouse model using a lung cancer cell line (Woodcock et al. 2015) and accelerated the healing of skin wounds, by targeting 14-3-3 function (Kular et al. 2015).

To investigate a therapeutic potential of 14-3-3ζ inhibition in mammary cancer in vivo, I established a mammary cancer model with 14-3-3ζ deficiency and utilised RB-012 compound, to investigate the effects of 14-3-3 inhibition in physiologically relevant pre-clinical model of breast cancer.

4.2. Hypothesis and Aims

I hypothesised that 14-3-3ζ plays a tumour-promoting role in mammary cancer progression.

The aims of the work presented in this chapter were to characterise tumour progression in a mouse model of mammary cancer of 14-3-3ζ deficiency and to evaluate the translational potential of 14-3-3 inhibition as a potential breast cancer treatment in vivo.

4.3. Results

4.3.1. *14-3-3ζ mRNA Expression is Upregulated in Human Breast Cancer Tissues*

To investigate the significance of the prognostic value and gene expression levels of 14-3-3ζ in the human breast cancer samples, I took advantage of the publicly available METABRIC dataset, which includes gene expression data derived from over 2,000 breast cancer samples (Curtis et al. 2012; Pereira et al. 2016).

My analyses revealed a significant association between high 14-3-3ζ mRNA expression and low five-year survival of patients with invasive breast cancer **(Figure 4.1A)** suggesting that there is a prognostic value in the levels of 14-3-3ζ mRNA expression in breast cancer.

The levels of the 14-3-3ζ mRNA were significantly higher in the breast tumour samples compared to the normal tissue **(Figure 4.1B)**. Interestingly, I observed a wide spread of expression values of 14-3-3ζ mRNA in tumour samples, suggesting the potential for further stratification of the patients based on their levels of 14-3-3ζ mRNA expression.

Further analysis of the METABRIC dataset revealed several informative associations. I observed that 14-3-3ζ mRNA expression levels increased with the increase in the tumour grade **(Figure 4.1C)**. Tumour grade is indicative of the advanced stage of the disease and poor prognosis in breast cancer (Malhotra et al. 2010). This observation suggests that 14-3-3ζ mRNA levels could be indicative of the advanced stage of the disease, with possible utility as a diagnostic marker in staging and should also be evaluated as a possible prognostic marker.

I then grouped 14-3-3ζ mRNA expression levels based on the currently accepted molecular subtypes of the breast tumours. Basal, HER2 and Luminal B subtypes showed significantly higher expression of 14-3-3ζ mRNA compared to Normal-like and Luminal A subtypes **(Figure 4.1E)**. I have previously described (Chapter 3 of this thesis), how in normal mammary

development, 14-3-3ζ deletion depletes basal progenitor populations. It is possible to hypothesise from these observations, that during cancer progression 14-3-3ζ may skew the mammary cell populations towards basal tumour progression. Furthermore, ~80% of basal-like tumours are referred to as triple negative (TNBC), which do not express receptors for estrogen or progesterone and lack expression of HER2, a receptor for epidermal growth factor (Gregorio et al. 2018; Gusterson & Eaves 2018). These tumours are high grade which is consistent with overexpression of 14-3-3ζ **(Figure 4.1C)**. Moreover, ~30% of basal-like breast cancers termed "Claudin-low" have a signature of elevated markers of epithelial to mesenchymal transition, immune response genes, and 'stem' cells (Gusterson & Eaves 2018). Consistent with these prior findings, my analysis of the TNBC cohort revealed significantly higher levels of 14-3-3ζ mRNA in TNBC patients **(Figure 4.1D)** compared to all breast cancers, suggesting that 14-3-3ζ potentially has a functional role in these subtypes.

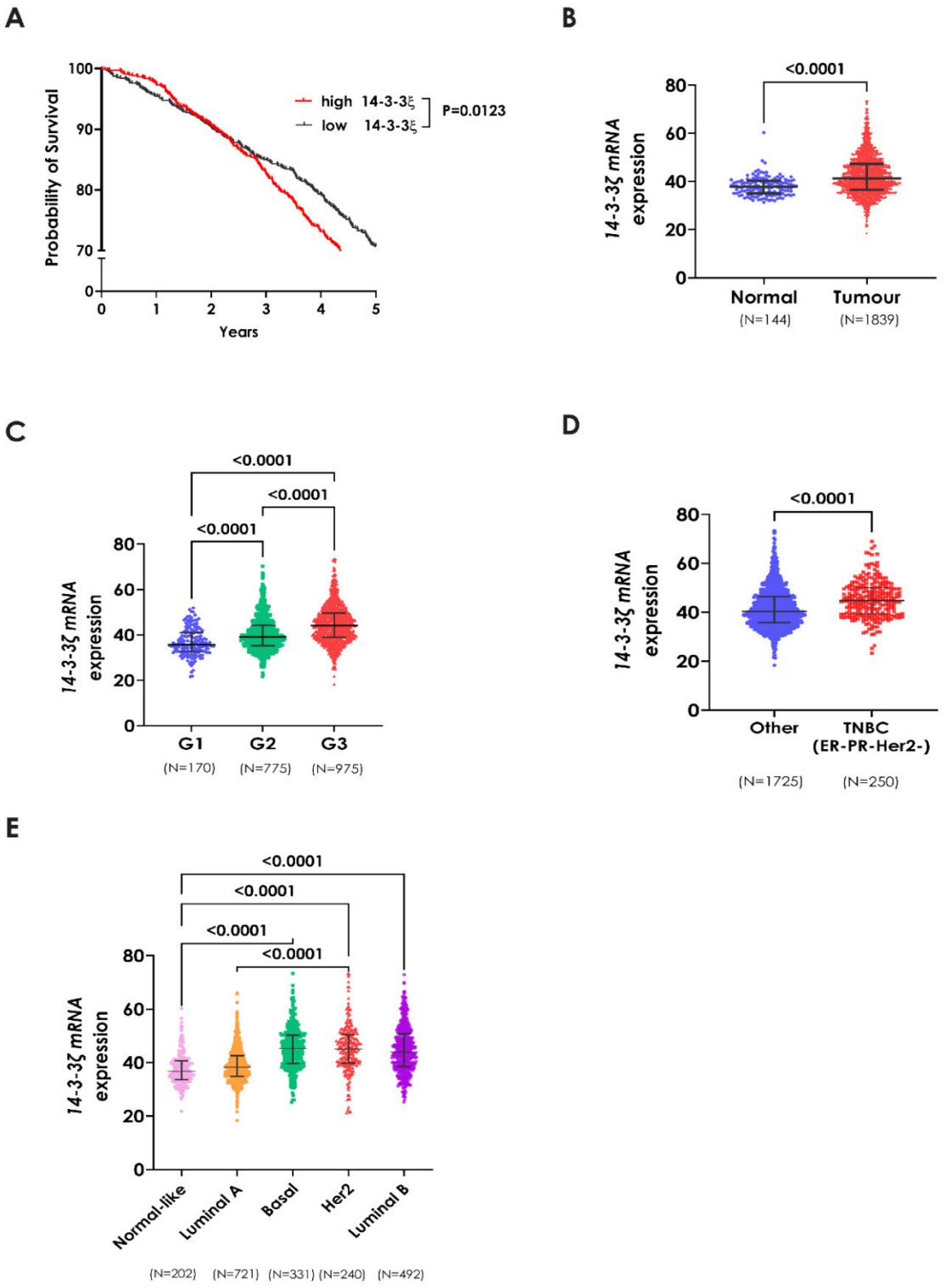


Figure 4.1: 14-3-3ζ mRNA is significantly upregulated in human breast cancer tissues

(A) Five-year Kaplan–Meier survival probability curve for invasive breast cancer patients with high or low 14-3-3ζ mRNA expression (above or below the median value) using METABRIC dataset. P-value calculated using Mantel–Cox test; **(B)** Comparison of 14-3-3ζ mRNA expression levels between normal breast and tumour tissues. Data is Median±IQR; two-sided Mann–Whitney test. **(C)** Comparison of 14-3-3ζ mRNA expression levels against tumour grade. Data is Median±IQR; Kruskal-Wallis test. **(D)** Comparison of 14-3-3ζ mRNA expression levels between triple-negative breast cancer (TNBC) subset and other breast

cancer subtypes. Data is Median±IQR; two-sided Mann–Whitney test. **(E)** Comparison of 14-3-3ζ mRNA expression levels between PAM50 subsets. Data is Median±IQR; Kruskal-Wallis test. METABRIC dataset. The number of samples per category are indicated within brackets.

4.3.2. *14-3-3ζ Promotes MMTV-PyMT-Driven Mammary Carcinogenesis*

In Chapter 3, I demonstrated an important role for 14-3-3ζ in normal mammary gland development, showing that the elaboration of the mammary epithelial tree was significantly delayed in the 14-3-3ζ KO animals compared to WTs or 14-3-3ζ HETs. Since the process of epithelial growth is impaired in 14-3-3ζ KO mice, it makes these mice unsuitable for the study of mammary cancer development since it would be difficult to distinguish between the roles of 14-3-3ζ in development and in cancer. However, I also demonstrated that in 14-3-3ζ HET mice there is an overall normal mammary gland development phenotype and therefore they can be used to understand whether 14-3-3ζ heterozygosity modulates mammary tumour growth.

To investigate the role of 14-3-3ζ in the mammary carcinogenesis I inter-crossed 14-3-3ζ HET females with MMTV-PyMT;14-3-3ζ HET males to produce progeny of MMTV-PyMT;14-3-3ζ WT (PyMT-ζWT) or MMTV-PyMT;14-3-3ζ HET (PyMT-ζHET) genotypes. This breeding strategy was necessary because female mice expressing PyMT within the mammary gland cannot be used for breeding as they develop mammary tumours. All mice were on the FVB/NJ strain background.

I monitored the onset of tumours in these two strains by palpation and assessed tumour growth using calliper measurements. The onset of the palpable tumours was significantly delayed in PyMT-ζHET mice compared to PyMT-ζWT mice **(Figure 4.2A)** suggesting that deletion of just one copy of 14-3-3ζ significantly delays tumour initiation in this model.

Furthermore, the cumulative tumour volume **(Figure 4.2B)** and weight **(Figure 4.2C)** were ~50% lower in HET group compared to WT controls. Overall tumours from PyMT-ζHET cohort were smaller than in PyMT-ζWT group and less vascularised **(Figure 4.2D).**

Together, these observations suggest that 14-3-3ζ has a tumour-promoting role in the mammary cancer progression, particularly at the early stages of the tumour development.

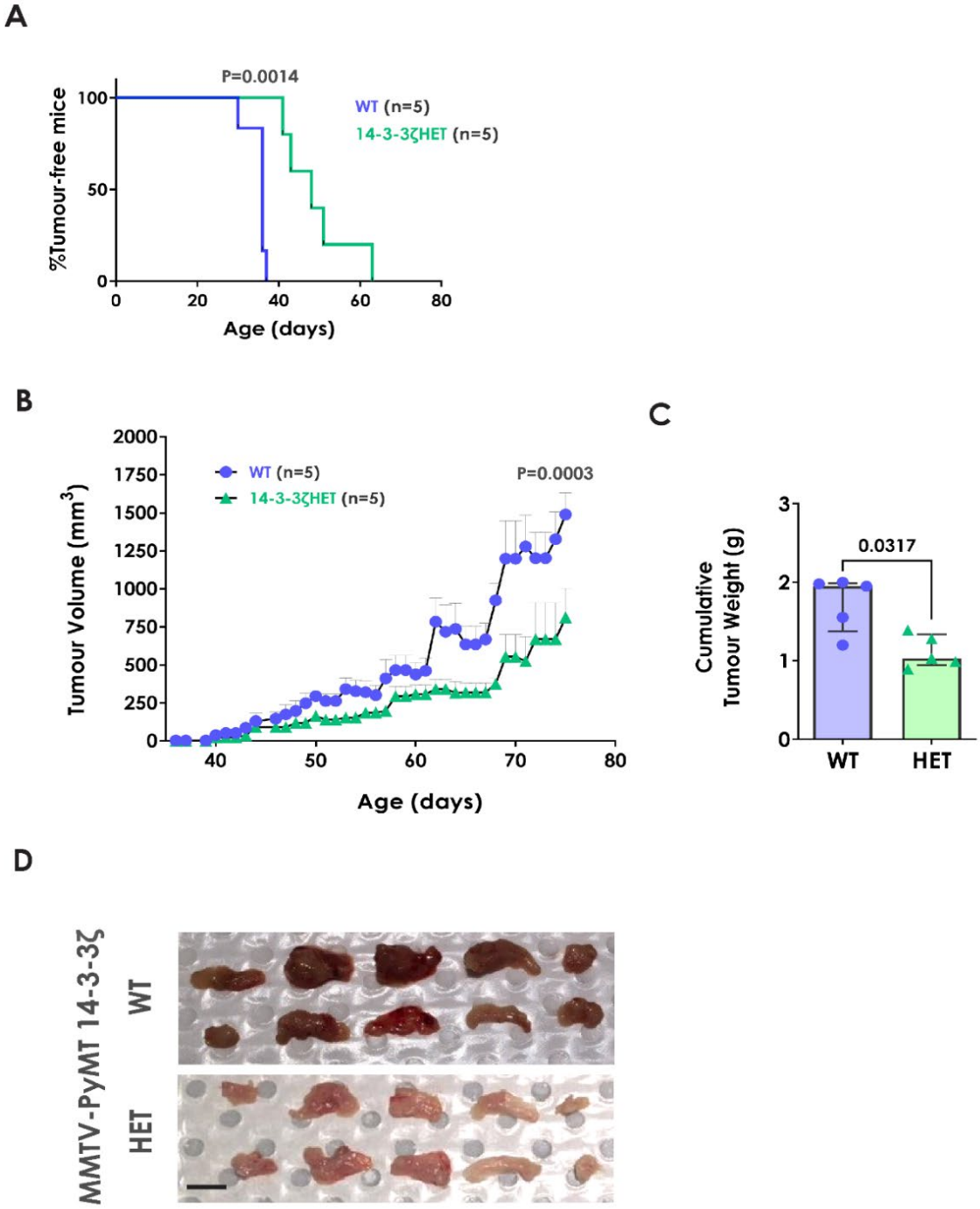


Figure 4.2: 14-3-3ζ promotes MMTV-PyMT-driven mammary carcinogenesis

(A) Kaplan–Meier curves show tumour-free survival in PyMT-ζWT and PyMT-ζHET mice compared using the Mantel–Cox test (n=5 per genotype). **(B)** Cumulative tumour volume of PyMT-ζWT and PyMT-ζHET mice (n=5 per genotype). Data are Median±IQR; two-way ANOVA. **(C)** Cumulative tumour weight taken at 11 weeks of age (n=5 each). Data are Median±IQR; two-sided Mann–Whitney test. **(D)** Representative images of all mammary glands with tumours from mice at 11 weeks of age. Scale bar=10 mm.

Histological analysis of the tumour tissues at the end point revealed that there was a significantly lower cancer cell proliferation rate in the PyMT-ζHET tumour tissues compared to PyMT-ζWT **(Figure 4.3A)** and no difference in the rate of apoptosis **(Figure 4.3B)** as analysed for Ki-67 by immunohistochemistry and TUNEL assay by fluorescence, respectively.

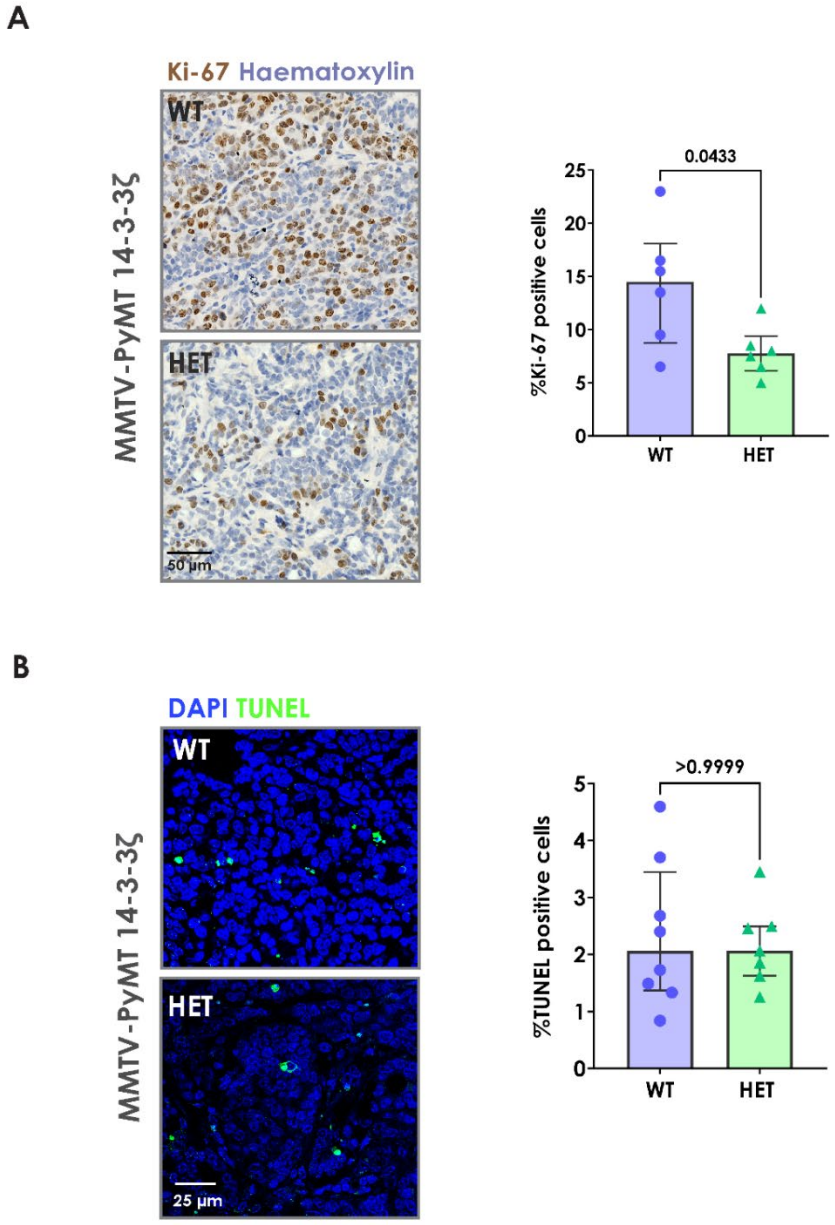


Figure 4.3: 14-3-3ζ reduction decreases epithelial cell proliferation rate and has no effect on apoptosis in mammary tumours at advanced stages of tumourigenesis

(A) Representative images of IHC analysis of Ki-67 in 11-week tumours from PyMT-ζWT and PyMT-ζHET mice, shown quantified as Median±IQR; two-sided Mann–Whitney test. **(B)** Representative images TUNEL fluorescence and quantification of percent of apoptotic cells per lesion area. Data are Median±IQR; two-sided Mann–Whitney test.

4.3.3. *Reverse-Phase Protein Array (RPPA) Analysis of PyMT-ζWT and PyMT-ζHET Tumours*

To investigate the molecular changes that could explain the significant differences in tumour growth between the two genotypes, we performed reverse-phase protein array (RPPA) analysis of the whole tumour tissues. RPPA is a high-throughput dot blot technique that allows quantitatively measurement of hundreds of signalling proteins (including phosphoproteins) in biological samples under the same experimental conditions (Boellner & Becker 2015; Paweletz et al. 2001). A total of 58 proteins and phosphoproteins were analysed (please refer to **Table A.1** in Appendix A for summary of data). The RPPA analysis revealed reduced levels of MAPK signalling effectors activated ERK1 and ERK2 **(Figure 4.4A)**; activated c-Jun **(Figure 4.4B)** and activated Yes-associated protein (YAP) **(Figure 4.4C)** in PyMT-ζHET tumours**.** The level of prohibitin **(Figure 4.4D)** was upregulated in PyMT-ζHET tumours. These results strongly suggest that 14-3-3ζ affects key signalling pathways that are associated with hallmark characteristics of breast cancer cells, such as cellular proliferation (MAPK, c-Jun, prohibitin) (Liu, P et al. 2017; Mebratu & Tesfaigzi 2009; Vleugel et al. 2006), anti-apoptotic activity (c-Jun) (Han, JY et al. 2008), and tumour suppressive properties of YAP that keep in check migratory and invasive properties (Yuan et al. 2008). These observations are consistent with my previous observations that show that 14-3-3ζ heterozygosity leads to decreased cellular proliferation in mammary tumours **(Figure 4.3A)** and is associated with reduced tumour growth **(Figure 4.2)**. A more elaborate discussion of these results is included at the end of this chapter.

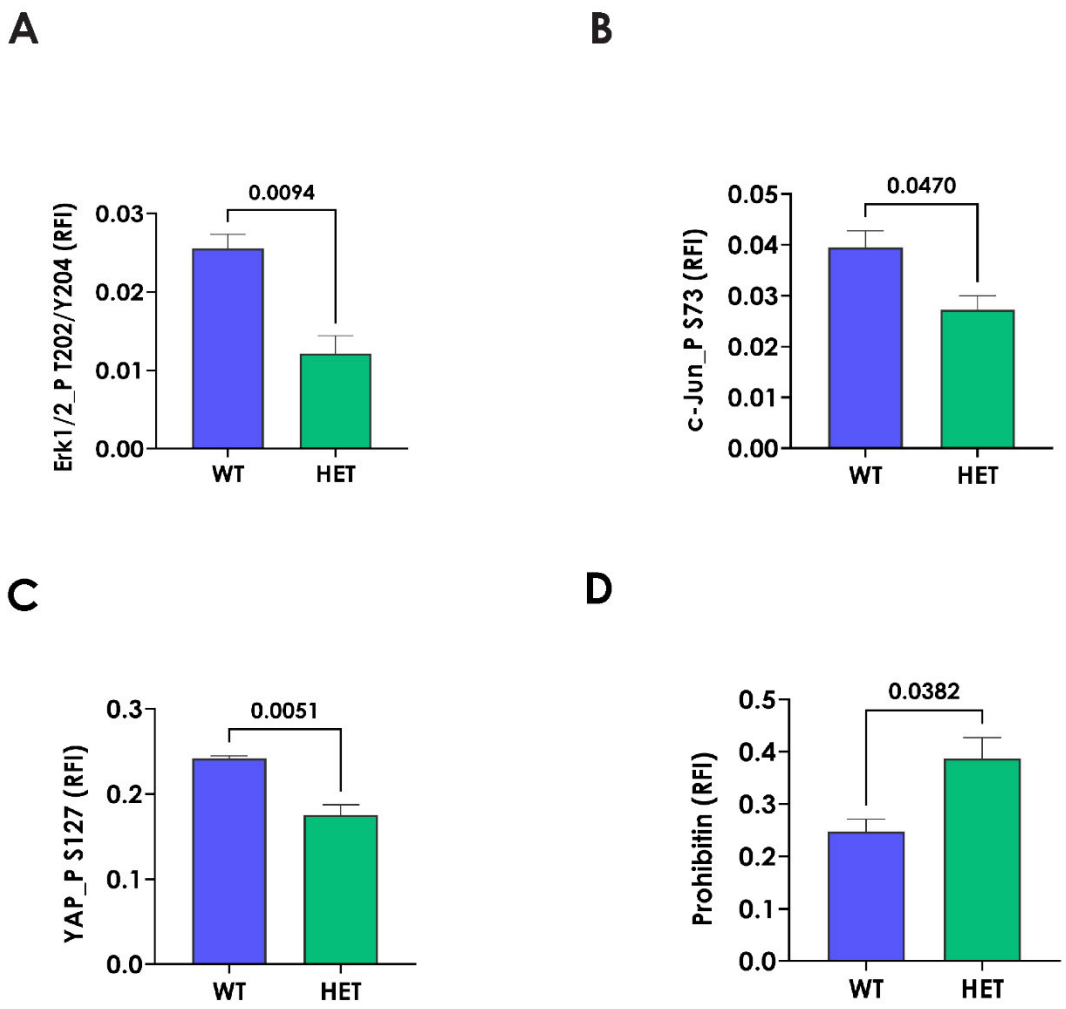


Figure 4.4: RPPA analysis of PyMT-ζWT and PyMT-ζHET tumours

Relative fluorescent intensity (RFI) values from RPPA analysis for active forms of Erk1/2 (A), c-Jun (B), YAP (C) and levels of prohibitin (D). Data is Mean±SEM; P values were determined using a student t-test.

4.3.4. *14-3-3ζ Heterozygosity Delays Tumour Initiation in MMTV-PyMT-Driven Mammary Tumourigenesis*

Since the tumour onset was significantly delayed in PyMT-ζHET animals compared to controls **(Figure 4.2A)**, I investigated whether 14-3-3ζ is important in earlier stages of tumourigenesis. I compared the histology of the 4th mammary fat pads (MFP) of PyMT-ζWT and PyMT-ζHET mice at eight weeks of age. Gross morphological assessment of whole-mount MFPs indicates overall fewer tumour lesions in PyMT-ζHET compared to PyMT-ζWT MFPs **(Figure 4.5A)**. Quantification of tumour lesions per area of mammary epithelium revealed significant lower

tumour area in PyMT-ζHET MFPs compared to the PyMT-ζWT MFPs **(Figure 4.5B).** These observations strongly suggest that 14-3-3ζ plays a tumour-promoting role at the early stages of mammary cancer development and potentially affects tumour cell proliferation.

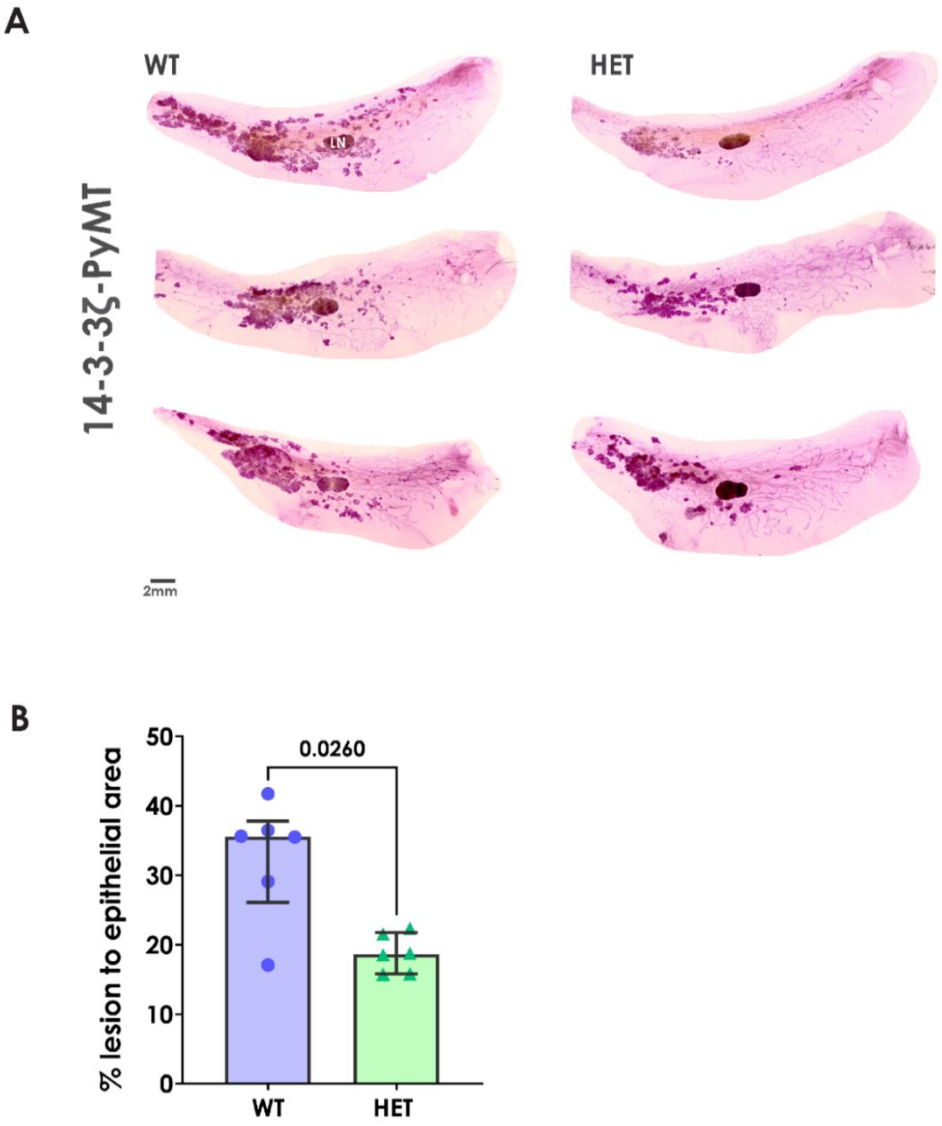


Figure 4.5: 14-3-3ζ heterozygosity delays tumour initiation in MMTV-PyMT-driven mammary tumourigenesis

(A) Representative whole-mount images of tumour lesions in PyMT-ζWT and PyMT-ζHET mice (n=6). Scale bar=2mm. **(B)** Quantification of tumour lesion area to total mammary fat pad epithelial area at eight weeks of age. Data are represented as Median±IQR and P values derived using the two-sided Mann–Whitney test.

4.3.5. *14-3-3ζ Heterozygosity Limits Epithelial Cell Proliferation but Does not Influence Apoptosis in Mammary Tumours at Early Stages of Tumourigenesis*

Two major hallmarks of cancer cells are unrestrained proliferation and resistance to programmed cell death (Hanahan & Weinberg 2011). Since 14-3-3 protein family members are known regulators of proliferative and apoptotic pathways (Morrison 2009), I investigated the rate of cell proliferation and apoptosis in PyMT-ζWT and PyMT-ζHET tumours.

I observed a two-fold lower proliferation rate in PyMT-ζHET tumour cells compared to PyMT-ζWT as assessed by BrdU incorporation **(Figure 4.6A).** These observations suggest that 14-3-3ζ levels strongly influence in vivo tumour cell proliferation in the PyMT model of mammary cancer. I next examined the level of apoptosis in PyMT-ζWT and PyMT-ζHET tumours using the TUNEL assay **(Figure 4.6B)**. Although there was a trend toward increased apoptosis in PyMT-ζHET tumours, the difference was not statistically significant, suggesting that apoptosis is not strongly influenced by 14-3-3ζ reduction at this stage of tumourigenesis.

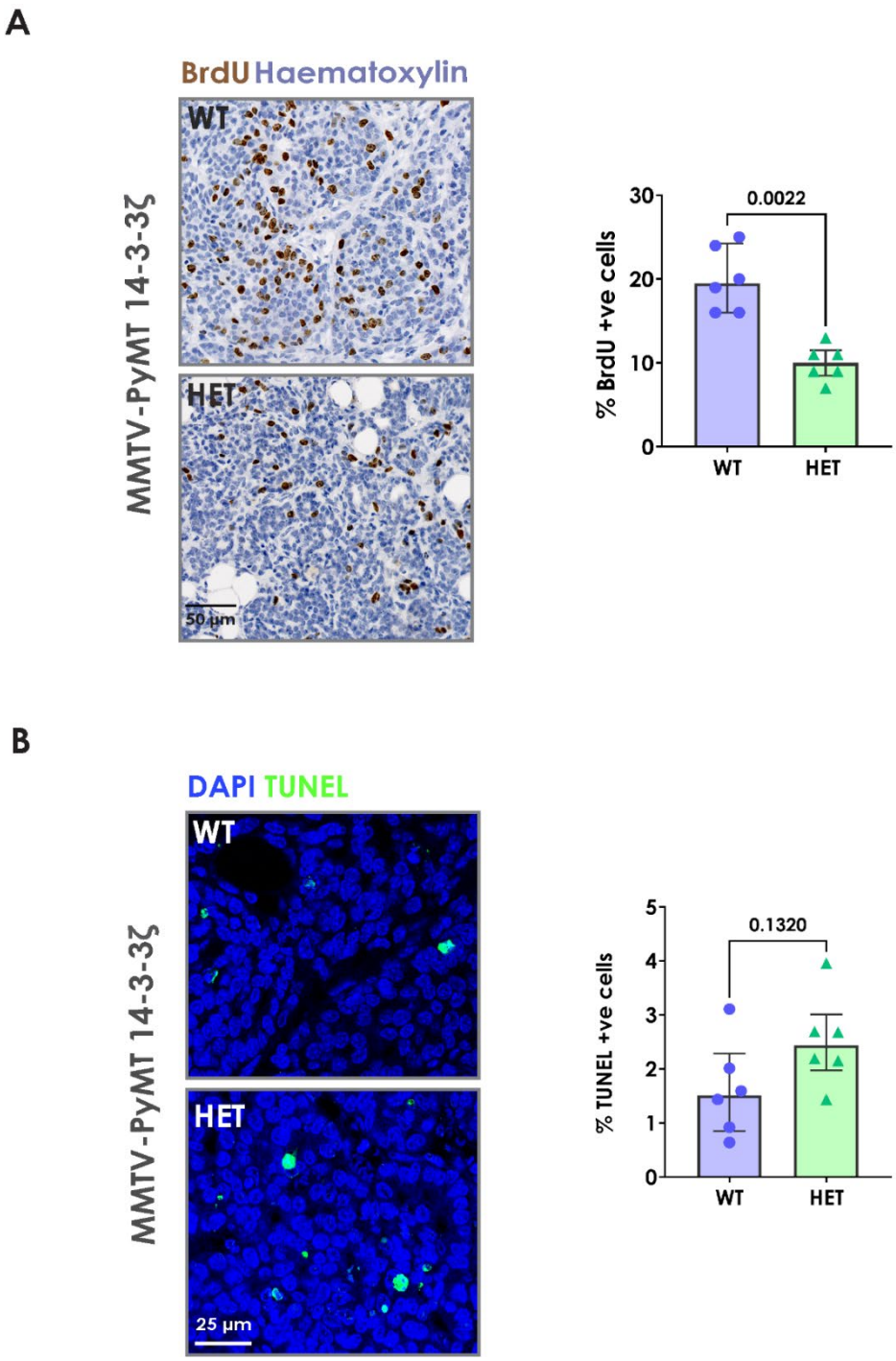


Figure 4.6: 14-3-3ζ reduction decreases epithelial cell proliferation rate and has no effect on apoptosis in mammary tumours

(A) Representative images of IHC analysis of BrdU incorporation assay in PyMT-ζWT and PyMT-ζHET tumours at eight weeks and quantification of the rate of proliferation. Data is Median±IQR; two-sided Mann–Whitney test. **(B)** Representative images of immunofluorescent analysis of TUNEL and quantification of percent of apoptotic cells per lesion area. Data is Median±IQR; two-sided Mann–Whitney test.

4.3.6. *14-3-3ζ Reduction in Mammary Tumours Does not Affect Rho/ROCK Signalling Pathway*

Our laboratory previously established a key role for 14-3-3ζ as a negative regulator of the Rho/ROCK signalling pathway in the skin (Kular et al. 2015). We have also recently established that Rho/ROCK signalling is enhanced in advanced mammary cancers relative to early lesions, and that enforcing enhanced signalling through the Rho/ROCK pathway promotes tumour progression in mammary cancers (Boyle et al. 2020). Therefore, I investigated whether the Rho/ROCK signalling pathway is altered in mammary tumours heterozygous for 14-3-3ζ.

I assessed ROCK activity by measuring the phosphorylation of ROCK substrates, MYPT at ROCK-target site Thr-696 (Feng, J et al. 1999) **(Figure 4.7A)** and MLC at ROCK-target site Ser-19 (Amano et al. 1996) **(Figure 4.7B)** by quantitative immunofluorescence analysis. These analyses revealed that Rho/ROCK signalling flux in PyMT-ζHET tumours was comparable to that in PyMT-ζWT controls. This observation suggests that the negative regulatory role of 14-3-3ζ in Rho/ROCK signalling pathway is tissue and context specific and does not play a role in mammary tumourigenesis. 14-3-3ζ-mediated enhancement of mammary tumourigenesis is therefore independent of Rho/ROCK signalling and likely mediated by alternative signalling pathways.

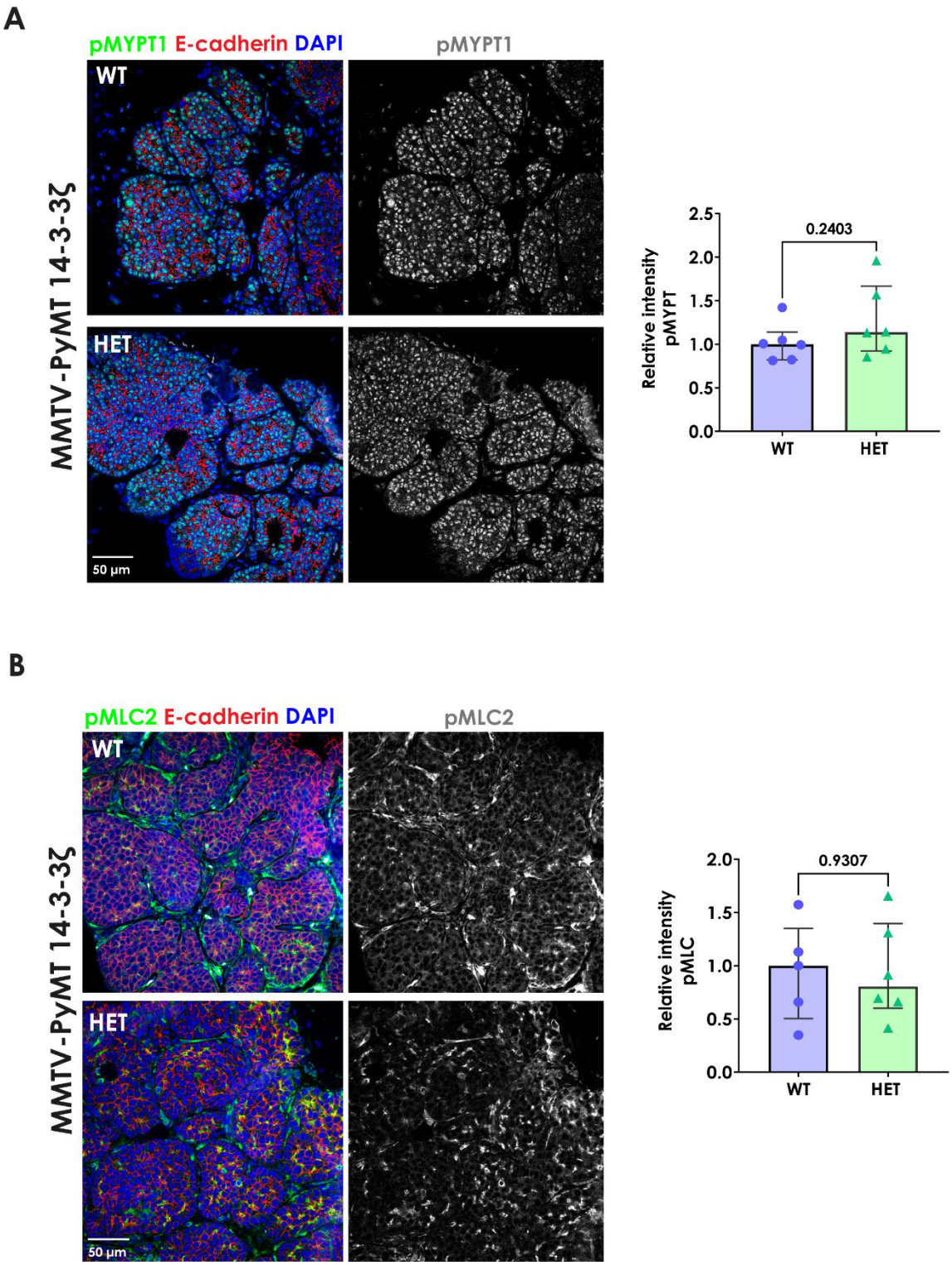


Figure 4.7: 14-3-3ζ heterozygosity does not affect signalling through the Rho/ROCK pathway in PyMT mammary tumours

Representative IF images of downstream effectors of Rho/ROCK signalling pathway pMypt1 **(A)** and pMlc2 **(B)** at 11 weeks with quantitative analysis of signal intensity per tumour area normalised to PyMT-ζWT control. Data are shown as Mean±SEM; two-sided Mann–Whitney test.

4.3.7. *14-3-3ζ Reduction in Mammary Tumours Does not Affect Extracellular Matrix Composition*

It is well-established that ECM components are important players in the process of mammary tumourigenesis (Gritsenko, Ilina & Friedl 2012; Levental et al. 2009; Northey et al. 2020; Provenzano et al. 2008) and our laboratory has demonstrated that mammary cancer cells corrupt fibroblasts to produce and remodel of ECM within their microenvironment (Boyle et al. 2020). I therefore assessed the levels of ECM components such as collagen which is known to promote tissue stiffness in mammary cancers (Levental et al. 2009) and periostin which has tumour-promoting functions and is associated with more invasive breast cancer phenotype (Kyutoku et al. 2011; Nuzzo et al. 2016).

I also assessed the levels of vimentin, the intermediate filament protein that is highly upregulated in the invasive breast cancer samples (Khillare et al. 2019) and was upregulated in mammary tumours during tumour progression and used as a CAF marker (Boyle et al. 2020). Vimentin is also required for CAF motility that assists tumour cell invasion (Richardson et al. 2018).

I found no differences in the levels of collagen **(Figure 4.8A)**, periostin **(Figure 4.8B)** or vimentin **(Figure 4.8C)** in 11-week mammary tumours in PyMT-ζWT or PyMT-ζHET mice by SHG and immunofluorescence analyses of comparably sized tumours. These observations, taken together with those from Section 4.3.6 (page 86). demonstrate that neither the production of extracellular matrix nor signalling through the Rho/ROCK pathway are affected in 14-3-3ζ heterozygous mice. These data suggest that the tumour-promoting role of 14-3-3ζ is independent of the Rho-ROCK signalling pathway and its function in regulating ECM production within the microenvironment.

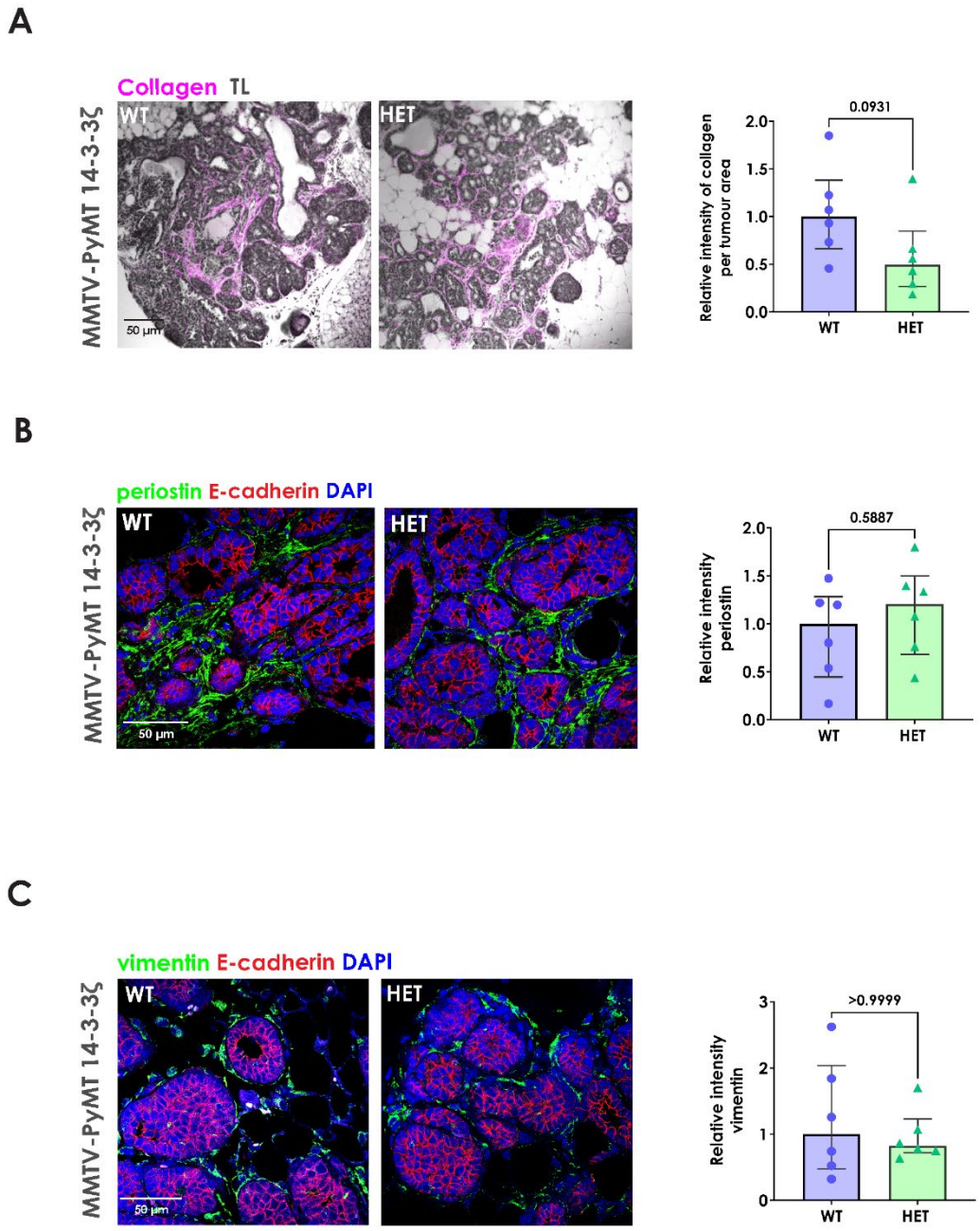


Figure 4.8: 14-3-3ζ heterozygosity does not affect extracellular matrix composition of mammary tumours

Collagen **(A)**, periostin **(B),** vimentin **(C)** levels are comparable PyMT-ζHET test and PyMT-ζWT control tumours. **(A)** Representative images of SHG analyses and Quantification of SHG signal per tumour area. **(B), (C)** Representative IF images. Quantification of intensity of stain (integrated density) per tumour area. Data are shown as Mean±SEM; P values show on graphs were determined using a two-sided Mann–Whitney test.

At this point of my investigation, Joshi et al reported on their investigations into the role of 14-3-3ζ in both the MMTV-Neu and MMTV-PyMT models of mammary cancer (Joshi et al. 2017). Consistent with my observations, the authors reported a transient delay in mammary development in whole body 14-3-3ζKO animals confirming the importance of this protein in

the process of mammary branching morphogenesis. Despite a clear developmental delay in epithelial branching, the authors went on to characterise tumourigenesis using PyMT-ζKO mice. They reported that two major signalling pathways, Ras/Raf/Erk and PI3K/Akt were found to be differentially regulated between WT and KO tumours and responsible for reduced tumour growth in KO animals. Yet, due to developmental delay in the 14-3-3ζKO animals, these results are difficult to interpret since it is not possible to be certain whether the reduced tumourigenesis is due to the developmental delay in KO mice and the resulting attenuated epithelia, or due to cancer-specific functions of 14-3-3ζ that suppress tumour growth.

Interestingly, the authors did not observe suppression of tumour growth in PyMT-ζHET mice as I did **(Figure 4.2A)**. Furthermore, the authors show that proliferation of tumour cells is significantly lower in PyMT-ζHET tumours compared to the WT group but surprisingly, this did not affect the overall tumour growth. This is different to my findings, which clearly indicate that 14-3-3ζ heterozygosity reduced proliferation of tumour cells **(Figure 4.6A)** which is associated with lower tumour burden compared to that in PyMT-ζWT mice **(Figure 4.2B)**.

Interestingly, Joshi et al. did not investigate whether it was 14-3-3ζ within the tumour cells or the microenvironment that enhanced tumour growth. Notably, we have previously highlighted an important role for 14-3-3ζ in the tumour microenvironment in a model of cutaneous squamous cell carcinoma (Kular et al. 2015), whereas there is no published research regarding the role of 14-3-3ζ in the mammary tumour microenvironment. I therefore pivoted to investigate whether 14-3-3ζ protein expressed within the microenvironment plays a role in mammary tumourigenesis.

4.3.8. *14-3-3ζ has a Tumour Promoting Role in the Mammary Tumour Microenvironment*

To investigate the role of 14-3-3ζ in the mammary tumour microenvironment, I established an engraftment model using primary mammary epithelial cells. I chose to use a primary cell engraftment approach to continue my investigations, since it recapitulates the genetic heterogeneity of human cancers and is therefore a high-quality model with clinical relevance (Kurpios et al. 2013; Lin, EY et al. 2003).

I first derived primary tumour cells from PyMT-ζWT and PyMT-ζHET donors and engrafted these separately into the 4th mammary fat pads of either ζWT or ζHET recipient female mice as detailed in Section 2.2.2.2 (page 43). Following engraftment, I traced tumour growth for a

period of 47 days, at which point the largest tumours had reached the ethical endpoint at which the experiment needed to be terminated. A time course of tumour size revealed a significant reduction in the tumour growth **(Figure 4.9A)** in PyMT-ζHET→ζWT group compared to the control PyMT-ζWT→ζWT group, and this was confirmed by final tumour weights **(Figure 4.9B)**. This observation is consistent with my previously observed slow tumour growth in the PyMT-ζHET genetic model **(Figure 4.2B)**, confirming that reduction the levels of 14-3-3ζ in cancer cells significantly reduces mammary tumour growth.

Intriguingly, in the PyMT-ζWT→ζHET engraftment group I observed an almost two-fold slower tumour growth (**Figure 4.9A)** and final weight **(Figure 4.9B)** compared to that in the control PyMT-ζWT→ζWT group, suggesting that 14-3-3ζ deficiency within mammary tumour microenvironment also suppresses tumour growth. Representative tumour images are shown in (**Figure 4.9C)**. This novel observation strongly implicates microenvironment-derived 14-3-3ζ in mammary tumourigenesis.

Taken together, my experimental observations suggested that 14-3-3ζ has tumour promoting roles in both tumour cells and in the microenvironment. Given this dual tumour-promoting role for 14-3-3ζ in the tumour and its microenvironment, I therefore decided to investigate whether there is therapeutic potential in pharmacologically inhibiting 14-3-3 in vivo using the MMTV-PyMT model of breast cancer.

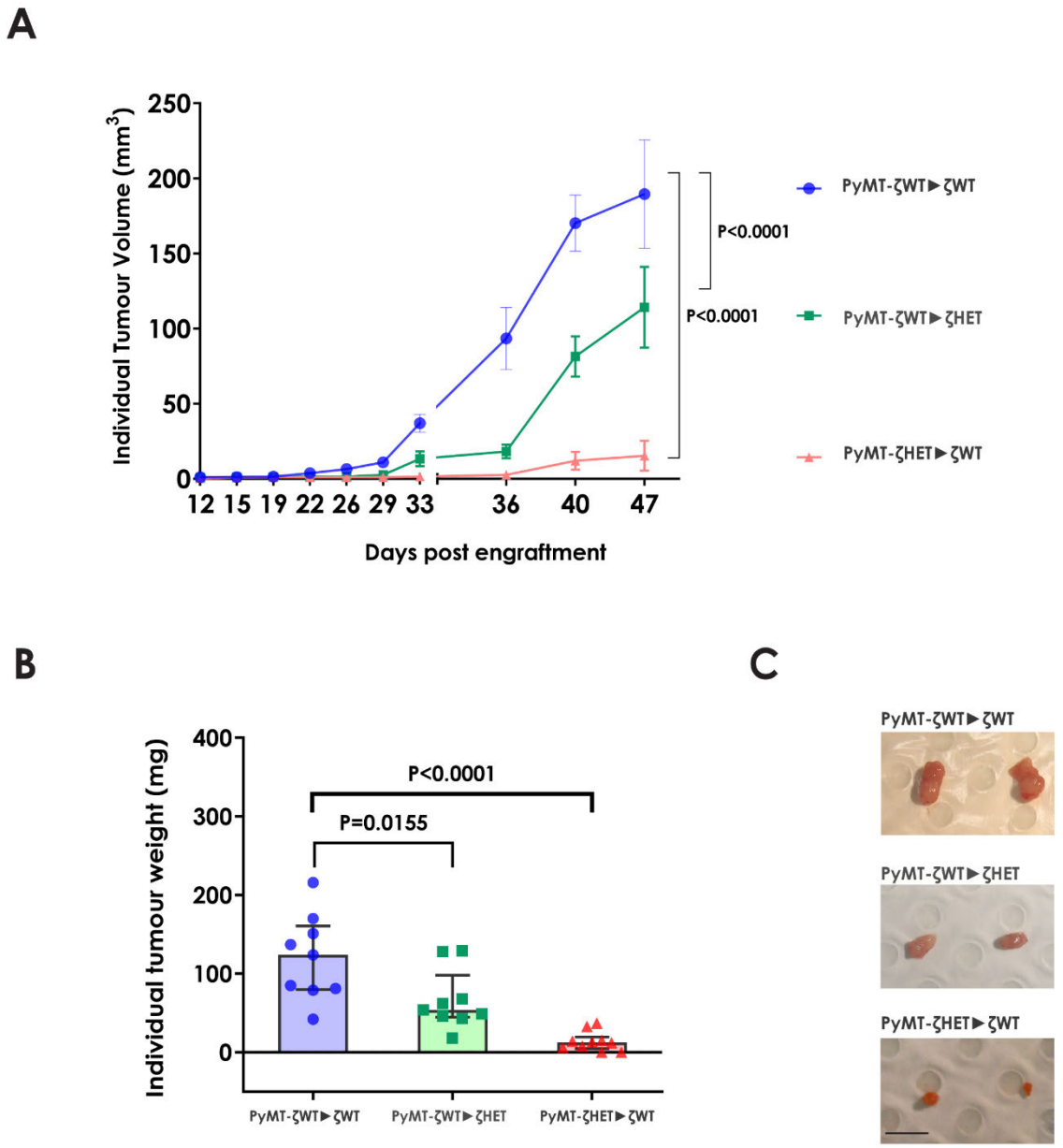


Figure 4.9: 14-3-3ζ has a tumour-promoting role in mammary tumour cells and also in the mammary tumour microenvironment

(A) Tumour growth curves of individual tumour volume in engraftment cohorts (n=5). Data are Median±IQR; two-way ANOVA. **(B)** Final individual tumour weights at humane endpoint. Data are Median±IQR; one-way ANOVA. **(C)** Representative images of tumours. Data is representative of two independent experiments of n=5 animals per cohort. Scale bar =10 mm

4.3.9. *Pharmacological Inhibition of 14-3-3 Reduces Mammary Tumour Burden in the MMTV-PyMT Model of Breast Cancer*

14-3-3ζ inhibition is proposed to be of therapeutic value in breast cancer, however no studies have investigated the effects of 14-3-3 inhibition in mammary cancer models in mice (Hartman & Hirsch 2017; Matta, Siu & Ralhan 2012; Pennington et al. 2018).

In my investigation of pharmacological inhibition of 14-3-3 in mammary cancer, I made use of RB-012 - a novel compound developed in our laboratories. RB compounds are derivatives of FTY720 (Fingolimod) and target 14-3-3 proteins by disrupting the 14-3-3 dimer. Since 14-3-3 proteins act as obligate dimers, disrupting dimer formation renders them inactive. Suppression of signalling through the PI3K-AKT and MAPK by RB-012 and its homologues has been biochemically demonstrated. RB-012 shows potent in vivo reduction of tumour burden in xenograft models using a lung cancer cell line (Woodcock et al. 2015). We have also previously found that that pharmacological 14-3-3 inhibition using RB-011, a close analogue of RB-012, phenocopies 14-3-3ζ deficiency in treatment of skin wounds (Kular et al. 2015).

I first tested the effect of RB-012 on the mammary epithelial cells by assessing pAKT and total AKT levels. Primary mammary epithelial cells derived from MMTV-PyMT mice were incubated in media supplemented with different concentrations of RB-012. I observed strong dose-dependent reduction of pAKT levels in primary mammary epithelial cells upon RB-012 treatment as indicated by representative Western Blot analysis **(Figure 4.10).**

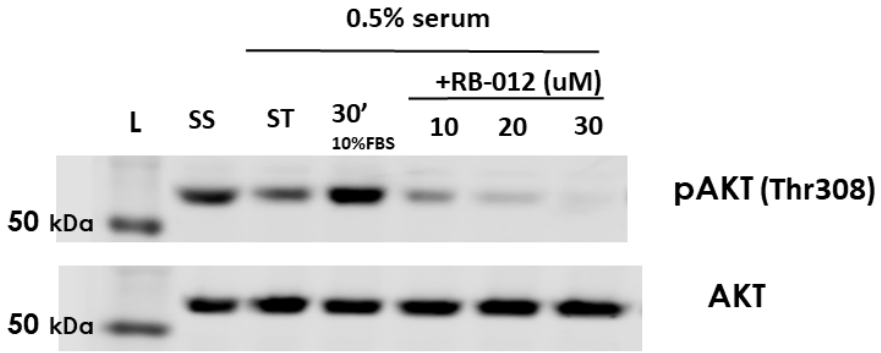


Figure 4.10: 14-3-3 inhibition downregulates pAKT signalling in primary mammary epithelial cancer cells

Western blot analysis pAKT (Thr308) and total AKT in the MMTV-PyMT primary mammary epithelial cells treated with RB-012 at the concentrations indicated. L – ladder; SS – steady state; ST – starved (0.5% serum for 3 hrs); 30'+10%FBS - 30-min stimulation with 10% FBS; concentrations of 10, 20, and 30 μM of RB-012 compound.

Next, I evaluated the effect of RB-012 administration on mammary tumourigenesis. The MMTV-PyMT mouse model, which develops spontaneous mammary tumours in up to all 10 mammary glands, is a highly relevant pre-clinical model for therapeutic testing (Attalla et al. 2021). Cohorts of MMTV-PyMT mice at 8 weeks of age (early carcinoma stage) when tumours were already established, were treated for 23 days with daily intraperitoneal (i.p). injections of RB-012 or vehicle control.

Tumour-bearing mice treated with RB-012 exhibited reduced tumour growth **(Figure 4.11A)** and a two-fold lower median final tumour weight **(Figure 4.11B)** compared to tumour-bearing mice treated with vehicle, demonstrating that 14-3-3 inhibition suppresses tumour growth in this model. This important observation opens a possibility that the signalling pathways mediated by 14-3-3ζ or/and other family members, could be of potential therapeutic value. Thus, it would be important to further to enhance our understanding of 14-3-3-mediated downstream molecular interactions that are inhibited by RB-012 by RPPA and RNAseq analyses.

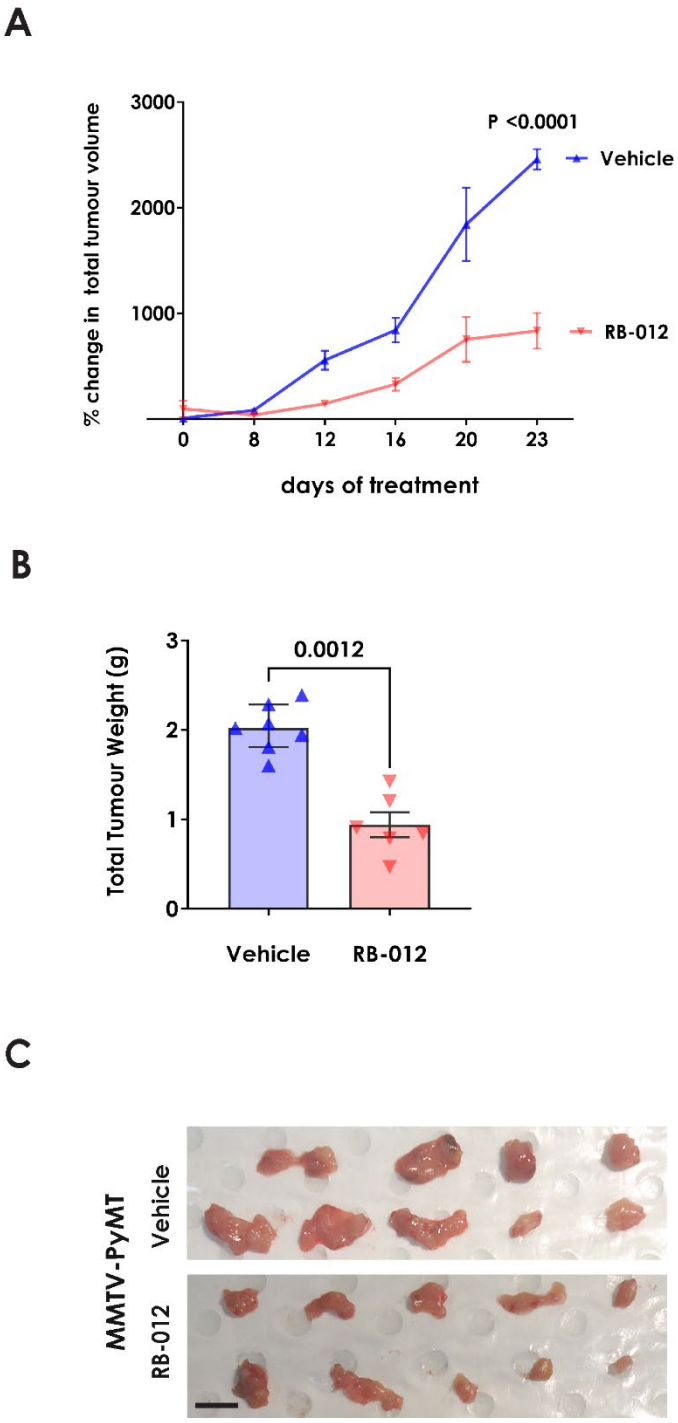


Figure 4.11: Pharmacological inhibition of 14-3-3 reduces mammary tumour burden in the MMTV-PyMT model of breast cancer

(A) Percentage change in total tumour volume. Data are shown as Median±IQR; two-way ANOVA. Representative graph of two independent experiments is shown. **(B)** Final total tumour weight per mouse at the endpoint n=7 (vehicle), n=6 (RB-012). Data are Median±IQR; two-sided Mann–Whitney test. **(C)** Representative images of the tumours at the endpoint. Data are representative of two independent experiments.

To further characterise the effect of the RB-012 treatment on PyMT-driven mammary tumours, I assessed cell proliferation and death in the tumours at the endpoint of the experiments. I observed a significantly smaller number of proliferating cells in tumours from RB-012-treated mice compared to controls as assessed by BrdU incorporation and immunohistochemistry **(Figure 4.12A)**. These observations suggest that inhibition of 14-3-3 potentially reduces in vivo tumour cell proliferation in the PyMT model of mammary cancer. I then examined the level of apoptosis in these tumours by TUNEL **(Figure 4.12B)**. The number of apoptotic cells in the tumours of both cohorts of mice at the endpoint of this experiment were the same, suggesting that apoptosis is not affected by 14-3-3 inhibition at least at the end point.

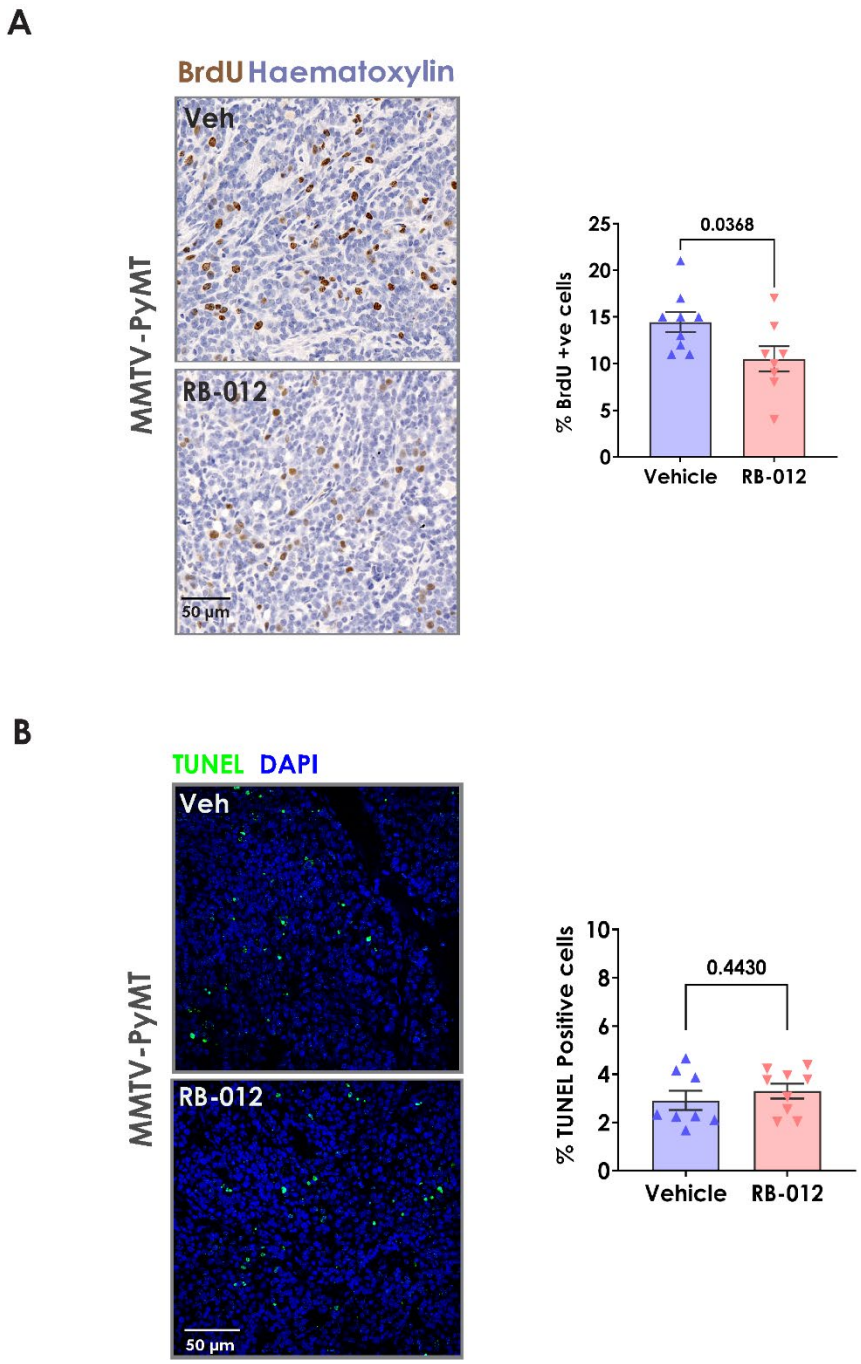


Figure 4.12: Pharmacological inhibition of 14-3-3 suppresses cancer cell proliferation and does not affect apoptosis in mammary cancers

(A) Representative images of IHC analysis of BrdU incorporation assay in Vehicle or RB-012 treated cohorts and quantification % proliferating cells per lesion area. Data are Median±IQR; two-sided Mann–Whitney test. **(B)** Representative images of TUNEL and quantification of percent of apoptotic cells per lesion area. Data are Median±IQR; two-sided Mann–Whitney test.

4.4. Discussion

In this chapter I investigated the role of 14-3-3ζ in mammary tumourigenesis. I established that 14-3-3ζ plays a tumour-promoting role in mammary carcinogenesis using the MMTV-PyMT mammary tumour model **(Figure 4.2)**. When 14-3-3ζ is reduced in mammary cancers a reduction in proliferation is observed at two crucial stages of tumour development: the early carcinoma stage at eight weeks of age **(Figure 4.6A)** and the late carcinoma stage at eleven weeks of age **(Figure 4.3A)** (Attalla et al. 2021). In the human disease, these stages correspond to ductal carcinoma in situ (DCIS) and invasive ducal carcinoma (IDC) respectively. Thus 14-3-3ζ may play an important role in regulating the proliferative capacity of cancer cells at these two stages and also potentially during the transition from DCIS to IDC. It may be exploitable as a therapeutic target for preventing the conversion to invasive disease. The transition from DCIS to IDC is the key step of malignancy and therapeutic intervention at this stage could potentially prevent malignancy.

My observations are consistent with a previously published report that 14-3-3ζ overexpression increases tumour cell proliferation in vivo in a carcinogen-induced mammary cancer model (Rehman et al. 2014). Furthermore, a report investigating the role of 14-3-3ζ in MMTV-PyMT mouse model highlighted that the Ras/Raf/Erk and PI3K/Akt pathways were downregulated upon 14-3-3ζ deletion in mammary cancers (Joshi et al. 2017).

There is an interesting divergence between this last published study and my work presented here. Surprisingly, Joshi et al. did not observe reduced tumour growth in the MMTV-PyMT model on the background of 14-3-3ζ heterozygosity as I did **(Figure 4.2B)** even though they observed, as I did **(Figure 4.6A)** that the proliferation of tumour cells is significantly lower in 14-3-3ζHET tumours compared to the WT group, and that apoptosis is increased. Given that my findings clearly indicate that reduced 14-3-3ζ levels suppressed proliferation of tumour cells **(Figure 4.3A)** while levels of apoptosis were comparable **(Figure 4.3B)** my observation of a reduced tumour burden **(Figure 4.2C)** in PyMT-ζHET compared to PyMT-ζWT is consistent with an imbalance in tissue homeostasis.

My RPPA analysis uncovered several pathways that are differentially regulated between the PyMT-ζWT and PyMT-ζHET tumours. Unsurprisingly, given the roles of 14-3-3 proteins in proliferative and apoptotic pathways, I have observed increased levels of activated Erk1/2 **(Figure 4.4A)**, activated c-Jun **(Figure 4.4B)** and YAP **(Figure 4.4C)** in wildtype tumours compared to heterozygous counterparts. Consistent with observations from the study by Joshi

et al., the Ras/Raf/Erk pathway is downregulated in PyMT-ζHET tumours relative to PyMT-ζWT tumours. Active Erk1/2 is a known regulator of cellular proliferation and cell death (Mebratu & Tesfaigzi 2009) and thus reduction in proliferative capacity in PyMT-ζHET tumours could be attributed to the reduction signalling through this pathway. Furthermore, activation of c-Jun has been previously reported in the invasive breast cancers and is associated with increased proliferation and angiogenesis (Vleugel et al. 2006) which is consistent with the lower proliferation in PyMT-ζHET tumours. YAP is a known interactor of 14-3-3 proteins which sequester it outside the nucleus, preventing its function as a transcriptional coactivator (Basu, S et al. 2003). Interestingly, YAP silencing in breast cancer cells is associated with increased migration and invasion (Yuan et al. 2008). Thus, the normal association of 14-3-3ζ with YAP in PyMT-ζWT tumours may inhibit YAP signalling, resulting in the enhanced tumour progression observed in this situation compared to that in PyMT-ζHET tumours. This could be tested by evaluating YAP nuclear localisation by immunohistochemistry.

A novel finding revealed by my RPPA analysis is the upregulation of prohibitin in PyMT-ζHET tumours relative to PyMT-ζWT tumours. High levels of prohibitin have not been previously reported in the context of 14-3-3ζ reduction. Interestingly, low levels of prohibitin in human breast cancer samples are positively correlated with a poor prognosis. Prohibitin is found to inhibit breast cancer cell proliferation (Liu, P et al. 2017) which is consistent with my observations. Indeed, Scansite (http://scansite4.mit.edu) predicts at least one 14-3-3ζ binding site (Thr-160) on mouse prohibitin and it has previously been demonstrated to have a role in regulating RAF activity via 14-3-3 proteins (Fischer et al. 2009). This could be a potential axis for further investigation in this model, given the important role of MAPK signalling in breast cancer.

Taken together my observations as well as published literature confirm that the cell proliferative capacity of mammary cancers is strongly dependent on 14-3-3ζ levels in vivo and this strong dependency of tumour cells on the levels of 14-3-3ζ may be exploitable as a therapeutic approach in breast cancer.

My work has revealed that 14-3-3ζ heterozygosity inhibits mammary tumourigenesis, strongly suggesting that reducing the levels of 14-3-3ζ may suppress mammary cancer progression and hinting that inhibiting 14-3-3ζ may have therapeutic utility in breast cancer. This is a highly significant finding, because complete loss of 14-3-3ζ has several deleterious consequences (Cheah et al. 2012). Therefore, suppressing rather than abolishing 14-3-3ζ function, such as might be possible by pharmacological means, may produce positive therapeutic outcomes for breast cancer patients.

I further confirmed the therapeutic potential for targeting 14-3-3ζ pharmacologically in the treatment of mammary cancers (Figure 4.11), using pre-clinical models of breast cancer and a novel 14-3-3- inhibitor, RB-012. At the end point of the experiment, in RB-012-treated mice, I observed significant reduction of tumour cell proliferation but not apoptosis (Figure 4.12). Interestingly, the pharmacological inhibition of 14-3-3 in this model closely phenocopies the transgenic model of the 14-3-3ζ heterozygosity, suggesting that it is the targeting of the 14-3-3ζ family member pharmacologically that may be responsible for the suppression of tumours. Given context specific roles of 14-3-3s it would be crucial to further dissect the key molecular targets that are affected by 14-3-3 inhibition, both in cancer cells and in the microenvironment. Therefore, to further investigate putative signalling pathways and the targets of 14-3-3 inhibition, a proteomic and phosphoproteomic analysis is required in tumour cells and the isolated components of the tumour microenvironment.

Furthermore, previous studies on the role of 14-3-3ζ in mammary cancer focused on the cancer cell specific roles (Lu, J et al. 2009; Xu et al. 2015). It is timely to expand these studies to the tumour microenvironment, given the growing appreciation of multiple tumour-promoting roles for the components of the microenvironment in breast cancer (Soysal, Tzankov & Muenst 2015). Since 14-3-3ζ promotes many tumourigenic and chemoresistance pathways in cancer and serves as a central node in major tumour cell signalling networks such as PI3K/AKT, ERK/MAPK, TGF-β, IGF-IR, and β-catenin (Pennington et al. 2018), it is highly likely that 14-3-3ζ could regulate several components of the tumour microenvironment through multiple pathways, which remain to be uncovered.

In this study, I established an orthotopic primary mammary cell engraftment model for studying the microenvironment-specific effects of 14-3-3ζ in immune competent animals. My investigation revealed a novel and previously uncharacterised in vivo tumour-promoting role for 14-3-3ζ in the mammary tumour microenvironment.

In other cancers, a few reports have suggested a tumour-promoting role for 14-3-3ζ in the tumour microenvironment. In human hepatocellular carcinoma, 14-3-3ζ promotes tumour progression by impairing immune cell infiltration (Wang, X et al. 2018). Another report on human pancreatic adenocarcinoma reveals a tumour-promoting role for 14-3-3ζ secreted by macrophages that then mediates chemoresistance in tumour cells (D'Errico et al. 2019). I have investigated the role of 14-3-3ζ in the mammary cancer microenvironment further and have presented this in Chapter 5.

14-3-3ζ upregulation is associated with the advancement in the disease in many cancers and it is suggested that cancer cells preferentially rely on the 14-3-3ζ family member to coordinate complex changes in the growth requirements and metabolic stress that occurs during tumourigenesis (Pennington et al. 2018). Future experiments focusing on the therapeutic potential of 14-3-3 inhibition using RB-012 in specific types of mammary cancers, such as triple negative orthotopic model using 4T1 murine cell line (Arroyo-Crespo et al. 2019) have the potential to reveal subtype specific pathways that would be affected in the context of 14-4-3 inhibition in both cancer cells and in the tumour microenvironment. Another important axis of investigation would be combination of pharmacological inhibition of 14-3-3 and other chemotherapeutic drugs, since 14-3-3ζ has been reported to promote chemotherapy resistance (Bergamaschi, Christensen & Katzenellenbogen 2011). This could potentially reveal cancer cell vulnerabilities and enhance the effect of current chemotherapies.

4.5. Conclusion

Here, I have demonstrated that mammary tumourigenesis is suppressed in 14-3-3ζ heterozygous mice and that this is associated with a lower proliferation rate of the 14-3-3ζ heterozygous tumour cells compared to their 14-3-3ζ wild-type counterparts. Therapeutic inhibition of 14-3-3 greatly reduces mammary tumour burden and phenocopies 14-3-3ζ heterozygosity that I had demonstrated in a genetic model of mammary cancers. Importantly, in this study, I revealed a novel tumour-promoting role of 14-3-3ζ in the mammary tumour microenvironment which has not been previously reported.

Taken together these observations strongly suggest that targeting 14-3-3 in breast cancer may not only potentially suppress tumour cell proliferation but also inhibit tumour permissiveness of the microenvironment. Since 14-3-3ζ promotes many tumourigenic and chemoresistance pathways in breast cancer and serves as a central node in major oncogenic signalling networks it is crucial to continue dissecting molecular mechanisms of inhibition of this protein in the context of breast tumourigenesis so that the key signalling nodes affected may be revealed and could be targeted in the therapeutic settings.

Chapter 5. The Role of 14-3-3ζ in the Mammary Cancer Microenvironment

5.1. Introduction

Experimental work that I have presented in Chapter 4, together with insights published by others firmly establish a tumour promoting role for 14-3-3ζ in mammary tumourigenesis. Whereas most studies have focused on the role of 14-3-3ζ in tumour cells, my findings presented in Chapter 4 also strongly suggest a role for 14-3-3ζ in the mammary tumour microenvironment. However, unlike in the case of cutaneous squamous cell carcinoma (Kular et al, 2015), I observed no changes in ECM composition or CAF infiltration of mouse mammary tumours from 14-3-3ζ-deficient or 14-3-3 inhibitor-treated mice. In this chapter, I therefore investigate whether 14-3-3ζ has functions in tumour immune cells.

5.1.1. *General Introduction*

The tumour microenvironment plays a crucial role in the establishment and the progression of mammary cancers. A major component of the breast cancer tumour microenvironment are tumour-infiltrating lymphocytes (TILs) (Ali et al. 2016). Tumour infiltrating lymphocytes can adopt tumour-promoting or tumour-suppressive functions depending upon the tissue context and the overall biochemical milieu, both local and systemic (Tower, Ruppert & Britt 2019). It is therefore crucial to understand which molecular factors regulate the recruitment and functional capacity of the anti-tumour TILs to develop better breast cancer therapies that precisely target the tumour permissive microenvironment (Loi et al. 2019).

5.1.2. *Cytotoxic T cells in the Breast Cancer Microenvironment*

Within the breast cancer microenvironment, one of the most prominent subsets of TILs are the $CD8^+$ cytotoxic T cells (Salgado et al. 2015). In human breast cancer biopsies, substantial infiltration of $CD8^+$ T cell into the tumours is strongly associated with better patient survival (Mahmoud et al. 2011) and is a powerful predictor of response to chemotherapy (Seo et al. 2013).

Yet, we do not precisely understand either the global or specific molecular regulators that contribute to the recruitment and function of $CD8^+$ T cells in the mammary microenvironments in patients. A clear understanding of the factors that guide the recruitment, trafficking, and functional capacities of cytotoxic $CD8^+$ T cells in mammary microenvironments will inform breast cancer therapy and permit the appropriate adoption of new approaches to immunotherapy.

5.1.3. *14-3-3ζ in Breast Cancer Progression and Mammary Tumour Microenvironment*

Ex vivo overexpression studies have established that 14-3-3ζ plays crucial roles in key signalling pathways that exhibit hallmarks of dysregulation in cancer cells, such as PI3K/AKT signalling, which regulates survival and proliferation (Neal et al. 2012); the MAPK/c-JUN pathway, which regulates proliferation (Rehman et al. 2014) and the TGF-β pathway, which facilitates epithelial to mesenchymal transition (EMT) (Xu et al. 2015), a key developmental process co-opted in tumourigenesis, that increases cancer cell invasiveness and metastatic potential (Felipe Lima et al. 2016).

As 14-3-3 family members are known to be important signalling modulators of survival and proliferative signalling pathways in many cancer types (Pennington et al. 2018), there are ongoing efforts to assess 14-3-3 inhibition in multiple cancer settings (Aghazadeh & Papadopoulos 2016; Freeman & Morrison 2011). Specifically, family member 14-3-3ζ is a subject of intense interest, given its overexpression in multiple cancer types (Gan, Ye & He 2020; Matta, Siu & Ralhan 2012), and there are now several reports confirming anti-tumour properties of 14-3-3 inhibition in vivo (Stevers et al. 2018; Woodcock et al. 2015).

Most of the tumour-promoting roles attributed to 14-3-3ζ are via cancer-cell specific mechanisms, with roles of 14-3-3ζ associated with the secretion of the protein by cancer cells via extracellular vesicles (EVs) to modulate the microenvironment (Su et al. 2018). Thus far, only a handful of studies show that there is a tumour cell-specific role for 14-3-3ζ in cancer that is associated with tumour microenvironment. For instance, it has been shown in ovarian cancer patients that macrophages secrete 14-3-3ζ, contributing to the immune-inflammatory cell environment (Kobayashi et al. 2009). Also, in pancreatic ductal adenocarcinoma (PDAC), tumour associated macrophages (TAMs) secrete 14-3-3ζ, which causes the resistance of treatment-naïve PDAC cells to chemotherapy-induced apoptosis (D'Errico et al. 2019). However, there are as yet no reports implicating 14-3-3ζ in the regulation of mammary cancer immune microenvironment, even though 14-3-3ζ has established roles within the immune system (see Section 5.1.4 on page 105). This is attributable to the complexity of the 14-3-3ζ signalling interactome, which renders such studies extremely difficult to undertake in vivo.

5.1.4. *14-3-3ζ-Immune Axis: Evidence From Inflammatory Disease and the Tumour Microenvironments*

14-3-3ζ is known to play a significant role in the immune system. One of its first immune-related functions to be identified was in the context of autoimmune and chronic inflammatory conditions. A peptide, 14-3-3ζδ, derived by the proteolytic processing of 14-3-3ζ was found to inhibit T cell trafficking in inflamed tissues of type 1 diabetes and rheumatoid arthritis patients (Chimen et al. 2015). Another intriguing study found that exogenously applied 14-3-3ζ promoted IFN-γ and IL-17 production in T cell populations from human donors (McGowan et al. 2019). Interestingly, investigations using an oral squamous cell carcinoma (OSCC) cell line revealed that 14-3-3ζ knock down (KD) up-regulates expression of inflammatory factors (TNF-α, IL-6, IFN-β, and RANTES (CCL5)) in these cells and the supernatants from 14-3-3ζ KD OSCC cells promoted IL-2 and IFN-γ expression, and proliferation in T cells (Han, X et al. 2015). These studies highlight the role of 14-3-3ζ in tumour immunity, and suggest that the precise mechanism is dependent upon tissue context, cell type and the microenvironment.

While there is evidence that 14-3-3ζ can play immunomodulatory roles in the tumour stroma in other cancers (D'Errico et al. 2019; Han, X et al. 2015; Salazar et al. 2020; Su et al. 2018), we do not yet know whether 14-3-3ζ has functions in the mammary cancer immune microenvironment. I therefore investigated the role of 14-3-3ζ in the mammary tumour microenvironment.

5.2. Hypothesis and Aims

Following on from my observation (detailed in Chapter 4) that 14-3-3ζ has a tumour-promoting role in the mammary microenvironment, I hypothesised that 14-3-3ζ plays an inhibitory role in infiltration or function of TILs during tumour progression.

The overall aim of the pilot experiments detailed in this chapter was therefore to evaluate the role of 14-3-3ζ in mammary tumour immunity using a transgenic mouse model of breast cancer in the context of an intact immune system and assemble preliminary data to inform experimental strategy.

5.3. Results

5.3.1. *Immune Infiltration is Increased in the 14-3-3ζHET Tumour Microenvironment*

To investigate whether host-expressed 14-3-3ζ has an impact on immune cell infiltration in established mammary tumours, I performed CD45 immunohistochemical analysis of tumour tissue from 14-3-3ζWT and 14-3-3ζHET recipients that had been engrafted primary mammary tumour cells derived from MMTV-PyMT mice to determine the proportion of infiltrating $CD45^+$ cells relative to the total cell number in each tumour. At the endpoint of the experiment, 47 days post-engraftment, I observed markedly higher $CD45^+$ infiltration in the tumours from 14-3-3ζHET recipients **(Figure 5.1)**. These data suggest that the levels of 14-3-3ζ within the microenvironment may influence the recruitment of immune cells into mammary tumours.

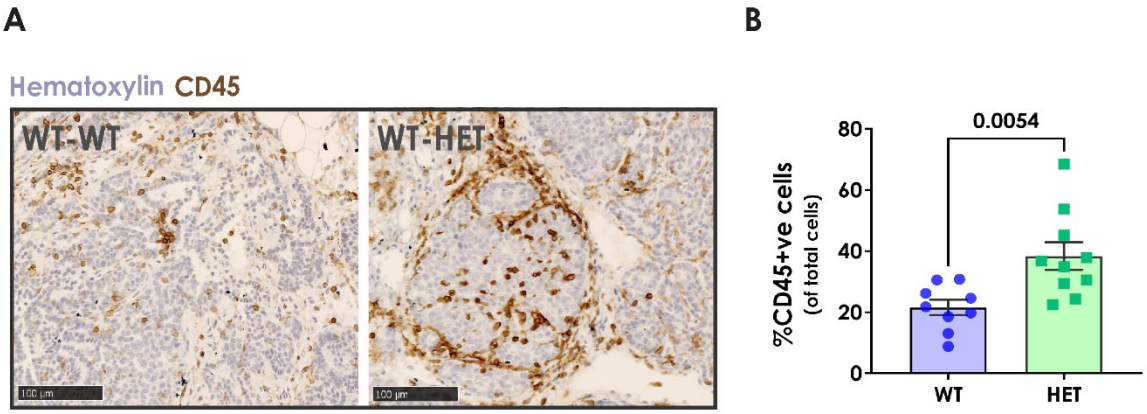


Figure 5.1: $CD45^+$ immune cell infiltration is increased in mammary tumours allografted into 14-3-3ζhet recipients

(A) Representative images of CD45 immunohistochemistry in the mammary tumours from 14-3-3ζWT or 14-3-3ζhet recipients. **(B)** Quantification of the percent of $CD45^+$ cells relative to total nucleated cells in the tumour. Data is Mean±SEM; unpaired student's t-test. Each data point represents an individual tumour WT (n=9), HET (n=10).

Consistent with my CD45 immunohistochemical analysis, I observed an almost two-fold higher frequency of $CD45^+$ tumour infiltrating immune cells in the tumours grown in a 14-3-3ζHET microenvironment compared to those grown in a 14-3-3ζWT microenvironment when

analysed by flow cytometry **(Figure 5.2A)**. There was also a trend towards a higher frequency of CD8$^+$ T cells in tumours grown in 14-3-3ζhet recipients compared to controls **(Figure 5.2B)**.

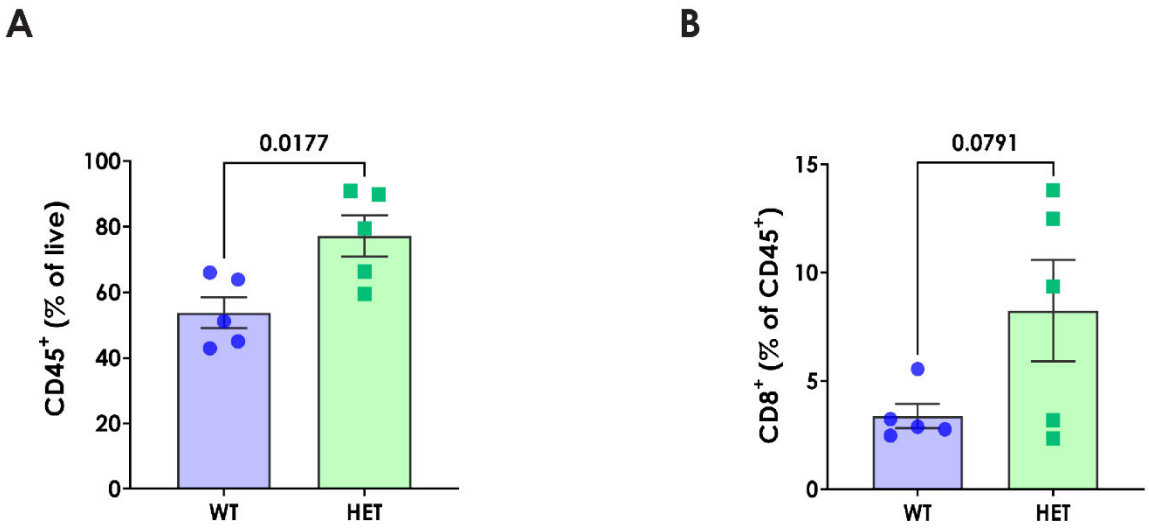


Figure 5.2: 14-3-3ζ heterozygosity of recipient mice enhances CD45$^+$ tumour infiltrating leukocytes and CD8+ T cells in the mammary tumours

(A) Bar graphs indicating frequency of CD45$^+$ tumour infiltrating leukocytes, pre-gated on live cells. **(B)** Bar graphs indicating frequency of CD8+ T cells, pre-gated on live, CD45$^+$ cells. Data are represented as Mean±SEM; unpaired student's t-test. Each data point represents two tumours pooled from an individual mouse (n=5).

Taken together, the IHC and flow cytometry analyses suggest that 14-3-3ζ depletion in the microenvironment enhances immune cell infiltration into mammary tumours at the advanced stages of breast cancer tumourigenesis. I observed that there is a potential role for 14-3-3ζ in suppressing CD8$^+$ T cell populations in the microenvironment in mammary tumours.

Yet, in the engraftment model described above, with tumours assessed at 47 days after engraftment, the functional assessment of CD8$^+$ T cells could be problematic due to T cell exhaustion, a state of T cell dysfunction that arises during chronic inflammatory condition such as cancer (Dolina et al. 2021) and could interfere with the functional readouts of T cell population. Since it has been shown that the MMTV-PyMT mouse mammary cancer model shows peak numbers of CD8$^+$ T cell infiltration at ~21 days post engraftment (Allen et al. 2020), I decided to choose an earlier time point to consistently address the infiltration and functional properties of CD8$^+$ T cells in tumours from 14-3-3ζ-WT and 14-3-3ζHET hosts, bypassing potential interference from T cell exhaustion.

5.3.2. *Experimental Strategy for Investigating CD8$^+$ T Cell Infiltration in Early Tumour Lesions*

To systematically address whether 14-3-3ζ expressed by the microenvironment influences infiltration and/or the functional capacity of the CD8$^+$ T cell populations, I designed an experimental model of early tumourigenesis using a cell line derived from tumours arising in an MMTV-PyMT mice on the FVB/NJ strain background, made available via SEARCHBreast (Blyth et al. 2016).

An established mouse cell line was used in these studies to minimise the variability of tumour growth observed when using primary mammary tumour cells. Primary mammary cells exhibit batch to batch variation in growth potential, which could influence tumour growth and as a result differentially affect the immune response (Gross et al. 2017). Thus, we used the cell line to induce uniform, reproducible immune responses in a mammary cancer model.

Previous studies show that in the orthotopic models of mammary cancer, the highest infiltration of CD8$^+$ T cell populations is observed at ~21 days post-engraftment (Allen et al. 2020), thus the 21-day endpoint for this experimental model was chosen.

Briefly, 5x10^4 tumour cells were injected per mammary fat pad of the 14-3-3ζ WT or 14-3-3ζHET recipient animals. Tumour lesions and draining lymph nodes (dLNs) were harvested 21 days post engraftment and their cell composition was analysed by flow cytometry.

To assess the cytotoxic potential of the T cells derived from the engrafted tumours, I used a method for ex vivo re-activation of the T cells using phorbol 12-myristate 13-acetate (PMA) and ionomycin and a multicolour flow cytometry analysis for established markers of cytotoxic potential (Granzyme B, IFN-γ, TNF-α, CD107a) to assess functional differences between CD8$^+$ T cells derived from 14-3-3ζWT or 14-3-3ζHET tumour microenvironments (for details, see Section 2.2.3; page 43). These studies provided pilot data to facilitate the planning of future experiments and are therefore hypothesis generating.

5.3.3. *The Cytotoxic Potential of Tumour Infiltrating CD8$^+$ T Cells Trends Higher in Tumour-Bearing 14-3-3ζ Heterozygous Recipients Compared to WT Recipient Mice*

In the early events of tumourigenesis (21 days post engraftment), I observed no marked difference between the proportions or the absolute numbers of the CD45$^+$ cells and infiltrating CD8$^+$ T between the two engraftment groups **Figure 5.3.**

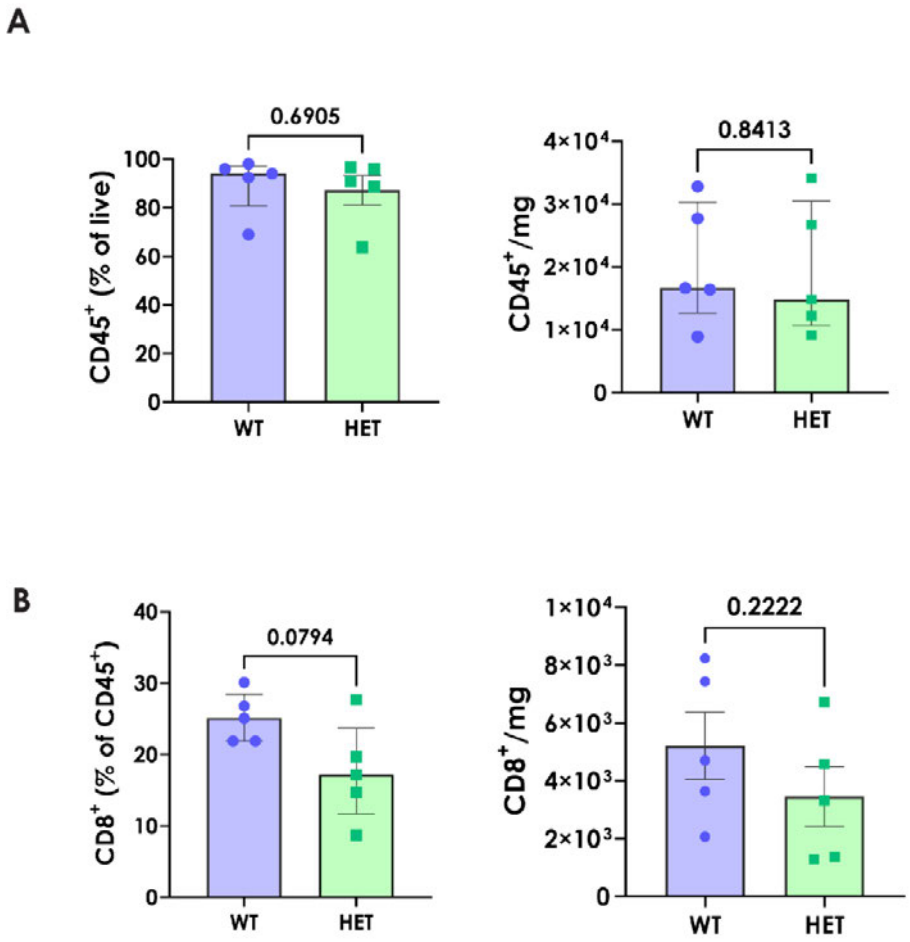


Figure 5.3: 14-3-3ζ levels in the microenvironment do not affect the CD45$^+$ or CD8$^+$ T cell infiltration in early stages of the mammary tumourigenesis

(A) Bar graph shows frequency and number of CD45$^+$ tumour infiltrating leukocytes, pre-gated on live cells. **(B)** Bar graph shows frequency and number of CD8$^+$ T cells, pre-gated on live, CD45$^+$ cells. Data are pooled from two independent experiments and expressed as Median±IQR; two-sided Mann–Whitney test. Each data point represents two glands pooled from an individual mouse (n=5).

However, there was a trend towards reduction in both frequency and number of $CD8^+$ T cell in the lesions extracted from 14-3-3ζHET microenvironment **(Figure 5.3B)**. These data contrast with my observations in advanced tumours (47 days post engraftment) **(Figure 5.2B)**, suggesting that the enhanced $CD8^+$ T cell infiltration could be associated with late tumourigenesis.

My flow cytometry panel also permitted the assessment of surrogates for cytotoxic potential in $CD8^+$ T cells derived from these tumours. I therefore assessed the levels of TNF-α, IFN-γ and CD107a since these intracellular molecules are crucial mediators of the anti-tumour response of $CD8^+$ T cells (St Paul & Ohashi 2020).

Further analysis of ex vivo re-stimulated immune cells extracted from tumours 21 days post engraftment revealed no statistically significant difference in the percentage of $CD8^+$ T cells with intracellular TNF-α **(Figure 5.4A)** present in the two microenvironments.

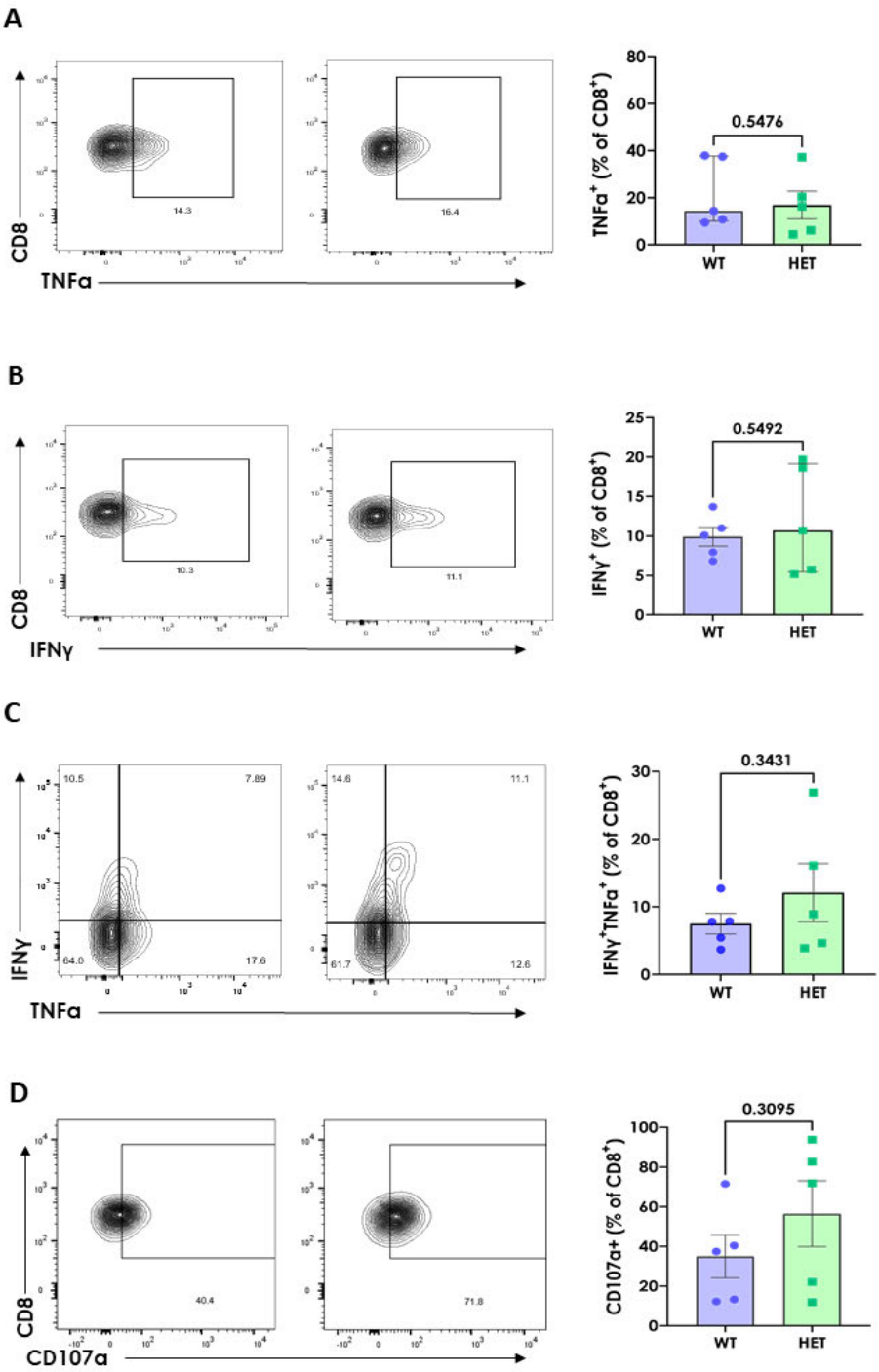


Figure 5.4: 14-3-3ζ reduction may enhance the cytotoxic potential of tumour infiltrating CD8$^+$ T cells in a proportion of early mammary cancer lesions

Representative flow cytometry and bar graphs of frequency of TNF-α producing CD8$^+$ T cells **(A)**, IFN-γ-producing CD8$^+$ T cells in tumours **(B)**, polyfunctional (IFNγ$^+$TNFα $^+$) CD8$^+$ T cells **(C)** and CD107$^+$ degranulating CD8$^+$ T cells **(D)**. Data are pooled from two independent experiments and expressed as Median±IQR; two-sided Mann–Whitney test Each data point represents two tumours pooled from an individual mouse (n=5). Data are pooled from two independent experiments.

IFN-γ production is associated with cytotoxic $CD8^+$ T cell-mediated tumour cell killing. I observed that there was a slightly higher frequency of IFN-γ production from $CD8^+$ T cells in two out of five tumours from 14-3-3ζHET recipients compared to their WT counterparts, consistent with enhanced local antitumour activity **(Figure 5.4B)**. There was a trend towards increase in the frequency of polyfunctional ($IFN\gamma^+TNF\alpha^+$) $CD8^+$ T cells in the 14-3-3ζHET tumours **(Figure 5.4C)**.

During the process of degranulation the lytic granule membrane, containing lysosomal associated membrane glycoproteins (LAMPs), merges with the plasma membrane of the activated $CD8^+$ T cells, and as a result LAMP-1 (CD107a) is incorporated into the plasma membrane of $CD8^+$ T cell (Betts & Koup 2004). I therefore compared the degranulation capacity of the cytotoxic $CD8^+$ T cells by measuring the levels of CD107a on the surface of the ex vivo re-stimulated $CD8^+$ T cells from tumours xenografted into 14-3-3ζWT or 14-3-3ζHET hosts. There was a trend in the frequency of CD107a suggesting a potential for enhanced capacity of cytotoxic activity in 14-3-3ζHET tumours at early stages of tumourigenesis (**Figure 5.4D)**.

Importantly, the assessment of lymphocytes from the non-tumour bearing 14-3-3ζWT or 14-3-3ζHET mice revealed no significant differences between the total spleen weight **(Figure 5.5A)**, or the frequencies of either $CD8^+$ **(Figure 5.5B)** or $CD4^+$ (**Figure 5.5F**) T cells and their effector ($CD44^+$) (**Figure 5.5C, G**) and activated ($CD69^+$) **(Figure 5.5D, H)** populations determined by flow cytometry. This data strongly suggests that the immune phenotype in the heterozygous mice is tumour-specific, and is not due to inherent developmental differences.

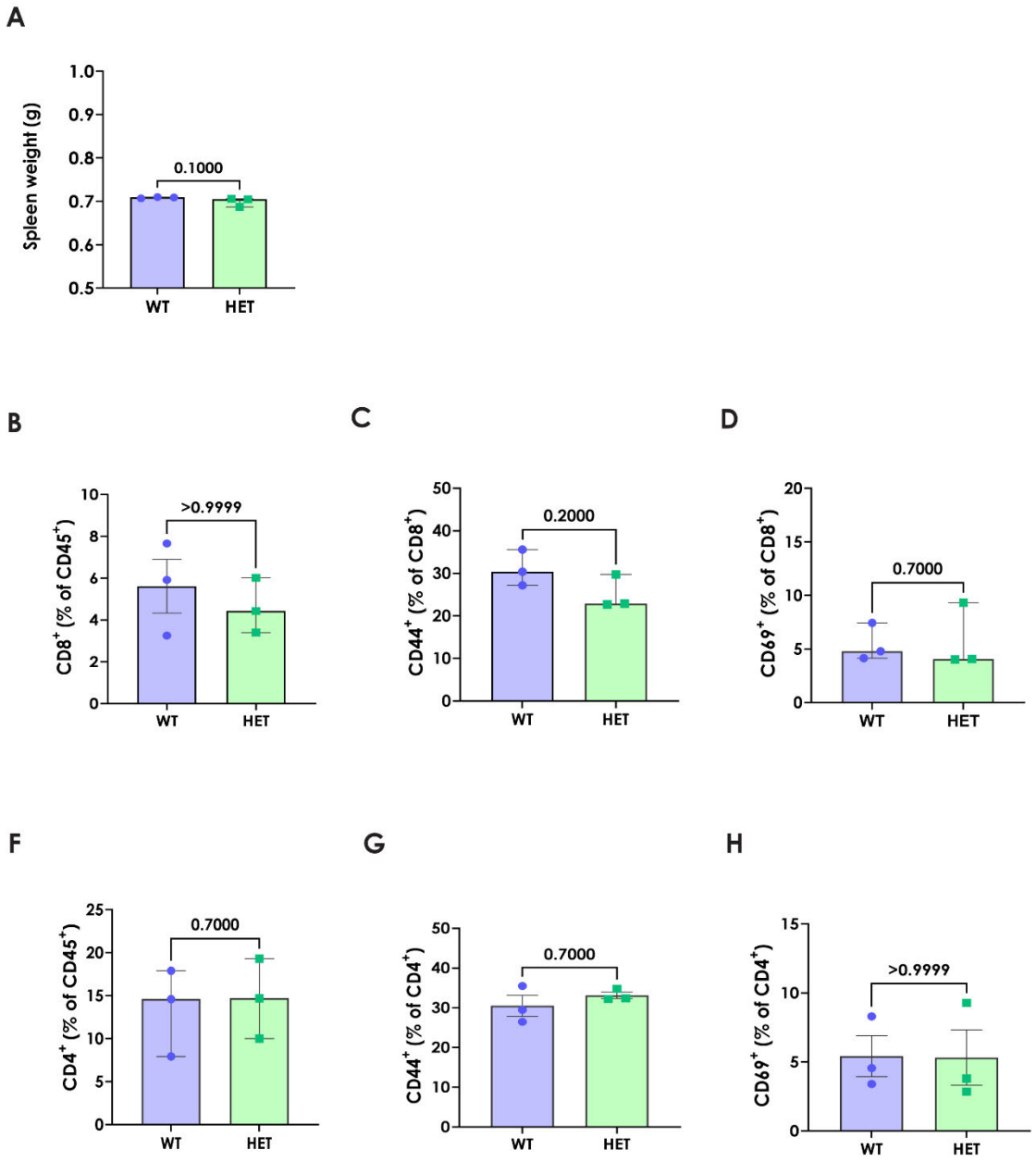


Figure 5.5: 14-3-3ζ heterozygosity has no effect on spleen weight and lymphocyte populations in non-tumour bearing mice.

(A) Bar graphs of individual spleen weight (g), from PyMT negative 14-3-3ζ WT or HET mice. Bar graphs of the frequency of total $CD8^+$ **(B)** and $CD4^+$ **(F)** T cells and their effector ($CD44^+$) **(C, G)** and activated ($CD69^+$) **(D, H)** populations determined by flow cytometry. Data are Median±IQR; two-sided Mann–Whitney test. Each data point represents one spleen from an individual mouse (n=3).

Together the data described in this section suggest that there may be a role for 14-3-3ζ in $CD8^+$ T cell mediated cancer specific immune response in the early stages of mammary tumourigenesis.

5.3.4. *The Cytotoxic Potential of Lymph-Node Derived CD8$^+$ T cells is Enhanced in Tumour-Bearing 14-3-3ζ Heterozygous Recipient Mice*

Lymph nodes are secondary lymphoid organs that are sites of antigen presentation and are therefore crucial for initiating anti-tumour immune responses (Chen, DS & Mellman 2013).

To assess the cytotoxic potential of the CD8$^+$ cells situated within dLNs that drain the mammary gland, I injected 5x10^4 cells per mammary fat pad of 14-3-3ζWT or 14-3-3ζHET recipients, harvested tumour draining LNs 21 days post-engraftment and analysed their cellular contents by flow cytometry.

Similar to my observations on the immune infiltrate within the lesions themselves, I observed that there was no difference in the proportion of CD8$^+$ T cells containing intracellular TNF-α **(Figure 5.6A)** between the two groups. However, there was a markedly higher proportion of IFN-γ producing CD8$^+$ T cells in 14-3-3ζHET recipients compared to 14-3-3ζWT counterparts **(Figure 5.6B)** similar to that found in the tumour lesions **(Figure 5.4B)**. There was no marked difference between the frequency of CD107a$^+$ degranulating CD8$^+$ T cells suggesting that the degranulation capacity of CD8$^+$ T cells is not affected by the 14-3-3ζ levels in dLN **(Figure 5.6C)**. These data suggest a potential role for 14-3-3ζ in the IFN-γ mediated responses of CD8$^+$ T cells in both the secondary lymphoid organs and the lesions arising from the engraftments.

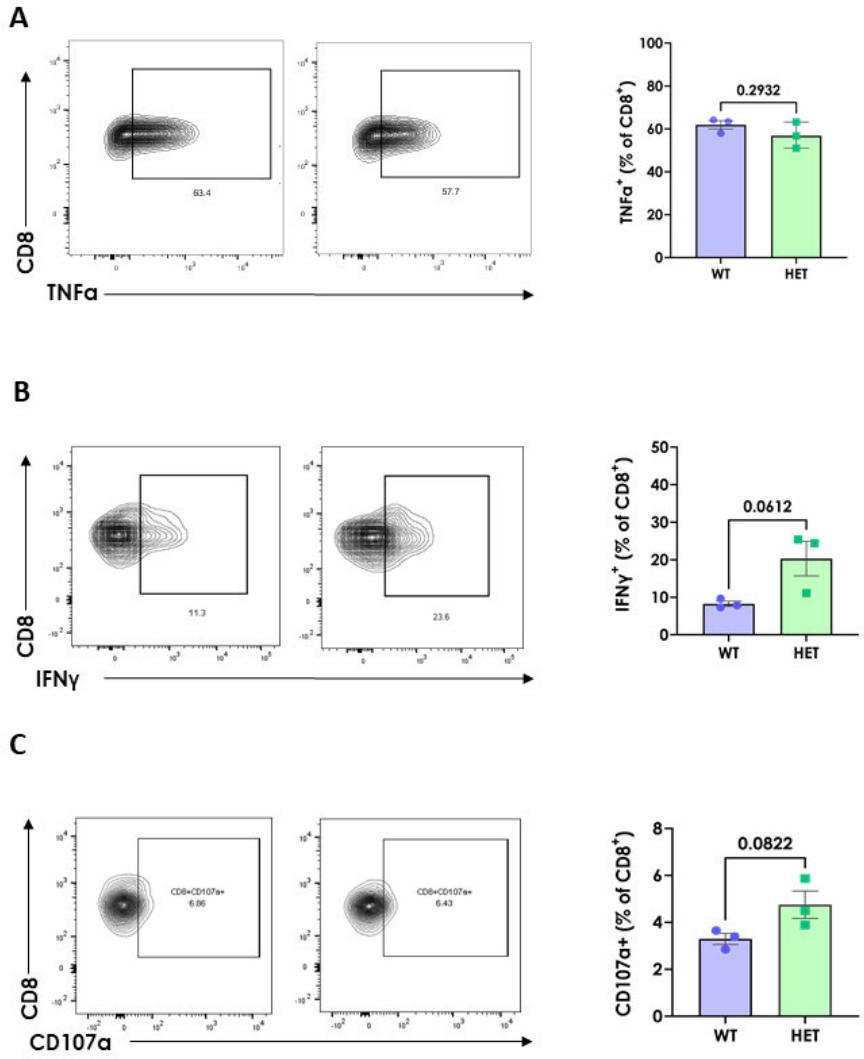


Figure 5.6: 14-3-3ζ heterozygosity in hosts increases cytotoxic potential of lymph-node derived CD8$^+$ T cells

Representative flow cytometry and bar graphs of frequency of TNF-α producing CD8$^+$ T cells **(A)**, IFN-γ-producing CD8$^+$ T cells in dLNs **(B),** CD107$^+$ degranulating CD8$^+$ T cells **(C)**. Data are Median±IQR; two-sided Mann–Whitney test Each data point represents two dLNs pooled from an individual mouse (n=3).

Furthermore, preliminary analysis of the lymphocyte populations from LNs from the non-tumour bearing 14-3-3ζWT or 14-3-3ζHET mice revealed no significant differences between the frequencies of total CD8$^+$ T cells **(Figure 5.7A)**, nor in their intrinsic capacity for TNF-α **(Figure 5.7B)** or IFN-γ responses **(Figure 5.7C)**, suggesting that the immune phenotype previously observed in the LN **(Figure 5.6B)** could be attributed to the tumour-specific immune responses.

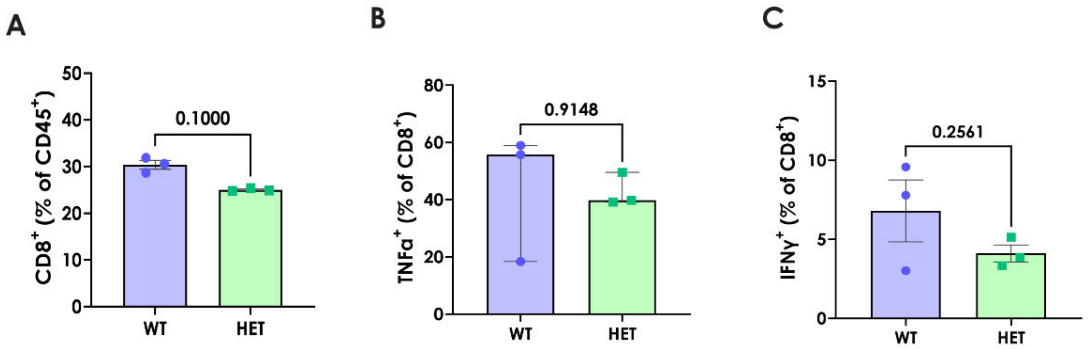


Figure 5.7: 14-3-3ζ heterozygosity does not affect intrinsic lymphocyte responses in non-tumour-bearing mice.

Bar graphs of the frequency of total $CD8^+$ T cells **(A),** TNF-α producing $CD8^+$ T cells **(B)** and IFN-γ-producing $CD8^+$ T cells **(C)** in LNs from 14-3-3ζWT or HET non-tumour bearing mice. Data are Median±IQR; two-sided Mann–Whitney test. Each data point represents brachial, axillary, and inguinal LN populations pooled from an individual mouse (n=3).

5.4. Discussion

The aim of the pilot experiments set out in this chapter was to investigate the role of 14-3-3ζ in mammary tumour immunity. I established that at the advanced stages of tumour progression, there was a significantly higher leukocyte infiltration into the tumours where 14-3-3ζ was reduced in the microenvironment **(Figure 5.1)**. I also observed that there was an increase in $CD8^+$ T cell infiltration in the tumours where 14-3-3ζ was reduced **(Figure 5.2B)**. This data is consistent with the observed reduced tumour growth and burden in the mammary glands with 14-3-3ζ-reduced microenvironment **(Figure 4.9)**. $CD8^+$ T cells are one of the most important cell-types mediating anti-tumour immunity, as they specifically recognise and directly kill tumour cells.

I, therefore, hypothesised that 14-3-3ζ plays an inhibitory role in TIL infiltration or function during tumour progression. To dissect the potential role of 14-3-3ζ in $CD8^+$ T cell infiltration I established an experimental strategy for investigating $CD8^+$ T cell infiltration in early tumour lesions and assessed their surrogate cytotoxic capacity ex vivo.

In the early events of tumourigenesis, the number or proportion of infiltrating leukocytes and $CD8^+$ T cells were not different between the wildtype and 14-3-3ζ-reduced recipients **(Figure 5.3)**. However, in tumour lesions from 14-3-3ζHET recipients, there were trends for higher frequency of IFN-γ-producing $CD8^+$ T cells; higher frequency of polyfunctional ($IFN\gamma^+TNF\alpha$

$^{+}$) $CD8^{+}$ T cells; and higher frequency of $CD107^{+}$ degranulating $CD8^{+}$ T cells **(Figure 5.4B and C)**. These results indicate that $CD8^{+}$ T cell from 14-3-3ζhet recipients may have increased anti-tumour capacity and indicate that 14-3-3ζ may play an inhibitory role in the $CD8^{+}$ T cell anti-tumour responses.

Another important observation in this study was that in the dLNs of early tumour lesions, in 14-3-3ζHET recipients there were trends for higher frequency of $IFN\gamma^{+}$ producing $CD8^{+}$ T cells **(Figure 5.6B)**. These observations could indicate that there could be an increased priming of the $CD8^{+}$ T cells by antigen-presenting cells occurring in the dLN when 14-3-3ζ is reduced. Thus, in the draining lymph nodes in the early mammary lesions 14-3-3ζ may play an important role in mediating IFN-γ responses of the $CD8^{+}$ T cells. This is important since the immune modulation in the sentinel LNs is becoming an attractive therapeutic target in cancer therapy applications (Ma, L et al. 2019; O'Melia et al. 2020).

This pilot study is the first report of the putative role of 14-3-3ζ-mediated IFN-γ responses in the mammary cancer model. 14-3-3ζ has previously been shown to be regulated downstream of IFN-γ signalling pathway (Nava et al. 2014). My observation that $CD8^{+}$ T cell production of IFN-γ is regulated by 14-3-3ζ suggests the existence of a reciprocal feed-back loop between 14-3-3ζ and IFN-γ in IFN-γ-mediated immune responses in cancer and potentially also other pathological conditions

It is important to bear in mind that, the power calculations performed to assess the number of animals required for a comprehensive flow cytometric analysis of the cytotoxic potential of T-cell populations extracted from mammary tumours is 174 to attain statistical significance of $p<0.05$ with 90% probability. The high number of animals required for this type of analyses, suggests that the methodology should be reconsidered and the research strategy refined. For example, cell-sorting could be performed to extract pure a CD8+ population from these tumours that can be expanded ex vivo to obtain sufficient numbers of cells to perform flow cytometry analysis (Lewis et al. 2015).

My observations that IFN-γ responses of $CD8^{+}$ T are enhanced when 14-3-3ζ is reduced are consistent with several studies published in other tumour models. For example, one study assayed human TILs ($CD3^{+}$) isolated from patients with hepatocellular carcinoma. The TILs were grouped into $14\text{-}3\text{-}3\zeta^{high}$ and $14\text{-}3\text{-}3\zeta^{low}$ based on median IHC score in tissues. When TILs were extracted and analysed for the levels of cytokines, $14\text{-}3\text{-}3\zeta^{low}$ TILs expressed higher levels of inflammatory cytokines (IFN-γ, IL-12, IL-2) and reduced levels of anti-inflammatory cytokines (TGF-β, IL-10 and IL-4). This study also reported that that $14\text{-}3\text{-}3\zeta^{high}$ $CD8^{+}$ T cells

displayed reduced activation, proliferation and anti-tumour functions compared to $CD8^+$ T cells expressing lower levels of 14-3-3ζ (Wang, X et al. 2018), which is consistent with my observations.

Interestingly, another study with oral squamous cell carcinoma examined the effects of media conditioned by tumour cells in which 14-3-3ζ had been knocked down by RNAi, on the activity of T cells. Levels of IFN-γ and IL-2 expression were significantly enhanced in T cells treated with conditioned medium from 14-3-3ζ-reduced cells (Han, X et al. 2015). This suggests that there is a possibility that in the mammary tumours similar mechanisms could be at play, and that the reduction of the level of 14-3-3ζ in the tumour cells leads to the secretion of a specific mediator that could then affect the $CD8^+$ T cell function in vivo.

Thus, based on my observations and the current literature, there is a possibility that 14-3-3ζ is a novel regulator of IFN-γ-mediated $CD8^+$ T cell responses in the early stages of tumourigenesis in the mammary cancers.

To further dissect the role of 14-3-3ζ in the mediation of $CD8^+$ T cell responses and function in mammary tumours, several key ex vivo and in vivo experiments are required.

An ex vivo approach would be to use a cytotoxicity assay to assess $CD8^+$ T cell capacity for direct killing. Isolated $CD8^+$ T cells from the tumour lesions from either 14-3-3ζ WT and het hosts can be co-cultured with luciferase tagged PyMT+ tumour cells (Olivo Pimentel et al. 2020). This experiment could establish whether 14-3-3ζ levels in the $CD8^+$ T cells affects their direct killing capacity. This could be performed in the presence and absence of IFN-γ depleting antibody to confirm whether IFN-γ-mediated responses are 14-3-3ζ dependent. These results would help strengthen my observation that $CD8^+$ T cell cytotoxic capacity in 14-3-3ζHET recipients is mediated via IFN-γ secretion **(Figure 5.4B)**.

Another approach would be to perform an RNAseq on isolated $CD8^+$ T cells from the tumour lesions from 14-3-3ζ WT and het hosts to identify key genes and major signalling pathways that are affected by the reduction of the 14-3-3ζ. This approach would allow dissection of specific pathways or groups of pathways that could be affected by the reduction in 14-3-3ζ and as a result expand immunotherapeutic options in breast cancers.

To test whether the IFN-γ-mediated $CD8^+$ T responses are regulated by 14-3-3ζ in the mammary tumour microenvironment in vivo, tumour cell engraftment experiments into 14-3-3ζ WT and het hosts followed by treatment with depleting antibodies against CD8 alone or in combination with IFN-γ depleting antibodies could be informative as to whether the reduction

in 14-3-3ζ influences $CD8^+$ T responses via IFN-γ. If so, this would establish that there is link between the $CD8^+$ T responses via IFN-γ and 14-3-3ζ reduction in vivo.

However, given the complexity of the immune crosstalk occurring in the tumour microenvironment, it is most likely that in this system, other immune cell types or chemokines could contribute to the $CD8^+$ T cell responses. It is possible that $CD4^+$ T helper (Th-1) cells could be secreting high amounts of proinflammatory cytokines (IL-2, TNF-α and IFN-γ). This can promote activation and cytotoxic $CD8^+$ T cell activity (Gonzalez, Hagerling & Werb 2018). Thus, a comprehensive immunophenotyping panel (Yu, YR et al. 2016) to identify other immune cell types that may be regulated by 14-3-3ζ in addition to T cells including macrophages, DCs, NK cells, T-regs and myeloid-derived suppressor cells (MDSCs) (Garner & de Visser 2020; Thorsson et al. 2018) would assist with additional understanding of the infiltration and potential crosstalk between various immune cell populations that are affected by the 14-3-3ζ reduction.

It is possible that in the mammary tumour microenvironment 14-3-3ζ regulates the cytokine and/or the chemokine milieu of the mammary tumours, which strongly influences the function of the $CD8^+$ T cells. For example, local availability of IFN-γ affects the motility of $CD8^+$ cytotoxic T cells. The deprivation of either autocrine or paracrine IFN-γ or blockade of IFN-γ signalling to cytotoxic T cells reduces their cytotoxic function, motility, and survival (Bhat et al. 2017). Therefore, a detailed analysis of cytokines and chemokines in the tumour tissues by ELISA testing for important regulators of $CD8^+$ T cell activity in the tumour microenvironment such as TGF-β, IL-10 and CX3CL1, CXCL9, (Esquivel-Velazquez et al. 2015; Liu, H et al. 2020) would help us to establish the cytokine/chemokine profile of the tumours in 14-3-3ζ wildtype hosts and could be informative of the effects that this milieu has on $CD8^+$ T cells in view that the uncoupling of such interaction could be investigated for therapeutic purposes.

Interestingly, I also observed that the frequency of polyfunctional ($IFN\gamma^+TNF\alpha^+$) $CD8^+$ T cells was increased in lesions with reduced 14-3-3ζ. Polyfunctional T cells can produce more than one cytokine simultaneously (e.g., $IFN\gamma^+$ $TNF\alpha^+$ $CD8^+$ T cells) and in the mammary tumour setting the polyfunctionality of the effector $CD8^+$ T cells is an important parameter that predicts the efficiency of T cell responses against tumours (Egelston et al. 2018). However, the factors that regulate polyfunctionality of the T cells remain mostly unknown (Imai et al. 2020). This axis could be a potential angle of investigation defining the roles of 14-3-3ζ in the polyfunctionality of $CD8^+$ T cells in the mammary cancers.

5.5. Conclusion

This pilot study demonstrates that 14-3-3ζ levels in the mammary tumour microenvironment significantly impact the $CD8^+$ T cell anti-tumour immune responses and opens the door to a more comprehensive understanding of the role of 14-3-3ζ in the tumour microenvironment, specifically in the modulation of anti-tumour immune responses. I have demonstrated that the reduction of 14-3-3ζ levels in the advanced stages of tumourigenesis leads to increased leukocyte infiltration into the tumours and increases $CD8^+$ T cell infiltration associated with reduced tumour burden. Furthermore, my observation revealed that reduced 14-3-3ζ levels in the tumour microenvironment affect IFN-γ-mediated $CD8^+$ T cell responses in the early stages of tumourigenesis in mammary cancers.

This is the first study to demonstrate that 14-3-3ζ could be a novel regulator of IFN-γ-mediated cytotoxic $CD8^+$ T cell responses and establishing the mechanistic basis for this function could have significant therapeutic implications.

Chapter 6. Final Discussion

6.1. Introduction

In this chapter, I summarise the key conclusions from my investigations and discuss future research directions that will assist in our understanding of how 14-3-3ζ regulates both mammary gland development and mammary tumorigenesis.

6.2. Key Conclusions and Future Directions

6.2.1. *A Role for 14-3-3ζ in Mammary Gland Development*

In Chapter 3, I showed that:

- 14-3-3ζ significantly contributes to branching morphogenesis of the mammary epithelium.
- 14-3-3ζ is required to maintain normal mammary epithelial cell proliferation.
- 14-3-3ζ maintains the basal epithelial cell content in developing mammary ducts.
- Levels of 14-3-3ζ gene expression in the mammary gland impact the levels of *Notch1, Pygo2, Dach1,* and *Gata3,* all important regulators of branching morphogenesis.

Further studies are required to dissect the specific role of 14-3-3ζ in mammary gland homeostasis, and some of these may require more tractable models. For instance, the limitations of our model being the whole body knock out system need to be addressed. The overall deletion of 14-3-3ζ in the whole mouse may cause systemic perturbations in endocrine signalling that could play a role in the mammary development in this model. It is also important to point out that in the full body KO mouse model, the systemic and tissue specific effects could not be separated and thus, given that the mammary tissue is hormone-responsive, the roles of systemic estrogen and other sex hormones in relation to 14-3-3ζ function should be also investigated (Brisken & Ataca 2015). An approach to understand whether 14-3-3ζ is required in mammary epithelial cells or the rest of the mouse could be to inject primary mammary epithelial cells from 14-3-3ζWT or KO animals into the cleared mammary fat pads of 14-3-3ζWT or KO recipients to study the mammary epithelial outgrowths (Lawson et al. 2015). Furthermore, it is not possible to infer from our observations whether the effect of 14-3-3ζ-deficiency on mammary gland development arises from a local requirement for this protein, and if so in which cell type (e.g., in mammary epithelial cells, adipocytes, mesenchymal cells, immune cells etc.), or a systemic requirement for it (e.g. in the bone

marrow). The specific role of 14-3-3ζ could be investigated using a conditional model that uses doxycycline-inducible tissue-specific Cre-expression to permit temporal and spatial control of the 14-3-3ζ gene expression in specific cell populations in vivo including mammary epithelial cells (Sakamoto et al. 2020).

It is not known whether 14-3-3ζ has a role in mammary stem cell regulation or differentiation. However, a role for 14-3-3ζ in intestinal stem cells has been suggested whereby it facilitates signalling through the Wnt/β-catenin pathway downstream of Akt activation (Tian et al. 2005). As this mechanism appears to be predominantly active within the intestinal stem cells, it is worth investigating whether 14-3-3ζ may also have similar function in the mammary stem cells. Another interesting area for investigation would a potential link between 14-3-3ζ and the YAP/TAZ signalling pathway. This pathway has important roles in stem/progenitor cells differentiation in the mammary gland (Panciera et al. 2016) and TAZ is regulated by 14-3-3 binding (Kanai et al. 2000), suggesting that a potential 14-3-3ζ/TAZ signalling axis could be worth investigating in mammary stem cell differentiation.

Importantly, it would be necessary to perform further analysis to understand the contribution of 14-3-3ζ to the maintenance of the basal epithelial population in the developing mammary gland. To dissect the contribution of 14-3-3ζ to mammary stem cell differentiation into specific lineages, the immunophenotyping analysis of mammary epithelial cells by flow cytometry will give information regarding the relative proportions of basal ($Lin^-CD24^+/CD29^{high}/CD49f^{high}/Sca1^{low}$) and luminal ($Lin^-CD24^+CD29^{low}CD49f^{low}$) populations (Fu et al. 2020). These populations could also be further characterised by RNAseq. This would allow for the identification of the proportion of cells within distinct populations along the lineage and identify the points at which 14-3-3ζ deficiency causes a bottleneck. Furthermore, the information gained by such a RNAseq experiment could informed antibody selection for subsequent RPPA for a candidate-based approach to identify key molecular pathways affected by 14-3-3ζ. The basal epithelial cell populations of the mammary gland contain a population of mammary stem cells (MaSCs) that are capable of regenerating the entire functional mammary gland (Shackleton et al. 2006; Stingl et al. 2006) and thus there is a possibility that 14-3-3ζ may contribute to the maintenance of the mammary stem cell population.

In this thesis, I have demonstrated that 14-3-3ζ heterozygous mice, which have only half the WT levels of this protein, exhibit normal mammary gland development and mammary epithelial tree elaboration. I therefore took full advantage of the opportunity to use 14-3-3ζ heterozygous mice to understand process of mammary epithelial tumourigenesis. I reasoned that 14-3-3ζ heterozygous mice may be used to study MMTV-PyMT-driven tumourigenesis to

investigate the role of 14-3-3ζ in mammary cancer without encountering potential confounding effects that a reduced epithelial cell compartment, as observed in 14-3-3ζ KO mice, may have on tumour growth.

6.2.2. *Mammary Tumourigenesis*

In Chapter 4, I showed that:

- 14-3-3ζ deficiency slows cancer cell proliferation in spontaneous mammary tumours.
- 14-3-3ζ deficiency delays tumour onset and slows PyMT-driven spontaneous mammary tumour growth. This is the first demonstration of the therapeutic window for targeting reduction in 14-3-3ζ levels in the context of mammary cancers given that abolition of 14-3-3ζ expression leads to developmental defects
- Pharmacological inhibition of 14-3-3 phenocopies 14-3-3ζ deficiency by lowering tumour burden in the PyMT-driven mammary cancer model.

Concurrent with my findings, others reported on the role of 14-3-3ζ in tumour growth using the MMTV-PyMT mouse model (Joshi et al. 2017). This study reported an inhibitory role for the 14-3-3ζ gene in mammary tumourigenesis, consistent with our findings. However, they only observed impaired tumour growth in PyMT-ζKO animals. In their hands, PyMT-ζHET mice exhibited tumour growth rates comparable to those of PyMT-ζWT mice. This is surprising since they also observed lower levels of epithelial cell proliferation in PyMT-ζHET tumours compared to PyMT-ζWT tumours, consistent with my results. Interestingly, Joshi et al. also reported reduced mammary epithelium in 14-3-3ζ KO animals, which would have certainly contributed to the lower tumour burden they observed in PyMT-ζKO mice. Therefore, it is difficult to conclude from the work of Joshi et al., that the tumour burden was reduced because of reduced cancer cell proliferation in PyMT-ζKO tumours. This is because we do not know to what extent the smaller number of pre-existing epithelial cells from which the tumours arose contributed to the low tumour burden. Joshi et al. observed Ras/Raf/Erk and PI3K/Akt signalling to be differentially regulated between WT and KO tumour tissues. However, in this study the authors did not distinguish between the roles of 14-3-3ζ in the tumour microenvironment vs. in epithelial cells since whole tumour tissue lysates were used for analysis. My findings, while to some degree congruent with those reported by Joshi et al.,

provide a more comprehensive and detailed characterisation of the role of 14-3-3ζ in mammary tumourigenesis.

However, given the complexity of the 14-3-3ζ interactome and its regulatory roles in proliferation, apoptosis, cell cycle and metabolism, systematic approaches should be used to dissect key molecular pathways affected in this model (Gan, Ye & He 2020; Pennington et al. 2018). Future studies will benefit from analysis of separate fractions of epithelial and stromal cells preparations from the tumour tissues using RNAseq and proteomics to identify major pathways and cellular processes that are affected by 14-3-3ζ deficiency in this model (Collins et al. 2013).

14-3-3ζ levels are associated with chemoresistance in several cancers. Intriguingly, 14-3-3ζ reduction sensitizes lung cancer cells to cisplatin (Fan, T et al. 2007), and multiple myeloma cells to bortezomib treatment (Gu, Y et al. 2018). Since 14-3-3ζ upregulation is associated with chemoresistance in endocrine sensitive breast cancer cells and high level of 14-3-3ζ is associated with poor clinical outcome for women on tamoxifen (ER-targeted endocrine therapy in breast cancer) (Bergamaschi, Christensen & Katzenellenbogen 2011; Bergamaschi & Katzenellenbogen 2012) I hypothesise that 14-3-3ζ inhibition could restore endocrine sensitivity of the tumour cells and reduce the risk of disease recurrence.

6.2.3. ***Tumour Microenvironment***

In Chapter 5, I showed that:

- 14-3-3ζ is tumour-promoting in the mammary microenvironment
- Reduced 14-3-3ζ in recipient mice resulted in smaller tumours and more tumour infiltrating $CD8^+$ T cells.
    - These $CD8^+$ T cells were likely to be IFN-γ-producing, a characteristic that is associated with high cytotoxic capacity.
    - $CD8^+$ T cells from inguinal mammary gland draining lymph nodes of tumour-bearing 14-3-3ζ HET mice were also more likely to produce IFN-γ.

While the roles of 14-3-3ζ in the immune system are yet to be comprehensively delineated, and its role in cancer immunity are still unclear, some clues as to potential avenues of investigation are available from the study of other conditions. For example, it is known in the context of autoimmune inflammatory responses that T cell trafficking can be suppressed by B-cell-

derived 14-3-3ζ (Chimen et al. 2015). Furthermore, there is in vitro evidence to suggest that 14-3-3 inhibition in tumour cells may have immunomodulatory effects. It has been demonstrated that the T cells had higher levels of IL-2, IFN-γ expression and higher T cell activity when treated with the medium conditioned by oral squamous cell carcinoma cells in which 14-3-3ζ had been depleted by RNAi (Han, X et al. 2015). These findings are highly significant because they demonstrate that 14-3-3ζ in tumour cells has the capacity to regulate their immunomodulatory functions. Some evidence also exists for a supporting a role for 14-3-3ζ in the tumour microenvironment - in tumour infiltrating lymphocytes (TILs). In one study, TILs derived from hepatocellular carcinoma patient samples were stratified into two categories based on the levels of 14-3-3ζ expression and analysed for several key properties. TILs with higher levels of 14-3-3ζ expression displayed reduced activation, proliferation and anti-tumour functions compared to the 14-3-3ζ-low expressing TILs. The 14-3-3ζ-high TILs also had increased levels of T cell exhaustion markers (PD-1, TIM-3, LAG-3 and CTLA-4) associated with impaired anti-tumour functions (Wang, X et al. 2018). Taken together, the above fragments of evidence suggest separate but important roles for 14-3-3ζ not only in tumour cells themselves, but also within the tumour microenvironment. While the findings discussed above suggest a role for 14-3-3ζ in tumour cell immunomodulation in oral squamous cell carcinoma cells and TILs in hepatocellular carcinoma, no role for 14-3-3ζ in mammary tumour immunity has been identified so far. My findings are therefore the first to demonstrate a potentially important role for this 14-3-3 family member in the immune responses to mammary cancer, which are possibly exploitable therapeutically. Future studies should include a comprehensive immunophenotyping panel (Yu, YR et al. 2016) to identify other immune cell types that may be regulated by 14-3-3ζ in addition to T cells, as other immune cell types are well-established to infiltrate breast cancers, including macrophages, DCs, NK cells, T-regs and myeloid-derived suppressor cells (MDSCs) (Garner & de Visser 2020; Thorsson et al. 2018). It is possible that 14-3-3 proteins regulate the chemokine and/or cytokine milieu of mammary tumours, which strongly influence the breast cancer microenvironment and future investigations should therefore include a detailed analysis of these signalling molecules in tumour tissues (Esquivel-Velazquez et al. 2015; Liu, H et al. 2020).

6.3. Overall Conclusion

14-3-3ζ protein levels are upregulated in breast cancers (Gan, Ye & He 2020). Several studies provide evidence for the potential prognostic value of 14-3-3ζ expression levels and the therapeutic merit of 14-3-3ζ inhibition in mammary cancer cells (Lu, J et al. 2009; Xu et al.

2015). Nevertheless, to date no other studies have demonstrated the therapeutic potential of 14-3-3ζ inhibition in mammary cancers either in pre-clinical models or in patients.

In the work presented in this thesis, I have demonstrated using the MMTV-PyMT mouse model of mammary cancers, that a 50% reduction in 14-3-3ζ levels via genetic means greatly suppresses mammary tumour progression. Furthermore, I have assessed the therapeutic utility of 14-3-3 blockade in this model of mammary cancers and have demonstrated a significant reduction of tumour growth by pharmacological inhibition of 14-3-3 using the RB-012 compound, thus opening up avenues for developing 14-3-3 targeting breast cancer therapies.

Importantly, my work uncovered novel and unexpected tumour-promoting roles for 14-3-3ζ in the mammary microenvironment suggesting a new role for this protein in inhibiting cytotoxic $CD8^+$ T cell mediated anti-tumour responses.

Further studies are required to assess the putative immunomodulating properties of 14-3-3ζ that my work has uncovered, and its potential for therapeutic inhibition in mammary cancers, which could lead to improvements to current breast cancer immunotherapies.

14-3-3ζ is centrally positioned in pro-tumour growth, survival signalling, autophagy, and glucose metabolism in multiple cancers, including oral cell squamous carcinomas, lung, breast, liver, and others. Therefore, 14-3-3 inhibition is an attractive tumour-targeting strategy and merits further investigation in other cancers as an approach capable of concurrently disrupting multiple tumour-promoting pathways while facilitating anti-tumour immune responses.

Appendix A Summary of RPPA data

Table A.1: Summary of data from Reverse-Phase Protein Array.

The RPPA analysis was performed on the whole mammary tumour tissue from eleven weeks old MMTV-PyMT FVB/NJ (WT) and 14-3-3ζ-/+ MMTV-PyMT FVB/NJ (HET) mice. The fold change was calculated by dividing the mean RFI value of the treated group by the mean RFI value of control group (Het/WT). p-values obtained by Welch two sample t-test. Antibody ID refers to the testing facility's antibody number and the Run Status is indicative of the validity of the experimental run.

Aantibody ID	Antibody Name	RunStatus	Treatment	FoldChange	p.value
12	mTOR	Passed	Het/WT	1.727215	0.096086
179	PI3 Kinase p85	No signal	Het/WT	1.707994	0.702956
41	Prohibitin	Passed, Donuts	Het/WT	1.564794	0.049785
142	IBa_P S32	No signal	Het/WT	1.564209	0.388566
135	CK2-a	Passed	Het/WT	1.479182	0.281239
241	4EBP1	Passed	Het/WT	1.376036	0.27068
101	p21 CIP/WAF1	Low signal	Het/WT	1.375037	0.269946
9	Bcl-xL	Passed	Het/WT	1.277841	0.258849
43	4E-BP1_P S65	Passed	Het/WT	1.247146	0.181997
2	Src	Passed	Het/WT	1.200744	0.389182
105	p38 MAPK	Passed	Het/WT	1.181817	0.554996
124	TAZ	Passed	Het/WT	1.134923	0.41662
74	GSK-3-?	Passed	Het/WT	1.129866	0.546553
4	rpS6_P S240;S244	Passed	Het/WT	1.126605	0.22569
32	Akt	Passed	Het/WT	1.108137	0.443843
10	Akt_P T308	Passed	Het/WT	1.107559	0.593317
83	ERK1/2	Passed	Het/WT	1.092757	0.442844
11	mTOR_P S2448	Passed	Het/WT	1.083695	0.59152
107	rpS6	Passed	Het/WT	1.079253	0.882097
22	Stat3	Passed	Het/WT	1.070449	0.746353
94	Tuberin	Low signal	Het/WT	1.0338	0.570598
40	β-Catenin	Passed	Het/WT	1.031481	0.895488
25	Mcl-1	Passed	Het/WT	1.01133	0.955265
44	4E-BP1_P T37;T46	Passed	Het/WT	0.996138	0.986528
36	PKC pan_P ?II S660	Passed	Het/WT	0.980501	0.952712
56	Caspase 3	Passed	Het/WT	0.975129	0.898922
26	c-Myc	Passed	Het/WT	0.958764	0.778669
49	Bad_P S112	Passed	Het/WT	0.919784	0.668449
60	cdc2_P Y15	Passed	Het/WT	0.917068	0.734088
51	Bax	Passed	Het/WT	0.900591	0.525015
3	rpS6_P S235;S236	Passed	Het/WT	0.864684	0.403498
120	PI3 Kinase p110?	No signal	Het/WT	0.838338	0.803403
53	?-Catenin_P S33;S37;T41	Low signal	Het/WT	0.832541	0.227473
29	Stat3_P Y705(Ab29)	Passed	Het/WT	0.806254	0.278674
159	ZEB1	Low signal	Het/WT	0.788549	0.731713
93	Stat3_P Y705(Ab93)	Low signal	Het/WT	0.788145	0.193279
31	Akt_P S473(Ab31)	Passed	Het/WT	0.785139	0.243894
191	JNK1/2/3	No signal	Het/WT	0.749461	0.636324
133	CDK6	No signal	Het/WT	0.7383	0.454737
118	YAP_P S127	Passed	Het/WT	0.726279	0.023555
134	IKK a/β_P S176;S180	Low signal	Het/WT	0.711018	0.40653
95	Tuberin_P T1462	Low signal	Het/WT	0.704913	0.399867
106	p38 MAPK_P T180;Y182	Passed	Het/WT	0.690565	0.302693
62	c-Jun_P S73	Passed, Donuts	Het/WT	0.689826	0.048368
127	Src_P Y527	No signal	Het/WT	0.668133	0.273528
18	Akt_P S473(Ab18)	Passed	Het/WT	0.653377	0.125804
113	Erk1/2_P T202/Y204(Ab113)	Passed	Het/WT	0.642098	0.605937
50	Bad_P S136	Low signal	Het/WT	0.631291	0.027117
57	Casp3_Clvd D175	Passed	Het/WT	0.612507	0.338215
30	p70 S6 Kinase	Passed	Het/WT	0.607517	0.178718
1	Src Family_P Y416	Low signal	Het/WT	0.517262	0.046007
137	p27 Kip1	No signal	Het/WT	0.481415	0.333964
20	Erk1/2_P T202/Y204(Ab20)	Passed	Het/WT	0.47555	0.01079
104	YAP	Low signal	Het/WT	0.457796	0.439773
108	p70 S6 Kinase_P T389	No signal	Het/WT	0.427911	0.429773
98	XIAP	Low signal	Het/WT	0.424361	0.131891
132	CDK2	No signal	Het/WT	0.263417	0.070151
119	Bcl-2	No signal	Het/WT	0.215266	0.006474

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