# Development of a Novel Therapeutic Strategy for Estrogen Receptor Negative Breast Cancer

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

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# List of Abbreviations

| | |
|---|---|
| ADT | Androgen deprivation therapy |
| AIs | Aromatase inhibitors |
| AMBIC | Ammonium bicarbonate |
| AR | Androgen receptor |
| ARA | Androgen receptor associated protein |
| ARA70 | AR-associated protein 70 |
| AREs | Androgen response elements |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| Bak | BCL2 antagonist/killer 1 |
| Bax | BCL2 associated X |
| BCL2 | B-cell lymphoma 2 |
| BRD4 | Bromodomain-containing protein 4 |
| BSA | Bovine serum albumin |
| CAK | CDK-activating kinase |
| CBP | CREB binding protein |
| CCND1 | Cyclin D1 |
| CDK | Cyclin-dependent kinase |
| $CH_3COOK$ | Potassium acetate |
| ChIP-seq | Chromatin immunoprecipitation sequencing |
| Cip/Kip | CDK interacting protein/Kinase inhibitory protein |
| CKI | CDK inhibitors |
| CPA | Cyproterone acetate |
| CTD | C-terminal domain |
| DAB | 3-3′-diaminobenzidine chromogen |
| DBD | DNA binding domain |
| DEG | Differentially expressed genes |
| DHEA | Dehydro-epi-androsterone |
| DHT | 5α-dihydrotestosterone |
| DMEM | Dulbecco's Modified Eagle’s Medium |

| | |
|---|---|
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPX | Dibutylphthalate polystyrene xylene |
| DSIF | DRB-sensitivity-inducing factor |
| E1 | Estrone |
| E2 | Estradiol |
| E2F | E2 family transcription factor |
| E3 | Estriol |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| EGTA | Ethylene glycol-bis (β-aminoethyl ether)-tetraacetic acid |
| EMEM | Eagle's Minimum Essential Medium |
| ER | Estrogen receptor |
| ERK | Extracellular signal-regulated kinase |
| ET | Endocrine therapies |
| EtOH | Ethanol |
| FBS | Fetal bovine serum |
| FC | Fold change |
| FSH | Follicle stimulating hormone |
| GO | Gene ontology |
| GRHL2 | Grainyhead like transcription factor 2 |
| GRIP | Glucocorticoid receptor interacting protein |
| GSEA | Gene set enrichment analysis |
| GSK3β | Glycogen Synthase Kinase 3 β |
| GSN | Gelsolin |
| HAT | Histone acetyltransferase |
| HCE | Human capping enzyme |
| HCL | Hydrochloric acid |
| HER2 | Human epidermal growth factor receptor-2 |
| HEXIM1 | Hexamethylene bis-acetamide inducible 1 |
| hg19 | Human genome 19 |
| HMBA | Hexamethylene bisacetamide |
| HSD | Hydroxysteroid dehydrogenase |
| HSP | Heat-shock proteins |

| | |
|---|---|
| IGF-1 | Insulin-like growth factor 1 |
| IGFR1 | Insulin-like growth factor receptor 1 |
| IgG | Immunoglobulin G |
| IHC | Immunohistochemistry |
| INK4 | Inhibitors of CDK4 |
| IP | Immunoprecipitation |
| ITS | Insulin transferrin sodium selenite |
| KCl | Potassium chloride |
| Kd | Kilodaltons |
| KOH | Potassium hydroxide |
| LAR | Luminal-AR |
| LB | Lysis buffer |
| LBD | Ligand binding domain |
| LCoR | Ligand Dependent Nuclear Receptor Corepressor |
| LH | Luteinizing hormone |
| LTP | Luciferase-Tomato-Puro |
| LVs | Lentiviral particles |
| mA | Milliampere |
| MAD2 | Mitotic arrest deficient 2 |
| MAGE-11 | Melanoma antigen gene protein |
| MCL1 | Myeloid cell leukemia |
| MEM | Minimum Essential Medium Eagle |
| MgCl2 | Magnesium chloride |
| MgSO4 | Magnesium sulfate |
| MHT | Menopause hormone therapy |
| MIND | Mammary intraductal xenografts |
| mL | Millilitre |
| mM | Millimolar |
| MPA | Medroxyprogesterone acetate |
| mRNA | Messenger-RNA |
| MSigDB | Molecular signature database |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| NCoA | Nuclear receptor coactivator |

| | |
|---|---|
| NCoR | Nuclear receptor corepressor |
| NELF | Negative elongation factor |
| Ng | Nanogram |
| NGS | Next generation sequencing |
| NLS | Nuclear localisation signal |
| nM | Nanomolar |
| NP-40 | Nonionic polyoxyethylene surfactant |
| NTD | N terminal domain |
| PBS | Phosphate buffered saline |
| PCA | Principle component analysis |
| PCR | Polymerase chain reaction |
| PDX | Patient derived xenografts |
| PEI | Polyethylenimine |
| PI3K | Phosphoinositide 3-kinase |
| PIC | Pre-initiation complex |
| PKA | Protein kinase A |
| PKC | Akt and protein kinase C |
| PLA | In situ proximity ligation assay |
| PP1α | Protein phosphatase 1α |
| PP2B | Protein phosphatase 2B |
| PR | Progesterone receptor |
| P-TEFb | Positive transcription elongation factor |
| qPLEX-RIME | Quantitative multiplexed RIME |
| qRT-PCR | Quantitative real time-polymerase chain reaction |
| Rb | Retinoblastoma tumour suppressor protein |
| RIME | Rapid immunoprecipitation mass spectrometry (MS) of endogenous protein |
| RIPA | Radioimmunoprecipitation assay buffer |
| RNA | Ribonucleic acid |
| RNAPII | RNA polymerase II |
| RNA-seq | RNA sequencing |
| RRM | Ribonucleotide reductase M |
| SAC | Spindle assembly checkpoints |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |

| | |
|---|---|
| Ser | Serine |
| SERDs | Selective ERα down-regulators |
| SERMs | Selective ERα modulators |
| SHBG | Sex hormone binding globulin |
| SILAC | Stable isotope labelling using amino acids in cell culture |
| siRNA | Small interfering RNA |
| SMRT | Silencing mediator of retinoid and thyroid hormone receptor |
| SOC | Super optimal broth with catabolites repression |
| SRC | Steroid receptor coactivator |
| STAR | Steroidogenic acute regulatory protein |
| TAMR | Tamoxifen-resistant |
| TBP | TATA-box-binding protein |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline-Tween |
| TCF7L2 | Transcription factor 7 like 2 |
| TFAP-2 | Transcription factor activating protein-2 |
| TFIIF | Transcription factor IIF |
| Thr | Threonine |
| TMT | Tandem mass tags |
| TNBC | Triple negative breast cancer |
| TSS | Transcription start site |
| Wnt | Wingless-type |
| XIAP | X-Linked inhibitor of apoptosis |
| µL | Microlitre |
| µg | Microgram |
| 17β-HSD | 17β-hydroxysteroid dehydrogenase |
| 3β-HSD | Androstenedione via 3β-hydroxysteroid dehydrogenase |
| 3β-HSD | 3β-hydroxysteroid dehydrogenase |

# Abstract

Estrogen receptor-α negative (ERα-) tumours are a highly aggressive and heterogeneous class of breast cancer (BC) with higher prevalence in younger women. ERα- BC is associated with a worse prognosis and limited therapeutic options. Therefore, discovery of new therapeutic targets is a clinical imperative. This thesis investigated three therapeutic targets for ERα- BC: the androgen receptor (AR), transcription factor AP-2β (TFAP-2β) and cyclin-dependent kinase 9 (CDK9).

AR is expressed in 20-50% of ERα- BC, but preclinical evidence about its role is equivocal, indicating both proliferative and anti-proliferative effects. To inform this controversy, genomic parameters associated with differential AR-mediated growth effects were examined in two ERα-/AR+ breast cancer cell lines with differing proliferative responses to the natural AR ligand 5α-dihydrotestosterone (DHT). Comparing the AR-regulated transcriptome of MDA-MB-453 (growth stimulated by DHT) with MFM-223 (growth inhibited by DHT) breast cancer cells revealed that DHT regulates a discrete set of genes associated with distinct biological functions in the two models. DHT increased expression of genes associated with metabolism, development, and cell growth in MDA-MB-453 cells. In MFM-223 cells, DHT induced expression of genes with tumour suppressor activity. Analysis of AR cistrome showed that lack of AR enrichment at tumour suppressor genes exclusively upregulated by DHT in MFM-223 cells did not explain the inability of AR to induce transcription of these genes in MDA-MB-453 cells. We therefore hypothesised that differential DHT-regulated transcriptomes are driven by distinct interactions with AR co-

regulatory proteins. Unbiased proteomic analysis of the AR interactomes identified TFAP-2β as a candidate factor of interest. High TFAP-2β expression specifically clustered molecular apocrine tumours (ERα-/AR+/HER2+) and TFAP-2β was required to sustain proliferative capacity, cell viability and expression of C-MYC and HER2 oncogenes in a representative cell line. Cistrome analysis revealed substantial co-localization of TFAP-2β and AR (at approximately 40% of total loci) following treatment with DHT. However, this interaction was not a critical determinant of oncogenic AR signalling. We conclude that TFAP-2β has AR-independent oncogenic effects and represents a novel new target for the molecular apocrine sub-type of ERα- BC.

CDK9 is a transcriptional cyclin that increases RNA Polymerase II (RNAPII) activity to sustain expression of normally short-lived oncogenic and anti-apoptotic proteins. Targeting CDK9 has been hampered by poor selectivity of existing inhibitors (CDK9i). Here, we report a novel CDK9i, D-11, which exhibited high potency against CDK9 (Ki=8nM) and displayed remarkable selectivity over other CDKs and 369 human kinases. D-11 suppressed proliferation and triggered apoptosis in ERα- BC cells and these effects were ascribed to the reduction of p-RNAPII, C-MYC and MCL1 levels, indicative of targeted CDK9 inhibition. *In vivo*, D-11 inhibited ERα- BC tumour growth without affecting body weight or histology of normal tissues, indicating its potential for clinical translation as a treatment for ERα- BC.

Overall, this thesis expands current knowledge about targeting AR in ERα- BCs, provides preclinical evidence to support TFAP-2β as a novel therapeutic

target and D-11 as a highly selective, non-toxic CDK9 inhibitor ready for clinical development.

# Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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# CHAPTER ONE

# INTRODUCTION AND REVIEW OF LITERATURE

## 1.1 Breast anatomy and development

The mammary gland is a modified, specialised apocrine gland that shares the same embryological origin with salivary and sweat glands (Javed & Lteif 2013). Breast tissue is composed of glandular, stromal and adipose tissues (Sharma et al. 2010), with the glandular portion consisting of 15–20 sections called lobes in humans, which are in turn comprised of smaller units called lobules (Zhu, W & Nelson 2013) (Figure 1.1 A-C). Each lobule is made up of grape-like clusters of acini (alveoli), the hollow sacs that produce and store milk during lactation (Noor, Hseon & Jeffrey 2016). Mammary gland lobes are connected to a network of ducts called the lactiferous sinus, which funnel milk to the nipple (Noor, Hseon & Jeffrey 2016).

The mammary ducts and alveoli are lined with two types of epithelial cells, the inner luminal secretory cells, and outer myoepithelial cells (Lund et al. 1996). Two histological sub-types of luminal secretory cells have been identified: basal cells, which have relatively clear cytoplasm and an oval nucleus lacking a visible nucleolus, and luminal cells that harbour darker, basophilic cytoplasm (Pontén et al. 2008). Myoepithelial cells (a hybrid of smooth muscle ('myo') and epithelial cells) form a continuous layer that separates luminal cells from the basement membrane (Omar 2014). Upon contraction, the myoepithelial cells decrease the length and increase the diameter of the ducts to eject the milk during lactation (Omar 2014). Moreover, myoepithelial cells are responsible

for synthesising basement membrane components and play an important role in mammary morphogenesis (Gudjonsson et al. 2005).

The stromal compartment of the breast is made up of two mesenchymal lineages: adipocytes and fibroblasts, as well as infiltrating immune and endothelial cells (Dzięgelewska & Gajewska 2018; Zhu, W & Nelson 2013). The stroma accounts for about 60% of the total breast tissue volume and provides the glandular tissues with support, structure, nutrients and immune defense (Dzięgelewska & Gajewska 2018). Several studies have shown that mammary stroma is an important regulator of mammary epithelial cell proliferation, survival, polarity, and differentiation, via paracrine, physical, and reciprocal signalling between epithelial cells and underlying stromal cells (Arendt et al. 2010; Maller et al. 2010; Mueller et al. 2002; Polyak & Kalluri 2010). Vascular endothelial cells are important for transporting nutrients and oxygen to breast tissues and represent a vital component of the epithelial microenvironment in breast cancer (Ingthorsson et al. 2010).

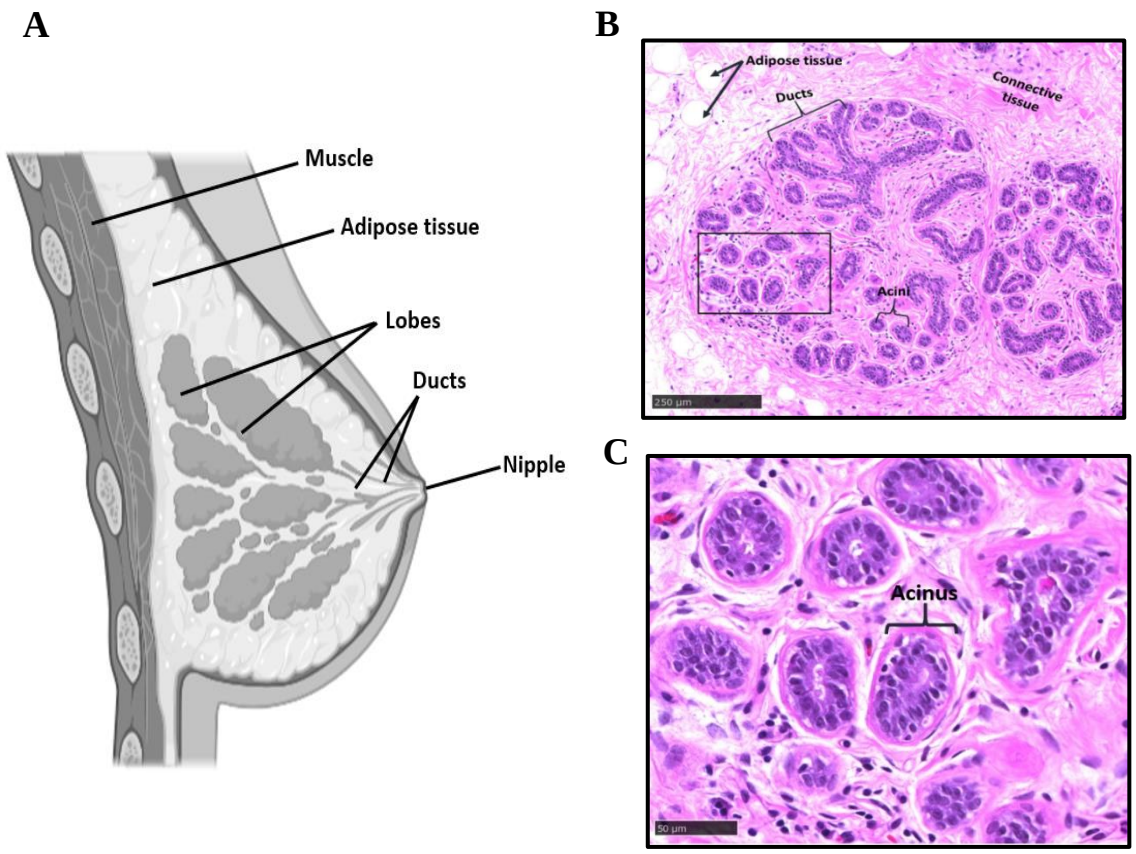



**Figure 1.1:** Anatomy of the human mammary gland. **(A)** The mammary gland contains 15–20 lobes, each lobe containing a series of branched ducts that drain into the nipples. **(B)** Hematoxylin and eosin-stained section of human breast tissue showing a terminal ductal lobular unit comprised of ducts and acini embedded in a fibrous connective tissue stroma. **(C)** Higher magnification view of boxed area in (B), showing acinus. Scale bar=50µm.

The mammary gland undergoes dynamic changes throughout various female developmental stages (Macias & Hinck 2012). At birth, the mammary glands are rudimentary structures consisting of very few ducts (Javed & Lteif 2013). During puberty and under the effect of ovarian sex steroid hormones (estrogen and progesterone) and pituitary gland growth hormone, the rudimentary mammary ducts elongate and branch out (Brisken & O'Malley 2010; Gusterson & Stein 2012). Once ovulation and menstruation begin, maturation of the breast tissue begins with the formation of secretory glands at the end of the milk ducts (Gusterson & Stein 2012; Javed & Lteif 2013). The breast tissue and ductal system continues to grow and mature, with the development of a number of glands and lobules (Dzięgelewska & Gajewska 2018; Inman et al. 2015; Javed & Lteif 2013). Another stage of mammary gland development occurs upon exposure to elevated hormone levels during pregnancy (Brisken & O'Malley 2010), when estrogen, progesterone, and prolactin levels rise (Brisken & O'Malley 2010). Estrogen stimulates proliferation of ductal cells and promotes secretion of prolactin and progesterone (Javed & Lteif 2013; Macias & Hinck 2012), which are the hormones responsible for formation and growth of milk-producing cells (Brisken & O'Malley 2010; Javed & Lteif 2013; Macias & Hinck 2012). After weaning, these hormones return to pre-pregnancy levels and the mammary gland undergoes post-lactational regression leading to collapse of the alveolar structures, and removal of the secretory epithelial and myoepithelial cells by apoptosis (Lund et al. 1996). With the onset of menopause, the ductal and glandular elements of the breast begin to shrink (Aranda-Gutierrez & Diaz-Perez 2019; Hassiotou & Geddes 2013), triggered by declining ovarian function and reduced levels of estrogen and

progesterone (Aranda-Gutierrez & Diaz-Perez 2019; Hassiotou & Geddes 2013). As menopause progresses, the breast connective tissues (fat and stromal elements) lose elasticity, leading to breast shrinkage and loss of shape (Hassiotou & Geddes 2013).

## 1.2 Breast cancer

### 1.2.1 Pathology and epidemiology

Breast cancer has a major impact on public health globally. It is one of the most common malignancies in women and the leading cause of cancer-related deaths among women worldwide (Bray et al. 2018). Each year, over 1.1 million women are diagnosed with breast cancer and more than 410,000 women die from the disease worldwide (Siegel, Miller & Jemal 2016). For Australian women, the lifetime risk of developing breast cancer by the age of 85 is one in seven (Australian Institute of Health and Welfare 2019a), and in 2020 it is estimated that 19,998 Australian women will be diagnosed with breast cancer, an average of 55 cases each day, with 3049 deaths (Australian Institute of Health and Welfare 2019b).

Breast cancer is an uncontrolled growth of neoplastic breast epithelial cells that arises due to genetic alterations/mutations in proto-oncogenes and/or tumour suppressor genes (Hartwell & Kastan 1994). Breast cancer most commonly develops in epithelial cells that line the glandular tissue of the breast, primarily within the milk-producing lobules (lobular carcinoma) and the milk ducts (ductal carcinoma) (Benson et al. 2009). The earliest recognisable form of breast cancer is non-invasive, *in situ* carcinoma, which is defined as a tumour that resides within the duct or lobules of the breast (Pinder 1995) (Figure 1.2).

Pure *in situ* carcinoma represents approximately 20% of all diagnosed breast cancers (Pinder 1995). In contrast, most (~80%) breast cancers are identified once they have become invasive or infiltrating (Sharma et al. 2010), where they have broken through the walls of the glands or ducts and diffused into surrounding breast tissues (Sharma et al. 2010) (Figure 1.2). Cancer cells can then spread (metastasise) to other parts of the body through the bloodstream or lymphatic system (Nguyen, Bos & Massagué 2009). It is this metastatic form of breast cancer that is most lethal, with many essential organs (e.g., liver, and brain) succumbing to metastatic cancer growth.

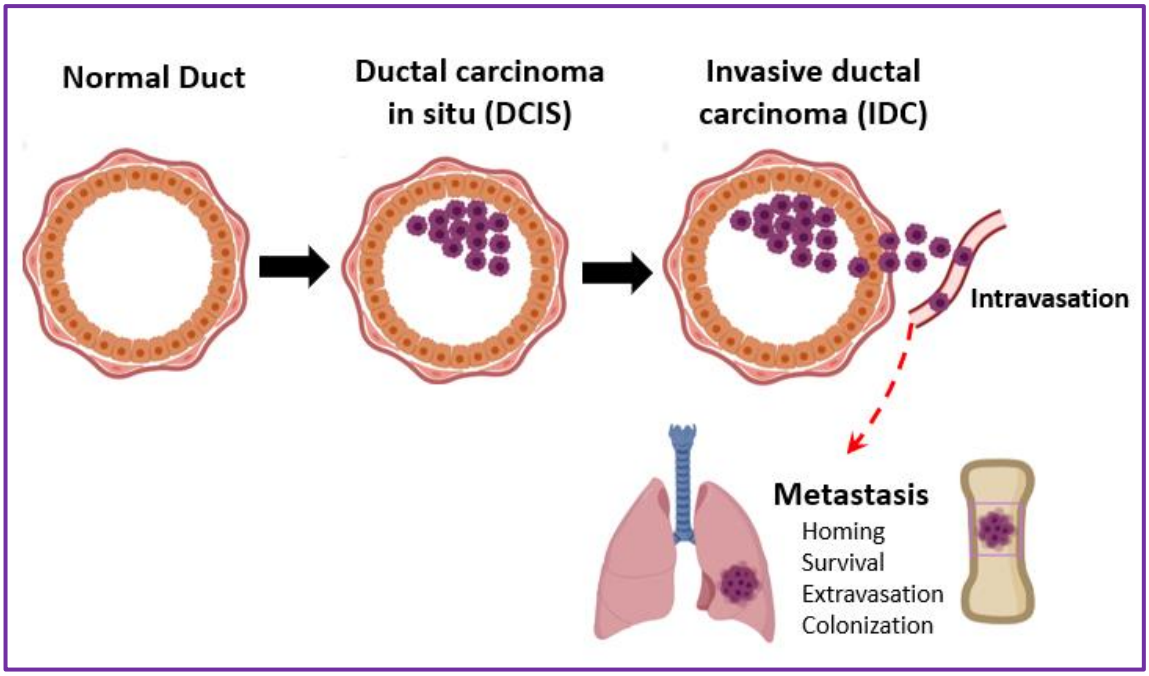



**Figure 1.2:** A schematic depicting the various pathologically defined stages of breast tumour progression, as well as various functions associated with metastatic spread. *In situ* carcinoma is tumour growth that remains within the duct or lobules of the breast. Invasive carcinoma occurs when the malignant cells break the walls of the glands or ducts where they originated and diffused into surrounding breast tissues. Metastasis, the final stage of cancer progression, occurs when cancer cells travel via the bloodstream or lymph vessels to other parts of the body.

### 1.2.2 Risk factors

More than three decades of epidemiologic studies have identified many risk factors that increase the likelihood of developing breast cancer. One of the strongest risk factors is age (Howlader et al. 2019; Singletary 2003), whereby the risk of developing breast cancer increases with increasing age in women (Howlader et al. 2019; Singletary 2003). The incidence of breast cancer for a woman in her 30s is approximately one in 227, compared with approximately one in 28 for a woman in her 70s, and more than three-quarters of breast cancer cases were diagnosed in women over the age of 50 (Howlader et al. 2019). Another important risk factor is genetics. Hereditary breast cancers are responsible for one-third of all breast cancer cases and up to 30% of all early-onset breast cancer (Mehrgou & Akouchekian 2016). The best-described breast cancer susceptibility genes are *BRCA1* and *BRCA2* (Langston et al. 1996). *BRCA1* and *BRCA2* act as tumour suppressor genes and play a role in the maintenance of genome integrity by repairing double-strand DNA breaks via the homologous recombination repair (HRR) pathway (Langston et al. 1996; Roy, R, Chun & Powell 2012). Women carrying *BRCA1* or *BRCA2* genetic mutations have a 60–80% lifetime risk of developing breast cancer (Mehrgou & Akouchekian 2016). Inherited mutations in other genes critical for genome integrity, including *CHEK2*, *PALB2*, *ATM*, *p53*, *PTEN*, *STK11*, *CDH1*, are also associated with increased risk of breast cancer (Launonen 2005; Lei, Haixin et al. 2002; Olivier, Hollstein & Hainaut 2010; Walsh & King 2007), albeit they are less common than *BRCA* mutations.

Lifetime exposure to ovarian hormones (e.g., estrogen) also influences risk for breast cancer, as early menarche, late menopause, and older age at first pregnancy all increased risk of breast cancer (Dall & Britt 2017; Li, CI et al. 2008; MacMahon et al. 1970).

For each one year delay in onset of menopause, the risk of breast cancer increases by 3% (Surakasula, Nagarjunapu & Raghavaiah 2014), while each one year delay in menarche and each additional birth decreases the risk of breast cancer by 5% and 10%, respectively (Dall & Britt 2017; Surakasula, Nagarjunapu & Raghavaiah 2014). Such increases in risk might be explained by a longer lifetime exposure to elevated sex steroid hormones, which can increase the proliferation of the mammary cells and suppress apoptosis (Dall & Britt 2017).

Estrogens are a group of steroid hormones that regulate female reproductive function and secondary sex characteristics, including breast development (Darbre 2015). Increases in the endogenous concentration of circulating estrogens is strongly associated with increased risk of breast cancer in postmenopausal women (Baglietto et al. 2010; Thomas, HV et al. 1997; Travis & Key 2003; Zeleniuch-Jacquotte, A et al. 2004). Postmenopausal women with high serum estrogen concentrations have a nearly two-fold risk of breast cancer compared with postmenopausal women with low serum concentrations of estrogens (Travis & Key 2003). Exogenous estrogens administered as part of oral contraception or menopause hormone therapy (MHT) can stimulate mammary epithelial cell proliferation and thereby increase breast cancer risk with long term use (Lancet 2019; Marjoribanks et al. 2012; Perkins, Louw-du

Toit & Africander 2018). Evidence from a large meta-analysis study looking at the relationship between estrogen-alone MHT and breast cancer risk, reported that current users had a 1.14 fold (95% confidence interval (CI) = 1.05–1.22) excess risk for developing breast cancer compared to women that never used MHT (Wang, K et al. 2017).

Androgen hormones, a primary focus of this thesis, also have a role in the aetiology of breast cancer. Androgens are generally considered as classical male sex hormones, but androgens are also made by reproductive and adrenal organs in women and play important biological roles, in part as precursors for the production of estrogen hormones (Dimitrakakis & Bondy 2009). In postmenopausal women, most prospective epidemiologic studies reported that higher levels of androgens including testosterone, dehydroepiandrosterone-sulphate (DHEAS) and androstenedione (A4) were significantly associated with an increased breast cancer risk (Kaaks, Rinaldi, et al. 2005; Key T 2002; Missmer et al. 2004; Zeleniuch-Jacquotte, A et al. 2004). However, the association between increased androgen levels and risk of breast cancer does not hold up when factoring in the conversion of testosterone to estrogen (Adly et al. 2006; Hankinson et al. 1998; McNamara, KM et al. 2014). In other words, conversion of testosterone to estrogen by aromatase activity could contribute to the association between testosterone and breast cancer risk (McNamara, KM et al. 2014). In premenopausal women, the association between circulating androgens and breast cancer risk is not so clear, with studies reporting either no association (Helzlsouer et al. 1992; Lee, SH et al. 1999) or a positive association (Kaaks, Rinaldi, et al. 2005; Key T 2002; Missmer et al. 2004; Zeleniuch-Jacquotte, A et al. 2004). A more detailed

review of epidemiological data relating to androgens in breast cancer is presented in Section 1.4.4.

Taken together, these studies provide evidence that sex steroid hormones (i.e., estrogens and androgens), play an important role in breast cancer.

## 1.3 Estrogen signalling in breast cancer.

### 1.3.1 Estrogen and the estrogen receptor axis

Notable attention has been given to the role of estrogen in breast cancer since British surgeon George Beatson (Beatson 1896) published his findings on the beneficial effects of ovariectomy in a premenopausal patient with advanced breast cancer. Beatson's findings signified that ovarian hormones (i.e., estrogens) are critical determinants of breast cancer growth.

Estrogens are a class of sex steroid hormones that are secreted mainly by the ovaries (Nelson & Bulun 2001). Estrogens are also produced at peripheral sites (extragonadal sites) such as breast, adipose tissue, adrenal glands, bone, and brain (Nelson & Bulun 2001) in humans. In the ovaries, estrogens are ultimately synthesised from cholesterol by a series of biochemical reactions collectively called steroidogenesis (Figure 1.3), where they can act locally as well as being secreted into circulation. Estrogens are transported through the circulation bound to sex hormone binding globulin (SHBG) to act on distal target tissues in an endocrine manner (Nelson & Bulun 2001; Zhao, H et al. 2016). About 55% of estrogens in women are bound to circulating SHBG (Laurent et al. 2016). Within extragonadal sites, estrogens are also locally produced by the aromatisation of circulating androstenedione or testosterone

via the aromatase (P450arom) enzyme and act in an intracrine manner (Nelson & Bulun 2001). In postmenopausal women, when the ovaries cease to produce estrogen and circulating estrogen levels precipitously drop, tissue levels of estrogen remain relatively constant due to intracrine activity (Yaghjyan, Colditz & Control 2011). Van Landeghem *et al* (1985) published one of the earliest studies showing that estrogen levels were similar in normal breast tissue from both pre- and postmenopausal women (Van Landeghem et al. 1985), which may be attributed to an increase in aromatase activity in the peripheral tissues.

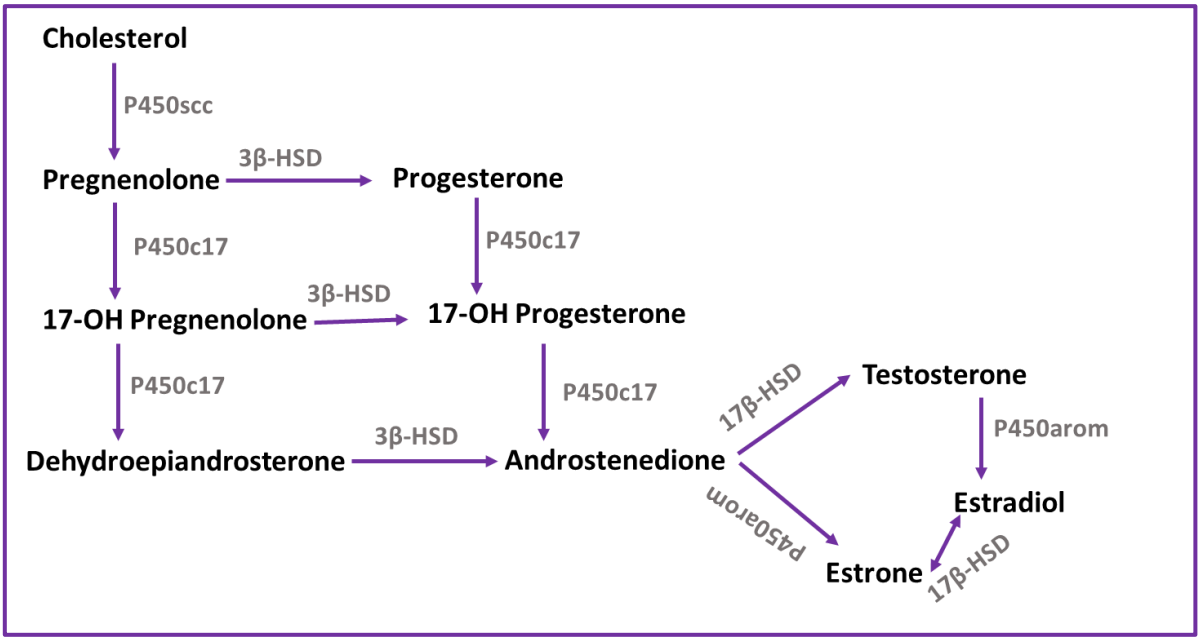



**Figure 1.3**: Estrogen biosynthesis in the ovaries. Estrogen production begins with the conversion of cholesterol into progestogens, androgens, and finally estrogens. Progesterone is converted to androgens via cytochrome P450 17α-hydroxylase (P45017) and 17-beta-hydroxysteroid dehydrogenase (17β-HSD). Androgen is converted to estrone, and estradiol via aromatase (P450arom). P450scc, cholesterol side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase.

Naturally, estrogens occur in three structurally related forms; estrone (E1), estradiol (E2, or 17β-estradiol), and estriol (E3) (Cui, Shen & Li 2013). E2 is the most common and potent circulating estrogen in females (Cui, Shen & Li 2013). Estrogens exert their effects via binding to the estrogen receptor (ER) (Enmark et al. 1997). There are two major isoforms of ER, ERα and ERβ, that govern a wide range of physiological processes including development of the female reproductive system, maintenance of bone mass, and protection of cardiovascular tissue (Enmark et al. 1997; Harvell et al. 2008). ERα and ERβ are encoded by two unique genes that reside on distinct chromosomes, *ESR1* and *ESR2* on the sixth and fourteenth chromosome (6q25.1 and 14q23.2), respectively (Enmark et al. 1997). While the role of ERβ in breast cancer initiation and progression is still debated, the role of ERα as a key transcriptional regulator and oncogenic driver of the majority of breast cancers is well established (Kurebayashi et al. 2000; Shoker et al. 1999). Like all steroid receptors, ERα is a ligand-activated nuclear transcription factor that regulates transcription of target genes responsible for controlling cell proliferation, maturation, and death of breast epithelial cells (Kurebayashi et al. 2000; Shoker et al. 1999). Deletion of ERα in mice results in a rudimentary mammary ductal system that fails to branch out (Bocchinfuso et al. 2000). A study by Feng *et al* (2007) showed that ERα not only regulates ductal morphogenesis during puberty but is also involved in alveologenesis during pregnancy and lactation (Feng, Yuxin et al. 2007) .

Aberrant ERα signalling is the key driver of breast carcinogenesis, responsible for the initiation and progression of at least 70% of all breast cancers (Zhou, Z et al. 2014). Activation of ERα by E2 stimulates growth of breast cancer cells

*in vitro* and *in vivo* (Bocchinfuso et al. 2000; Couillard et al. 1998; Huseby, Maloney & McGrath 1984). Preclinical studies showed that estrogen-induced proliferation of breast cancer cell lines is driven by ERα modifying expression of hormone-responsive genes involved in the cell cycle and/or apoptosis (Altucci et al. 1996; Carroll et al. 2006; Eeckhoute et al. 2006; Foster & Wimalasena 1996). E2 stimulated the growth of ERα positive cell lines, MCF-7, ZR-75-1, and T47-D, by inducing expression of the master transcription factor and oncogenes C-MYC, C-MYB and the cell cycle regulator cyclin D1 (Abdullah et al. 2018; Altucci et al. 1996; Drabsch et al. 2007; Dubik & Shiu 1988; Eeckhoute et al. 2006; Foster & Wimalasena 1996; Watts et al. 1994). Cyclin D1 regulates progression of cells through the G1 phase of the cell cycle (Allen-Petersen & Sears 2019), and overexpression of cyclin D1 is strongly associated with ERα expression in breast cancer (Mohammadizadeh et al. 2013), present in approximately 60% of ductal carcinomas (Pietras & Márquez-Garbán 2007). Other *in vitro* studies have shown that E2 can inhibit breast cancer cell apoptosis by regulating the expression of anti-apoptotic proteins such as BCL2, and MCL1 (Gompel et al. 2000; Schacter, Henson & Gibson 2014). Moreover, ERα interacts with several key growth stimulatory proteins, including c-Src, (the p85 subunit of phosphoinositide 3-kinase (PI3K)), epidermal growth factor receptor (EGFR), insulin-like growth factor receptor 1 (IGFR1), and human epidermal growth factor receptor-2 (HER2) (Driggers & Segars 2002; Manavathi et al. 2013; Pietras & Márquez-Garbán 2007; Stoica et al. 2003). These interactions activate multiple signal transduction pathways, such as MAPK/ERK and phosphoinositide 3-kinase (PI3K)/Akt, which are known to promote tumour angiogenesis, proliferation,

invasion, and metastasis (Driggers & Segars 2002; Manavathi et al. 2013; Pietras & Márquez-Garbán 2007; Stoica et al. 2003).

Although aberrant ERα signalling plays a crucial role in breast cancer, there are also some breast cancers that develop in the absence of ER signalling. Therefore, breast cancer can be broadly categorised into two main subtypes: ERα-positive (ERα+) and ERα-negative (ERα-).

### 1.3.2 ERα-positive and ERα-negative subtypes of breast cancer

Breast tumours that are positive for ERα and/or progesterone receptor (PR; a biomarker of ERα activity) by immunohistochemistry (IHC) are classified as luminal breast cancers (Yersal & Barutca 2014). Luminal tumours represent approximately 65% and 80% of tumours diagnosed in premenopausal and postmenopausal women, respectively (Dai et al. 2016; Rakha et al. 2010). Women with luminal tumours have longer relapse-free and overall survival rates compared to those with ERα-negative tumours (Dai et al. 2016; Zhang, MH et al. 2014). Using gene expression profiling, luminal breast cancers were further divided into two molecular subgroups, luminal A and B (Sørlie et al. 2001; Yaşar et al. 2017; Yersal & Barutca 2014). The luminal A subtype is characterised by higher levels of ERα, PR positivity, lack of HER2 overexpression, and lower levels of proliferation-related genes (Szostakowska et al. 2019; Yersal & Barutca 2014). The luminal B subtype is ERα positive and either PR positive or negative and HER2 positive or negative, with high levels of proliferation-related genes (e.g., *MYB*, *CCNB1*, and *GGH)* compared to luminal A breast cancer (Szostakowska et al. 2019; Yaşar et al. 2017; Yersal & Barutca 2014). Endocrine therapies (ET) are a key treatment modality in the

management of ERα-positive breast cancers (Martinkovich et al. 2014). ET is a general term for drugs that target ERα, including aromatase inhibitors (AIs), selective ERα modulators (SERMs), and selective ERα down-regulators (SERDs) (Szostakowska et al. 2019). AIs attenuate ER signalling by decreasing estrogen production (Fabian 2007; Lønning 2009). SERMs competitively block the effect of estrogen at the receptor level, as they can interfere with the binding of estrogen to the ER, with interaction between ERα and its co-activators and/or binding of ERα to the promoter elements of its target genes (Szostakowska et al. 2019). For many decades, the SERM tamoxifen was the gold standard for treating both pre- and postmenopausal patients with hormone receptor-positive breast cancer and has vastly improved survival outcomes for those patients (Howell et al. 2004; Lønning 2009). However, *de novo* and acquired resistance to these treatments led to generation of a new class of endocrine therapy drugs called SERDs (Howell et al. 2004; Wardell et al. 2015). SERDs such as fulvestrant are competitive antagonists whose interaction with ER induce proteasome-dependent degradation (Howell et al. 2004; Wardell et al. 2015). In part, response to an ET underpins the greater chance of survival in women with ERα-positive disease compared to ERα-negative disease (Zhou, Z et al. 2014).

ERα-negative breast cancer, which represents up to 30% of all cases, is a highly aggressive and heterogeneous class of breast tumours with a higher prevalence in younger women, and is associated with a poorer prognosis compared to ERα-positive breast cancer (Barcellos-Hoff 2013). ERα-negative breast cancer can be divided into HER2-enriched and basal-like, the latter also called triple negative breast cancer (TNBC) (Chen, J-Q & Russo 2009; Yersal

& Barutca 2014). ERα-negative/HER2-enriched tumours are characterised by amplification or overexpression of HER2 and can be treated with HER2-targeting agents such as trastuzumab (Yersal & Barutca 2014). However, the presence of primary or acquired resistance to anti-HER2 treatments remains a significant challenge for ERα-negative/HER2-enriched breast cancer patients (Wahdan-Alaswad, Liu & Thor 2020). TNBC tumours represent 15–20% of all diagnosed breast cancer cases, and lack ERα, HER2 and PR expression (Chen, J-Q & Russo 2009). Women diagnosed with TNBC usually present with much more severe disease states, including larger tumours, higher pathology grade and lymph node metastasis (Chen, J-Q & Russo 2009; Haffty et al. 2006). The absence of well-defined molecular targets means TNBC patients will not respond to most currently available ERα and HER-2 targeted therapies (Yao et al. 2017). They are mainly managed with standard chemotherapy treatment, which is associated with a high rate of local and systemic relapse (Chen, J-Q & Russo 2009; Yao et al. 2017) .

Microarray gene expression studies have led to the discovery of the molecular apocrine subtype of ERα-negative breast cancer, distinguished by elevated androgen receptor (AR) signalling (Farmer et al. 2005). Molecular apocrine tumours overlap with ERα-negative/HER2-enriched tumours (Doane et al. 2006; Farmer et al. 2005). The molecular apocrine sub-type also overlaps with the "Luminal-AR (LAR)" subtype of TNBC as defined in the Vanderbilt TNBC classification metric (Lehmann et al. 2011). Molecular apocrine tumours lack the expression of ERα and PR but have high expression of AR, as well as genes known to be regulated by AR in prostate cancers (Farmer et al. 2005; Lehmann et al. 2011). Preclinical reports have shown that AR likely

plays a role in the growth of molecular apocrine tumours (Doane et al. 2006; Lehmann et al. 2011; Naderi & Hughes-Davies 2008; Ni et al. 2011), instigating widespread interest in targeting AR in this breast cancer sub-type. However, the exact role of AR in clinical molecular apocrine tumours, or indeed, any sub-type of ERα-negative breast cancer remains unclear. The role of AR in breast cancer is discussed in more detail in Section 1.4.

What is clear, is that ERα-negative tumours pose a significant therapeutic challenge due to the limited available molecular targets for drug development. Thus, there is an urgent need to develop new target therapies and therapeutic strategies to improve outcomes for ERα-negative breast cancer patients. The overarching aim in this PhD project is to test novel and distinct therapeutic targets for the ERα-negative subtype of breast cancer including the AR, and cyclin-dependent kinase 9 (CDK9), as discussed in the sections below.

## 1.4 Androgen signalling in ERα-negative breast cancer.

### 1.4.1 Androgen sources and synthesis in females

Androgens are cholesterol-derived steroid hormones primarily responsible for the development and maintenance of masculine characteristics (Mooradian, Morley & Korenman 1987). Androgens are also produced in females and have important physiological roles that include regulation of mammary gland growth, precursors for estrogen production (Figure 1.3), anabolic effects, stimulation of bone formation, and erythropoietin production in the kidneys (Shahani et al. 2009; Vanderschueren & Bouillon 1995). The five major androgens present in the systemic circulation, listed in descending order of

serum concentration, include dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone, and 5α-dihydrotestosterone (DHT) (Labrie 2004). In women, androgen synthesis takes place primarily in the ovaries and adrenal glands but can also be synthesised in peripheral tissues (Greenblatt et al. 1976; Labrie 2004) (Figure 1.4). In premenopausal women, 25% of testosterone is produced by the ovaries, 25% by the adrenals, and 50% by peripheral conversion (Lobo, Kelsey & Marcus 2000; Longcope et al. 1986). In postmenopausal women, 50% of testosterone is produced by the ovaries, 10% by the adrenals and 40% by peripheral conversion (Laughlin et al. 2000; Lobo, Kelsey & Marcus 2000). Ovaries and adrenal glands synthesise androgens from cholesterol through enzymatic conversions (Figure 1.3) and these are transported through the circulation bound to SHBG or albumin to reach their intended target tissues, where they exert tissue-specific effects (Antoniou-Tsigkos et al. 2019; Miller, Geller & Rosen 2006). In the cytoplasm of target tissue cells, testosterone can bind to the AR, or can be converted to the more potent androgen DHT, by the cytoplasmic enzyme 5α-reductase (Miller, Geller & Rosen 2006). In women, approximately 66% of total circulating testosterone is bound to SHBG, and alterations in the level of SHBG have a dramatic effect on the free levels of testosterone in serum (Bartsch et al. 1980; Botwood et al. 1995). Increased levels of E2 and thyroxine increase SHBG, whereas growth hormone, insulin-like growth factor 1 (IGF-1), androgens, prolactin, and obesity suppress SHBG (Bartsch et al. 1980; Enriori et al. 1986; Plymate et al. 1990).

Peripheral tissues such as adipose, breast, muscles, and liver produce androgens from circulating steroid precursors (Labrie et al. 2001; Labrie et al.

2000) (Figure 1.4). The adrenal glands mostly secrete the steroid precursors DHEA and DHEAS, which are converted within peripheral tissues to androstenedione via 3β-hydroxysteroid dehydrogenase (3β-HSD), and then to testosterone via 17β-HSD (Labrie 2004; Labrie et al. 2001). Androgens produced in peripheral tissues exert mostly local actions (intracrine) within the cells of synthesis, with only a minimal amount released into circulation (Sasano et al. 2008). Therefore, the serum levels of androgens are not necessarily indicative of their bioactivity in target tissues.

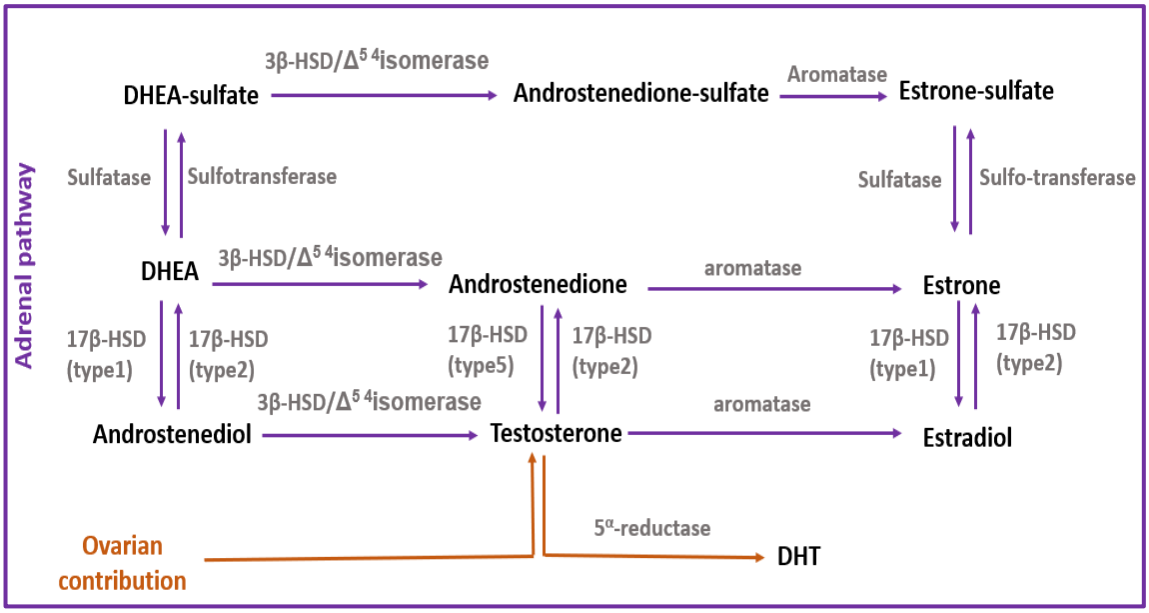
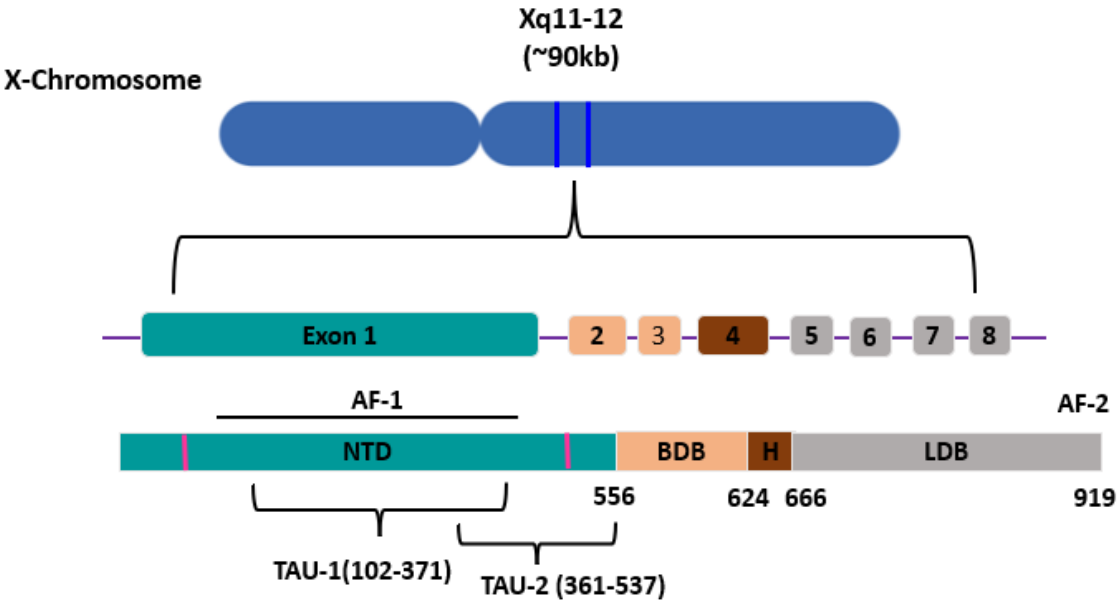



**Figure 1.4:** The classic androgen synthesis pathway, as established in the adrenal cortex and peripheral tissues. AIs, aromatase inhibitors; DHEA, dehydro-epi-androsterone; DHT, dihydrotestosterone; HSD, hydroxysteroid dehydrogenase.

### 1.4.2 AR structure and signalling

The biological action of androgens is mediated through binding to the androgen receptor (AR), a ligand-inducible transcription factor that belongs to the nuclear receptor superfamily (Tilley et al. 1989). AR is encoded by a single copy gene (>90 kb) located on the X chromosome at position Xq11-12 (Brown, C et al. 1989; Tilley et al. 1989) (Figure 1.5). The coding sequence of AR is comprised of 8 exons, which produce a protein of approximately 917 amino acids at a molecular weight of about 98.8 kD (Brown, C et al. 1989; Tilley et al. 1989) depending on the size of two polymorphic microsatellite regions in Exon 1 of the *AR* gene (Brown, C et al. 1989; Edwards et al. 1992). Post-translational modifications, such as phosphorylation, increase the apparent molecular weight of the AR (~110 kD) when analysed by SDS-polyacrylamide gel electrophoresis (Gioeli et al. 2002). Like other members of the nuclear receptor superfamily, AR exhibits a modular structure composed of three functional regions: amino-terminal domain (NTD), DNA binding domain (DBD), and ligand binding domain (LBD) (Brinkmann et al. 1989) (Figure 1.5).

**A**




**B**

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  1 MEVQLGLGRV YPRPPSKTYR GAFQNLFQSV REVIQNPGPR HPEAASAAPP GASLLLLQQQ
 61 QQQQQQQQQQ QQQQQQQQQQ ETSPRQQQQQ QGEDGSPQAH RRGPTGYLVL DEEQQPSQPQ
121 SALECHPERG CVPEPGAAVA ASKGLPQQLP APPDEDDSAA PSTLSLLGPT FPGLSSCSAD
181 LKDILSEAST MQLLQQQQQE AVSEGSSSGR AREASGAPTS SKDNYLGGTS TISDNAKELC
241 KAVSVSMGLG VEALEHLSPG EQLRGDCMYA PLLGVPPAVR PTPCAPLAEC KGSLLDDSAG
301 KSTEDTAEYS PFKGGYTKGL EGESLGCSGS AAAGSSGTLE LPSTLSLYKS GALDEAAAYQ
361 SRDYYNFPLA LAGPPPPPPP PHPHARIKLE NPLDYGSAWA AAAAQCRYGD LASLHGAGAA
421 GPGSGSPSAA ASSSWHTLFT AEEGQLYGPC GGGGGGGGGG GGGGGGGGGG GGGEAGAVAP
481 YGYTRPPQGL AGQESDFTAP DVWYPGGMVS RVPYPSPTCV KSEMGPWMDS YSGPYGDMRL
541 ETARDHVLPI DYYFPPQKTC LICGDEASGC HYGALTCGSC KVFFKRAAEG KQKYLCASRN
601 DCTIDKFRRK NCPSCRLRKC YEAGMTLGAR KLKKLGNLKL QEEGEASSTT SPTEETTQKL
661 TVSHIEGYEC QPIFLNVLEA IEPGVVCAGH DNNQPDSFAA LLSSLNELGE RQLVHVVKWA
721 KALPGFRNLH VDDQMAVIQY SWMGLMVFAM GWRSFTNVNS RMLYFAPDLV FNEYRMHKSR
781 MYSQCVRMRH LSQEFGWLQI TPQEFLCMKA LLLFSIIPVD GLKNQKFFDE LRMNYIKELD
841 RIIACKRKNP TSCSRRFYQL TKLLDSVQPI ARELHQFTFD LLIKSHMVSV DFPEMMAEII
901 SVQVPKILSG KVKPIYFHTQ
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**Figure 1.5:** AR gene and protein structure. **(A)** Schematic overview of the different functional domains of the human AR. Numbers below the bars refer to the amino acid residues which separate the domains starting from the N-terminus (left) to C-terminus (right). NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain; AF, activation function, TAU, transcription activation units. **(B)** The amino acid sequence of the human AR protein, based on NCBI accession number P10275. The diagram highlights sequences representing the domains by colour, NTD (green), DBD (light orange), hinge region (dark orange) and LBD (gray).

The NTD accounts for more than half of the AR protein (residues 1–555), and its entirety is encoded by exon one (Tan et al. 2015). The NTD contains an activation function (AF)-1 region that plays an important role in transactivation, dimerisation, and recruitment of co-regulatory proteins crucial for AR's transcriptional function (Alen et al. 1999; Jenster et al. 1995; Onate et al. 1998; Yu, Xinzhe et al. 2020). The AF-1 of the AR is composed of two units, known as transcription activation units 1 and 5 (Tau-1, 102-371 and Tau-5, 361-537) (Jenster et al. 1995). Tau-1 is crucial for the transcriptional activity of AR when the receptor is activated by androgens (Jenster et al. 1995). Although Tau-5 is less well characterised, it has been shown to be more important than Tau-1 when activation occurs via androgen independent mechanisms in androgen independent cell lines and mouse xenograft models of prostate cancer (Dehm et al. 2007; Metzger et al. 2003). Moreover, the first 20 amino acids of the NTD are highly conserved across species and contain an FxxLF motif (where x is any amino acid) important for interactions with the AR LBD, termed the AR N-C interaction (Steketee et al. 2002; Yu, Xinzhe et al. 2020). This is a key interaction for normal receptor function, stabilising the bound ligand, and is crucial for AR-dependent gene regulation, since mutations that disrupt this interaction impair AR activity (Langley, Kemppainen & Wilson 1998). The AR DBD, (residues 556–623) represents a cysteine-rich region that is highly conserved among steroid hormone receptors (Chang, Kokontis & Liao 1988; Lubahn et al. 1988; Tilley et al. 1989). The DBD contains two zinc finger motifs, each of them consisting of four cysteine residues that incorporate a zinc ion (Zilliacus et al. 1995). A conserved amino acid motif (P-Box) within the first zinc finger directs AR DNA binding to

androgen response elements (AREs) that are inverted hexameric DNA half-sites that are spaced by 3 base pairs (e.g. CC***AGAACA***TCA***AGAACA***C) in the regulatory regions of target genes (Davey & Grossmann 2016; Zilliacus et al. 1995). The second zinc finger, containing the D-box motif, stabilises the DNA-bound receptor complex and mediates dimerisation of AR monomers (Centenera et al. 2008; Davey & Grossmann 2016; Zilliacus et al. 1995). Located at the carboxyl terminus of AR, the LBD (residues 666–919) mediates high affinity binding of AR to its natural androgenic ligands DHT and testosterone (Kd = 0.2-0.5nM) (Grino, Griffin & Wilson 1990; Kemppainen et al. 1999), as well as synthetic androgens such as R1881 (Kd = 0.6nM) and mibolerone (Kd = 0.53nM) (Kemppainen et al. 1999; Murthy et al. 1986). A ligand-dependent AF-2 function is located within the LBD (He, B et al. 1999; Moilanen et al. 1997). AF-2 is involved in the interaction between AR and co-regulators containing the LxxLL motif (He, B et al. 1999), where mutation of this region is found to dramatically reduce ligand-dependent AR activation (Buchanan et al. 2001). However, unlike other steroid hormone receptors, the LBD of AR has a weak affinity for factors containing LxxLL motifs, and instead, preferentially binds to aromatic-rich motifs that are found within the AR NTD and AR-specific co-regulators (He, B, Kemppainen & Wilson 2000). The hinge region (residues 624–665) is a flexible region that connects the DBD with the LBD (Haelens et al. 2007). The hinge region contains a nuclear localisation signal (NLS) that is exposed upon ligand-induced conformational changes to the AR structure, and contains important sites for receptor phosphorylation, acetylation, and degradation (Clinckemalie et al. 2012; Haelens et al. 2007).

In the absence of its ligand, AR resides primarily in the cytoplasm of cells as part of a large multi-protein complex, which consists of receptor polypeptide and molecular chaperones including heat shock proteins, co-chaperones, and cytoskeletal proteins , maintaining structural integrity of the AR and keeping it in an inactive form (Pratt & Toft 1997) (Figure 1.6). Upon ligand binding, AR adopts an active conformation that facilitates the dissociation of chaperones, formation of N/C termini interaction, exposure of the NLS and translocation of AR into the nucleus (Cutress et al. 2008; Tyagi et al. 2000; van Royen et al. 2012). Once in the nucleus, AR forms a homo-dimer (i.e. two AR proteins bound together) which facilitates its binding to AREs within the promoter and enhancer regions of AR target genes (Shaffer et al. 2004). A study by Van Royen *et al* (2012) suggests that dimerisation of the AR only occurs after nuclear translocation and may require prior binding to the DNA (van Royen et al. 2012). On the other hand, AR DNA binding events may not be limited to loci containing classic AREs. Previous genome-wide profiling studies have shown that 16-22% of AR-DNA binding sites do not contain AREs, which may suggest that AR tethering to other factors bound to DNA. Prior to development of ChIP-seq technology to profile genome-wide DNA binding patterns, it was thought that AR predominantly bound to the promoters of target genes. However, ChIP-seq studies revealed that most AR binding sites were greater than 10kb from the transcription start site (TSS) of target genes (Massie et al. 2007; Wang, Q et al. 2007). Therefore, the current paradigm involves AR binding to enhancer sites. AR-DNA binding allows recruitment of the basal transcription machinery (e.g. TATA box-binding protein (TBP) and transcription factor IIF (TFIIF)) as well as other regulators such as CREB-

binding protein (CBP), transcription-activated p300 and AR-binding protein 70 (ARA70) to AR (Frønsdal et al. 1998; McEwan & Gustafsson 1997; Zhou, Z-x et al. 2002). AR binds to enhancer sites that are generally distant from the promoter, but AR at these sites interacts with gene promoters by chromatin looping (Wang, Q, Carroll & Brown 2005; Wu, D et al. 2011). Wang and colleagues showed that RNA-polymerase II tracks through a large DNA loop formed between AR target gene enhancers and promoters (Wang, Q, Carroll & Brown 2005). Transcription elongation then occurs via RNA-polymerase II to transcribe AR target genes (Massie et al. 2007; Wang, Q et al. 2007). After completion of its transcriptional function, free AR receptors are exported back to the cytoplasm for a new round of protein importation, activation, and gene transcription (Massie et al. 2007; Wang, Q et al. 2007).

Although the aforementioned processes are considered as the canonical AR signalling mechanism, not all cellular responses to androgens fit within this genomic model, and not all require transcription mediated by AR. This is because ligand-transformed AR can associate with molecular substrates in the cytoplasm and cell membrane, triggering release of intracellular calcium and activation of kinases such as MAPK (ERK), protein kinase A (PKA), Akt and protein kinase C (PKC) (Baulieu & Robel 1995; Foradori, Weiser & Handa 2008; Michels, G & Hoppe 2008). This mode of action is referred to as rapid, non-genomic signalling of AR. The non-genomic signalling by AR is distinguished from genomic signalling by speed, with response times being seconds to minutes, as opposed to genomic signalling which requires at least 30 min (Michels, G & Hoppe 2008), indicating a lack of transcription and

translation from androgen-responsive genes (Boonyaratanakornkit & Edwards 2007; Michels, G & Hoppe 2008).

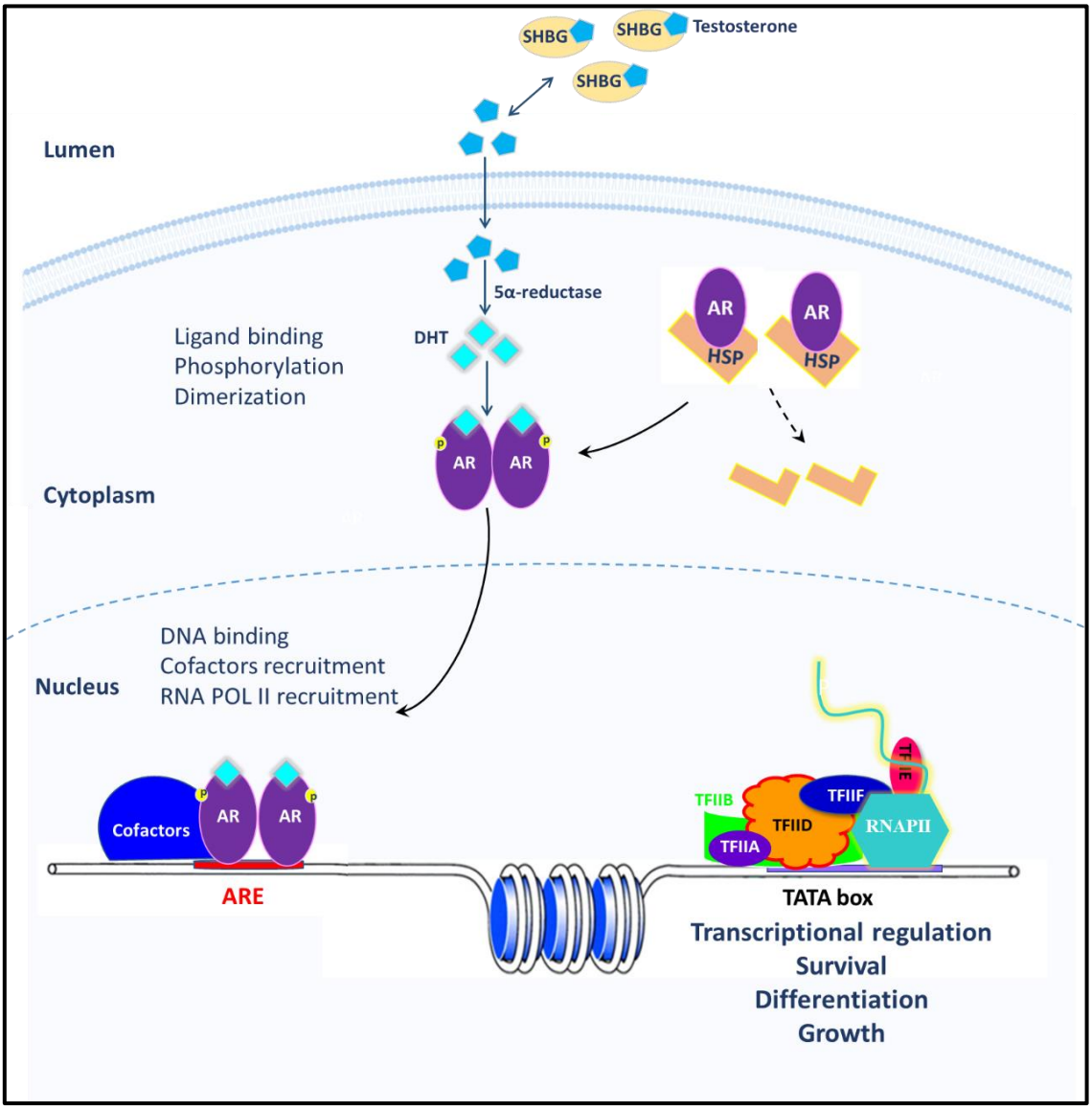



**Figure 1.6**: Simplified model of the AR canonical pathway in androgen target tissue. Testosterone (T) enters the cell and is converted by 5α-reductase into dihydrotestosterone (DHT). Upon steroid binding, AR undergoes a conformational change and releases heat-shock proteins (HSP). The AR translocates to the nucleus where DNA binding, and the recruitment of coactivators occur. Target genes are transcribed (mRNA) and translated into proteins (Bennett et al. 2010).

### 1.4.3 Androgen signalling in normal breast epithelial cells.

In the luminal epithelial cells of primate mammary glands, AR protein expression is high (59-75% AR positive) and remains stable during the menstrual cycle and early stages of pregnancy, only showing a significant decrease during the late stage of pregnancy and lactation (Cheng, G et al. 2005). In contrast, the expression level of ERα is much lower (0-12.5% of epithelial cells are positive) and changes dramatically during different stages of menstruation and pregnancy (Cheng, G et al. 2005). Similar patterns of AR and ERα expression are found within the normal breast epithelia of women without breast cancer (Hickey, T et al. 2012). Since breast epithelia have high expression of AR, the role of androgen signalling in regulating proliferation and development of normal mammary glands has been investigated in several studies (Forsbach et al. 2000; Hofling et al. 2007; Peters et al. 2011). Compelling evidence suggests that androgens normally inhibit mammary epithelial proliferation and breast growth (Forsbach et al. 2000; Hofling et al. 2007; Peters et al. 2011; Zhou, J et al. 2000). Administration of testosterone to ovariectomised rhesus monkeys was able to inhibit E2-induced mammary cell proliferation (Zhou, J et al. 2000). Inhibition of androgen signalling in normal cycling monkeys by treatment with the AR antagonist flutamide resulted in more than a two-fold increase in the proliferation of mammary epithelial cells (Dimitrakakis et al. 2003), supporting a growth inhibitory role for this steroid receptor.

Similar results were obtained in mice by Peters *et al*. (2011), whereby treatment of females at mid-puberty (5 weeks) with DHT or flutamide altered mammary gland development/morphology, with stimulation of the AR pathway resulting in reduced ductal extension, a known estrogen-dependent process (Peters et al. 2011). A potential explanation for the inhibitory effect of androgens in intact animals is the feedback effect of androgens on the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH), which in turn decreases E2 levels (Marques et al. 2018). Furthermore, the influence of circulating testosterone on the proliferation of breast epithelial cells is in part dependent upon the relative expression and activity of aromatase and 5α-reductase (Li, X 2010), the enzymes responsible for conversion of testosterone to E2 or DHT, respectively. In studies of transgenic mice with overexpression of the aromatase gene (*AROM+*), males undergo abnormal breast development due to excess exposure to E2, highlighting the importance of the estrogen/androgen balance in regulating proliferation in mammary epithelia.

Observations from other studies indicate similar effects of androgens on breast development in humans (Forsbach et al. 2000; Hofling et al. 2007; Quigley et al. 1995). Androgen excess due to an adrenal tumour or hyperplasia suppresses normal breast development in girls, despite apparently adequate estrogen levels (Forsbach et al. 2000). In male patients with hypogonadism and androgen deficiency, gynecomastia (enlargement of breast tissue in males) is often observed (Quigley et al. 1995). Moreover, the effect of testosterone in combination with estrogen and a synthetic progestin (norethindrone) as part of MHT in postmenopausal women was studied by Hofling et al. (2007). While

the estrogen and progestin combination MHT enhanced breast epithelial cell proliferation, no significant changes were observed when testosterone was added (Hofling et al. 2007). Taken together, these studies demonstrate that in normal, pre- and post-menopausal breast tissue, AR activity is critical to counter-balance E2-induced cell proliferation, ultimately influencing normal glandular development.

### 1.4.4 Androgens and breast cancer risk

The association of androgen levels with risk of developing breast cancer has been deliberated in several studies (Kaaks, Berrino, et al. 2005; Zeleniuch-Jacquotte, A et al. 2004). In postmenopausal women, most of the prospective epidemiologic studies have shown that circulating androgens are positively associated with increased breast cancer risk (Kaaks, Berrino, et al. 2005; Zeleniuch-Jacquotte, A et al. 2004). Higher levels of testosterone, DHEAS and androstenedione, were associated with increased breast cancer risk (Kaaks, Berrino, et al. 2005; Zeleniuch-Jacquotte, A et al. 2004). A pooled analysis of nine prospective studies, including up to 663 incident cases of breast cancer and 1102 controls, reported a two-fold increase in breast cancer risk between women in the upper versus the lower quartiles of sex steroid serum concentrations (estrogen and testosterone) (Key T 2002). This positive correlation is most likely due to the fact that circulating androgens are substrates for estrogen production. Indeed, Adly and Colleagues (2006) showed that breast cancer risk is linked only to higher serum levels of estrogens, independently of androgen levels (Adly et al. 2006), suggesting that

circulating androgen hormones might only influence breast cancer risk indirectly through conversion to E2 by aromatase activity (Adly et al. 2006).

In premenopausal women, the association between androgens and breast cancer risk is also not so clear, with studies reporting either no association (Helzlsouer et al. 1992; Lee, SH et al. 1999), a positive association (Kaaks, Rinaldi, et al. 2005; Key T 2002; Missmer et al. 2004; Zeleniuch-Jacquotte, A et al. 2004) or negative association (Helzlsouer et al. 1992; Lee, SH et al. 1999). A case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) included 370 cases of premenopausal breast cancer and 726 individually matched controls and showed significantly higher levels of serum testosterone in breast cancer patients compared with controls (Kaaks, Berrino, et al. 2005). The same study reported that serum testosterone concentration was positively associated with breast cancer risk, where women in the highest quartile of serum testosterone had approximately 80% greater breast cancer risk than women in the lowest quartile (Kaaks, Berrino, et al. 2005). The results of this study were supported by two other prospective cohort case studies conducted in America and Colombia (Dorgan et al. 2010; Zeleniuch-Jacquotte, Anne et al. 2012). Other studies on premenopausal women have reported an inverse relationship between levels of androgens and breast cancer risk (Helzlsouer et al. 1992; Lee, SH et al. 1999). A study compared the levels of androgens measured in urine samples from benign and malignant breast tumours with normal samples, and found lower androgen levels in breast cancer patients compared to controls (Lee, SH et al. 1999), indicating a protective role of androgens on the breast. The protective effect of androgens is further supported by clinical studies showing that male breast

cancer (which accounts for <1% of all breast cancers) is associated with deficient androgen production (Hsing et al. 1998; Thomas, DB et al. 1992). In contrast to the above-mentioned studies, Thomas et al (1997) and Page et al (2004) found no association between levels of circulating androgens and the risk of breast cancer (Page et al. 2004; Thomas, HV et al. 1997)

As outlined above, studies to date have not provided consensus on the association between androgens and breast cancer risk. The discrepancies in the reported results are likely to be attributed to limitations in the methodology used to measure androgens and differences in experimental conditions (e.g. time of samples collection, sample size, and type of fluid measured - serum versus urine) (McNamara, KM et al. 2014). The androgen assays (mainly immunoassays) used were developed mainly to measure the higher levels found in men and lack the sensitivity and specificity in low ranges found in women (Harwood & Handelsman 2009; McNamara, KM et al. 2014). Testosterone levels vary hourly in response to diurnal rhythm, diet, stress, and exercise, so a single value may be inadequate to assess true tissue exposure (Bui et al. 2013; Opstad & Metabolism 1992; Rothman et al. 2011). The sample size may also contribute to contradicting results as larger studies have more statistical power to detect smaller differences as significant (McNamara, KM et al. 2014). New studies using a more accurate and sensitive method (such as mass spectrometry) and an appropriate experimental design to measure androgens, accounting for their conversion to estrogen before assessing the association of circulating androgens to breast cancer risk, are needed. Such studies would be informative in developing a rationale for targeting AR in breast cancer.

### 1.4.5 Clarifying the role of androgen signalling in ERα- negative breast cancer

Androgen therapy has been used historically to treat advanced breast cancer (Goldenberg 1964; Kennedy 1958). However, this therapeutic strategy was eventually phased out due to virilizing side effects (e.g. hirsutism, and deepening of the voice) and the advent of drugs that oppose ER signalling (e.g. Tamoxifen) (Lønning 2009). While anti-estrogenic therapies are effective in the majority of breast cancer cases, 30% of breast cancers are negative for ER (previously described in section 1.3.2) and cannot benefit from this treatment strategy (Harvell et al. 2008). The characterization of AR expression in most breast cancers has renewed interest in androgen therapy for this disease, especially since non-virilising selective AR modulators (SARMs) are now clinically available (Chen, F, Rodan & Schmidt 2002). The AR is expressed in approximately 20-50% of ERα and PR negative breast cancers, and its expression is correlated with a lower Nottingham grade and apocrine differentiation (Liu, YX, Zhang & Tang 2018; McNamara, K et al. 2013; Niemeier et al. 2010; Xu, M et al. 2020), but controversy over how best to therapeutically target AR in this disease context hampers clinical advancement of androgen therapy.

In contrast to ERα-positive breast cancer in which AR independently and reproducibly predicts a better survival outcome (Ricciardelli et al, Clin Can Res, 2019), there have been no consistent findings linking AR expression to patient survival in ER-negative breast cancer. Where some studies reported no association between AR expression and patient survival (Adamo et al. 2017; Gonzalez et al. 2008; Hu, Rong et al. 2011; Peters et al. 2009; Sunar et al.

2018; Xu, M et al. 2020), other studies have shown a positive correlation with good prognosis and improved overall survival (Adamo et al. 2017; Agoff et al. 2003; Guiu et al. 2018; Kucukzeybek et al. 2018; Luo et al. 2010; Ricciardelli et al. 2018; Witzel et al. 2013). Moreover, some studies have suggested that AR expression is associated with increased mortality among women with ERα-negative tumours (Choi, JE et al. 2015; Hu, Rong et al. 2011). Park and co-investigators (2011) have shown a trend toward poorer outcomes in AR-positive, ERα-negative breast cancers (Park, S et al. 2011). A recent study by Liu et al (2018) showed that AR expression had no correlation with disease-free and overall survival of patients with TNBC; rather, it predicted a poor survival of patients specifically with stage III TNBC in comparison with those at earlier stages (Liu, YX, Zhang & Tang 2018). Disparate results may be attributed to the vast heterogeneity of the ERα-negative subset of breast cancer (McNamara, KM et al. 2014). ERα-negative breast cancers show a high degree of tissue and molecular heterogeneity (Yersal & Barutca 2014), especially at the AR level, as some groups exhibit higher AR expression (molecular apocrine) than others (basal) (Farmer et al. 2005). Accordingly, the percentage of these different sub-groups in the study population may affect the results. Therefore, studying the association of AR with patient survival necessitates very large cohort numbers to determine the role of AR expression on patient survival in specific ERα-negative breast cancer subtypes.

Like the discrepancy in the above-mentioned clinical studies, pre-clinical studies are also equivocal, indicating that AR signalling may exert proliferative or anti-proliferative effects in ERα-negative breast cancer cell line models (Birrell, S et al. 1995; Doane et al. 2006; Farmer et al. 2005; Hackenberg et al.

1991; Ni et al. 2011; Robinson, JL et al. 2011; Thakkar et al. 2016; Zhu, A et al. 2016). Targeting AR with antagonists in ERα-negative disease is considered a promising therapeutic strategy since preclinical data suggests that AR has oncogenic activity in at least a subset of ERα-negative subtypes (Birrell, S et al. 1995; Naderi & Hughes-Davies 2008; Ni et al. 2011; Robinson, JL et al. 2011). However, the role of AR in ERα-negative disease is still debated. Discrepancies among studies are detailed below and highlight the need for a better understanding of AR in this disease context. To date, clinical trials of AR antagonists for ERα-negative disease have shown little efficacy (Bonnefoi, H et al. 2016; Gucalp et al. 2013; Traina et al. 2018), likely due to the lack of understanding the biological role of AR in this disease. This PhD project aims to determine the molecular mechanisms that underpin divergent AR-mediated growth effects in ERα-negative breast cancers to facilitate development of rational clinical AR target strategies.

### 1.4.6 Androgens induce divergent proliferative responses in ERα-negative/AR-positive breast cancer models.

Androgenic effects have been most widely studied in the molecular apocrine subgroup of ERα-negative breast cancer, which is characterised by high levels of AR and high expression of genes known to be regulated by AR signalling in prostate cancer cells (Doane et al. 2006; Farmer et al. 2005). A sub-set of these genes are also ERα regulated genes in ERα-positive breast cancers (Doane et al. 2006; Robinson, JL et al. 2011), suggesting a plasticity of steroid receptor-mediated regulation.

The most commonly used cell line model of molecular apocrine breast cancer is MDA-MB-453, shown to be growth stimulated by DHT long before identification of this breast cancer subtype (Bentel et al. 1999; Birrell, S et al. 1995). Subsequent studies using this cell line *in vitro* (Cochrane et al. 2014; Doane et al. 2006; Naderi & Hughes-Davies 2008; Ni et al. 2011) and *in vivo* (Cochrane et al. 2014; Huang, R et al. 2017; Ni et al. 2011) support the concept that AR signalling may have an oncogenic role in molecular apocrine breast cancers. Similarly, androgens have been shown to increase the *in vitro* growth of other, less commonly used cell line models of this disease, including HCC-202, SUM190, and SUM185PE (Naderi & Hughes-Davies 2008; Ni et al. 2011). Consistent with these findings, blocking AR action by treatment with AR antagonists (e.g. bicalutamide or enzalutamide) abolished the *in vitro* growth stimulatory effect of natural and synthetic AR ligands in MDA-MB-453 and SUM185PE cells (Cochrane et al. 2014; Doane et al. 2006; Lehmann et al. 2011; Naderi, Chia & Liu 2011) and decreased *in vivo* growth of cell line xenografts (Huang, R et al. 2017; Lehmann et al. 2011; Naderi, Chia & Liu 2011; Speers et al. 2017). In contrast to these findings, a study by Wang et al. (2011) showed that DHT inhibits proliferation of MDA-MB-453 cells, whereas knockdown of AR by siRNA promoted cell growth (Wang, Y et al. 2011). The different proliferative outcomes in this study compared to the others could be attributed to their differences in experimental conditions that might impact on AR activity, such as cell culture conditions (particularly medium composition and fetal bovine serum type), nature of the AR ligand used, and the time and dosing of treatment schedules. However, due to insufficient methodology reporting by Wang et al (2011), determining the reasons for this discrepancy is

problematic. Despite this, the anti-proliferative effect of AR signalling in the MDA-MB-453 cells is supported by other preclinical data demonstrating that the androgenic progestin medroxyprogesterone acetate (MPA) has high affinity for AR and inhibits cell proliferation in an AR dependent manner (Bentel et al. 1999). MPA is a synthetic progestin once frequently used as a second line hormonal therapy for the treatment of tamoxifen-resistant metastatic breast cancer. Although MPA was thought to exert its therapeutic effect by acting exclusively through the PR, clinical response to MPA was correlated to AR expression in the tumours and non-responders were shown to have inactivating AR mutations (Birrell, SN et al. 1995). Hence, MPA has clinical relevance as an AR agonist in breast cancer cells. (Birrell, SN et al. 1995). Collectively, these findings suggest that AR-mediated proliferative effects in the MDA-MB-453 model of molecular apocrine breast cancer are context-dependent. There are also inconsistencies in the literature concerning the proliferative effect of AR signalling in other ERα-negative breast cancer cell lines. Proliferation of MFM-223 and CAL-148 cell lines, which are both genetically similar to MDA-MB-453 as they transcriptionally cluster with this cell line due to high expression of AR and known AR target genes, is inhibited by androgens i*n vitro* (Hackenberg et al. 1993; Hackenberg et al. 1991; Thakkar et al. 2016). Similarly, a recent study showed that activation of AR signalling via DHT or the androgenic progestin D-Norgestrel inhibits growth of MFM-223 xenograft tumours (Thorek et al. 2019).

The divergent proliferative effects of androgens on different cell line models of ERα-negative/AR-positive breast cancer likely reflects the inherent heterogeneity of the disease and suggests that not all will benefit from therapies

that inhibit AR activity. This is further supported by the variable results of clinical trials involving androgen deprivation therapy (ADT) in women with molecular apocrine breast cancers (Bonnefoi, H et al. 2016; Gucalp et al. 2013; Kumar et al. 2017; Traina et al. 2018). These clinical studies have given inconclusive results, where about 25% of patients showed clinical benefit, including some prolonged responses, but most did not (Venema et al. 2019). Moreover, AR expression did not predict response in these trials. This lack of efficacy may be explained partly by problems with patient selection, but a more fundamental problem is a lack of understanding around the mechanistic basis of AR signalling in different ERα-negative breast cancer contexts. Based on all the described data, we can say that AR expression alone is not sufficient to identify patients suitable for AR-targeting therapies, and characterisation of additional markers of favourable response to these therapies is required.

The different AR-mediated growth effects observed in the molecular apocrine cell lines (as described above) might be attributed to disparities in the expression profile of AR co-regulatory proteins. Considerable evidence from prostate cancer cell lines and clinical studies indicates that altered expression of AR co-regulators that either promote or repress target gene expression can modulate cellular responses to androgens or non-androgenic steroids (Chmelar et al. 2007; Lonergan & Tindall 2011; Wasmuth et al. 2020). Although breast and prostate cancer arise in separate organs, they are both endocrine driven cancers and have many parallels in their biological characteristics (Risbridger et al. 2010). Indeed, AR action in prostate cancer is similar to that observed in some models of ER-/AR+ breast cancer, and it is therefore reasonable to argue that AR co-regulatory proteins could have a similarly important role in

determining AR action in ER-/AR+ breast cancers. The MDA-MB-453, HCC202, SUM190 cell lines could express different AR co-regulators, or differentially interact with the same AR co-regulators, compared to other molecular apocrine cell lines, potentially mediating oncogenic activity of AR in those cells. Altered growth responses to androgens might also be influenced by the mutational status of AR. The *AR* gene in MDA-MB-453 cells contains a G-T transversion in exon 7. This results in an AR protein variant consisting of a glutamine to histidine substitution at amino acid 865 (Q865H) of the ligand binding domain (Moore et al. 2012a). Compared to wild type AR, mutant AR decreases sensitivity to DHT and alters the growth response of MDA-MB-453 cells to androgens (Moore et al. 2012a). Molecular modelling of this mutant-AR protein shows conformational changes likely to influence the structure of the ligand binding pocket, which affects AR function (Moore et al. 2012a). Therefore, mutant variants of the AR could alter growth responses to androgen by recruiting a discrete subset of mutant-AR coregulators that mediate AR oncogenic effects. To date, this line of investigation has not been pursued.

**Table1.1**: Effect of AR targeting on proliferation of ERα-negative breast cancer cell lines *in vitro.*

| Citation | Cell line | Medium | Type of FBS | AR agonist | Dose | Length of treatment (Days) | Effect on proliferation | AR antagonist reverse AR agonist effect |
|---|---|---|---|---|---|---|---|---|
| (Birrell, S et al. 1995) | MDA-MB-453 | DMEM/F12 | DCC-FBS | DHT or Mibolerone (synthetic androgen) | 0.1-10 nM | 6-18 | ↑* | ✓ |
| (Bentel et al. 1999) | | DMEM/F12 | DCC-FBS | MPA | 100nM | 12 | ↓* | ✓ |
| (Doane et al. 2006) | | MEM | DCC-FBS | R-1881 (synthetic androgen) | 0.1–10 nM | 7 | ↑* | ✓ |
| (Naderi & Hughes-Davies 2008) | | DMEM | DCC-FBS | Testosterone | 1µM | 4 | ↑* | ✓ |
| (Ni et al. 2011) | | DMEM | DCC-FBS | DHT | 10nM | 5 | ↑* | ✓ |
| (Wang, Y et al. 2011) | | ND | ND | DHT | 10nM | 5 | ↓* | ND |
| (Cochrane et al. 2014) | | Leibovitz's L-15 | FBS | DHT | 10nM | 10 | ↑* | ✓ |
| (Park, IH et al. 2019) | | Leibovitz's L-15 | DCC-FBS | DHT | 10 nM | 3 | NS* | ND |
| (Ni et al. 2011) | HCC-202 | RPMI | DCC-FBS | DHT | 10nM | 7 | ↑* | ND |
| (Ni et al. 2011) | SUM185PE | Hams-F12 | DCC-FBS | DHT | 10nM | 5 | ↑* | ✓ |
| (Naderi & Hughes-Davies 2008) | SUM-190 | DMEM | DCC-FBS | Testosterone | 1µM | 4 | ↑* | ✓ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| (Hackenberg et al. 1993) | MFM-223 | MEM | DCC-FBS | DHT | 0.1 &10 nM | 7 | ↓* | ✓ |
| (Thakkar et al. 2016) | | MEM | DCC-FBS | DHT/ Enobosarm | 10nM / 100 nM | 8-10 | ↓* | ND |
| Thakkar et al. 2016) | CAL-148 | MEM | DCC-FBS | DHT/ Enobosarm | 10nM / 100 nM | 8-10 | ↓* | ND |
| **AR antagonist effect** | | | | | | | | |
| **Citation** | **Cell line** | **Medium** | **Type of FBS** | **AR antagonist** | **Dose** | **Length of treatment (Days)** | **Effect on proliferation** | **AR antagonist reverses AR agonist effect** |
| (Naderi, Chia & Liu 2011) | MDA-MB-453 | Leibovitz's L-15 | FBS | Hydroxyflutamide | 5-30µM | 2 | ↓* | ND |
| (Speers et al. 2017) | | RPMI | FBS | Enzalutamide | 0.1 &1 µM | 6 | ↓* | |
| (Naderi, Chia & Liu 2011) | HCC-202 | RPMI | ND | Hydroxyflutamide | 20-100µM | 2 | ↓* | |
| (Speers et al. 2017) | SUM-185PE | ND | ND | Enzalutamide | 0.1 &1 µM | 6 | ↓* | |
| (Speers et al. 2017) | ACC-422 | MEM | FBS | Enzalutamide | 0.1 &1 µM | 6 | ↓* | |
| (Speers et al. 2017) | ACC-460 | ND | ND | Enzalutamide | 0.1 &1 µM | 6 | ↓* | |

(DCC-FBS) Dextran coated charcoal fetal bovine serum

(NS) no significant change in baseline proliferation

(ND) Not determined

Growth assay

* Cytotoxicity assays (MTT & MTS)

* Cell counting

* Others (Crystal violet, Methylene blue binding assay, & Clonogenic assay

**Table 1.2**: Effect of AR targeting on proliferation of ERα -negative breast cancer cell line xenografts.

| Citation | Xenograft | Treatment | Dose | Length of treatment | Growth response | AR antagonist reverses AR agonist effect |
|---|---|---|---|---|---|---|
| (Ni et al. 2011) | MDA-MB-453 | DHT | 60-day release pellet | ~45 day | ↑ | ✓ |
| (Cochrane et al. 2014) | | DHT | 1.5 mg 60-day release pellet | ~35 day | ↑ | ✓ |
| (Huang, R et al. 2017) | | DHT | 60-day release pellet | 49 day | ↑ | ✓ |
| (Thorek et al. 2019) | MFM-223 | DHT | 12.5 mg 60-day release pellet | 65 days | ↓ | ND |
| (Naderi, Chia & Liu 2011) | MDA-MB-453 | Hydroxyflutamide | 25 mg/kg/ 60-day release pellet | 30 days | NS | ND |
| (Speers et al. 2017) | | Enzalutamide | 10 mg/kg/day | 45 days | ↓ | |
| (Huang, R et al. 2017) | | Bicalutamide | 10 mg/kg/day | 49 day | ↓ | |
| (Lehmann et al. 2011) | SUM185PE | Bicalutamide | 100 mg/kg/day | 21 day | ↓ | |
| (Lehmann et al. 2011) | CAL-148 | Bicalutamide | 100 mg/kg/day | 21 day | ↓ | |

(NS) no significant change in baseline proliferation

(ND) Not determined

### 1.4.7 AR co-regulatory proteins

Over 300 proteins have been identified as AR-interacting co-regulators that can influence transcription of AR target genes either positively (co-activators) or negatively (co-repressors) (Figure 1.7) (Chmelar et al. 2007; DePriest et al. 2016; Heemers & Tindall 2007; Heinlein & Chang 2002; van de Wijngaart et al. 2012). AR co-regulators have been extensively studied in the context of prostate cancer. Based on their functional characteristics, co-regulators are broadly categorised into two main types. Type I co-regulators function primarily with AR at the promoters of target genes to facilitate DNA occupancy, chromatin remodelling, histone modifications, as well as recruitment of the basal transcriptional machinery (Aarnisalo, Palvimo & Jänne 1998; Lemon, Tjian & development 2000; Marshall et al. 2003). Examples of this type of co-regulator are p160 coactivators, cAMP-response element-binding protein (CREB)-binding protein (CBP)/p300, and the SWI/SNF chromatin remodelling complex (Aarnisalo, Palvimo & Jänne 1998; Bevan et al. 1999; Marshall et al. 2003). The p160 family of co-regulators consists of 3 160-kDa proteins, namely steroid receptor coactivator 1 (SRC1); nuclear receptor coactivator 2 (NCoA-2) and its mouse homolog glucocorticoid receptor interacting protein 1 (GRIP1); and steroid receptor coactivator-3 (SRC-3) (Montell 2003; Zhou, H-J et al. 2005). The p160 coactivators influence AR transactivation capacity directly via their intrinsic histone acetyltransferase (HAT) activity, by which they are able to add an acetyl group to the lysine residues of histones, leading to a more open chromatin structure that is more accessible to the transcriptional machinery (Aarnisalo, Palvimo & Jänne 1998; Ogryzko et al. 1996; Spencer et al. 1997). P160 coactivators also

function as bridging factors to recruit secondary coactivators that also possess chromatin remodelling and HAT capabilities, such as (CBP)/p300 and pCAF, or protein methyltransferase activity like CARM1 or PRMT1, and basal transcription factors, TFIIB and TBP (Aarnisalo, Palvimo & Jänne 1998; Chakravarti et al. 1996; Stallcup et al. 2003). Ablation of SRC-1 in androgen-dependent LNCaP prostate cancer cells represses activation of AR target genes and reduces AR-dependent cellular proliferation (Agoulnik et al. 2005). NCoA-2 amplification is commonly observed in prostate cancer and may sensitize AR to be activated by low levels of androgen (Taylor et al. 2010; Zhou, XE et al. 2010). SRC-3 is a known AR co-activator in prostate cancer (Zhou, XE et al. 2010), and increased SRC-3 expression is associated with a more aggressive phenotype and worse prognosis (Gnanapragasam et al. 2001; Zhou, H-J et al. 2005). The SWI/SNF chromatin remodelling complex regulates activity of transcription factors generally by re-organising chromatin structure through either facilitating nucleosome condensation (which induces transcriptional repression) or nucleosome dispersion (assisting in transcriptional activation) (Montecino et al. 2007). A study by Marshall *et al* (2003) reported that complete ligand-dependent activation of two AR target genes, *KLK3* and probasin, requires SWI/SNF function (Marshall et al. 2003).

Type II co-regulators include molecular chaperones that coordinate AR maturation and movement, as well as coordinators of transcription (Chmelar et al. 2007). Examples of type II co-regulators are androgen receptor associated protein 70 (ARA70), ARA55, melanoma antigen gene protein (MAGE-11), supervillain, gelsolin (GSN), and the TRAP-mediator complex (Chmelar et al. 2007). ARA70 has been shown to enhance AR transcriptional activity while

only slightly enhancing the transcriptional activity of other steroid receptors (i.e., glucocorticoid, progesterone and estrogen receptors) (Yeh, S & Chang 1996). ARA70 enhanced AR transcriptional activity by ten-fold in the presence of $10^{-10}$ M DHT or $10^{-9}$ M testosterone in prostate cancer cells (Yeh, S & Chang 1996). ARA55 binds to the AR-LBD in a hormone-dependent manner through its C-terminal LIM domains, and results in increased AR activity (Fujimoto et al. 1999). ARA55 mRNA expression is lower in normal prostate tissue compared with adjacent malignant tissue, and in hormone refractory prostate cancer compared with untreated tumours (Mestayer et al. 2003). High ARA55 expression has been associated with shorter recurrence free survival and overall survival in hormone refractory prostate cancer patients (Miyoshi et al. 2003). MAGE-11 binds the AR N-terminal FxxLF motif that mediates the androgen-dependent N/C interaction with AF2 in the AR LBD (Bai et al. 2005). MAGE-11 forms a stable complex with the ligand-free AR that results in increased AR stability, and in the presence of the DHT it competes with the N/C interaction, thereby increasing the exposure of the LBD coactivator groove to the recruitment and activation by other coactivators (Bai et al. 2005). AR activity is also enhanced by cytoskeletal proteins, such as supervillin and GSN, which regulate AR trafficking into the nucleus (Nishimura et al. 2003; Ting et al. 2002). Overexpression of GSN enhances AR transcriptional activity in the presence of either androgen or the AR antagonist flutamide (Nishimura et al. 2003). The TRAP-mediator complex consists of over 12 polypeptides and is recruited to AR in a ligand-dependent manner via a 220kDa component, referred to as PBP, that contains two alternatively utilised LxxLL NR interaction motifs (Wang, Q et al. 2002; Zhu, Y et al. 1997). The TRAP-

mediator complex enhances AR transcriptional activity by recruiting the RNA polymerase II holoenzyme and core transcription factors to the promoter (Wang, Q et al. 2002; Zhu, Y et al. 1997). A recent study by our group identified a new AR coregulator, Grainyhead Like Transcription Factor 2 (GRHL2) (Paltoglou et al. 2017). *GRHL2* mRNA level is amplified in primary and metastatic tissues of prostate cancer and elevated in malignant compared to non-malignant prostate tissues (Paltoglou et al. 2017). The same study showed that GRHL2 is important to maintain AR expression in many prostate cancer models, and to enhance AR transcription activity, and is co-located with AR at specific sites on chromatin to regulate genes relevant to prostate cancer progression (Paltoglou et al. 2017).

Besides co-activators, AR can also recruit co-repressors. Corepressors can inhibit transtractional activity of AR in different ways, such as inhibition of AR DNA binding or nuclear translocation, recruitment of histone deacetylases, or interrupting the interaction between AR and its co-activators (Chmelar et al. 2007). Well characterised AR corepressors are the nuclear receptor corepressor (NCoR1) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT/NCoR2) (Wang, L, Hsu & Chang 2005). NCOR1 and SMRT interact directly with AR in the absence or presence of agonist/antagonist (Wang, L, Hsu & Chang 2005). In addition to the recruitment of histone deacetylases (HDACs), NCOR1 and SMRT may repress AR transactivation through other mechanisms, such as inhibition of the AR N/C interaction or competition with the key coactivators, including the p160 family, for the same AR binding surfaces (Alland et al. 1997; Berrevoets et al. 2004; Cheng, S et al. 2002; Choi, KC & Yoon 2006; Liao et al. 2003). Other AR corepressors, such as cyclin D1,

LCoR and Glycogen Synthase Kinase 3 β (GSK3β) have also been identified (Burd, Morey & Knudsen 2006). The C-terminal domain of cyclin D1 binds to the hinge region of AR in a hormone-dependent manner, and results in decreased AR activity (Coqueret 2002; Knudsen, Cavenee & Arden 1999). Cyclin D1-mediated inhibition of AR may result from its capacity to inhibit the association between coactivator (CBP)/p300 and the AR, as both cyclin D1 and the AR can bind to the same domains of (CBP)/p300 (Knudsen, Cavenee & Arden 1999; Reutens et al. 2001). LCoR (Ligand Dependent Nuclear Receptor Corepressor) interacts with AR and is recruited to chromatin in an androgen-induced manner (Asim et al. 2011). Overexpression of LCoR in prostate cancer cell lines repressed AR activation by the natural agonist DHT and the anti-androgen cyproterone acetate (CPA) (Asim et al. 2011). The protein expression level of LCOR is found to be lower in human prostate carcinoma cell lines such as LNCaP, C2-4B, 22RV1, and PC3 compared with the RWPE1 cell line, a non-tumorigenic, normal prostate epithelial model (Asim et al. 2011). GSK3β is a serine/threonine protein kinase that phosphorylates a broad range of substrates, including transcription factors such as C-MYC and c-Jun (Rogatsky, Waase & Garabedian 1998; Sears et al. 2000). It has been shown that GSK3β can phosphorylate the AR N-terminus and thereby suppress AR transactivation (Wang, L et al. 2004).

Collectively, these studies show that AR relies on functional and structural interactions with multiple co-regulatory proteins to ensure transcription of its target genes and highlight the fact that most of these studies were conducted in prostate cancer. Very little is known about co-regulators of AR in breast cancer

in general and in ERα-negative /AR positive breast cancer in particular. Addressing this knowledge gap formed part of this PhD thesis.

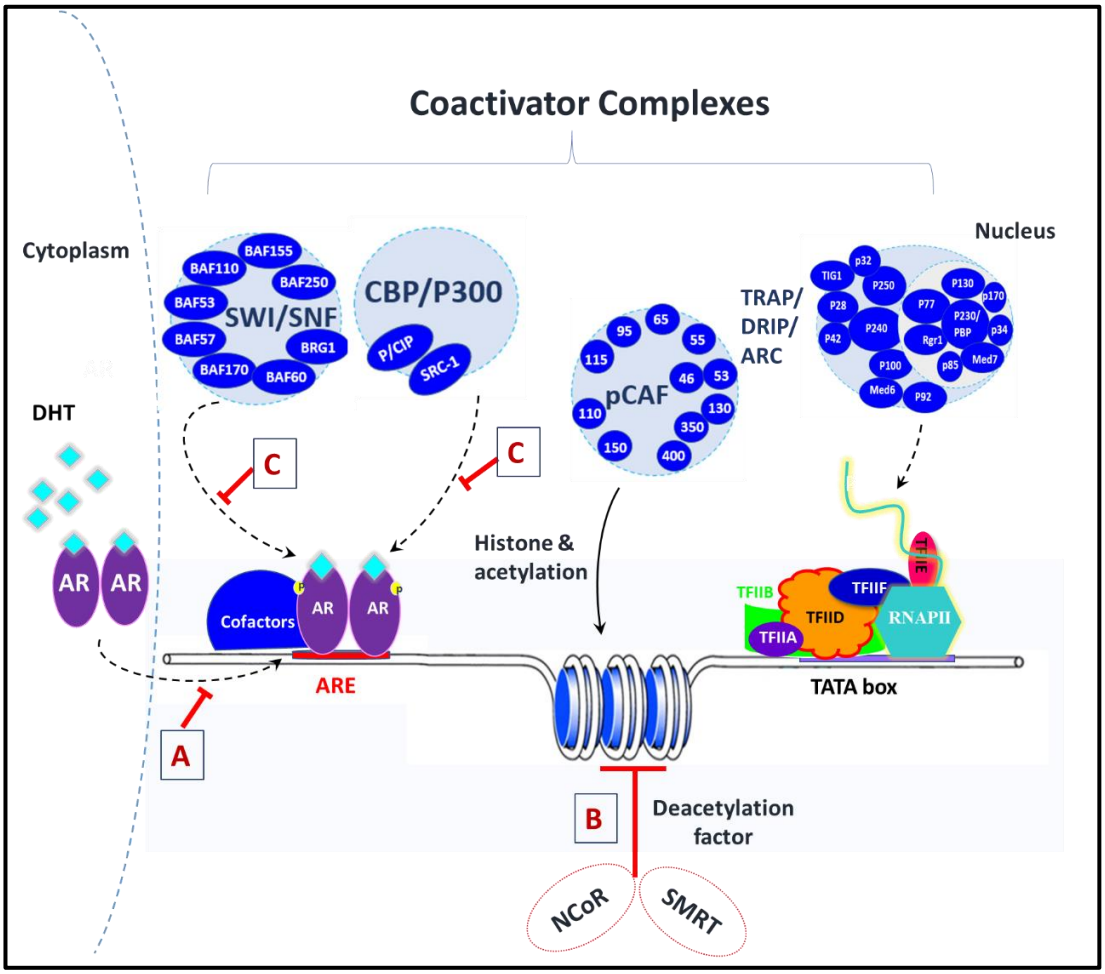



**Figure 1.7:** Co-regulator complexes in AR-mediated transcription. Coactivator complexes include factors involved in chromatin remodeling (SWI/SNF), histone modification (CBP/SRC-1/p/CAF) and recruitment of core transcription factors (TRAP/DRIP/ARC). Corepressor complexes include factors that (A) inhibit AR DNA binding or nuclear translocation, (B) recruit histone deacetylases, and (C) interrupt the interaction between AR and its coactivators. Figure adapted from (Glass & Rosenfeld 2000).

### 1.4.8 Molecular mechanisms implicated as mediators of divergent AR activity in ERα- /AR+ breast cancer.

In addition to AR co-regulators, pioneer factors that collaborate with AR are also important for androgen responsive gene expression. FOXA1, a member of the forkhead family of transcription factors, is considered a 'pioneer factor' because it is able to bind to regions on the DNA that have compacted chromatin, opening it up for other transcription factors to access DNA (Zaret & Carroll 2011). For example, FOXA1 is required for ERα to bind chromatin in ERα-positive breast cancers (Hurtado et al. 2011) and is a major regulator of AR chromatin binding in prostate cancer (Mirosevich et al. 2006; Robinson & Carroll 2012) as well as the ERα-negative MDA-MB-453 cell line model of molecular apocrine breast cancer (Ni et al. 2011; Robinson, JL et al. 2011). AR activation in MDA-MB-453 cells stimulates a FOXA1-dependent AR transcriptional program that is similar to transcriptional programs associated with ERα agonist activity in ERα positive breast cancer cell lines (Robinson, JL et al. 2011). Loss of FOXA1 in MDA-MB-453 cells *in vitro* decreased colony formation and blocked androgenic induction of the oncogenic transcription factor MYC (Robinson, JL et al. 2011). In the absence of AR activation in ERα-negative breast cancer cell lines, transcription factor 7 like 2 (TCF7L2) physically interacts with FOXA1 on DNA to mediate transcriptional repression of specific AR target genes, including *MYC* (Ni et al. 2013). Upon DHT stimulation, AR replaces TCF7L2 in the FOXA1 complex, leading to transcriptional upregulation of *MYC* (Ni et al. 2013).

AR signalling can also crosstalk with other molecules that have been suggested as potential biological therapeutic targets in breast cancer (Chia et al. 2011;

Naderi, Chia & Liu 2011; Ni et al. 2011). Several studies have shown crosstalk between AR and HER2 signaling in molecular apocrine breast cancer cells (Chia et al. 2011; Ni et al. 2011) and *HER2* amplification is commonly reported in molecular apocrine breast cancers (Farmer et al. 2005; Sanga et al. 2009). In molecular apocrine tumours without *HER2* amplification, activating mutations are found in *HER2* in 9% of cases, suggesting that activation of the HER2 pathway may be a universal feature of this subtype (Jiang, Y-Z et al. 2019). In molecular apocrine disease, AR activation induces extracellular signal-regulated kinase (ERK) phosphorylation and kinase activation through a HER2-dependent pathway (Chia et al. 2011). This study by Naderi and colleagues showed that the AR-mediated induction of ERK requires HER2, and AR activity, in turn, regulates HER2 expression as an AR target gene (Chia et al. 2011). A concurrent study by Ni and colleagues added to this mechanistic insight by demonstrating androgen-dependent stimulation of wingless-type (Wnt)/β-catenin and HER2 oncogenic signalling pathways by transcriptional up-regulation of the canonical Wnt ligand WNT7B and HER3 (Ni et al. 2011). Wnt signalling is required for normal development of the mammary gland (Turashvili et al. 2006), and high expression of WNT7B has been reported in approximately 10% of breast cancer cases (Huguet et al. 1994). The canonical Wnt pathway involves the binding of Wnt proteins to the cell surface and inactivation of glycogen synthase kinase 3β (GSK-3β), which phosphorylates the cell adhesion molecule β-catenin (Lange et al. 2007; Wang, Zhishan et al. 2005). Hypo-phosphorylated β-catenin translocates to the nucleus, where it binds to transcription factors of the LEF/TCF family and activates downstream target genes (Wang, Zhishan et al. 2005). In the

molecular apocrine cell lines MDA-MB-453 and SUM185PE, ligand-dependent activation of AR induces WNT7B expression, leading to activated nuclear translocation of β-catenin (Ni et al. 2011). This in turn leads to the direct interaction of β-catenin with AR, which induces expression of *HER3*, an activating binding partner of HER2 (Ni et al. 2011). Inhibition of AR, Wnt/β-catenin, or HER2 signalling pathways attenuates growth of MDA-MB-453 and SUM185PE cells (Ni et al. 2011). These findings were further supported by *in vivo* results showing that treatment with the AR antagonist bicalutamide inhibited DHT-stimulated growth of MDA-MB-453 xenografts, diminished activation of HER3, and induced apoptosis (Ni et al. 2011).

The significance of HER3 in HER2 positive breast cancer is well known for its ability to form heterodimers with HER2 to upregulate the PI3K/AKT pathway (Ni et al. 2013). This is interesting since in MDA-MB-453 cells, the PI3K/AKT pathway enhances transcriptional activity of oncogenic MYC via phosphorylation and degradation of MAD1, disrupting the MAD1-MAX complex and increasing levels of MYC/MAX heterodimers (Ni et al. 2013). In contrast to these findings, Wang et al. (2011) found that in MDA-MB-453 cells, DHT up-regulated expression of the tumour suppressor gene PTEN, via AR binding to an androgen-responsive element in the PTEN upstream promoter (Wang, Y et al. 2011). While induction of this critical tumour suppressor gene through AR activation is consistent with the anti-proliferative effect observed in this study, this finding has not been replicated in the literature.

The precise mechanisms by which androgenic stimulation may inhibit proliferation of some molecular apocrine cell lines but promote the proliferation of others are not clearly understood. The studies described above support the concept that AR interacting proteins are critically important determinants of AR action in ERα-negative breast cancer. With the advent of new technology and development of contemporary models of breast cancer, which I will describe in chapter three, this PhD project provides the perfect opportunity to better define how AR acts in ERα-negative/AR positive breast cancer. Understanding the intricacies of AR signalling will better inform AR targeting strategies for treatment of ERα-negative breast cancer and could lead to discovery of novel biomarkers of therapeutic response.

## 1.5 Targeting cyclin dependent kinase 9 (CDK9) for treatment of ERα-negative breast cancer.

### 1.5.1 Overview of CDK proteins

At most, 50% of ERα-negative breast tumours express the AR, and of these, not all are expected to respond to AR-targeted therapies (Narayanan & Dalton 2016). Additional therapeutic targets are essential to improve outcomes for ERα-negative breast cancer patients, independent of their AR status. Targeting cyclin-dependent kinase 9 (CDK9) is emerging as a potential treatment strategy for ERα-negative breast cancer patients (Sonawane et al. 2016). As a transcriptional regulator, CDK9 can enhance growth of multiple cancers, including ERα-negative breast cancer, by promoting sustained transcription of normally short-lived oncogenic and anti-apoptotic gene transcripts (i.e., *C-MYC, BCL2, MCL1, and XIAP*), leading to abnormally high expression of their

associated proteins (Franco et al. 2018; McLaughlin et al. 2017; Morales & Giordano 2016; Polier et al. 2011; Rajput et al. 2016). CDK9 is a member of the broad CDK family, which consists of conserved serine (Ser) /threonine (Thr) protein kinases (Sonawane et al. 2016). These protein kinases mediate transfer of a phosphoryl group (–PO3) from adenosine triphosphate (ATP) to hydroxyl groups in the side chains of Ser or Thr of the substrate (Sonawane et al. 2016). CDK's activate their target substrates upon binding to a regulatory cyclin subunit bound to that substrate (Shapiro 2006). Cyclins are a group of proteins generally characterised by having an oscillating (cyclic) pattern of expression and a cyclin box motif (a domain of approximately 100 amino acid residues) that forms a stack of five α-helices (Pines 1995).

CDK/Cyclin complexes were first identified in starfish, xenopus and yeast species and cloned in the 1970s–1980s as gene products involved in regulation of the cell cycle (Hartwell et al. 1974; Labbe et al. 1988; Labbe et al. 1989; Lee, MG & Nurse 1987; Lohka, Hayes & Maller 1988). These pioneering studies and other follow-up studies showed that CDK/Cyclin complexes represent *bona fide* regulators of cell growth and division through phosphorylation of substrates involved in DNA replication, chromatin condensation, assembly of the mitotic spindle and disassembly of the nuclear envelope (Elledge & Spottswood 1991; Hartwell et al. 1974; Labbe et al. 1988; Labbe et al. 1989; Lee, MG & Nurse 1987; Lohka, Hayes & Maller 1988; Matsushime et al. 1992; Meyerson et al. 1992; Nurse 1990). To date, twenty different CDKs have been discovered in mammalian cells and about the same number of cyclins (Table 1.3) (Malumbres & Barbacid 2005). Notably, not all of them are regulators of cell cycle progression (Lolli 2010). Biological

research has revealed the existence of a transcriptional class of CDKs (CDK 7, 9, 10, 12, and 19), which have a pivotal role in controlling transcription, since they determine the initiation and elongation of mRNA transcripts by phosphorylating the C-terminal domain (CTD) of RNA polymerase II (RNAPII) (Cao, L et al. 2014; Elmlund et al. 2006; Malumbres & Barbacid 2005; Nigg 1996; Tassan et al. 1995; Wang, S & Fischer 2008). A more detailed review of *the bona fide* cell cycle and transcriptional CDKs is presented in Sections 1.5.3 & 1.5.4.

**Table 1.3:** Cyclin dependent kinases with corresponding cyclins and their main function. Adapted from (García-Reyes et al. 2018).

| Name | Cyclin | Function | Name | Cyclin | Function |
|---|---|---|---|---|---|
| **CDK1** | Cyclin A<br>Cyclin B<br>Cyclin D<br>Cyclin E | Cell cycle (G2–M) | **CDK11** | Cyclin L | Splicing |
| **CDK2** | Cyclin A<br>Cyclin B<br>Cyclin D<br>Cyclin E | Cell cycle (G1–S)<br>Cell cycle (S–G2) | **CDK12** | Cyclin K<br>Cyclin L | Transcription regulation<br>Splicing |
| **CDK3** | Cyclin C<br>Cyclin E | Cell cycle (G1–S) | **CDK13** | Cyclin K<br>Cyclin L | Splicing |
| **CDK4** | Cyclin D | Cell cycle (G1–S) | **CDK14** | Cyclin D<br>Cyclin Y | Wnt signaling |
| **CDK5** | Cyclin I | Senescence | **CDK15** | Unknown | Unknown |
| **CDK6** | Cyclin D | Cell cycle (G1–S) | **CDK16** | Cyclin Y | Vesicle trafficking |
| **CDK7** | Cyclin H | Transcription regulation | **CDK17** | Unknown | Unknown |
| **CDK8** | Cyclin C | Wnt pathway modulation | **CDK18** | Cyclin A<br>Cyclin E | Genome integrity |
| **CDK9** | Cyclin K<br>Cyclin T1 | Transcription regulation | **CDK19** | Cyclin C | Transcription regulation |
| **CDK10** | Cyclin M | Transcription regulation | **CDK20** | Unknown | Unknown |

### 1.5.2 Structure and regulation of CDKs

The structure of CDKs is conserved throughout this family of kinases (Echalier et al. 2010) and exhibits the classical bi-lobal structure, with an amino-terminal lobe (N-lobe), carboxy-terminal lobe (C-lobe) with the ATP-binding site (active or catalytic site) sandwiched in-between (Endicott, Noble & Johnson 2012; Wood & Endicott 2018). The N-lobe is composed mainly of β-sheets, containing five anti-parallel β-strands, and one conserved α-helix (also called C-helix) (Endicott, Noble & Johnson 2012; Wood & Endicott 2018). The C-helix contains the cyclin consensus sequence, which is important for cyclin binding (Echalier et al. 2010; Lim & Kaldis 2013). The C-lobe is rich in α-helices and contains the activation segment (also referred to as the T-loop) (Lolli 2010). Full activation of CDKs requires the phosphorylation of highly conserved threonine residues in the T-loop region, mainly via CDK-activating kinase (CAK) (Endicott, Noble & Johnson 2012). Close to the activation segment is a functionally opposite segment, the inhibitory loop, named the glycine-rich loop (G-loop) because of its primary sequence that includes three highly conserved glycine residues (Gu, Rosenblatt & Morgan 1992). The G-loop includes two possible target sites for drug inhibition, Thr14 and Y15 (Where Y is any amino acid; examples of Y: Tyrosine in CDK2, alanine in CDK4, and phenylalanine in CDK9) (Gu, Rosenblatt & Morgan 1992). The two terminal domains are connected through a single peptide strand which acts as a hinge linker to ensure that the two lobes can rotate with respect to each other without disruption of the secondary structure (Endicott, Noble & Johnson 2012).

Activity of CDKs is tightly controlled by cyclin binding, phosphorylation, and binding of proteins that directly inhibit CDK action (Lim & Kaldis 2013; Pavletich 1999). CDK protein levels are constant throughout the cell cycle, whereas levels of cyclins rise and fall depending upon the stage of the cell cycle, thus resulting in the periodical activation of CDKs (Lim & Kaldis 2013). Without cyclin, the T-loop blocks the entrance of the catalytic cleft (which contains the ATP and substrate binding sites) and prevents kinase activity (Endicott, Noble & Johnson 2012). Furthermore, within the inactive CDK, several amino acids at the active sites are incorrectly positioned, so that phosphates of ATP are not ideally oriented for an optimal kinase reaction (Crosby 2007). Cyclin binding induces a conformational change and positional switch of the T-loop, which opens the catalytic cleft, affects the orientation of the putative substrate binding site of CDKs, and leads to appropriate exposure of T-loop Thr residues (e.g. Thr160 in CDK2 and Thr-186 in CDK9) for phosphorylation (Gu, Rosenblatt & Morgan 1992; Nekhai, Petukhov & Breuer 2014). Subsequent phosphorylation of Thr residues induces further conformational changes in the T-loop and C-terminal domain, which stabilises the substrate binding site, enabling the kinase to be a fully functional enzyme (Nekhai, Petukhov & Breuer 2014).

Phosphorylation of CDKs can also negatively regulate kinase activity (Crosby 2007). Phosphorylation of Thr14 and Y15 residues of the G-loop results in the inhibition of CDK activity, even in the presence of CAK (Gu, Rosenblatt & Morgan 1992; Malumbres & Barbacid 2005; Nekhai, Petukhov & Breuer 2014). Wee1 and Myt1 have been identified as the kinases responsible for the

phosphorylation of the inhibitory sites (Malumbres & Barbacid 2005; Pavletich 1999).

CDK activity may also be regulated by CDK inhibitors (CDKi), which form stable complexes with the CDK prior to cyclin binding, preventing it from binding with its cyclin partner (Lim & Kaldis 2013). Two distinct families of CDKi have been identified, the INK4 (Inhibitors of CDK4) family and the Cip/Kip (CDK interacting protein/Kinase inhibitory protein) family (Echalier et al. 2010; Pavletich 1999). The INK4 family includes $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$, which specifically inactivate CDK4 and CDK6 (Pavletich 1999). The second family of inhibitors, the Cip/Kip family, includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, pan-inhibitors that can inactivate any CDK family member (Borriello et al. 2011; Ma et al. 2010)

### 1.5.3 Cell cycle CDKs

Uncontrolled proliferation mediated by dysregulation of many cell cycle pathways is extremely common in cancer cells, and thus, regulators of the cell cycle have long been considered attractive targets for cancer therapy (Diaz-Padilla, Siu & Duran 2009; Hanahan & Weinberg 2011; Thu et al. 2018). The eukaryotic cell cycle contains two distinct stages: interphase and mitosis (Hartwell & Kastan 1994). Interphase is further divided into three sub-phases, gap 1 (G1), DNA synthesis (S), and gap 2 (G2) (Alberts et al. 2018; Bertoli, Skotheim & De Bruin 2013). During the G1-phase, the cell doubles in size and accumulates proteins and enzymes required for the S-phase (Bertoli, Skotheim & De Bruin 2013; Hartwell & Kastan 1994). During S-phase, DNA is duplicated, histones are synthesised, and the cell moves into the G2-phase

(Bertoli, Skotheim & De Bruin 2013; Hartwell & Kastan 1994). In G2-phase, protein synthesis continues, microtubules are assembled, any errors in DNA replication are rectified and the cell then enters mitosis (Hartwell & Kastan 1994). In the mitotic phase, chromosomes are segregated and the cell divides, resulting in formation of two daughter cells each having a nucleus containing the same number of chromosomes as the mother cell (Hartwell & Kastan 1994). Cell cycle CDKs (CDK 1-6) govern cell cycle transitions and eventual cell division (Ding et al. 2020; Thu et al. 2018) (Figure 1.8). When cells are stimulated to replicate, expression of D-type cyclins (D1, D2 and D3) is induced, and the resulting proteins associate with and activate their partner kinases CDK4 and 6, which were kept in an inactive state by interaction with INK4 family members ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$) or by $p21^{cip1}$ and $p27^{Kip1}$ (Ding et al. 2020; Echalier et al. 2010; Malumbres & Barbacid 2001). The main function of both CDK 4 and 6 within the cell cycle is to phosphorylate the retinoblastoma tumour suppressor protein (Rb) (Ding et al. 2020; Echalier et al. 2010). Under normal circumstances, the Rb protein recruits co-repressors to suppress the transcription of target genes regulated by the E2 family (E2F) transcription factor, in order to inhibit the G1/S transition (Malumbres & Barbacid 2001). Therefore, phosphorylation of Rb by CDK4/6 leads to a conformational change within the protein, resulting in the release of E2F (Malumbres & Barbacid 2001). In turn, E2F induces the transcription of G1/S mediators including cyclin A, cyclin B, cyclin E, ribonucleotide reductase M1 (RRM1), RRM2, spindle checkpoint protein (mitotic arrest deficient 2 (MAD2)), and BUB1 mitotic checkpoint serine/threonine kinase (BUB1) (Asghar et al. 2015; Ding et al. 2020; Malumbres & Barbacid 2001).

During the late G1-phase, cyclins E1 and E2 are activated, thereby binding and activating CDK2, which until then is held in check by CDK inhibitors $p21^{cip1}$ and $p27^{Kip1}$ (Watanabe, Broome & Hunter 1995). The CDK2/Cyclin E complex phosphorylates Rb at additional Ser/Thr sites, which leads to total inactivation of Rb (Tsai et al. 1993). Subsequently, the hyper-phosphorylated Rb no longer binds E2F, which results in a further increase of E2F levels within the cell (Tsai et al. 1993). Furthermore, active CDK2 can phosphorylate several proteins required for DNA replication (e.g., replication factors A and C), duplicate the centrosome (e.g., nucleophosmin (NPM)) and synthesise histones (e.g., nuclear protein, coactivator of histone transcription (NPAT)) (Ding et al. 2020; Okuda et al. 2000; Sever-Chroneos et al. 2001). Later in the S-phase, cyclin E is no longer needed and is decomposed via the ubiquitin proteasome pathway (Ding et al. 2020). The newly biosynthesised cyclin A then associates with CDK2 to terminate the S-phase by phosphorylating and deactivating E2F. This initiates the cell-cycle transition from S- to G2-phase, in which cyclin A subsequently activates CDK1, leading cells to enter transition to the mitotic phase (Asghar et al. 2015; Ding et al. 2020). Upon mitosis, CDK1 activity is maintained by cyclin B (Gavet & Pines 2010). Active CDK1 phosphorylates a large number of substrates that mediate breakdown of the nuclear envelope, condensation of chromosomes, and assembly of the mitotic spindle (Gavet & Pines 2010; Murray 2004). The mitotic metaphase to anaphase transition is controlled by spindle assembly checkpoints (SAC), and anaphase is dependent on decreased activity of CDK1 via the degradation of cyclin B via the cyclosome (Gavet & Pines 2010). The reduced expression of CDK1 enables chromosome separation and completion of mitosis (Murray 2004).

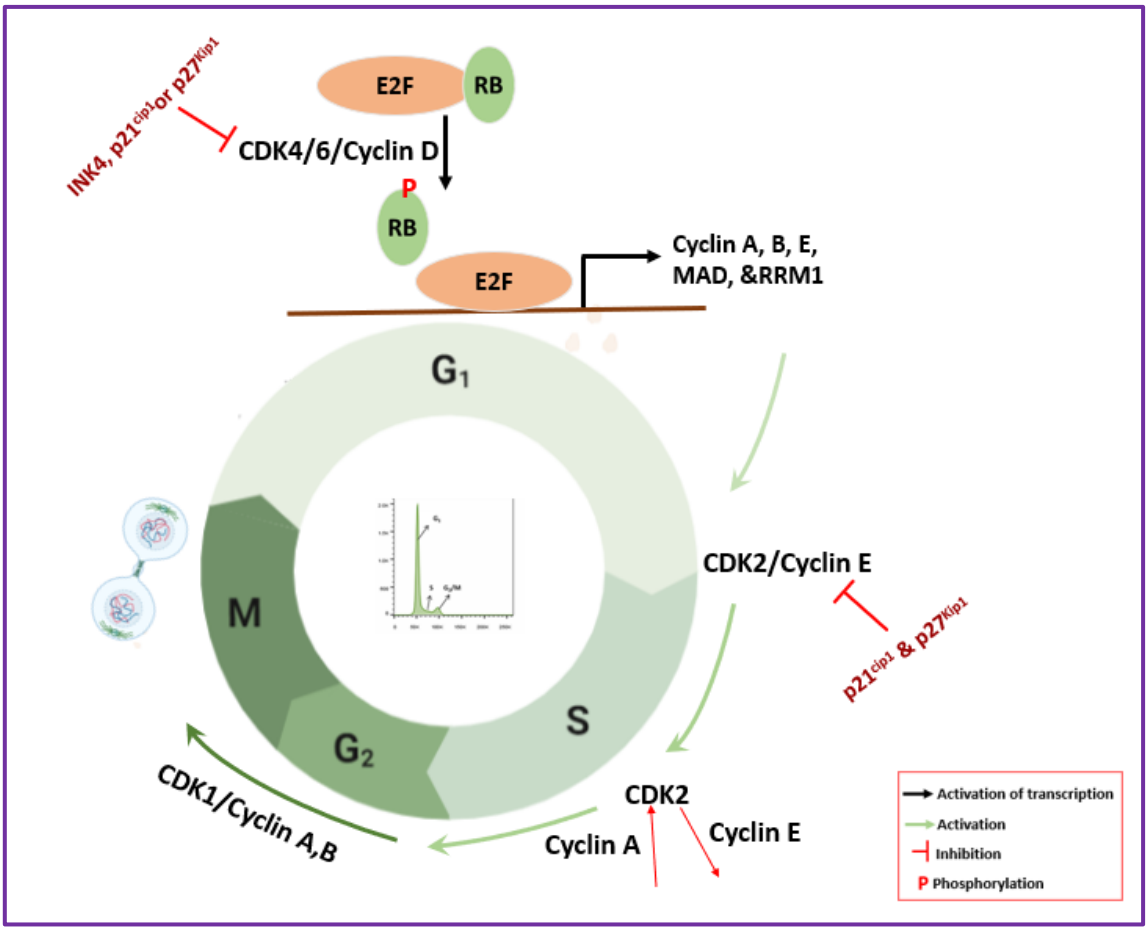



**Figure 1.8:** A schematic view of the cell cycle regulation by cyclin-dependent kinases (CDKs) and associated cyclins. Each phase in cell cycle progression is regulated by CDKs and their regulatory partner cyclins, as well as CDK inhibitors. G1 (Gap1), G2 (Gap2), S (DNA synthesis), Rb (retinoblastoma tumour suppressor protein), E2F (E2 family transcription factor), INK4 (Inhibitors of CDK4), $p21^{cip1}$ & $p27^{Kip1}$ (Kinase inhibitory proteins), RRM1 (ribonucleotide reductase M1), & MAD (spindle checkpoint protein).

In breast cancer, alterations in cell cycle regulatory proteins have been described and have been associated with tumorigenesis and progression of disease (An et al. 1999; Buckley et al. 1993; Ding et al. 2020; Malumbres & Barbacid 2009; Malumbres & Barbacid 2005). Dysregulation of the cyclin D/CDK4/6/INK4/Rb axis, in particular, has been extensively characterised in breast cancer (An et al. 1999; Bohn et al. 2010; Buckley et al. 1993). Amplification of *CCND1* (cyclin D1 gene) is observed in up to 15-20% of breast cancers (Buckley et al. 1993), while the cyclin D1 protein is overexpressed in over 50% of breast cancers (Buckley et al. 1993; Dickson et al. 1995; Gillett et al. 1994). Overexpression of cyclin D1 demonstrates a positive correlation with ERα expression, whereby 58% of ERα-positive breast cancer cases harboured cyclin D1 overexpression compared with 21% of ERα-negative breast cancer cases (Kenny et al. 1999; Umekita et al. 2002). Crosstalk between ERα signalling and cyclin D1 is well established. Cyclin D1 is an ERα target gene and an important mediator for estrogen induction of cell cycle progression in breast cancer (Butt et al. 2005; Musgrove et al. 2011). High cyclin D1 mRNA levels and amplification of *CCND1* in ERα positive breast cancers were strongly correlated with increased risk of relapse, local recurrence, metastasis, and death (Kenny et al. 1999; Roy, PG et al. 2010; Utsumi et al. 2000). Amplification of the *CDK4* and *CDK6* genes were also detected in about 16% of primary breast cancers (An et al. 1999; Hamilton & Infante 2016). A study by An and colleagues (1999) reported that the mean proliferative index (measured by IHC of the proliferative marker Ki-67) in breast tumours with *CDK4* gene amplification was significantly higher than in those without (An et al. 1999).

Loss of CDK inhibitor $p16^{INK4a}$ expression was reported in 49% of primary breast cancers, particularly in ERα-positive breast tumours (Campbell, I et al. 2000; Dick & Rubin 2013). Given these observations, preclinical and clinical studies have been conducted to evaluate the effect of targeting CDK4/6 using pharmacological CDK inhibitors in breast cancer (Finn, Aleshin & Slamon 2016; Rizzolio et al. 2010; Skowron et al. 2020). The most extensively investigated CDK4/6 inhibitor is flavopiridol (Finn, Aleshin & Slamon 2016). Flavopiridol is an intravenously administered CDK inhibitor that reduces CDK4/6 activity, with half-maximal inhibitory concentration ($IC_{50}$) values of 3.66 and 20.6nM in cell free assay, respectively (Sedlacek 2001). However, Flavopiridol can inhibit activity of other kinases including CDK1, 2, 7 and 9 with $IC_{50}$ values ranging from 20 to 170 nM in cell free assay (Finn, Aleshin & Slamon 2016). Flavopiridol underwent clinical testing in ERα-positive breast cancer and gave largely disappointing results (Fornier et al. 2007; Rizzolio et al. 2010). It was shown to be non-selective (i.e. off target effects), with flavopiridol having an unfavourable safety profile, causing a wide range of drug-mediated, dose-limiting side effects including neutropenia, hyperglycemia, cardiac and pulmonary dysfunction (Fornier et al. 2007; Ribnikar, Volovat & Cardoso 2019). Subsequently, more selective CDK4/6 inhibitors were developed including palbociclib (PD 0332991) and ribociclib (LEE011) (Dhillon 2015; Hortobagyi et al. 2016). Palbociclib, the first FDA (Food and Drug Administration) approved CDK4/6 inhibitor, is an orally active, potent, and highly selective CDK4/6 inhibitor with $IC_{50}$ value of 11 nmol/l and 15 nmol/l in cell free assay, respectively (Cadoo et al. 2014). Ribociclib, another selective CDK4/6 inhibitor that is currently approved for

clinical use, is a structural analogue of palbociclib and shares its target selectivity and pharmacological characteristics (e.g. absorption, metabolism & bioavailability) (Kim, S et al. 2020). In preclinical studies, both palbociclib and ribociclib inhibit proliferation of ERα/Rb positive breast cancer cell lines and growth of xenograft tumours (Fry et al. 2004; Kim, S et al. 2020; Kishino et al. 2019). The inhibitory effects of these inhibitors were accompanied by G1 arrest, dephosphorylation of Rb as well as a decrease in E2F-dependent gene expression (Fry et al. 2004). Dean and colleagues showed that treatment of Rb-positive primary breast cancer tissues cultured *ex vivo* as explants with palbociclib significantly reduced proliferation (Dean et al. 2012). The same study also reported that Rb-negative breast tumours were less sensitive to palbociclib (Dean et al. 2012). Moreover, synergistic effects of combining palbociclib or ribociclib with the ERα antagonist tamoxifen in ERα-positive breast cancer cell lines were demonstrated (Finn et al. 2009; Tripathy, Bardia & Sellers 2017). These observations were followed by a translation of the laboratory findings into clinical studies (Finn et al. 2014; Im et al. 2019; Turner et al. 2015; Verma et al. 2016). A combination of palbociclib or ribociclib with the aromatase inhibitor letrozole or the anti-estrogen fulvestrant, prolonged progression free survival in ERα-positive/HER2-negative advanced breast cancer patients (Finn et al. 2014; Im et al. 2019; Turner et al. 2015; Verma et al. 2016).

In contrast to the impressive therapeutic efficacy of CDK4/6 inhibitors in ERα-positive breast cancer, ERα-negative breast cancers show less sensitivity to these inhibitors (Finn, Aleshin & Slamon 2016; Jennifer et al. 2020; Klein et al. 2018). The lack of efficacy of CDK4/6 inhibition in ERα-negative breast

cancer is likely attributed to the absence of Rb or overexpression of p16 $^{INK4a}$ that is common in this sub-type of disease (Bohn et al. 2010; Hashmi et al. 2018; Pandey et al. 2019). Rb is lost or mutated in approximately 20% of ERα-negative breast cancers (Witkiewicz et al. 2018). Overexpression of p16 $^{INK4a}$ in the presence of functional Rb also confers resistance to CDK4/6 inhibitors as a result of diminished CDK4 activity (Pandey et al. 2019). Collectively, preclinical, and clinical studies have shown that targeting CDK4/6 has good therapeutic efficacy for ERα-positive breast cancer, specifically through its combination with other treatment regimens. However, it is not efficacious in ERα-negative breast cancer, warranting investigation into alternative CDK therapeutic targets in this disease context.

### 1.5.4 Transcriptional CDKs

#### 1.5.4.1 Function in transcription

The transcription of protein-coding genes by eukaryotic RNA polymerase II is divided into discrete phases of initiation, elongation, and termination (Fuda, Ardehali & Lis 2009; Yan et al. 2019) (Figure 1.9). In the initiation phase, general transcription factors (TFIIA, TFIIB, TFIID, TFIIF, TFIIS, TFIIE, and TFIIH), and un-phosphorylated RNAPII are recruited to the promoter of a gene, forming the pre-initiation complex (PIC) (Buratowski 1994; Pietras & Márquez-Garbán 2007). Promoter recognition occurs via TFIID, a multi-subunit complex containing the TATA-binding protein (TBP) and at least 14 tightly associated factors (Buratowski 1994; Pietras & Márquez-Garbán 2007). The binding of TFIID acts as a platform for the nucleation of the remaining factors (Dvir et al. 2001). TFIIB and TFIIA enter and stabilise TFIID binding

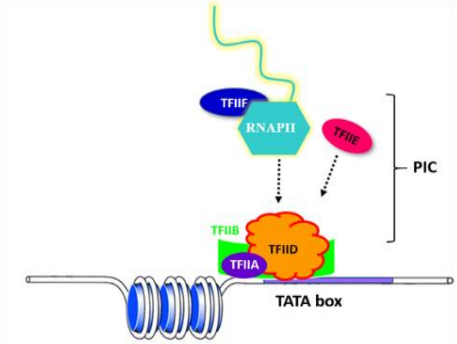
at the promoter by interacting directly with TFIID and DNA sequences flanking the TATA box (Buratowski 1994). TFIIB and TFIIA, in turn, recruit the RNAPII–TFIIF complex, but transcription cannot occur until TFIIE is incorporated and TFIIH recruited (Dvir et al. 2001). TFIIH has helicase and ATPase activities that unwind DNA and initiate transcription by RNAPII (Ohkuma et al. 1995). While the pre-initiation complex alone is able to drive basal transcription (Kanin et al. 2007; Serizawa, Conaway & Conaway 1993), the phosphorylation and activation of RNAPII via transcriptional CDKs in the initiation and elongation phases of transcription is required to achieve a continuous and high rate of transcription (Peissert et al. 2020). Five CDKs have been shown to be involved in controlling transcription: CDK7, CDK9, CDK10, CDK12, & CDK19. Among these, the role of CDK7 and CDK9 is the most well characterised (Fisher 2017; Glover-Cutter et al. 2009; Malumbres & Barbacid 2005; Peissert et al. 2020). CDK7, along with cyclin H and RING-finger protein MAT1, forms the CDK-activating kinase (CAK) complex, which associates with core subunits of TFIIH to form holo-TFIIH (Peissert et al. 2020). The first identified target of TFIIH-associated kinase activity was the carboxy-terminal domain (CTD) of Rpb1 (the largest subunit of RNAPII), which contains multiple repeats (52 in human Rpb1) of a heptad sequence (consensus: Y1S2P3T4S5P6S7) (Wong, Jin & Struhl 2014). Within the CTD repeats of RNAPII, CDK7 phosphorylates the Ser5 position (Glover-Cutter et al. 2009).

These phosphorylation events are thought to disrupt stable interaction between CTD and PIC components, thereby permitting RNAPII to be released from the promoter (Coin & Egly 2015). Before continuing to the elongation phase, RNAPII is paused by binding with negative elongation factor (NELF) and the DRB-sensitivity-inducing factor (DSIF) (Dvir et al. 2001). RNAPII pausing appears as a key regulation step in transcription, during which several quality-control processes take place, including mRNA 50 capping by the human capping enzyme (HCE), which protects pre-mRNA from decay during transcription (Akhtar et al. 2019; Kanin et al. 2007). The transition from the paused state to elongation is promoted by the positive transcription elongation factor (P-TEFb), a complex of CDK9 and cyclin T (Bacon & D'Orso 2019). CDK9 facilitates release of paused RNAPII into the elongation phase, leading to the synthesis of mRNAs through phosphorylation of three main substrates: 1) Ser2 residues located in the heptad repeats of the RNAPII CTD; 2) DSIF, transforming it into a positive elongation factor; and 3) NELF, resulting in its ejection from the pre-mRNA chain (Bacon & D'Orso 2019). The termination phase follows elongation, where RNAPII separates from the DNA template and releases mRNA (Rosonina et al. 2006).

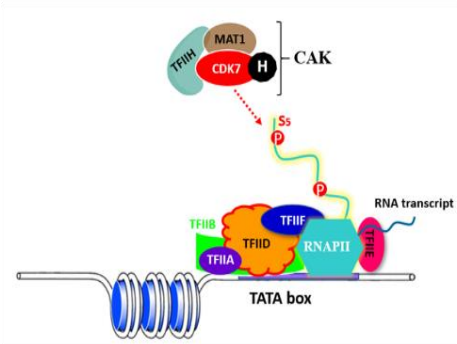
Unlike basal or low rates of transcription, where phosphorylation of the RNAPII CTD by P-TEFb is not required (Scafe et al. 1990; Serizawa, Conaway & Conaway 1993), the kinase function of P-TEFb plays a critical role in promoting expression of genes that are regulated by super enhancers, large clusters of DNA regulatory elements ("enhancers") that drive transcription of genes controlling cell identity and stimulating oncogenic transcription (Bacon & D'Orso 2019). Such genes include MYC (Boffo et al.

2018; Huang, C-H et al. 2014), C-MYB (Drabsch et al. 2007) and anti-apoptotic proteins e.g. MCL1 and BCL2 (Boffo et al. 2018; Cidado et al. 2020). By controlling gene expression of tumour driving genes, CDK9 is implicated in the initiation and progression of several types of cancer including leukemia (Boffo et al. 2018; Cidado et al. 2020), breast (McLaughlin et al. 2019; Mitra et al. 2016), colon (Rahaman, Lam, et al. 2019), and prostate (Falco, Giordano & therapy 2002; Franco et al. 2018; Rahaman et al. 2016). Since this study focuses on targeting CDK9 in the ERα-negative subtype of breast cancer, the following sections will provide an in-depth focus on the biological mechanisms of CDK9.

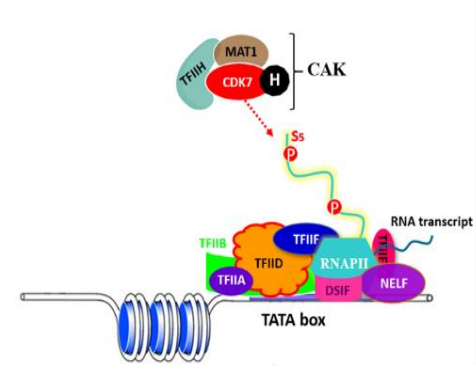
A. Formation of preinitiation complex (PIC)



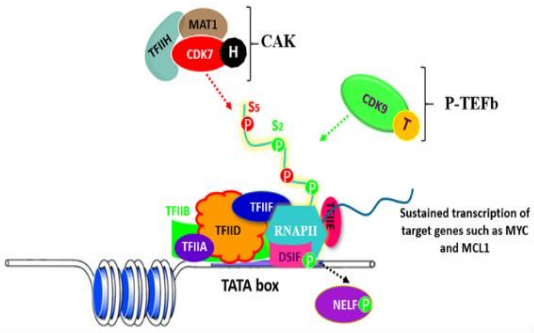

B. CDK-activating kinase (CAK)-TFIIH complex phosphorylates RNAPII CTD and initiate transcription




C. RNAPII pausing by negative elongation factor (NELF) and the DRB-sensitivity-inducing factor (DSIF)




D. CDK9/ Cyclin T promotes transcription elongation via phosphorylating RNAPII CTD & pausing factors




E. Transcription termination, RNAPII separates from the DNA template and releases RNA

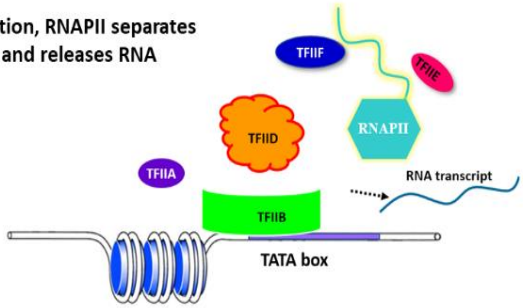
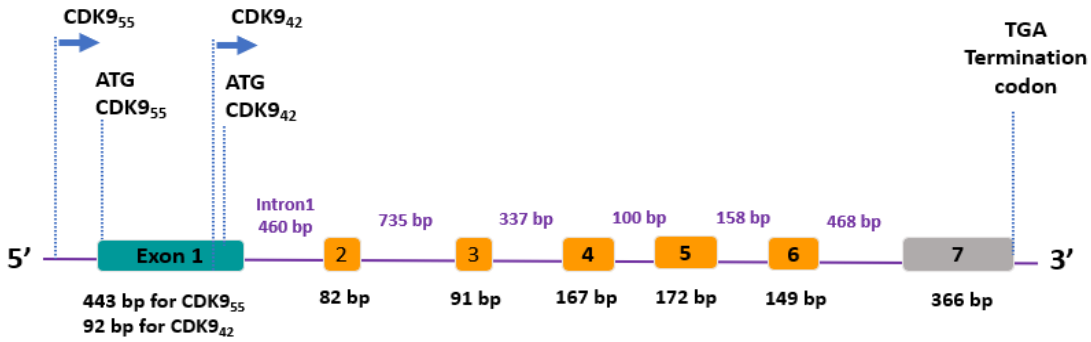


**Figure 1.9:** Schematic representation of transcription phases. **(A)** General transcription factors (TF) & RNAPII are recruited to the promoter of the gene, forming the pre-initiation complex (PIC). **(B)** Holo-TFIIH (TFIIH, RING-finger protein MAT1, CDK-activating kinase (CAK)) phosphorylates the c-terminal domain of RNAPII at Ser5 to initiate transcription. **(C)** RNAPII is paused by the negative elongation factor (NELF) and the DRB-sensitivity-inducing factor (DSIF). **(D)** Transition from the paused state to elongation is promoted by the positive transcription elongation factor (P-TEFb, a complex containing CDK9 & Cyclin T). **(E)** The termination phase occurs when RNAPII separates from the DNA template and releases RNA.

### 1.5.4.2 Isoforms and genetic aspects of CDK9

CDK9 exists in two isoforms in mammalian cells, a lighter 42 kDa protein ($CDK9_{42}$) and a heavier 55 kDa protein ($CDK9_{55}$) (Liu, H & Herrmann 2005). The $CDK9_{55}$ isoform has 117 additional amino acid residues in front of the amino terminus of $CDK9_{42}$ (Fu, T-J et al. 1999). The two CDK9 isoforms are encoded by the same gene, which is located at 9q34.1, near the telomeric end of the chromosome 9 long arm (chr9:127,785,679-127,790,792) (dos Santos Paparidis, Durvale & Canduri 2017). The CDK9 encoding gene has two different promoters, located more than 500 bp apart from each other (dos Santos Paparidis, Durvale & Canduri 2017; Shore et al. 2003) (Figure 1.10). $CDK9_{55}$ is transcribed from a TATA-containing promoter and has an additional 351 bp in its coding sequence (corresponding to the 117 exclusive N-terminal residues) (dos Santos Paparidis, Durvale & Canduri 2017). The CDK9 promoter that encodes for $CDK9_{42}$ mRNA does not have a functional TATA box, but contains a GC-rich transcription sequence, which is characteristic of promoters within constitutively active genes required for basic cell functions (termed housekeeping genes) (dos Santos Paparidis, Durvale & Canduri 2017; Shore et al. 2003).

**A**




**B**

```
  1  MQRDAPPRAP APAPRLPAPP IGAAASSGGG GGGGSGGGGG GASAAPAPPG LSGTTSPRGP
 61  GGGRRAEEAG SAPRGRKWPW RRKWRGRGGA WSAAAAGPGA GAAAAATGGG GGALEAAMAK
121  QYDSVECPFC DEVSKYEKLA KIGQGTFGEV FKARHRKTGQ KVALKKVLME NEKEGFPITA
181  LREIKILQLL KHENVVNLIE ICRTKASPYN RCKGSIYLVF DFCEHDLAGL LSNVLVKFTL
241  SEIKRVMQML LNGLYYIHRN KILHRDMKAA NVLITRDGVL KLADFGLARA FSLAKNSQPN
301  RYTNRVVTLW YRPPELLLGE RDYGPPIDLW GAGCIMAEMW TRSPIMQGNT EQHQLALISQ
361  LCGSITPEVW PNVDNYELYE KLELVKGQKR KVKDRLKAYV RDPYALDLID KLLVLDPAQR
421  IDSDDALNHD FFWSDPMPSD LKGMLSTHLT SMFEYLAPPR RKGSQITQQS TNQSRNPATT
481  NQTEFERVF
```

**Figure 1.10:** Gene structure and protein sequences of human CDK9. **(A)** Schematic overview of the CDK9 gene showing exons (orange blocks), introns (purple) and positions of start codons (ATG) for each CDK9 isoform. **(B)** Human CDK9 protein sequence. An additional 117 amino acid fragment (exclusive to CDK9$_{55}$) is marked in green, G-loop in blue, and T-loop in red.

The two isoforms of CDK9 display differences in subcellular localisation, tissue distribution and function (Liu, H & Herrmann 2005). $CDK9_{42}$ has been reported to localise to the nucleoplasm, whereas $CDK9_{55}$ localises to the nucleolus (Liu, H & Herrmann 2005). Moreover, substantial variations in relative abundance of the two isoforms were observed among various human tissues (Shore et al. 2005). For example, spleen, breast and testis tissues have high levels of $CDK9_{42}$ expression, whereas $CDK9_{55}$ expression is high in lung, liver and brain tissues (Liu, H & Herrmann 2005). Both isoforms are found to hyper-phosphorylate the CTD of RNAPII and mediate transcription elongation (Bacon & D'Orso 2019). However, preclinical studies have shown that $CDK9_{55,}$ but not $CDK9_{42}$, is able to bind to Ku70 (a DNA repair protein that binds to DNA double-strand break ends and helps repair DNA via the non-homologous end-joining) and is therefore involved in the DNA repair process (Liu, H et al. 2010; Yu, DS & Cortez 2011).

### 1.5.4.3 Regulation of CDK9 activity

The regulation of CDK9 function is a complex process that includes post-translational modifications as well as binding of the P-TEFb complex to specific factors that induce or inhibit kinase activity (dos Santos Paparidis, Durvale & Canduri 2017; Egloff et al. 2006). Phosphorylation at residue Thr186 of the activation (T-loop) segment is the most important modification that CDK9 undergoes because it is essential to kinase enzymatic activity (Lolli 2010). Phosphorylation of Thr186 triggers major conformational changes that opens the ATP binding pocket and substrate binding site, making CDK9 fully active (dos Santos Paparidis, Durvale & Canduri 2017). Unlike other CDKs,

Thr186 is not phosphorylated by the CDK-activating kinase (Kim, JB & Sharp 2001), and instead it has been suggested that it is an autophosphorylation site (Li, Q et al. 2005). In addition to Thr186, a cluster of CDK9's C-terminal residues (Ser-347, Ser-353, and Ser-357; Thr-350 and Thr-354) are also known to be auto-phosphorylated and have been reported to be important for CDK9/Cyclin T complex nuclear localisation (Mueller et al. 2002; Napolitano, Majello & Lania 2003).

Factors that inhibit CDK9 include 7SK and HEXIM1 (Kiss, Michels & Bensaude 2001; Michels, AA et al. 2003). 7SK is a small, abundant 331-nucleotide-length nuclear RNA (snRNA) showing high-sequence conservation in vertebrates (Kiss, Michels & Bensaude 2001). HEXIM1 was initially identified as a protein whose expression is stimulated in vascular smooth muscle cells in response to hexamethylene bisacetamide (HMBA) treatment, an inhibitor of proliferation (Michels, AA et al. 2003). In association with 7SK, HEXIM1 is able to bind P-TEFb and inhibit its kinase and transcriptional activity (Dey, Chao & Lane 2007; Michels, AA et al. 2003). HEXIM1 contains a peptide sequence that binds to the kinase to block the active site, thereby preventing CDK9 from binding to its substrates (Peterlin, Brogie & Price 2012; Sonawane et al. 2016). HEXIM1 can interact with P-TEFb and inhibit its kinase activity only in the presence of 7SK, suggesting that 7SK provides the structural scaffold for assembly of the kinase-inactive 7SK/HEXIM1/P-TEFb complex (Egloff et al. 2006). Upon transcription, release of the CDK9/Cyclin T complex from 7SK/HEXIM1 occurs through post-translational modifications of CDK9 (Chen, R et al. 2008; Chen, R, Yang & Zhou 2004). A study by Chen and colleagues (2008) showed that dephosphorylation of the phospho-Thr186

in the T-loop segment of CDK9 by PP1α (protein phosphatase 1α) and PP2B (protein phosphatase 2B) results in its release from 7SK (Chen, R et al. 2008). In addition to post-translational modifications, bromodomain-containing protein 4 (BRD4) can release P-TEFb from the HEXIM/7SK complex (Cassandri et al. 2020). BRD4 competes with the inhibitory complex HEXIM/7SK and recruits free P-TEFb to the transcription start site via histone acetylation (Yang, Z et al. 2005). However, the recruitment of BRD4 depends on the transcription factors that bind to the promoter and/or enhancer of the target gene (Yang, Z et al. 2005). Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) has been the first discovered transcription factor implicated in CDK9/BRD4 delivery to transcriptional complexes via interaction of CDK9 and the RelA subunit (Barboric et al. 2001). Moreover, C-MYC can also recruit and place CDK9 into promoter regions of its gene targets, with presence of CDK9 sufficient to drive expression of C-MYC-target genes (Cassandri et al. 2020; Galbraith, Bender & Espinosa 2019; Pereira et al. 2015).

### 1.5.4.4 CDK9 related pathways in cancer

The kinase activity of CDK9 facilities transcription of crucial transcription factors that are involved in several biological processes, including cell growth, protection against apoptosis, and DNA repair (dos Santos Paparidis, Durvale & Canduri 2017; Franco et al. 2018). The master regulator C-MYC, a well-known CDK9 target, controls the expression of several genes involved in cell cycle and proliferation (Bretones, Delgado & León 2015). Cell cycle-related genes cyclin D2, cyclin E1, CDK4, CDK6, Cdc25A, E2F1 were among the first

described C-MYC target genes (Amati, Alevizopoulos & Vlach 1998; Bretones, Delgado & León 2015; Mateyak et al. 1999; Meyer & Penn 2008). Moreover, one of the most important targets of C-MYC is the CDK inhibitor p27 (Mateyak et al. 1999; Yang, W et al. 2001). In lymphoid and breast cancer cells, C-MYC accelerates cell proliferation rates through its ability to antagonise p27 function as a CDK inhibitor (Chandramohan et al. 2008; Yang, W et al. 2001). In normal cells, expression and activity of C-MYC are tightly controlled to maintain the optimal levels to maintain homeostatic cell proliferation (Meyer & Penn 2008). In contrast to non-malignant cells, C-MYC expression is estimated to be elevated or dysregulated in up to 70% of human cancers (Dang, CVJC 2012). High levels of C-MYC expression have been linked to aggressive human prostate cancer and TNBC (Gurel et al. 2008; Palaskas et al. 2011). Since CDK9 is important to sustain C-MYC mRNA transcripts, inhibition of CDK9 has been investigated as one avenue to reduce production of the C-MYC protein in cancer cells (Cassandri et al. 2020; Lu et al. 2015; Rowland et al. 2016).

Short half-life anti-apoptotic BCL2 proteins such as MCL1, BCL2 and XIAP, which are determinants of cancer cell survival, also require CDK9 to maintain their transcription at high levels (Barille-Nion et al. 2012; Scherr et al. 2020; Xiao et al. 2015). Anti-apoptotic proteins restrain the apoptotic pathway by binding and sequestering pro-apoptotic factors (Williams et al. 2017). Specifically, anti-apoptotic proteins either 1) bind to BCL2 effectors (Bak and Bax) to block pore formation in the outer mitochondrial membrane caused by Bak/Bax oligomerisation (Emily et al. 2001; Willis et al. 2005), or 2) sequester BCL2 activators (e.g., Bim, Bid, and Puma), which facilitate Bak/Bax

oligomerisation (Emily et al. 2001). High expression of BCL2 and MCL1 have been reported in breast cancer (Dawson et al. 2010; Merino et al. 2016; Oakes et al. 2012) and high MCL1 levels were associated with poor prognosis in TNBC cancer (Campbell, KJ et al. 2018). Interestingly, levels of MCL1 and BCL2 gene products have been shown to decrease dramatically upon exposure to CDK9 inhibitors, leading to reduced cell proliferation and survival in several types of cancer, including breast cancer (Cidado et al. 2020; Luedtke et al. 2020; Mitra et al. 2016; Thomas, D et al. 2013).

An association of CDK9 with AR has reported, indicating CDK9 has the ability to phosphorylate AR at the Ser81 residue of the AR NTD in prostate cancer cells (Rahaman et al. 2016). Several investigations attribute the role of Ser81 phosphorylation to the regulation of AR stability, nuclear translocation, as well as binding to chromatin (Chen, S et al. 2012; Gioeli et al. 2002; Wu, K et al. 2014). According to Gordon et al. (2010), overexpression of CDK9 along with its partner cyclin T increased Ser81 phosphorylation of AR in prostate cancer cells *in vitro* (Gordon et al. 2010). Silencing the CDK9 protein in LNCaP cells resulted in a significant reduction in Ser81 phosphorylation even after androgen induction (Rahaman et al. 2016; Richters et al. 2021). Based on these observations, we hypothesise that inhibition of CDK9 could also help in inhibiting the oncogenic effects of AR in molecular apocrine breast cancer cells.

Together, these studies provide compelling evidence that CDK9 related pathways play a pivotal role in the initiation and/or progression of the

malignant cell phenotype. Hence, targeting CDK9 represents a potential therapeutic strategy for ERα-negative breast cancer.

### 1.5.4.5 CDK9 inhibitors and ERα- negative breast cancer

Pharmacological CDK9 inhibitors have been extensively investigated as therapeutics for a variety of hematologic cancers, as high CDK9 and MCL1 expression is correlated with poor survival in patients of blood cancers (Boffo et al. 2018; Li, X-x et al. 2019). Table 1.4 provides CDK inhibition profiles for the most common CDK9 inhibitors (Boffo et al. 2018). These inhibitors are deemed competitive inhibitors of the ATP-binding site, which is a highly conserved feature across the CDK family, and consequently, these CDK9 inhibitors lack specificity, incurring side effects in patients (Boffo et al. 2018; Mariaule & Belmont 2014; Sonawane et al. 2016).

Elevated CDK9 expression has been shown to be associated with worse overall survival in ERα-negative breast cancers (Brisard et al. 2018). However, very few studies have examined the effect of CDK9 inhibition in ERα-negative breast cancer models, and of these few studies the compounds that were used to inhibit CDK9 activity had the undesirable effect of also inhibiting several other related enzymes. Dinaciclib is a potent pan-CDK inhibitor with $IC_{50}$ of 1, 1, 3, 4, 60 and 70 nM for CDK2, CDK5, CDK1 ,CDK9, CDK6, and CDK7, respectively (Parry et al. 2010). Dinaciclib effectively inhibits growth of ERα-negative breast cell lines *in vitro* and *in vivo* (Rajput et al. 2016). The inhibitory effects of dinaciclib were accompanied by a decrease in C-MYC, as well as a decrease in protein levels of CDK1 and its partner cyclin B1 (Rajput et al. 2016). A Phase 1 clinical trial ( NCT01624441) began with dinaciclib in

patients diagnosed with advanced TNBC at the MD Anderson Cancer Center (USA), but was not completed due to toxicity of the treatment including neutropenia, syncope (a temporary loss of consciousness usually related to insufficient blood flow to the brain) and haemolytic anemia (Mitri et al. 2015). CYC065 is a second-generation, orally available ATP-competitive inhibitor of CDK2/CDK9 kinases with an $IC_{50}$ of 5 and 26 nM, respectively (Rao et al. 2017). CYC065 inhibits proliferation and viability of ERα-negative breast cancer cell lines and patient derived xenografts (PDXs) (Rao et al. 2017). Treatment with the CYC065 resulted in decreased CDK9 activity and C-MYC expression, along with increased G1 phase cell cycle distribution due to off target CDK2 inhibition (Rao et al. 2017). Collectively, these data demonstrate that targeting CDK9 has been hampered by the poor selectivity of existing drugs.

Herein, we evaluated the pre-clinical efficacy of a newly developed, highly selective, and potent CDK9 inhibitor called D-11 in ERα-negative breast cancer cell lines and xenografts. D-11 was developed by Professor Shudong Wang (Drug Discovery and Development Group, University of South Australia, Australia). To improve selectivity for CDK9, D-11 was developed by leveraging the striking difference in the way transcriptional CDK9 associates with Cyclin T compared to the way cell cycle CDKs associate with their cyclin partners (e.g., CDK2/Cyclin A) (Baumli et al. 2008). The orientation of cyclin T with respect to CDK9 is rotated by about 26°, resulting in a comparatively sparse number of contacts between CDK9 and Cyclin T (Baumli et al. 2008). The buried molecular surface area of CDK9/Cyclin T is 60% wider than the molecular surface area buried on complex formation of

CDK2/Cyclin A (Baumli et al. 2008). The big size of D-11 enables it to pass through the space that exists between CDK9 and cyclin T but does not enable it to reach the ATP binding site of other CDKs. Indeed, D-11 demonstrates superior selectivity towards CDK9 over the other known CDK9 inhibitors such as dinaciclib. D-11 inhibits the CDK9 catalytic activity with kinase inhibition ($K_i$) of 8 nM but is far less effective on other CDKs with $K_i$ of 1530, 430, 2620, 1540, and 600 nM for CDK1, CDK2, CDK4, CDK6, and CDK7, respectively.

In summary, selective CDK9 inhibition is an attractive therapeutic strategy for TNBC tumours since they are addicted to continuous transcription of short half-life oncogene and pro-survival factors. Therefore, a highly selective, potent, and orally bioavailable CDK9 inhibitor D-11 is a promising treatment candidate for preclinical investigation in TNBC.

**Table 1.4:** CDK inhibition profiles of the most common CDK9 inhibitors (Boffo et al. 2018; Yankulov et al. 1995).

| Compound | CDK inhibition ($IC_{50}$) | Mode of administration |
|---|---|---|
| **Dinaciclib** | CDK9: 4 nM<br>CDK1: 3 nM<br>CDK2: 1 nM<br>CDK5: 1 nM | Intravenous |
| **AT7519** | CDK9: 10 nM<br>CDK2: 47 nM<br>CDK4: 100 nM<br>CDK5: 13 nM<br>CDK6: 179 nM | Intravenous |
| **LY2857785** | CDK9: 11 nM<br>CDK8: 16 nM<br>CDK7: 246 nM | Intravenous |
| **P276–00** | CDK9: 20 nM<br>CDK1: 79 nM<br>CDK2: 224 nM<br>CDK4: 63 nM | Intravenous |
| **SNS-032** | CDK9: 4 nM<br>CDK2: 38 nM<br>CDK7: 62 nM | Intravenous |
| **TG02** | CDK9: 3 nM<br>CDK1: 9 nM<br>CDK2: 5 nM<br>CDK3: 8 nM<br>CDK5: 4 nM<br>CDK7: 37 nM | Oral |
| **CDKI-73** | CDK9: 6 nM<br>CDK1: 8 nM<br>CDK2: 3 nM<br>CDK4: 8 nM<br>CDK6: 37 nM<br>CDK7: 134 nM | Intravenous, oral |
| **DRB** | CDK9: 3µm<br>CDK7: 20 µm<br>CDK8: 20 µm | Oral |

## 1.6 Hypothesis and aims

It is evident that AR and CDK9 represent two very promising targets for therapeutic interventions in ERα- negative breast cancer. However, there are important issues that need to be addressed before these targets can be considered as novel effective therapeutic strategies for ERα- negative breast cancer. Targeting AR in ERα-negative breast cancer has both proliferative and anti-proliferative effects, and the precise mechanisms of these divergent effects are still unclear. Additionally, targeting CDK9 has been limited by the poor selectivity and toxicity of existing inhibitors.

### Hypothesis

Targeting the AR or CDK9 is an effective therapeutic strategy for ERα-negative breast cancer.

### Objectives of this thesis

**Aim 1: To define genomic parameters of AR activity associated with proliferative versus anti-proliferative cellular responses in ERα- negative/AR positive breast cancer.**

1.1 Bioinformatically mine unpublished genomic and proteomic datasets, generated by my host laboratory, of ERα-negative breast cancer cell lines with divergent responses to ligand activation *in vitro*.

1.2 Determine the effect of differential expression of candidate AR factors from Aim 1.1 on proliferation and AR chromatin binding in ERα-negative/AR positive cell lines *in vitro*.

**Aim 2: Examine the anti-tumour efficacy of CDK9 inhibition in ERα- negative breast cancer.**

2.1 Evaluate the effect of a novel and highly selective CDK9 inhibitor (D-11) on the proliferation, apoptosis, and cell cycle status of ERα- negative breast cancer cells *in vitro.*

2.2 Determine the mechanisms behind CDK9 targeted effects in ERα- negative breast cancer cells *in vitro*.

2.3 Evaluate the effect of D-11 on normal breast tissues cultured *ex vivo.*

**Aim 3: Assess AR or CDK9 treatment strategies in contemporary models of ERα-negative breast cancer.**

3.1 Evaluate anti-tumour effect of D-11 using cell-line xenograft model of ERα- negative breast cancer.

3.2 Examine AR treatment strategy in patient-derived models of ERα- negative /AR positive breast cancer.

The results of this project will provide important preclinical data for new therapeutic strategies that could change the standard of care in many women who would otherwise succumb to this disease.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Materials

**Table 2.1:** Reagents

| Name | Supplier | Catalogue number |
|---|---|---|
| DAB | Sigma | D9015 |
| Acetic acid | Sigma | 320099 |
| Agar | Sigma | A7002 |
| Ampicillin | Sigma | A8351 |
| Antimycotic-antibiotic | Sigma | A5955 |
| BD™ CS&T | BD | 642412 |
| Bovine serum albumin | Sigma | A9647 |
| Bromophenol blue | Sigma | B0126 |
| Chloroform | Sigma | C2432 |
| Citric acid monohydrate | Sigma | C7129 |
| Clarity Western ECL Substrate | Bio-Rad | 170-5061 |
| cOmplete protease inhibitor | Roche | 11697498001 |
| Criterion precast gel (4-12%) | Bio-Rad | 567-1084 |
| DAPI | Thermo Scientific | D1306 |
| DHT | Sigma | D-5027 |
| DMSO | Sigma | D2650 |
| D-luciferin | Promega | P1043 |
| Donkey serum | Sigma | D9663 |
| DPX | Sigma | 06522 |
| DMEM-High Glucose | Sigma | D5671 |
| Duolink II Detection Kit | Sigma | Duo92008 |
| Dynabeads™ Protein A | Thermo Scientific | D2214 |
| Dynabeads™ Protein G | Thermo Scientific | K1816 |
| E2 | Sigma | E2758 |
| EMEM | Sigma | M0325 |

| | | |
|---|---|---|
| Eosin | Australian Biosatin | AEPA |
| Ethanol | Scharlau | ET00110500 |
| EGTA | Sigma | E3889 |
| EDTA | Sigma | E5134 |
| Fetal bovine serum | Sigma | 12003C |
| Formaldehyde | Sigma | 8775 |
| Glucose | Sigma | G5400 |
| Glycerol | Chem Supply | GA010-2.5L-P |
| Glycine | Sigma | G8898 |
| Glycoblue | Thermo Scientific | AM9515 |
| Glycogen | Thermo Scientific | AM9516 |
| Goat serum | Sigma | G9023 |
| $H_2O_2$ | Chem -Supply | 01552 |
| Halt phosphatase inhibitor | Thermo Scientific | 862495 |
| HEPES | Sigma | H3375 |
| Horseradish streptavidin-peroxidase | DAKO | 20031449 |
| Human recombinant insulin | Sigma | I0516 |
| Hydrocortisone | Sigma | H0888 |
| IncuCyte® caspase-3/7 green apoptosis assay reagent | Essen Bioscience | 4440 |
| IncuCyte® NucLight rapid red reagent | Essen Bioscience | 4717 |
| ITS) | Sigma | I1884 |
| iQ SYBR green supermix | Bio-Rad | 170-8885 |
| iScript cDNA synthesis kit | Bio-Rad | 170-8891 |
| Isoflurane | Henry Schein | 5100XN |
| Isopropanol | Sigma | I9516 |
| L-glutamine | Sigma | G7513 |
| Lipofectamine RNA iMAX | Thermo Scientific | 13778150 |
| Lithium chloride | Sigma | L9650 |
| Luria broth | Sigma | L3522 |
| Magnesium chloride | Sigma | M-1028 |
| Magnesium sulfate | Sigma | M-9397 |
| Mayer's hematoxylin | Australian Biosatin | ALLM |
| Methanol | Chem Supply | MA004-2.5L-P |
| MOPS buffer | Bio-Rad | 161-0788 |
| Neutral buffered formalin | Chem Spply | 151116 |

| | | |
|---|---|---|
| Nitrocellulose membrane (0.4 μm) | Amersham | GEHE10600016 |
| N-laurylsarcosine | Sigma | L9150 |
| NP-40 | Roche | 11332473001 |
| Nuclease free water | Ambion | AM9937 |
| NucleoBond Xtra Maxi Plus kit | Macherey-Nagel | 740420.50 |
| Opti-MEM | Thermo Scientific | 31985070 |
| PBS | Gibco | 14190 |
| Phenol-chloroform-isoamyl alcohol | Sigma | P2069 |
| Pierce BCA protein assay kit | Thermo Scientific | 23225 |
| PLA mounting media | Dako | S3023 |
| PLA wash buffer A | Sigma | Duo82046 |
| PLA wash buffer B | Sigma | Duo82048 |
| Polyethylenimine | Poly-Sciences | 24765 |
| Ponceau | Sigma | P3504 |
| Potassium acetate | Sigma | P1190 |
| Potassium chloride | Sigma | P9541 |
| Potassium hydroxide | Sigma | P1767 |
| Precision plus protein dual colour standards | Thermo Scientific | 350001695 |
| Propidium Iodide | Sigma | P4170 |
| Proteinase K | Boehringer Mannheim | 1000144 |
| Puromycin | Thermo Scientific | Ant-pr |
| QIAquick plasmid miniprep kit | Qiagen | 27106 |
| RNase A | Sigma | R4642 |
| RPMI 1640 | Gibco | 13412 |
| Sodium chloride | Ajax Fine Chemicals | 465 |
| Sodium deoxycholate | Sigma | D6750 |
| Sodium dodecyl sulphate | Sigma | 75746 |
| Sodium hydroxide | Chem Supply | 8A178 |
| Sodium pyruvate | Sigma | S8636 |
| Stratagene MycoSensor qPCR kit | Agilent | 302106 |
| Tris-base | Sigma | T1378 |
| Tris-HCl | Sigma | T3253 |
| Triton X-100 | Sigma | T8787 |
| Trizol | Sigma | T9424 |
| Trypan blue | Sigma | T6146 |

| | | |
|---|---|---|
| Trypsin-EDTA | Sigma | T4049 |
| Tryptone | Sigma | T-7293 |
| Turbo-DNase kit | Thermo Scientific | AM1907 |
| Tween 20 | Sigma | P7949 |
| Yeast extract | Fluka Analytical | 92144 |
| β-mercaptoethanol | Sigma | 63689 |

**Table 2.2:** Solutions

| **Name** | **Preparation** |
|---|---|
| 0.4% Trypan blue | 0.4g Trypan blue<br>100mL PBS |
| Freezing media | 5%FBS<br>4%Growth media<br>1%DMSO |
| Propidium iodide staining solution | 50µg/mL PI<br>0.1% Triton X-100<br>100µg/mL RNase A |
| **Bacterial transformation and plasmid DNA extraction solutions** | |
| SOC media | 0.5%Yeast extract<br>2%Tryptone<br>10mM NaCl<br>2.5mM KCl<br>10mM MgCl2<br>10mM MgSO4<br>20mM Glucose |
| Luria broth | 25g LB<br>1L H2O<br>(Add 20g agar for plates) |
| P1 Buffer | 50mM Tris-HCl, pH 8.0<br>10mM EDTA<br>100µg/mL RNase A |
| P2 Buffer | 200mM NaOH<br>1%SDS |
| P3 Buffer | 3.0M potassium acetate, pH 5.5 |
| **Western blotting solutions** | |

| | |
|---|---|
| RIPA buffer | 50mM Tris-HCl pH 8.0<br>150mM NaCl<br>0.5% Sodium deoxycholate,<br>0.1% SDS<br>0.1% Triton X-100 |
| Loading buffer (6x) | 0.27M Tris-base<br>10.3% SDS<br>35% Glycerol<br>6% β-mercaptoethanol<br>0.05% Bromophenol blue |
| TBS (Tris-buffered saline) (10x) | 151.5g Tris-base<br>219g NaCl<br>in 2.5L H2O<br>Adjust pH into 7.4 |
| TBST (Tris-buffered saline, 0.1% Tween 20) (1x) | 2.5mL Tween 20<br>250mL 10x TBS<br>in 2.5L H2O |
| Transfer Buffer (10x) | 77.5g Tris -base<br>360g Glycine<br>in 2.5L H2O |
| Ponceau stain | 1% Ponceau S in<br>5% Acetic Acid |
| **Chromatin immunoprecipitation (ChIP) solutions** | |
| Solution A | 1% Formaldehyde<br>50mM HEPES-KOH, pH 7.5<br>100mM NaCl<br>1mM EDTA<br>0.5mM EGTA |
| Lysis buffer 1 (LB1) | 50mM HEPES-KOH, pH 7.5<br>140mM NaCl<br>10% glycerol<br>1mM EDTA<br>0.5% NP-40<br>0.25% Triton X-100 |
| Lysis buffer 2 (LB2) | 10mM Tris-HCl, pH 8.0<br>200mM NaCl<br>1mM EDTA<br>0.5mM EGTA |

| | |
|---|---|
| Lysis buffer 3 (LB3) | 10mM Tris-HCl, pH 8.0<br>100mM NaCl<br>1mM EDTA<br>0.5mM EGTA<br>0.1% Na-Deoxycholate<br>0.5% N-laurylsarcosine |
| RIPA buffer | 50mM HEPES-KOH, pH 7.5<br>500mM LiCl<br>1mM EDTA<br>1% NP40<br>0.7% Na-Deoxycholate |
| Elution buffer | 50mM Tris-HCl, pH 8.0<br>10mM EDTA<br>1% SDS |
| TE buffer | 10mM tris-HCL, pH 8.0<br>1mM EDTA |
| **Immunohistochemistry solutions** | |
| 10 mM citrate buffer (pH 6.5) | 2.10g Citric acid monohydrate in 1L H2O<br>Adjust pH to 6.5 |
| DAB solution | 105 mL of 56.5mM Tris-buffer (pH 7.4)<br>100mg DAB |

**Table 2.3:** Primers

| Primer name | Sequence | Annealing temperature |
|---|---|---|
| C-MYC F | 5′-TTCGGGTAGTGGAAAACCAG-3′ | 55°C |
| C-MYC R | 5′-CCCCCTCGTCGCAGTAGAAA-3′ | |
| MCL1 F | 5′-CCAAGAAAGCTGCATCGAACCAT-3′ | 60°C |
| MCL1 R | 5′- CAGCACATTCCTGATGCCACCT-3′ | |
| GAPDH F | 5′- TGCACCACCAACTGCTTAGC-3′ | 55 °C |
| GAPDH R | 5′- GGCATGGACTGTGGTCATGAG-3′ | |
| HER2 F | 5′- ACACGATTTTGTGGAAGGACAT-3′ | 60°C |
| HER2 R | 5′- AGCCCTTACACATCGGAGAA-3′ | |

| | | |
|---|---|---|
| ALDH1A3 F | 5′- TGGCACGAATCCAAGAGTGG-3′ | 55 ℃ |
| ALDH1A3 R | 5′- TTGTCCACGTCGGGCTTATC-3′ | |
| KMO F | 5′- TGCCATCCCTCTAATTGGAGA-3′ | 55 ℃ |
| KMO R | 5′- GCCCGCATTCATTCCTTGC-3′ | |
| CLCA2 F | 5′- ATGCAAGTACCCACAACCAAG-3′ | 60 ℃ |
| CLCA2 R | 5′- GATTACATCCCATGCACTTCTGA-3′ | |
| FAR2 F | 5′- CCGTGCAGTACAAGCTCTCC-3′ | 55 ℃ |
| FAR2 R | 5′- CTGCTCAGTCGTGTTCAAGTATT-3′ | |
| KMO ChIP-PCR F | 5′- ACAGCTGCTCTGCTGAAGGG-3′ | 60 ℃ |
| KMO ChIP-PCR R | 5′- TCCTGCTGTTCCCTCTGCCT-3′ | |
| ALDH1A3 ChIP-PCR F | 5′- CACCCGGGCTTGATCAGAAT-3′ | 55 ℃ |
| ALDH1A3 ChIP-PCR R | 5′- GTCACCCGCTTCAGATTGCT-3′ | |
| CLCA2 ChIP-PCR F | 5′- CAAGGCCAGAGGGAAAGGG-3′ | 60 ℃ |
| CLCA2 ChIP-PCR R | 5′- AATCCATTCTGCAGCCCAGT-3′ | |
| FAR2 ChIP-PCR F | 5′-TGCAGGACCTCATCAAGATACA-3′ | 60 ℃ |
| FAR2 ChIP-PCR R | 5′-GGCCTACCACGGGACCTAAG-3′ | |
| NC ChIP-PCR F | 5′- GTGAGTGCCCAGTTAGAGCATCTA-3′ | 55 ℃ |
| NC ChIP-PCR R | 5′- GGAACCAGTGGGTCTTGAAGTG-3′ | |

## 2.2 Methods

### 2.2.1 Test compounds

DHT (Sigma) was dissolved in 100% ethanol to a concentration of 10mM stock. D-11 and L-453 were provided by Professor Shudong Wang (Drug Discovery and Development group, Cancer Research Institute, University of South Australia, Australia). For the *in vitro* assays, concentrated 10mM stocks of D-11 and L-453 were prepared in 100% Dimethyl sulfoxide (DMSO). 10mM concentrated stocks of D-11 and L-453 were stored at -20 and 4°C, respectively, and diluted into growth media as required on the day of the treatment. For the *in vivo* study, D-11 was freshly formulated in 0.1M sodium acetate buffer, pH 4.5.

### 2.2.2 Cell culture

#### 2.2.2.1 Maintenance of cell lines

MDA-MB-453, MDA-MB-231, MDA-MB-468, and HEK 293T/17 cell lines were obtained from the American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (with high glucose) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 2mM sodium pyruvate. MFM-223 cell line was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, 2mM L-glutamine, and a 1x concentration of insulin–transferrin–sodium selenite (ITS)

supplement. All cell lines were kept at 37°C in a humified incubator with 5% $CO_2$. Cell lines were routinely tested for mycoplasma infection using Stratagene MycoSensor qPCR kit and cell line identity by short tandem repeat profiling (Cell Bank Australia).

### 2.2.2.2 Reviving, passaging, and freezing of cells.

To revive cells from frozen stocks stored in liquid nitrogen, cryo-vials were rapidly thawed by gently swirling in a 37°C water bath. In a laminar flow hood, cryo-vials were decontaminated by spraying with 70% ethanol, and cell suspension was transferred to a 15mL conical tube containing 10mL of growth media. Cells were centrifuged at 1500rpm for 5min, the supernatant was removed, and the cell pellet was resuspended in 12mL of growth media and transferred to a T-25 sterile tissue culture flask (Corning). T-25 flasks were incubated at 37°C in a humified incubator with 5% $CO_2$.

For regular passaging of cell lines, cell lines were grown in sterile T-75 tissue culture flasks (Corning) and were passaged at regular intervals when cells reached approximately 75% confluency. At the time of passaging cells, culture media was aspirated from the flask and cells were washed with 5mL pre-warmed 1x concentration phosphate-buffered saline (PBS). PBS was aspirated and 3mL of pre-warmed 0.25% (w/v) Trypsin-EDTA solution was added. Cells were incubated with trypsin at 37°C for 3-5 min (until cells detached from the flask). Trypsin was neutralized with 10mL pre-warmed media containing 10% FBS and collected in a 50mL conical tube. Cells were pelleted by centrifugation at 1500rpm for 5min and resuspended with 12mL growth media. Cell count was determined by diluting 30µL of cell suspension with 30µL of

0.4% Trypan blue dye and counting manually with a hemocytometer (Hirschmann). Cells were re-seeded at appropriate density into a new sterile T-75 tissue culture flask.

For the freezing protocol, cells were detached using trypsin, counted (as outlined above), and resuspended in freezing media (10% DMSO, 40% FBS, 50% culture media) at a concentration of 2-3 x $10^6$ cells/mL. The cells were mixed gently, and 1mL of cell suspension was added to each labelled cryo-vial (Corning) and placed in isopropanol filled Mr. Frosty freezing containers (Thermo Scientific) at -80°C. Cells were transferred to liquid nitrogen once frozen.

### 2.2.3 Transduction of human breast cancer cell lines with mKate2 and Luciferase-Tomato-Puro (LTP).

#### 2.2.3.1 Bacterial cell transformation

mKate2 and LTP plasmids (Appendix 1) were a kind gift from Prof. Richard Iggo (University of Bordeaux). Bacterial cell transformation was carried out by mixing 1.5µL of plasmid (mKate2 or LTP) with 10µL of Stbl3 E. coli cells to give a final concentration of 2 x $10^9$ cells/µg of DNA. The DNA-cell mixture was transferred into a chilled 1 mm-gap cuvette for electroporation at 1250 volt using Gene Pulser Xcell$^{Tm}$ Electroporation System. The cuvette was then flushed with 400µL SOC media (Super Optimal broth with Catabolite's repression) and the cells were recovered by incubation at 37 °C for 30min. After incubation, cells were streaked on Luria broth (LB) plates containing 100µg/mL ampicillin. Plates were incubated overnight at 37°C.

### 2.2.3.2 Preparation of plasmid DNA

Plasmid mini-cultures were prepared by inoculating a single bacterial colony containing the plasmid of interest (from section 2.2.3.1) into 3mL LB media containing 100µg/mL ampicillin and grown overnight at 37°C with vigorous shaking. Small-scale plasmid (mini-prep) preparations were performed using the QIAquick Plasmid Miniprep Kit according to the manufacturer's instructions. Briefly, 1mL of the bacterial culture was transferred to a 1.5mL microcentrifuge tube and centrifuged at 14,000g for 30sec followed by resuspension in 100µL of buffer P1 (containing 100µg/ml RNase A). For cell lysis, 100µL of P2 buffer (containing 1%SDS and 200mM NaOH) was added and mixed well by inverting the tube 4-6 times. To neutralise the pH of the cell lysate, 100µL of P3 buffer (3.0M potassium acetate, pH 5.5) was added and gently mixed by inverting the tube several times. Separation of cell debris from plasmid DNA was performed by centrifugation at 14,000g for 5min at room temperature (RT). After centrifugation, the upper phase was transferred into a new microcentrifuge tube and an equal volume of isopropanol was added, mixed well, and incubated for 10min at RT. DNA was pelleted by centrifugation at 12,000g at 4°C for 30min, washed with 70% ethanol, and resuspended in nuclease-free water. The resulting DNA concentration and purity were determined by spectrophotometry using a Thermo Scientific NanoDrop 2000.

Plasmid maxi-cultures were prepared in a volume of 250mL as described above by 1/1000 dilution of starter cultures. Plasmid DNA prep (large scale) was performed using the NucleoBond Xtra Maxi Plus kit (Macherey-Nagel)

according to the manufacturer's instructions. Bacterial cells were harvested by centrifugation of maxi-cultures at 4000g for 30min at 4°C. The supernatant was poured off and the cell pellet was resuspended with 8mL of resuspension buffer. Lysis solution (8mL) was added to the cells, mixed, and incubated at RT for 5min. Meanwhile, 15mL of equilibration buffer was applied to the column filter to equilibrate the NucleoBond® Xtra column. Following incubation, cell lysate was neutralised with 8mL of neutralisation buffer and mixed thoroughly by inversion. The neutralised bacterial cell lysate was slowly loaded into the column filter and allowed to pass through the filter by gravity flow. The filter was washed with 5mL of FIL-EF wash buffer and the filter was discarded after all the solution passed through the filter. The NucleoBond® Xtra column was then washed with 35mL of ENDO-EF wash buffer followed by 15mL of WASH-EF wash buffer. To elute the plasmid DNA from the column, 5mL of elution buffer was added into the column and the flowthrough elute containing the plasmid DNA was collected in a sterile 15mL conical tube. DNA was precipitated using isopropanol as described above and dissolved in 200µL of endotoxin- free water.

### 2.2.3.3 Transfection

Lentiviral particles (LVs) were produced by transfection of HEK 293T/17 cells with vector plasmid (mKate2 or LTP) and packaging plasmids (psPAX2, Addgene, #12260; pMD2-G Addgene, #12259). 293T/17 cells were seeded at a density of 2 x1 $0^6$ in T-75 tissue culture flasks and incubated until cells reached ~70% confluence. DNA solution was mixed with polyethylenimine (PEI), at a ratio of 1:3, allowed to form complexes for 15min at RT and then 1mL of the

complex mixture was added to the cells. The medium was replaced with Opti-MEM media after 12hr. Conditioned medium containing viral particles was harvested after 48hr, concentrated 200-fold using Vivaspin 20 Ultrafiltration columns (GE Healthcare) by centrifugation at 3000g at 10°C and stored at -80°C.

MDA-MB-453 and MFM-223 cells were seeded in 6-well cell culture plates (CoStar) at a seeding density of 4 x $10^5$ cells/well and infected with mKate or LTP viral particles at a multiplicity of infection equal to 1. After 48hr, the medium was refreshed with standard media with 2µg/mL puromycin. Cells were expanded under puromycin selection for at least two passages, then frozen into multiple cryovials and stored in liquid nitrogen for future assays.

### 2.2.4 Proliferation and apoptosis assays

Cells were seeded into 96-well cell culture plates (CoStar) in media containing IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent (final concentration 5µM) and IncuCyte® NucLight Rapid Red Reagent for Live-Cell (diluted 1:2000). For cell lines already labelled with mKate2, only Caspase-3/7 Green Apoptosis Reagent was added. Plates were imaged over a period of 5-7days, collecting four images per well every 3 hours at 10x magnification using the IncuCyte S3 (Essen Bioscience). The resultant images were analysed to determine the number of live and apoptotic cells using the IncuCyte S3 analysis software.

## 2.2.5 Western blot

### 2.2.5.1 Preparation of cell lysates

For protein extraction, cells were seeded in 6-well cell culture plates (CoStar), allowed to attach for at least 24hr and then treated as designated for each experiment. After treatment, plates were placed on ice, the media removed, and the cells were washed three times with 1x cold PBS. Cells were then harvested into 1.5mL Eppendorf tubes with radioimmunoprecipitation assay buffer (RIPA) supplemented with 1x cOmplete Protease Inhibitor and 1x Halt Phosphatase inhibitor using a cell scraper. Protein lysate was passed through a 27-gauge needle to shear DNA and centrifuged at 10,000g for 15min at 4°C to pellet cell debris. The supernatant (containing the protein) was transferred to a new 1.5mL microcentrifuge tube and stored at -80°C.

### 2.2.5.2 Protein concentration assay

The total protein concentration of cell lysates was quantified by the Pierce BCA protein assay kit (Thermo Scientific). Six different concentrations of bovine serum albumin (BSA, 0-5mg/mL) were used as protein standards. The appropriate volume of protein standards or 1µL of protein samples were added into each well of a flat bottom 96-well plate (CoStar). After that, 200µL of BCA working reagent (WR) (prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B) was added into each well and incubated at 37°C for 30min. Absorbance of protein samples was measured using a PolarStar microplate reader at a wavelength of 562nm. Protein concentration in the test samples was calculated against the standard curve.

### 2.2.5.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were prepared for electrophoresis by denaturing ~40µg of lysate in a final concentration of 1x loading dye buffer at 90°C for 5min. Samples were then loaded into 4-12% Bis-Tris gradient SDS-PAGE gels (Criterion$^{TM}$ Precast gel, Bio-Rad) for separation with 1x concentration MOPS running buffer (Bio-Rad) at a constant voltage of 85V for 20min followed by 150V for 40min (until the dye run off the gel). 5µL of Precision Plus Protein Dual Colour standard was used as size marker.

### 2.2.5.4 Protein transfer and immunoblotting

Transfer was performed using a standard wet transfer protocol using a nitrocellulose membrane (GE Healthcare life sciences). Resolving gel and nitrocellulose membrane were placed in-between wet filter papers (Whatman) and sponge pads (Bio-Rad) then placed into a transfer cassette (Bio-Rad), which was placed into a transfer tank (Bio-Rad Criterion Blotter) filled with chilled 1x transfer buffer and a frozen ice pack. Protein transfer was carried out at a constant current of 400mA for 60min. Ponceau staining of the nitrocellulose membrane was used to confirm transfer of proteins. Membranes were de-stained by washing with 1x TBST (Tris-buffered saline, 0.1% Tween 20) for 10min. The nitrocellulose membrane was blocked in TBS containing 5% skim-milk powder for 2hr at RT. The primary antibody was diluted to the optimal dilution in TBS buffer with 1% BSA and incubated with the membrane at 4ºC overnight on a rocking tray. After three washes with TBST, the

membranes were incubated with HRP-conjugated secondary antibody for 1hr at RT. Signals were visualised with Clarity Western ECL Substrate (Bio-Rad) as described by the manufacturer and imaged using ChemiDoc MP (Bio-Rad) imaging system. Protein expression was determined by densitometry using Image Lab Software (v5.2.1).

### 2.2.6 RNA isolation and quantitative real time-polymerase chain reaction (qRT-PCR)

#### 2.2.6.1 RNA extraction

Cells were grown in 6-well cell culture plates for RNA extraction, washed three times with 1x PBS and harvested into 2mL Eppendorf tubes with Trizol (1mL/well). Trizol samples were incubated at 37°C for 15min then mixed with 200mL chloroform and shaken vigorously for 15sec, followed by incubation for 3min at RT. Samples were centrifuged at 12,000g for 15min at 4°C. After centrifugation, the upper aqueous phase was transferred to a new 1.5mL microcentrifuge tube and mixed well with 500µL isopropanol. Samples were incubated at RT for 10min, and RNA was pelleted by centrifugation at 12,000g at 4°C for 30min. Pellets were washed with 70% ethanol, allowed to dry in air for 15-20min, and then dissolved with ~35µL nuclease-free water. Samples were stored at -80°C until future use.

#### 2.2.6.2 DNase treatment

Turbo-DNase kit (Thermo Scientific) was used for DNA digestion to eliminate genomic DNA contamination from the extracted RNA, according to the manufacturer's protocol. Briefly, 10µL of 10x Turbo DNase buffer and 1µL of

Turbo DNase were added to 2µg RNA and incubated at 37°C for 30min. To inactive DNase activity, 5µL of the inactivation reagent was added for 5min at RT with constant mixing. Samples were centrifuged at 10,000g for 1.5min and supernatant was collected. RNA precipitation was conducted by incubating samples with 50µL 75% isopropanol and 2µL glycogen, and incubated overnight at -80˚C. The following day, RNA was centrifugated at 12,000g at 4°C for 30min, washed with 70% ethanol and resuspended in ~35µL nuclease-free water. The resulting RNA concentration and purity were determined using Thermo Scientific NanoDrop 2000.

### 2.2.6.3 Reverse transcription

Total RNA was converted to cDNA using iScript Select cDNA Synthesis Kit (Bio-Rad) before performing qRT-PCR. 1µg of RNA was diluted with nuclease-free water to a volume of 15µL. Diluted RNA samples were mixed with 5µL iScript reaction buffer and 1µL of reverse transcriptase. A control containing all components except RNA was also included. iScript reactions were run in an iCycler thermocycler using the following conditions: 25˚C for 5min, 42°C for 30min, 85°C for 5min and 4°C hold. The cDNA was diluted 5-fold in nuclease free water and stored at -20°C until required.

### 2.2.6.4 Quantitative RT-PCR (qRT-PCR)

Quantitative RT-PCR was performed on a CFX384 Real-Time PCR Detection System (Bio-Rad). Master mixes were prepared with 0.5µL of 5µM forward and reverse primers, 5µL iQ SYBR Green Supermix (Bio Rad), 2µL nuclease free water, and 2µL cDNA. Quantitative RT-PCR conditions were as follows:

95 °C for 3min followed by 40 cycles of 95 °C for 15sec, 55 or 60 °C for the annealing temperature (dependent on the primer set used), 30sec and 72 °C for 30sec. Melt curve was determined by a temperature increase from 60˚C to 95˚C in 0.5˚C increments. qRT-PCR reaction primers and annealing temperature are listed in Table 2.3. Data were analysed using CFX Manager Software Version 3.0 (Bio-Rad). Relative gene expression was determined by the ΔΔCT method using GAPDH as an internal control for quantification analyses of gene targets.

### 2.2.7 Cell cycle analysis

#### 2.2.7.1 Fixation and staining

Cell cycle was monitored by measuring DNA content using the DNA-binding fluorescent dye propidium iodide (PI). For cell cycle analysis cells were grown in 6-well cell culture plates. On the day of harvest, growth media (containing dead cells) was collected in a 50mL conical tube, and cells were detached with trypsin and added to the same tube containing the media. Cells were centrifuged at 1500g for 5min, pellets were resuspended in 500µL PBS, slowly dropped into 4.5mL of pre-chilled 70% ethanol and incubated overnight at 4 °C. Fixed cells were washed with PBS, then pelleted and resuspended in 1mL of PI solution (50µg/mL PI 0.1% Triton X-100 and 100µg/mL RNase A) and incubated for 30min at RT.

#### 2.2.7.2 Flow cytometry analysis

PI-stained cells were analysed on FACS Canto II (BD Biosciences) running CellQuest software (BD Biosciences). Prior to sample run, the instrument was set up with calibration particles (BD™ CS&T beads) to perform quality control

of the instrument's optics, electronics, and fluidics. A blue laser operating at 488 nm was used for excitation and PI fluorescence was collected using the red channel (560-615nm). A medium flow rate (60 µL/min) was used, and 1 x $10^5$ nuclei were analysed from each sample. The flow rate and amplification settings were kept constant throughout the experiment. A PI fluorescence area (PI-A) vs. PI fluorescence width (PI-W) dot plot was drawn on a linear scale to eliminate clumps and aggregates using qualitative gating. A PI-A histogram was drawn to view nuclear DNA content. Data were processed using FlowJo V10.6.

### 2.2.8 Chromatin immunoprecipitation (ChIP) coupled with PCR (ChIP-PCR) or sequencing (ChIP-seq)

#### 2.2.8.1 Harvesting and cross-linking

The MDA-MB-453 or MFM-223 cell lines were seeded into 150mm tissue culture dishes (to a confluency of approximately 70%; 4 x dishes per treatment) in normal maintenance culture media, allowed to attach for 24-48 hrs, then washed with warm PBS and plated in culture media supplemented with 5% steroid-stripped FBS for three days, with daily media changes to ensure a steroid-free environment, and then treated as designed for each experiment. Three independent replicate experiments were performed, representing consecutive passages of cells. Cells were cross-linked with 1% formaldehyde in solution A for 10min and quenched with 2mM glycine. Cells were washed three times with ice-cold PBS and harvested into 1mL ice-cold PBS containing 1x concentration protease inhibitors cocktail (Roche) using a cell scraper. Cells

were centrifuged at 1,300g at 4°C for 3min, the supernatant was removed, and cell pellets snap-frozen on dry ice and stored at 80° C.

### 2.2.8.2 Lysis and sonication

Frozen cell pellets were thawed on ice for 30min and resuspended in cell lysis buffer (LB1), rotated for 10min at 4°C, pelleted at 2,000g at 4°C for 5min, discarding the supernatant. Cell pellets (containing the nuclear content) were resuspended in nuclear lysis buffer (LB2), incubated for 10min at 4°C and centrifuged at 2,000g for 5min. Chromatin was resuspended in lysis buffer 3 (LB3) and sonicated to produce chromatin fragments of 100-500bp. Sonication was performed with a Diagenode Bioruptor® Plus UCD 300-TO for 10min cycling with 30sec 'ON' and 30sec 'OFF'. Sonicated lysate was centrifuged for 10min at 20,000g at 4°C to remove debris. 1% of the sonicated chromatin was taken as input control. The rest of the chromatin samples were immunoprecipitated overnight with 5µg of mouse monoclonal TFAP-2β antibody (Santa Cruz, # SC-390119) bound to Protein G Dynabeads (Thermo Scientific, #10004D), 5µg of rabbit polyclonal AR antibody (Abcam, #ab108341) or 2µg rabbit polyclonal H3K27ac antibody (Abcam, # Ab4729) bound to Protein A Dynabeads (Thermo Scientific, #10002D). The following day, beads were washed six times with ice-cold RIPA buffer followed by one wash with cold TE buffer supplemented with 50mM NaCl to remove unbound DNA. Protein-DNA complexes were eluted from the beads by resuspension in 200µL elution buffer. 150µL of elution buffer was also added to the input sample and both the input and test (ChIP) samples were incubated overnight at 65°C to reverse protein-DNA cross links. The following day, the supernatant

was collected and diluted with a 200µL elution buffer, ready for DNA purification.

### 2.2.8.3 DNA purification

For DNA extraction, 8µL of 1mg/mL RNase A was added to ChIP and input samples and incubated at 37°C for 1hr. Proteins were digested by incubation with 20mg/mL Proteinase K for 2hr at 55°C. DNA was purified using 400µL of phenol-chloroform-isoamyl alcohol (25:24:1), vigorously mixed and spun at 10,000g for 5min to separate phases. The top aqueous phase was collected into a new microcentrifuge tube and DNA precipitation was performed with 2µL glycogen and 800µL 100% ethanol overnight at 80°C. DNA was pelleted at 14,000g for 30min, washed with 70% ethanol and air-dried. The pellets were resuspended in 20µL 10mM Tris-HCl pH 8.0. DNA concentration was measured using Qubit® 2.0 fluorometer (Invitrogen).

### 2.2.8.4 ChIP-PCR and ChIP-seq

ChIP-PCR reactions were prepared using iQ SYBR Green Supermix (BIO-RAD) and primers as listed in Table 2.3. Each qRT-PCR reaction contained 2µL ChIP DNA, 1µL primer mix (0.5µL of 5µM forward and reverse primers each), 5µL SYBR green master mix and 2µL of nuclease free water. PCR conditions were as follows: 95 °C for 3min followed by 40 cycles of 95°C for 15sec, 60°C for 30 sec and 72°C for 30sec. Negative controls (without addition of DNA) were included in each run. PCR was performed with the CFX384 Real Time PCR Detection System (Bio-Rad). ChIP-PCR data was analysed

using the %Input method, and further depicted as Fold Enrichment over negative control (NC).

For sequencing, 5ng of DNA (ChIP-enriched or input) was used for library preparation with an Illumina TruSeq ChIP Library Prep Kit (Illumina) and sequenced (75bp single-end reads) at the South Australian Health and Medical Research Institute, Genomics Facility, using an Illumina NextSeq 500. All computational analyses were performed using Galaxy Bioinformatics Platform (Jalili et al. 2020) (for more details see chapter three, section 3.2.2.2)

## 2.2.9 Immunohistochemistry

### 2.2.9.1 Preparation of tissue for paraffin embedding.

Extracted tissues were immediately fixed in 10% neutral buffered formalin and incubated overnight at 4°C. The following day, tissues were transferred into 70% ethanol and then processed for dehydration using the Tissue-Tek VIP automated tissue processor (Sakura, Torrance, CA). After processing, tissues were embedded in paraffin wax using the Leica embedding machine and stored at RT until required. Paraffin sections were cut using a Leica sledge microtome at 5µm and floated in 37°C water bath. The sections were collected on superfrost slides (Thermo scientific) or Adhesive microscope slides (TRAJAN), and incubated overnight at 50°C.

### 2.2.9.2 Immunohistochemistry staining

Sections were deparaffinized in three changes of xylene for 5min, followed by dehydration using 100% ethanol. The endogenous peroxidase activity was

blocked using 0.9% $H_2O_2$ in PBS for 15min followed by two washes in 1x PBS for 5min. Slides were subjected to heat-induced epitope retrieval using Decloaking Chamber (BioCare-medical) in 10mM citrate buffer (pH 6.5). Non-specific immunoglobulin binding was blocked by incubation with 5% goat serum in 1x PBS for 30min. The blocking solution was then replaced with the required primary antibody at the optimal concentration and incubated overnight in a humidified chamber at 4°C. After washing slides twice with 1x PBS, the secondary biotinylated antibody was applied for 1hr at RT. Horseradish streptavidin-peroxidase conjugate was applied for 1hr, then the sections were incubated with the peroxidase substrate, 3-3′-diaminobenzidine chromogen (DAB) in tris-buffer (pH 7.4) containing 0.01% $H_2O_2$ for 6min, to develop a brown reaction product. Slides were then washed twice with 1x PBS and counterstained with Mayer's hematoxylin for approximately 2min. Once stained, the sections were then dehydrated in 100% ethanol and placed in xylene. Mounting media was added to the tissue sections and a cover slip placed carefully to seal the tissue, minimising bubble formation. Appropriate positive and negative (minus primary antibody) controls were included in all runs. Slides were scanned using a NanoZoomer 2.0HT Microscopy System (Hamamatsu) and visualised using NDPView2 software.

### 2.2.9.3 Eosin and hematoxylin staining (H&E)

Paraffin-embedded tissue sections (5µM) were baked onto superfrost slides for 45min at 50°C before xylene deparaffinization and rehydration with 100% ethanol. Slides were washed three times with 1x PBS and incubated with hematoxylin for 5min. Excess hematoxylin was removed from the tissues by

several dips in acid alcohol and subsequently rinsed in tap water for at least 10min. Slides were stained with eosin (0.1%) and after 5min washed thoroughly with tap water. The sections were then dehydrated in 100% ethanol, placed in xylene, and mounted using coverslips (20 x 20 mm) and DPX (Dibutylphthalate Polystyrene Xylene) media. Slides were scanned using a NanoZoomer 2.0HT Microscopy System (Hamamatsu) and visualised using NDPView2 software.

### 2.2.10 Small interfering RNA (siRNA) transfection

siRNA transfection was performed with Lipofectamine RNAiMax reagent (Thermo Scientific) according to the manufacturer's instructions. Briefly, a 1:100 mixture of Lipofectamine RNAiMAX transfection reagent and pre-warmed Opti-MEM media was prepared and incubated at RT for 10min. siRNA oligomer (20μM) was added to the mixture at final concentrations of 2.5, 5, and 10nM and incubated for 20min at RT to allow formation of liposomes. Nonspecific siRNA (AllStars Negative Control siRNA) was used at a concentration of 10nM as a negative control. MDA-MB-453 cells were detached from the tissue culture flask using trypsin and counted using haemocytometer. For western blot, RNA extraction, and cell cycle analysis, 500μL of the siRNA-lipofectamine mixture was added to each well of a 6 well plate. For reverse transfection, 7.5 x $10^5$ cells were added into 2.5mL of media. Cells were harvested after 72hr for protein and cell cycle analysis and after 48hr for RNA extraction.

Cell proliferation assay and ChIP-seq were performed in 96-well cell culture plates and 150-mm cell culture dishes, respectively. Accordingly, the amount

of siRNA-lipofectamine mixture used was changed based on the setup of the experiment. For cell proliferation assays, 1.5 x $10^4$ cells were added in 100µL media to 20µL of siRNA-lipofectamine mixture/well. Proliferation assay was conducted using IncuCyte S3 (as described in section 2.2.4). For ChIP-seq experiments, 1.2 x $10^7$ cells in 15mL media were combined with 3mL of siRNA-lipofectamine mixture, incubated for 48hr at 37°C in a humified incubator with 5% $CO_2$, then treated with vehicle (EtOH) or 10nM DHT for 4hr and processed as described above in section 2.2.8.

### 2.2.11 *In situ* proximity ligation assay (PLA)

#### 2.2.11.1 Sample preparation

The PLA assay was performed on formalin fixed paraffin embedded tumour tissues and 2D adherent tissue culture cells using a Duolink II Detection Kit (Sigma). For tumour tissues, the standard IHC protocol was followed up until the protein blocking step. For 2D adherent cells, MDA-MB-453 cells were seeded on glass coverslips (20 x 20 mm) at a density of 3.5 x $10^5$ in 6-well cell culture plates and left to attach for 48hr. Where appropriate, MDA-MB-453 cells were treated with vehicle (EtOH) or DHT for 6hr prior to collection. Cells were fixed in 10% neutral buffered formalin for 10min at RT. Cellular membranes were permeabilised with 0.1% Triton X-100 for 1hr at RT and washed three times for 5min in 1x PBS.

Tumour tissues and cell line samples were protein blocked by adding 10% Donkey serum in PBS (matching the host species of the labelled secondary antibody) and incubated for 30min at RT in a humidity chamber. The blocking solution was then replaced with a 1:400 dilution of rabbit anti-AR (LifeSpan

Biosciences, #B3326) and 1:400 dilution of mouse anti-TFAP-2β (Santa Cruz, # SC-390119) antibodies and incubated overnight in a humidified chamber at 4°C. After three washes with 1x PBS, the samples were incubated with PLA probes supplied with the kit, diluted 1:5 in Duolink diluent for 1hr at 37°C in a humidity chamber. Samples were washed twice for 5min in 1x wash buffer A. PLA probes were ligated by incubating with ligase enzyme diluted 1:40 in ligation buffer for 30min at 37°C in a humidity chamber. Samples were again washed in 1x wash buffer A before incubation with polymerase enzyme that was diluted 1:80 in amplification buffer for 100min at 37°C in a humidity chamber. Following incubation, samples were washed twice for 10min in 1x wash buffer B and then washed once in 1:100 diluted wash buffer B for 1min. After that, samples were stained with DAPI (1/1000 in PBS) for 1min, washed twice for 5min in PBS in the dark, mounted with DAKO mounting medium, and stored in the dark at RT until analysis. The signal was visualized as a distinct fluorescent focus and was captured using Texas red and DAPI filters on the Olympus FV3000 confocal microscope (Adelaide Microscopy, The University of Adelaide).

### 2.2.11.2 PLA Image Analysis using ImageJ.

Analysis of PLA images was performed as previously described (Law et al. 2017). Images from single scans were analysed in ImageJ to calculate the number of PLA puncta. Images (at least four images/replicate) were first transformed to 8-bit, and a threshold was then selected manually to discriminate PLA puncta from background fluorescence (Image > Adjust > Threshold). Once selected, this threshold was applied uniformly to all images in the sample set. The built-in ImageJ macro "Analyze Particles" was then used to count and characterise all objects in the threshold image.

### 2.2.12 *Ex vivo* explant of normal breast tissue.

Ethical approval for this study was granted by the University of Adelaide Human Research Ethics Committee (approval#: H-2015-175). Normal breast tissues were obtained (with a written informed consent form) from women undergoing breast reduction surgery at the Flinders Medical Centre, Adelaide, South Australia. Tissues were cultured *ex vivo* as previously described (Centenera et al. 2018; Dean et al. 2012). In brief, tissues were transported on ice to the laboratory and washed with phenol red free RPMI 1640 medium to remove blood. Adipose tissue was removed using a sterile scalpel, and the remaining connective tissue containing glandular elements was dissected into approximately $1mm^3$ pieces. Tissue pieces were placed onto gelatin dental sponges (Ethicon) pre-soaked in phenol red free RPMI 1640 media supplemented with 10%FBS, 2mM L-glutamine, 10µg/mL human recombinant insulin, 10µg/mL hydrocortisone, 1x concentration antimycotic-antibiotic and 100nM E2 and incubated at 37°C with 5% $CO_2$. After 24hr, the medium was

removed and replaced with a culture medium supplemented with treatments, followed by culture for a further 48hr. Four tissue pieces from each case were randomly allocated to each treatment on a single sponge. Cultured tissue pieces were fixed in 10% neutral-buffered formalin at 4°C overnight, dehydrated, embedded in paraffin wax using the Tissue-Tek VIP automated tissue processor (Sakura, Torrance, CA) and used for H&E and Ki-67 IHC staining.

### 2.2.13 Mammary intraductal (MIND) xenografts

#### 2.2.13.1 Ethics approval and animals

Animal experiments were approved by the University of Adelaide Animal Research Ethics Committee (approval#: M-2018-088). Female NGS (NOD scid gamma mouse) mice were purchased from Animal Research Centre (Perth, Western Australia). All the animals used in this study were between 10 –12 weeks of age. Mice were housed at the Adelaide Health and Medical Sciences BioResources Facility, (The University of Adelaide). Mice were kept in a 12-hour light-dark cycle, temperatures of 19-23°C with 40-60% humidity, fed and watered *ad-libitum*, under pathogen-free conditions and were carefully monitored throughout the experiment, as per ethically approved clinical monitoring guidelines.

#### 2.2.13.2 MIND injection of breast cancer cells

The MIND model as established by Professor Iggo, as previously described (Richard et al. 2016; Sflomos et al. 2016). The day before injection, the mice were anesthetised using isoflurane (4-5%) and the fur coat on the lower

abdomen around the fourth inguinal nipples was removed using hair removal cream (Veet). The next day, the nipples were surgically cleaned with iodine solution followed by 100% ethanol, and the tip of the nipples was cleaved with spring scissors. A total of 2 x $10^5$ LTP labelled MDA-MB-453 cells were resuspended in 10µL normal growth media and injected into one of the fourth inguinal mammary ducts of NSG mice using a 50-gauge blunt-ended Hamilton syringe. Once all the 4th glands were injected, a topical analgesic (Lidocaine) was applied to the nipple for analgesic, and the mouse was placed on a heating pad for recovery to compensate a loss of body temperature. Paracetamol analgesic (300mg/kg) was given to the mice and removed 3 days post injection.

Tumours were allowed to establish for 5 days, and then mice allocated by simple randomisation to receive vehicle (n =10) or D-11 (10 mice received 8 doses of 200mg/kg/day and then 7 doses of 150mg/kg/day due to toxicity concerns) for 15 consecutive days. Tumour growth was monitored every week by injection of mice with 100µL of D-luciferin (30mg/mL) followed by bioluminescent imaging with the IVIS Lumina X5 imaging system (PerkinElmer). Photon counts per second were recorded using IVIS, and the injected mammary glands were harvested for IHC at the completion of the experiment. Spleen and liver tissues were collected and stained with H&E for pathological analysis of tissues.

### 2.2.14 Statistical analysis

Statistics were performed using Graphpad Prism 9.0. Two-tailed Student t tests, one-way or two-way ANOVA tests were used as indicated in the results sections. The half-maximal inhibitory concentration ($IC_{50}$) of CDK9i D-11 was

determined from the generated concentration-response curves using non-linear regression curve fit in GraphPad 9 software. All data are expressed as the mean ± SEM. A value of $P < 0.05$ was considered statistically significant.

# CHAPTER THREE

# MECHANISMS GOVERNING AR ACTIVITY IN ERα-NEGATIVE BREAST CANCER CELL LINES WITH DIVERGENT PROLIFERATIVE RESPONSES TO ANDROGENS

## 3.1 Introduction

Molecular apocrine and some TNBC tumours are sensitive to androgens as they express high levels of AR and target genes known to be regulated by AR in prostate cancer (Doane et al. 2006; Farmer et al. 2005; Lehmann et al. 2011). Although these tumours express high levels of AR, how AR signalling impacts their growth remains an area of controversy. The potent androgen DHT stimulates proliferation of the MDA-MB-453 and HCC-202 breast cancer cell lines, considered models of molecular apocrine breast cancer (Cochrane et al. 2014; Doane et al. 2006; Naderi & Hughes-Davies 2008; Ni et al. 2011; Park, IH et al. 2019), suggesting an oncogenic role. Nevertheless, this proliferative response to androgen is not seen in all models of ERα-negative/AR-positive breast cancer. Activation of AR by DHT inhibits proliferation of the TNBC cell lines MFM-223 and CAL-148 (Hackenberg et al. 1993; Thakkar et al. 2016; Thorek et al. 2019), which are both genetically similar to MDA-MB-453 as they transcriptionally cluster with this cell line due to high expression of AR and known AR target genes (Lehmann et al. 2011). The precise molecular mechanisms by which AR can induce divergent proliferative effects in ERα-negative breast cancer cells is unknown. This chapter bioinformatically assessed data generated from three contemporary genomic technologies to

characterise AR activity associated with proliferative versus anti-proliferative cellular responses in ERα-negative/AR-positive breast cancers, with the view to understanding the mechanistic basis of oncogenic versus tumour suppressive effects. The following paragraphs introduce the methodologies used to generate the data analysed for this chapter: RNA-seq, ChIP-seq and RIME.

RNA sequencing (RNA-seq) uses the capabilities of high-throughput sequencing technology to provide insight into genome-wide transcriptional profiles (termed the transcriptome) (Han et al. 2011). RNA-seq is widely regarded as superior to other gene expression analysis approaches (Han et al. 2011). Unlike hybridisation-based approaches (e.g. microarrays), RNA-seq does not require transcript-specific probes, thereby avoiding hybridisation issues and offering unbiased detection of known and unknown transcripts (Kukurba & Montgomery 2015; Wang, Zhong, Gerstein & Snyder 2009). Additionally, RNA-seq allows genome-wide analysis of transcription at single nucleotide resolution with low background signal, as the cDNA fragments generated from an RNA sample can be unambiguously mapped to unique regions on the genome (Kukurba & Montgomery 2015; Wang, Zhong, Gerstein & Snyder 2009). The benefits of this gene expression analysis technology have been evolving to better characterise gene expression profiles of breast cancer cells under different experimental conditions (Bi et al. 2020; Moore et al. 2012b). For example, a previous study from our laboratory used microarray technology to examine gene expression changes in response to DHT and the synthetic progestin, MPA, in the MDA-MB-453 cell line (Moore et al. 2012b). A recent study by Bi and colleagues used RNA-seq technology to screen transcriptomes of parental and tamoxifen-resistant (TAMR) MCF-7

breast cancer cells to identify gene expression changes associated with tamoxifen resistance in ERα-positive breast cancer (Bi et al. 2020). Herein, we used RNA-seq technology to examine AR transcriptional targets in two ERα-negative/AR-positive cell lines, MDA-MB-453 and MFM-223, with similar expression levels of AR but divergent growth responses to AR agonists.

Chromatin immunoprecipitation sequencing (ChIP-seq) is a powerful method for identifying genome-wide chromatin binding sites – termed the cistrome - of DNA-associated proteins (Schmidt et al. 2009). Before ChIP-seq, chromatin immunoprecipitation coupled with microarray (ChIP-chip) was the method for studying protein–DNA interactions (Buck & Lieb 2004). However, due to limitations of the ChIP-chip technology including low resolution, high signal to noise ratio, and a dependence on hybridisation (Laajala et al. 2009; Trapnell et al. 2010), ChIP-seq has become the technique of choice amongst the research community as it has many advantages (Cao, Jia et al. 2016). Like RNA-seq, ChIP-seq has high resolution at the single base-pair level and no limitation on genome coverage (Ho et al. 2011). Moreover, ChIP-seq has a higher signal-to-noise ratio compared to ChIP-chip due to the absence of the hybridisation step, and can work from a much smaller amount of starting material (Ho et al. 2011). The basic protocol of ChIP-seq involves fixation of cells or tissue with formaldehyde to form covalent linkages between DNA-binding proteins and DNA, cell lysis, chromatin purification and subsequent fragmentation, followed by isolation of specific DNA–protein complexes through immunoprecipitation with protein-specific antibodies for a factor of interest (Furey 2012). The DNA isolated from the immunoprecipitated complexes is

then amplified by a polymerase chain reaction (PCR) and sequencing-specific adapters ligated to the end products to form a library (Furey 2012). After this step, the amplicons are sequenced using next generation sequencing (NGS) technology (Mundade et al. 2014). The reads resulting from sequencing are mapped to the genome, and location of the sequenced DNA is identified, marking regions of protein-bound DNA for the target of interest (Mundade et al. 2014). ChIP-seq technology has been used to generate cistromes representing ERα (Carroll et al. 2006; Chi et al. 2019; Piggin et al. 2020; Ross-Innes et al. 2012; Singhal et al. 2016) and AR (Ni et al. 2011; Robinson, JL et al. 2011) in breast cancer cells. Through ChIP-seq technology Carroll and colleagues showed that ER cistrome was different in good versus poor outcome primary breast tumours and in endocrine sensitive versus resistant models (Ross-Innes et al. 2012). Cistrome analysis also furthered mechanistic understanding of AR signalling in the MD-MB-453 cell line (Ni et al. 2011; Robinson, JL et al. 2011). The study by Robinson *et al* (2011) mapped AR binding events for the first time in breast cancer cells and observed a greater overlap between the AR cistrome of MDA-MB-453 cells and the ERα cistrome of MCF-7 breast cancer cells compared to the AR cistrome of LNCaP prostate cancer cells (Robinson, JL et al. 2011). In contrast, Ni and colleagues found a larger overlap between the AR cistrome of MDA-MB-453 cells and the AR cistrome of LNCaP cells than the ERα cistrome of MCF-7 cells (Ni et al. 2011). Regardless of this discrepancy, which could be attributed to differences in experimental conditions, the studies concur that the potential oncogenic effect of AR in MDA-MB-453 cells involves AR acting like ERα in a breast cancer context but also acting

like AR in a prostate cancer context (Hickey, T et al. 2012). By using ChIP-seq technology, in this study, we investigated the DHT-stimulated AR cistromes of MDA-MB-453 and MFM-223 breast cancer cells.

Rapid immunoprecipitation (coupled with) mass spectrometry (MS) of endogenous protein (RIME) is an unbiased approach for identifying interacting proteins (defined as the interactome) of a chromatin-bound factor of interest (D'Santos et al. 2015). RIME is used to purify endogenous protein, rather than the use of exogenous tagged approaches and can be used to identify protein complexes from limited amounts of starting material (D'Santos et al. 2015). The basic RIME protocol is similar to the first stages of the ChIP-seq protocol and involves formaldehyde crosslinking performed prior to antibody-based immunoprecipitation of endogenous protein/DNA complexes, with subsequent release of DNA, peptide digestion and MS analysis (D'Santos et al. 2015; Mohammed et al. 2016). The RIME approach has been used to characterise composition of ERα interactomes, identifying novel ERα interacting proteins in ERα-positive breast cancer cells (D'Santos et al. 2015; Mohammed et al. 2013; Mohammed et al. 2015). Moreover, RIME coupled with an amino acid labelling technique (Stable isotope labelling using amino acids in cell culture; SILAC) has allowed quantitative characterisation of changes to the ERα and PR associated interactomes to identify novel coregulators in agonist and antagonist-liganded conditions in ERα-positive breast cancer cells (D'Santos et al. 2015; Mohammed et al. 2013; Mohammed et al. 2015). A recent study has introduced another modified RIME assay called quantitative multiplexed RIME (qPLEX-RIME) to monitor and quantify the dynamics of chromatin-associated complexes

(Papachristou et al. 2018). qPLEX-RIME uses Tandem Mass Tags (TMT), chemical isobaric reagents, to label RIME-generated peptides, which allows the simultaneous analysis of multiple conditions and biological replicates in a single experiment (Papachristou et al. 2018). In the latter study, qPLEX-RIME was applied to discover temporal changes to the ERα interactome in MCF-7 cells treated with tamoxifen and to identify the ERα interactome in patient-derived xenograft (PDX) breast tumours and primary human breast cancer tissues (Papachristou et al. 2018). Our study is the first to use the RIME approach to investigate differences in the AR interactomes of MDA-MB-453 & MFM-223 cells.

This part of my PhD project implemented these contemporary techniques (RNA-seq, ChIP-seq and RIME) to interrogate AR action in two models of ERα-negative/AR-positive breast cancer with divergent proliferative responses to the AR ligand DHT. By examining the AR-associated interactomes, cistromes and transcriptomes, this chapter aimed to identify candidate factors that mediate oncogenic versus tumour suppressive AR activity in ERα-negative breast cancer.

## 3.2 Methods

### 3.2.1 RNA-seq

#### 3.2.1.1 Treatment and sample preparation

RNA-seq data was previously generated by the host laboratory using the following protocol. MDA-MB-453 and MFM-223 cells were cultured in media supplemented with 5% steroid-stripped FBS and treated with either vehicle (EtOH) or 10nM DHT for 6hr. Six independent replicates representing consecutive passages of cells were generated per treatment condition for each cell line. Total RNA was extracted using the RNeasy® kit (Qiagen) according to the manufacturer's instructions. In short, cells were harvested by scraping into RLT buffer containing 1% (v/v) 2- mercaptoethanol. One volume of 70% ethanol was added, and the lysate was homogenized by pipetting. The lysates were transferred to a RNeasy spin column and centrifuged at 9,000g for 15sec. The flow through was discarded and the collection tube was replaced. Samples were washed with RW1 buffer and centrifuged at 9,000g for 15sec. The RNeasy column was placed into a new collection tube, buffer RPE was added, and the sample was centrifuged as above. RNA was eluted with 30-50μL of RNase-free water. The Turbo-DNase kit (Thermo Scientific) was used for DNA digestion to eliminate genomic DNA contamination from the extracted RNA according to the manufacturer's protocol (as described in chapter 2 section 2.2.6.2). Concentration and quality of the RNA samples were measured using the Thermo Scientific NanoDrop 2000. Sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA Library Prep High

Throughput Kit according to the manufacturer's protocol starting with 1µg total RNA. Briefly, purified mRNAs were fragmented and converted to cDNA with reverse transcriptase. The resulting single-stranded cDNAs were converted to double-stranded cDNAs and subjected to end-repair, A-tailing, and adapter ligation. The constructed libraries were amplified using 15 cycles of PCR (98°C for 10sec, 60°C for 30sec,72°C for 30sec,72°C for 5min, and hold at 4°C). Samples were sequenced (40bp single end reads) at the Genomics Core Facility, Cancer Research UK (CRUK), Cambridge Institute, UK, using an Illumina NextSeq.

### 3.2.1.2 RNA-seq data analysis

Bioinformatic analysis of RNA-seq data was conducted using R program (v2.15.1). Sequenced regions were aligned to Human genome 19 (hg19) using TopHat v2.0.9 (Trapnell et al. 2010). The reads were quantified into gene counts using HT-Seq v0.6.0. Read counts were normalised and tested for differential gene expression by the edgeR package from Bioconductor (Chen, Y et al. 2014). Differential expression analysis was conducted using DESeq (Anders & Huber 2010). A gene was considered to be differentially expressed if it exhibited a fold change (FC) of greater than 1.5 (up-regulated gene) or less than 0.6 (down-regulated gene) and if the gene had a p-value less than 0.05. Heat maps and volcano graphs were prepared using RStudio (v1.3.959).

### 3.2.1.3 Functional enrichment analysis of gene ontology

To identify biological processes associated with differentially expressed genes in MDA-MB-453 and MFM-223 cells, I performed functional enrichment analysis of gene ontology using the Gene set enrichment analysis (GSEA)

website (http://www.broad.mit.edu/gsea/index.jsp) and the molecular signature database v7.1 (MSigDB) as the reference gene set (Liberzon et al. 2011). MSigDB hosts an extensive collection of genes that were grouped based on certain criteria such as biological role, canonical pathways, regulatory target genes and cellular component.

## 3.2.2 ChIP-seq

### 3.2.2.1 Treatment and samples preparation

ChIP-seq data was previously generated by the host laboratory using the following protocol. The MDA-MB-453 or MFM-223 cell lines were seeded into 150mm tissue culture dishes at a density of 8 x $10^6$ (4x dishes per treatment) in normal maintenance culture media, allowed to attach for 24-48hr, then washed with warm PBS and plated in culture media supplemented with 5% steroid-stripped FBS for three day, with daily media changes to ensure a steroid-free environment, prior to treatment with either vehicle (EtOH) or DHT (10nM) for 4hr. Treated cells were immediately cross-linked with 1% formaldehyde for 10 min, quenched for 2min with 0.2M glycine, washed several times with cold PBS, and scraped into 1mL of cold PBS containing 1x concentration of a protease inhibitor cocktail. Cells were washed twice in cold PBS containing the protease inhibitor, liquid removed, and pellets frozen at -80C until further processing. For immunoprecipitation (IP), 100 µL of Dynabeads® Protein A beads were pre-absorbed with 5µg of rabbit polyclonal AR antibody (Abcam ER179; ab108341). Chromatin isolation and fractionation, IP and DNA purification were performed as described in chapter two, section 2.2.8. Three independent replicate experiments were performed,

representing consecutive passages of cells. 5ng of DNA (ChIP-enriched or input) was used for library generation with an Illumina TruSeq ChIP Library Prep Kit (Illumina) and sequenced (75bp single-end reads) at the South Australian Health and Medical Research Institute- Genomics Facility using an Illumina NextSeq 500.

### 3.2.2.2 ChIP-seq data processing

#### Quality control, alignment, and peak calling

All computational analyses were performed using the Galaxy Bioinformatics Platform (Jalili et al. 2020). Quality control assessment for the raw sequencing data (FASTQ) was conducted using FastQC (Galaxy v0.72+galaxy1). FastQC checked whether the raw sequencing reads contained PCR primers and adaptors and provided information about the base quality score (Brown, J, Pirrung & McCue 2017). The base quality score, also known as a Phred or Q score is an integer value representing the estimated probability of an error, i.e., that the base is incorrect (Wan, Anh & Asai 2012). The score generally range from 2 to 40 with higher scores indicating greater confidence in the call (Wan, Anh & Asai 2012). All reads were trimmed for adapter sequences and poor-quality bases using the default parameters of Filter by quality tool (Galaxy v1.0.2+galaxy0). Sequenced regions were aligned to hg19 using the Bowtie2 default parameters (Langmead et al. 2009), mapped reads with a minimum mapping quality (MAPQ) <10 and duplicate reads were removed using filter SAM/BAM tools (Galaxy v1.8+galaxy1). To identify genomic regions that were enriched with aligned reads (peak calling), I used the MACS2 tool (Galaxy v2.1.1.20160309.6) with a pooled input sample as the control.

## Consensus bed files

Consensus bed files containing peaks that appeared in at least two of the three replicates (rep) of a particular condition were generated. Intersect-intervals Bedtool (Galaxy v2.29.0) was used to intersect overlapping pieces of intervals between rep 1 and rep 2 (generating file A), rep 1 and rep 3 (generating file B), and rep 2 and rep 3 (generating file C), of a given treatment with a minimum overlap of 1bp. After that, intersected intervals (File A, B, & C) were concatenated together using Concatenate (Operate on Genomic Intervals, Galaxy v0.1.0) and merged to remove any duplicate reads using Bedtools/combine overlapping-nearby intervals into a single interval (Galaxy v2.29.2). Consensus files were used for downstream analysis.

## Peak annotation and motif analysis

Regions of AR chromatin binding (peaks) were annotated with respect to neighbouring genes (≤100 kb from the transcription start site) using CisGenome (v2). Peak annotation results were integrated with the outcomes of the differential expression analysis using Join two files (Galaxy Version 1.1.2) and Filter data (Galaxy Version 1.1.1) tools. Motif analysis was conducted using MEME-ChIP online tool, v5.1.1 (Machanick & Bailey 2011). As MEME-ChIP needs FASTA sequence as an input file, peak summits were extended +/-250bp around the summit using bedtools and FASTA sequence for each centered interval was extracted using Extract Genomic DNA tool (Galaxy V3.0.3+galaxy2). Extended FASTA files were used as an input for MEME-ChIP and motif analysis was performed using default parameters.

**Presentation of ChIP-seq results**

A principal component analysis (PCA) plot and heatmaps were generated from multiBigwigSummary using Generate PCA plots (Galaxy v3.3.2.0.0) and plotHeatmap (Galaxy Version 3.3.2.0.1) tools, respectively. ChIP-seq data was visualized using the Integrative Genomics Viewer (IGV) (Robinson, JT et al. 2011).

## 3.2.3 RIME

### 3.2.3.1 Sample preparation

RIME was previously performed by my supervisor, A/Prof Theresa Hickey, while she was working in the Carroll laboratory (Cancer Research UK, Cambridge Institute) as described (Paltoglou et al. 2017). Briefly, MDA-MB-453 and MFM-223 cells were plated at 8 x $10^6$ in maintenance culture media in 6 x 150 mm tissue culture dishes per condition. After 48hr, cells were cross-linked with 1% EM-grade formaldehyde for 7min, quenched with 2mM glycine for 2min, washed several times with ice-cold PBS, and harvested into ice-cold PBS containing a protease inhibitor cocktail using a cell scraper. Two cell pellets were created for each experiment, representing 6 plates of cells each, one for the AR IP and the other for an IgG control. Two independent replicates were prepared for MDA-MB-453, the first line to undergo investigation, but three independent replicate experiments were subsequently performed for MFM-223 cells. These replicates represented consecutive passages of cells. For IP, Dynabeads® Protein A beads were pre-absorbed with 10µg of rabbit polyclonal AR antibody (Santa Cruz Biotechnology, AR N20: sc-816) or anti-Rabbit IgG (Santa Cruz Biotechnology, sc-2027). For

chromatin isolation, cell pellets were resuspended in LB1 (50mM HEPES-KOH (pH 7.5), 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25%Triton X-100) followed by rotation mixing for 10 min at 4°C. Then, nuclei were pelleted and resuspended in LB2 buffer (10mM Tris-HCL (pH 8.0), 200mM NaCl, 1 mM EDTA and 0.5mM EGTA) and rotated at 4°C for 5min. The samples were then resuspended in LB3 buffer (10mM Tris-HCl (pH 8), 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Na-deoxycholate and 0.5% N-lauroylsarcosine) and sonicated in a water bath sonicator (Diagenode Bioruptor). The bead-bound antibody and sonicated chromatin were incubated overnight at 4°C. The following day, beads were washed 10 times with ice-cold RIPA buffer followed by one wash in freshly prepared 100mM AMBIC (ammonium bicarbonate) to remove unbound proteins. A 10µL trypsin solution (15ng/µL) prepared in 100mM AMBIC was added to the beads followed by overnight incubation at 37°C. The next day, trypsin solution was added for a second digestion step followed by incubation for 4h at 37°C. At the end of the second digestion step, the tubes were placed on a magnet and the supernatant solution was collected and acidified by the addition of 2µL 5% formic acid. Digested peptides were analysed at the Proteomics Core Facility, Cancer Research UK (CRUK) Cambridge Institute, UK, using an LTQ Orbitrap MS system (Thermo Scientific) as described (Paltoglou et al. 2017).

### 3.2.3.2 RIME data analysis

MS peptides reads were identified via the SwissProt human database using the Mascot search engine v2.3.0. Only co-precipitating proteins that occurred in all independent biological replicates were considered. These proteins were

identified using the 'comparing values of two-three lists' tool of BaRC (Bioinformatics & Research Computing) and then further filtering was achieved by excluding proteins that appeared in matching IgG controls. The molecular function of AR co-regulators was determined using Metascape (A Gene Annotation & Analysis Resource) (Zhou, Yingyao et al. 2019).

## 3.3 Results

### 3.3.1 Transcriptional targets of AR in MDA-MB-453 and MFM-223 cells

#### 3.3.1.1 mRNA expression of known AR target genes in the RNA-seq datasets

Results of the RNA-seq experiments were firstly examined to validate changes in mRNA expression of genes that are known to be regulated by androgens in breast and/or prostate cancer. FKBP5 acts as a co-chaperone that modulates steroid hormone receptor activity (Fries, Gassen & Rein 2017). *FKBP5* expression is often used as an indicator of functional AR signalling, as it reflects better than any other AR target gene, the presence of residual androgen after either short or long-term androgen deprivation therapy in prostate cancer (Zhou, Ye, Bolton & Jones 2015). Our results showed that treatment of MDA-MB-453 cells with DHT significantly increased *FKBP5* mRNA expression (FC=4.14, p-value= 6.6E-11) in comparison to vehicle treated cells. Similar results were observed in DHT-treated MFM-223 cells (FC=3.29, p-value= 9.6E-15). *C1orf116* is highly expressed in prostate cancer tissues and has been shown to be up-regulated by androgens in prostate cancer cell line models

(Steketee et al. 2004). Our RNA-seq data showed that DHT significantly induced mRNA expression of *C1orf116* by nine and four-fold in MDA-MB-453 and MFM-223 cells, respectively. Other genes which have been previously shown to be upregulated by DHT or the synthetic androgen R1881 in MDA-MB-453 cells via microarray, such as *SEC14L2*, *RANBP3L*, *UGT2B28*, and *WNT7B* (Moore et al. 2012b; Ni et al. 2013; Ni et al. 2011), also demonstrated increased mRNA levels upon DHT treatment in MDA-MB-453 and MFM-223 cells (Table 3.1). *KLK3* is a key downstream target gene of the AR in normal and malignant prostate tissues and is a serum biomarker to monitor prostate cancer progression (Saxena et al. 2012). Exposure of MDA-MB-453 cells to DHT did not induce expression of *KLK3*, an observation in agreement with previous studies that have reported that *KLK3* is not induced by androgens in this cell line (Magklara, Brown & Diamandis 2002; Magklara et al. 2000; Moore et al. 2012b). Interestingly, in MFM-223 cells, DHT caused a ~19 fold increase in *KLK3* mRNA level relative to the vehicle (Table 3.1). *CLDN8* encodes a protein that forms tight junctions in cell membranes (Ashikari et al. 2017; Zhang, Y et al. 2020) and is associated with several signalling pathways implicated in epithelial cell proliferation and has been identified as an androgen-regulated gene in prostate cancer (Tsukita et al. 2008). *CLDN8* expression was significantly increased (FC=9.44, p-value=8.3E-07) upon DHT treatment in MDA-MB-453 cells (Table 3.1).

Collectively, these examples provide confirmation that the RNA-seq data used in this study demonstrates responses to DHT treatment that are consistent with androgenic responses of previous studies in prostate, validating its use for

characterising transcriptomic differences that might underscore differential proliferation effects of AR in ERα- negative /AR-positive breast cancer cells.

**Table 3.1:** mRNA expression of known AR target genes in response to DHT in MDA-MB-453 & MFM-223 cells. Six independent replicates representing consecutive passages of cells were generated per treatment condition for MDA-each cell lines. p-value was generated using One Way ANOVA.

| **Ensembl gene ID** | **Gene symbol** | **MDA-MB-453 DHT versus vehicle** | | **MFM-223 DHT versus vehicle** | |
|---|---|---|---|---|---|
| | | FC | p-value | FC | p-value |
| ENSG00000096060 | *FKBP5* | 4.14 | 6.6E-11 | 3.29 | 9.6E-15 |
| ENSG00000182795 | *C1orf116* | 9.44 | 6.4E-12 | 3.89 | 2.8E-16 |
| ENSG00000100003 | *SEC14L2* | 41.44 | 1.1E-13 | 5.29 | 2.1E-16 |
| ENSG00000164188 | *RANBP3L* | 18.52 | 1.2E-11 | 16.36 | 5.5E-18 |
| ENSG00000135226 | *UGT2B28* | 32.09 | 1.1E-08 | 14.21 | 2.34E-07 |
| ENSG00000142515 | *KLK3* | ND | ND | 19.91 | 5.58E-05 |
| ENSG00000156284 | *CLDN8* | 9.44 | 8.3E-07 | ND | ND |

ND, not detected

### 3.3.1.2 Differential expression analysis of RNA-seq data

To identify differentially expressed genes (DEG) between vehicle and DHT treatments, the DESeq workflow was used. Hierarchical clustering of genes regulated by DHT versus vehicle was performed to check if the replicates of each treatment group had similar gene expression profiles. As shown in Figure 3.1, replicates of each treatment condition were clustered together in each cell line. Using the filtering criteria of fold change (FC) ≥1.5 and p-value of <0.05, 443 genes were up-regulated in the DHT treatment group compared with the vehicle group in MDA-MB-453 cells (Figure 3.2A). For the down-regulated genes, filtering criteria of FC < 0.6 and p-value of <0.05 were used. DHT significantly down-regulated expression of 304 genes in MDA-MB-453 cells compared with the vehicle (Figure 3.2A). In the MFM-223 cell line, DHT significantly induced mRNA expression of 393 genes (FC ≥1.5) and down-regulated mRNA levels of 323 genes (FC < 0.6) compared with the vehicle (Figure 3.2B). Unique and shared genes between MDA-MB-453 and MFM-223 cell lines were also assessed. About 15.2% (110 genes) of DHT up-regulated genes were common between MDA-MB-453 and MFM-223 cell lines (Figure 3.2C, Appendix 2A). DHT down-regulated genes showed less overlap, with only 37 genes (6.2%) shared between MDA-MB-453 and MFM-223 cells (Figure 3.2D, Appendix 2B). Whereas some genes were up or down regulated by DHT in both cell lines, the majority of DHT-regulated genes were modulated in only one of the two cell lines (Figure 3.2 C-D). In MDA-MB-453 cells, 333 genes (45.9%) were uniquely up-regulated, and 267 genes (45.3%) were uniquely down-regulated by DHT. In MFM-223 cells, treatment with

DHT uniquely up-regulated 283 genes (39%) and downregulated 286 genes (48.5%).

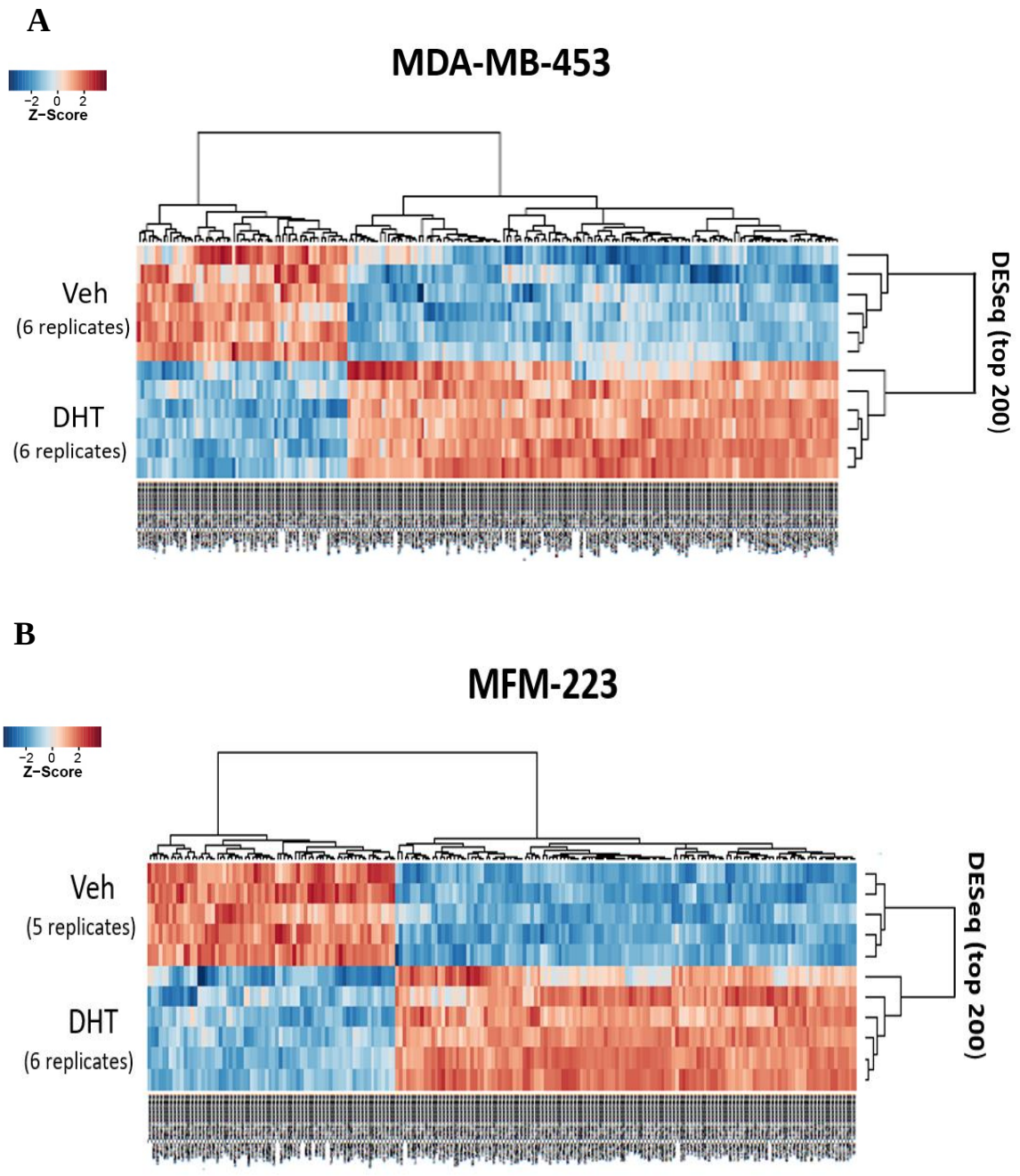



**Figure 3.1:** Heat maps depict the clustering of top 200 differentially expressed genes (DHT versus vehicle) in **(A)** MDA-MB-453, and **(B)** MFM-223 cells. Note that the Veh treatment group for MFM-223 cells only has 5 independent replicates representing consecutive passages of cells because the sequencing run failed for one replicate.

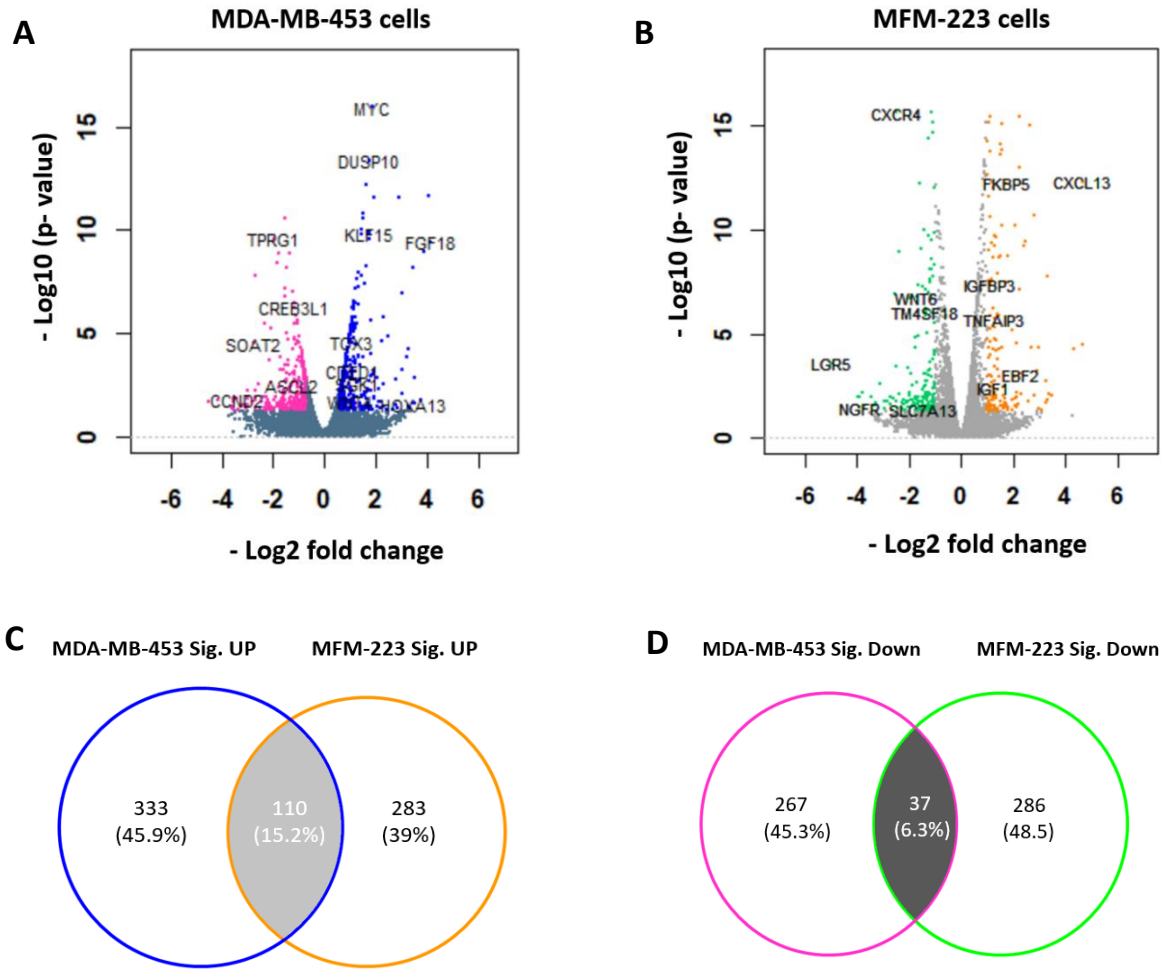



**Figure 3.2:** RNA-seq analysis of AR transcriptomes in MDA-MB-453 & MFM-223 cells. Volcano plots showing distribution of gene expression for DHT in MDA-MB-453 **(A)** and MFM-223 **(B)** cells compared to vehicle control. Blue or orange points represent significantly up-regulated genes (Sig. Up) with FC ≥1.5 & p-value <0.05; pink or green points represent significantly down-regulated genes (Sig. Down) with FC< 0.6 and p-value <0.05; petrol blue or light gray points represent genes with 1.5 > FC ≥ 0.6 (p-value <0.05) and/or p-value ≥ 0.05. (**C-D**) Venn diagrams showing the number of genes uniquely or commonly up-regulated (FC ≥1.5 & p-value <0.05) or down-

regulated (FC< 0.6 and p-value <0.05) by DHT in MDA-MB-453 and MFM-223 cells.

### 3.3.1.3 Functional annotation of DHT-regulated genes in MDA-MB-453 versus MFM-223 cells

**DHT-regulated genes unique to MDA-MB-453 cells**

Functional enrichment analysis of Gene Ontology (GO) terms was performed to provide a global view about biological processes associated with DHT-regulated genes in MDA-MB-453 versus MFM-223 cells (Figure 3.3 and Appendix 2C-F). Genes that are uniquely regulated by DHT in MDA-MB-453 and MFM-223 cells, potentially contribute to the divergent proliferative effects of this ligand in those two cell lines. Therefore, this section will first discuss GO terms of unique DHT-regulated genes, and then will address common genes. The identified DHT up-regulated genes that were unique to the MDA-MB-453 cell line fit mainly into four categories: (1) response to steroid hormones; 2) metabolism; 3) development and (4) growth (Figure 3.3A and Appendix 2C). In particular, DHT treatment significantly increased the expression of many genes associated with lipid metabolism in MDA-MB-453 cells including *FAR2*, *ACSL4*, *ACSL1*, *PDSS1*, *ALDH1A3*, *PLCB2*, *PLCH2*, and *HSPG2*. Genes associated with other metabolic processes were also enriched by DHT in this cell line, including amino acid, ribonucleoprotein, and carbohydrate metabolism. Examples of genes that were involved in these pathways are listed in Appendix 2C.

Genes associated with developmental processes such as epithelium & mesenchymal tissue development and major organ system development (such

as nervous, reproductive, circulatory, and immune system development) were also induced by DHT in MDA-MB-453 cells. Among these genes are *EPHA2*, *FGFR2*, *EPHA7*, *DDN*, *AFF3*, *NAV2*, *ISX* (Appendix 2C). The RNA-seq results also revealed DHT-induced expression of genes associated with cell survival/growth including *CAMKK1* (Brzozowski & Skelding 2019; Karacosta et al. 2012; Massie et al. 2011), *ALKBH2* (Fujii et al. 2013; Pilžys et al. 2019), *TOX3* (Dittmer et al. 2011; Seksenyan et al. 2015), *SGK1* (Lang et al. 2010; Shanmugam et al. 2007), and *WNT4* (Ni et al. 2011).

On the other hand, the GO analysis showed that DHT significantly suppressed mRNA levels of several genes that are associated with cell motility, adhesion, wound healing, and invasion pathways (Figure 3.3B) in MDA-MB-453 cells. Examples of these genes are: *COL5A3*, *CCR2*, *FBLN5*, *MMP-7*, *MMP-14*, *VTN*, *ADGRB1*, *SDK1*, *MCMAM*, *CDH13* and many other genes listed in Appendix 2D. Moreover, the data showed that DHT treatment decreased the expression of genes with purported tumour suppressor activity including *DLC1* (Plaumann et al. 2003; Song, YF et al. 2006; Ullmannova-Benson et al. 2009), *FBXW7* (Sailo et al. 2019; Yeh, C-H, Bellon & Nicot 2018), and *SFRP1* (Klopocki et al. 2004). The fold change and p-values for all genes discussed in this section were listed in Appendix 2C-D.

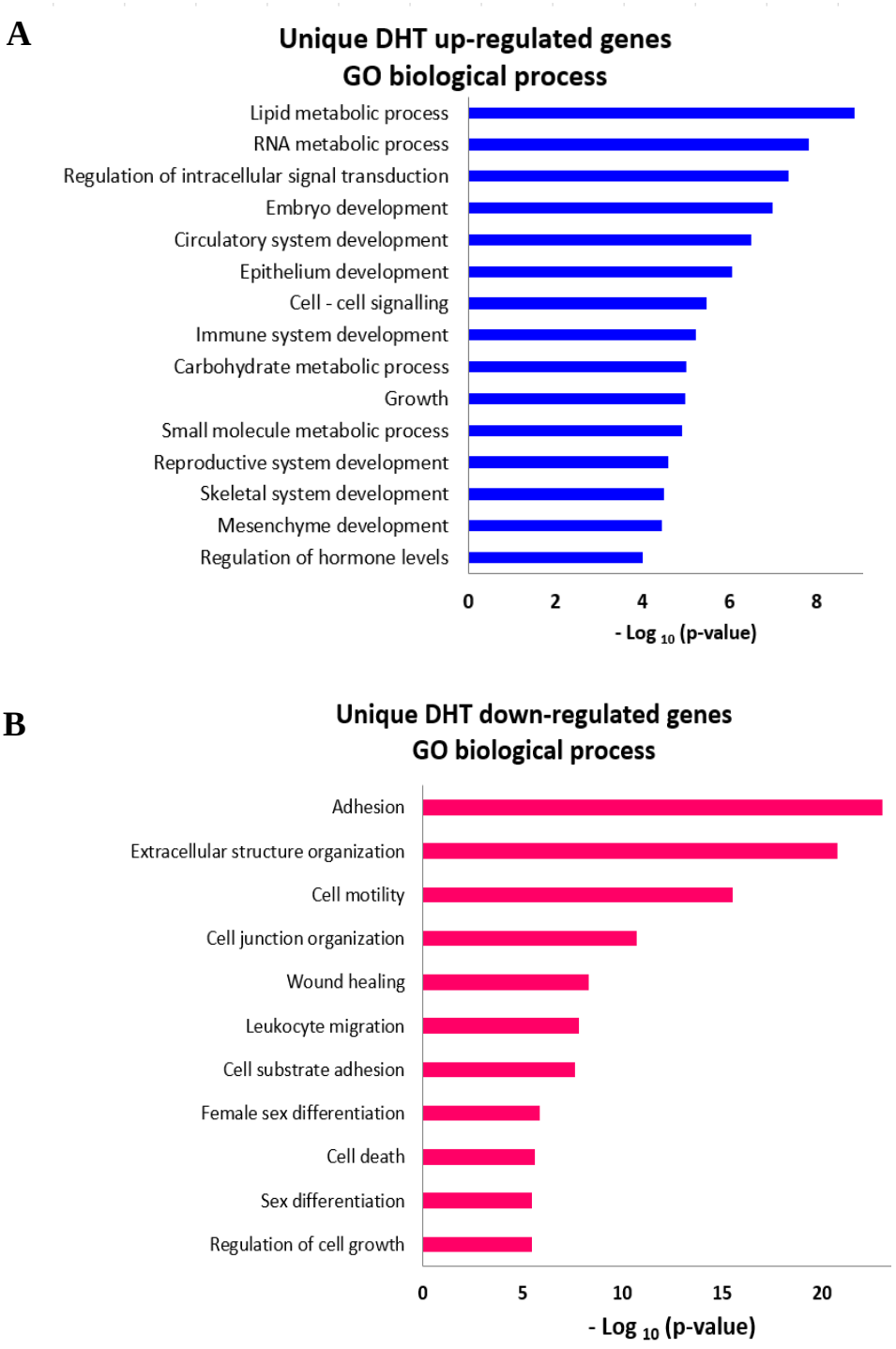



**Figure 3.3:** Functional annotation of genes uniquely regulated by DHT in the MDA-MB-453 cell line compared to the MFM-223 cell line. Bar graph **(A)** shows the biological processes associated with DHT up-regulated genes, while bar graph **(B)** shows biological pathways associated with DHT down-regulated genes in MDA-MB-453 cells.

**DHT-regulated genes unique to MFM-223 cells**

In MFM-223 cells, activation of AR by DHT induced mRNA levels of genes involved in ion and transmembrane transport, cell motility, response to hormones and regulation of proliferation & cell death (Figure 3.4A & Appendix 2E). We further investigated the genes that were enriched in regulation of proliferation & cell death pathways. Among these genes, we identified the transcriptional repressor *ZBTB16* and negative regulator of growth factor signalling *LRIG1*. *ZBTB16* and *LRIG1* are tumour suppressor genes in several human cancers including breast, prostate, bladder, colon, cervical, and lung (Cao, JingPing et al. 2013; Hedman & Henriksson 2007; Kikugawa et al. 2006; Krig et al. 2011; Li, Q et al. 2019; Lindquist et al. 2014; Ljuslinder et al. 2007). DHT also induced expression of other genes that have tumour suppressor activity in different types of cancer including *RGS2* (Cao, X et al. 2006), *ZNF689* (Hu, Ruozhen et al. 2011), *SPDEF* (Cheng, X-H et al. 2014), *TNFAIP3* (Honma et al. 2009), *IGFBP3* (Cai, Dozmorov & Oh 2020), *HIPK2* (Feng, Yuanyuan et al. 2017), *HPGD* (Wolf, I et al. 2006) and *Nkx 3.1* (Bhatia-Gaur et al. 1999).

Genes that were significantly and uniquely repressed by DHT in MFM-223 cells were involved mainly in development and homeostasis regulation (Figure 3.4B & Appendix 2F). Moreover, DHT decreased mRNA expression of genes involved in cell survival or with purported oncogenic activity such as *LGR5* (Xu, L et al. 2019; Yang, L et al. 2015), *TM4SF18* (Allioli et al. 2011; Cao, Jia et al. 2016; Huang, Y-K, Fan & Qiu 2016; Sun, Y et al. 2015; Zukauskas et al. 2011), *and BCL2* (Dawson et al. 2010; Merino et al. 2016). The fold change

and p-values for all genes discussed in this section were listed in Appendix 2E-F.

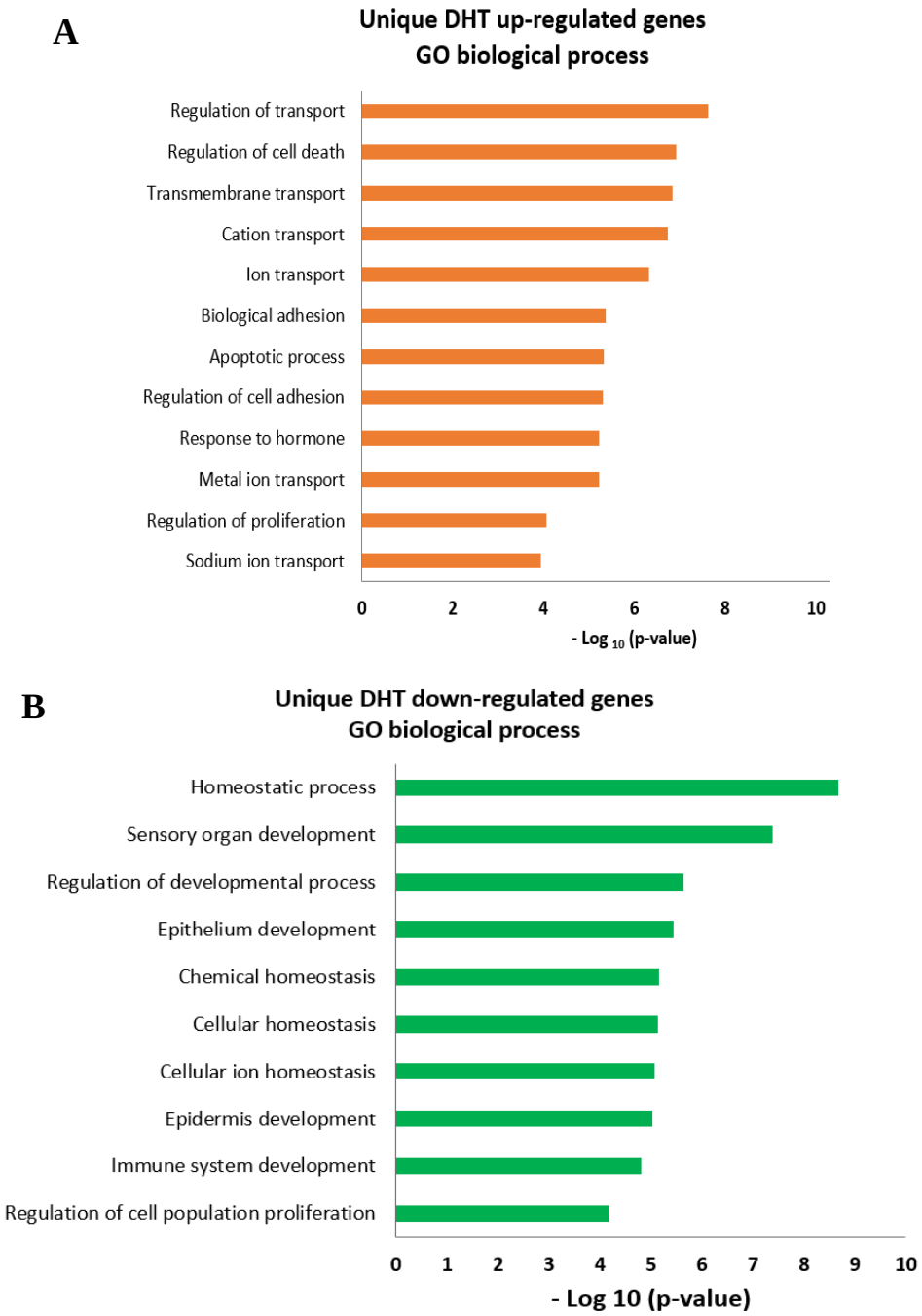



**Figure 3.4:** Functional annotation of genes uniquely regulated by DHT in the MFM-223 cell line compared to the MDA-MB-453 cell line. Bar graph **(A)** shows the biological processes associated with DHT up-regulated genes, while bar graph **(B)** shows biological pathways associated with DHT down-regulated genes in MFM-223 cells.

**DHT-regulated genes common to MDA-MB-453 and MFM-223 cells**

Since the number of genes that were regulated by DHT in both cell lines was low (up-regulated genes =110 and down-regulated genes =37), Gene Ontology analysis did not show significant enrichment of these genes in specific biological processes. However, among the most significantly up-regulated genes are *UGT2B28*, *UGT2B11*, *CYP4F8*, and *SLC26A3* (Appendix 2A). *UGT2B28* and *UGT2B11*, encode proteins that maintain intracellular steroid hormone homeostasis by inactivating steroids through the addition of a sugar moiety to a hydroxyl group (glucuronidation) within the steroid molecule (Barbier et al. 2008; du Toit & Swart 2016). The glucuronidation of androgens renders them unable to bind the AR, allowing the secretion of conjugated steroids (Barbier et al. 2008; du Toit & Swart 2016). *CYP4F8* encodes a member of the cytochrome P450 superfamily of enzymes that catalyse synthesis of cholesterol, steroids and other lipids (Vainio et al. 2011). CYP4F8 silencing significantly reduced cell viability in LNCAP prostate cancer cells (Vainio et al. 2011). SLC26A3 is a transmembrane glycoprotein that transports chloride ions across the cell membrane in exchange for bicarbonate ions (Dorwart et al. 2008). While the role of SLC26A3 as an AR target gene has not yet been studied, AR signalling has shown to be able to enhance transcription of members of the solute carrier family that transport lipid molecules, glucose, amino acids, and other nutrients such as SEC14L2, SLC1A3, SLC16A6 and SLC2A5 (Moore et al. 2012b; Sakakibara et al. 2021; Xu, Y et al. 2006). Moreover, DHT significantly up-regulated expression of *C-MYC* and *WNT7B* in both MDA-MB-453 and MFM-223 cells. Previous studies have examined the role of *C-MYC* and *WNT7B* genes in AR signalling in MDA-MB-453 (Ni

et al. 2013; Ni et al. 2011). As previously discussed in Chapter 1 (Section 1.4.8), DHT stimulates the expression of *WNT7B* and *HER3* in MDA-MB-453 cells, leading to activation of the Wnt/β-catenin and HER2 signalling pathways, which are required for androgen-induced growth of these cells (Ni et al. 2011). DHT-induced HER2 signalling also induces PI3K/AKT pathway activation, which in turn leads to degradation of MAD1 (MXD1), which disrupts the MAD1–MAX complex (Ni et al. 2013). This allows MAX to interact with MYC, leading to enhance oncogenic function of MYC (Ni et al. 2013). Here, we also showed that DHT stimulates expression of the *MYC* and *WNT7B* in MFM-223 cells, which their growth is inhibited by DHT. This may indicate that MYC and WNT7B signalling are not the main signalling pathways mediating oncogenic effects of AR in ERα-negative/AR-positive breast cancer or it may also indicate that MFM-223 cells lack some factors that mediate oncogenic effects of those signalling pathways.

In both MDA-MB-453 and MFM-223 cell lines, DHT significantly suppressed mRNA levels of genes associated with cell migration including *CCR1*, *CXCR4*, *WNT6*, *GBP2*, *AMIGO2*, and *LURAP1L*. Moreover, the *AR* gene itself was significantly down-regulated by DHT in MDA-MB-453 and MFM-223 cells. Negative autoregulation of AR mRNA by androgens has been reported for prostate (Wolf, DA et al. 1993; Yeap, Krueger & Leedman 1999) and breast cancer cell lines (Hall, R et al. 1994; Hall, RE et al. 1992; Yeap, Krueger & Leedman 1999).

Moreover, few genes were found to be regulated in the opposite way by DHT in MDA-MB-453 and MFM-223 cells (Table 3.2). An example of a gene that was up-regulated by DHT in MDA-MB-453 cells but down-regulated in MFM-

223 cells is *PLCB2*. PLCB2 catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and generates 2 important downstream materials: diacylglycerol (DAG) and inositol1,4,5-trisphosphate (IP3) (Lopez et al. 2001). DAG and IP3 involve in a great diversity of signalling pathways, such as the Ras signalling pathway and Rho signaling pathway (Lopez et al. 2001; Wang, X et al. 2018). *PLCB2* knockdown inhibits proliferation of PC3 and DU145 prostate cancer cell lines via the PTEN/AKT signalling pathway (Wang, X et al. 2018). An example of a gene that was down-regulated by DHT in MDA-MB-453 cells but up-regulated in MFM-223 cells is *CXCR7*. CXCR7, a chemokine receptor able to bind the chemokines CXCL12/SDF-1 and CXCL11, the primary regulator for cell migration, invasion, and expression of metalloproteinase (Sun, X et al. 2010). Similar to the effect of DHT on mRNA expression of CXCR7 in MDA-MB-453 cells, treatment of LNCaP prostate cancer cells with 1 nM DHT for 48 hr reduced the expression of CXCR7 by 70% (Yu, L, Yu & Wang 2015). The fold change and p-values for all genes discussed in this section were listed in Appendix A-B.

**Table 3.2:** mRNA expression of genes regulated in the opposite way by DHT in MDA-MB-453 and MFM-223 cells.

| Ensembl gene ID | Gene symbol | MDA-MB-453 DHT versus vehicle | | MFM-223 DHT versus vehicle | |
|---|---|---|---|---|---|
| | | FC | p-value | FC | p-value |
| ENSG00000143153 | ATP1B1 | 2.182048 | 5.73E-07 | 0.5163 | 6.56E13 |
| ENSG00000211448 | DIO2 | 2.916672 | 0.009179 | 0.516783 | 0.002321 |
| ENSG00000137841 | PLCB2 | 4.789161 | 2.31E-05 | 0.445167 | 8.74E-07 |
| ENSG00000163638 | ADAMS9 | 0.404246 | 0.019345 | 2.231762 | 0.026749 |
| ENSG00000122786 | CALD1 | 0.299778 | 0.000514 | 2.39396 | 4.54E06 |
| ENSG00000144476 | CXCR7 | 0.502467 | 0.037541 | 2.080464 | 0.000128 |
| ENSG00000144285 | SCN1A | 0.196063 | 3.25E-06 | 2.410777 | 1.61E05 |

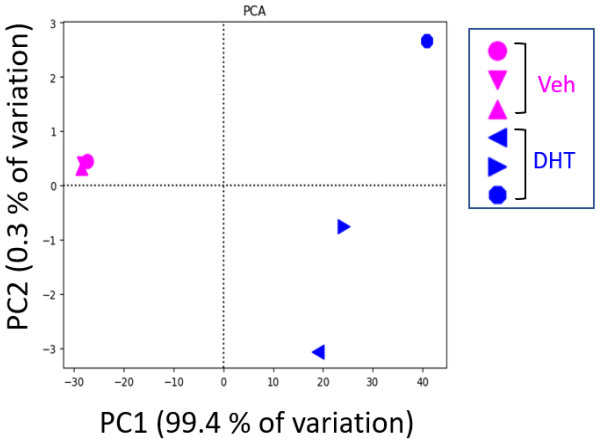
### 3.3.2 Genome-wide identification of AR binding sites (ARBS) in MDA-MB-453 versus MFM-223 cells

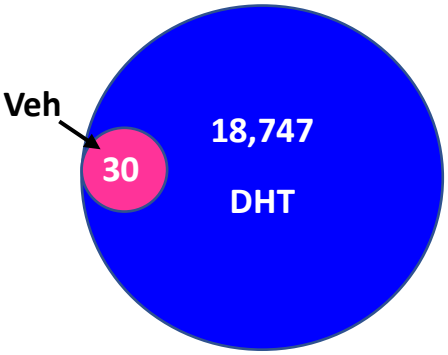
#### 3.3.2.1 Overview of AR ChIP-seq data

To examine whether differences in the DHT-regulated transcriptomes in MDA-MB-453 and MFM-223 cells are due to differences in their AR cistromes, the unpublished AR ChIP-seq data of MDA-MB-453 and MFM-223 cells treated with vehicle or 10 nM DHT for 4 hours was analysed. An overview of the number of the reads that passed through the quality control assessment steps, mapping efficiency, and number of called ChIP peaks for each sample are shown in Appendix 2G. Overall, all samples had a base quality score (Phred score) ≥ 34 and unique aligned rate (%) ≥ 74, indicating the high quality of the data. PCA was used to check if the replicates of each treatment condition had similar peak profiles. There was a strong correlation between ChIP-seq replicates in each treatment group; PCA identified two distinct clusters separated along PC2 in each cell line corresponding to the vehicle and DHT treatments (Figure 3.5A). To compare the DHT-stimulated AR cistromes associated with the MDA-MB-453 and MFM-223 cell lines, I created consensus peak-sets containing reproducible binding regions identified in at least two of the three replicates for each condition (i.e., vehicle and DHT). As expected, the consensus AR cistrome was markedly expanded in the presence of DHT in both cell lines due to ligand-induced receptor activation (Figure 3.5B). AR was recruited to 18,777 consensus DNA binding sites in MDA-MB-453 cells and 14,493 consensus binding sites in MFM-223 cells (Figure 3.5B). Among these AR binding sites (ARBS), 11,043 MDA-MB-453 specific ARBS (MDA-MB-453 unique sites) that were not called as peaks in the MFM-223 cell line, 6,759 MFM-223 specific ARBS (MFM-223 unique sites), and 7734 common ARBS (Figure 3.5 C and Figure 3.6). Figure 3.6B shows examples of AR bound loci located near previously-characterised AR gene targets.

**A**

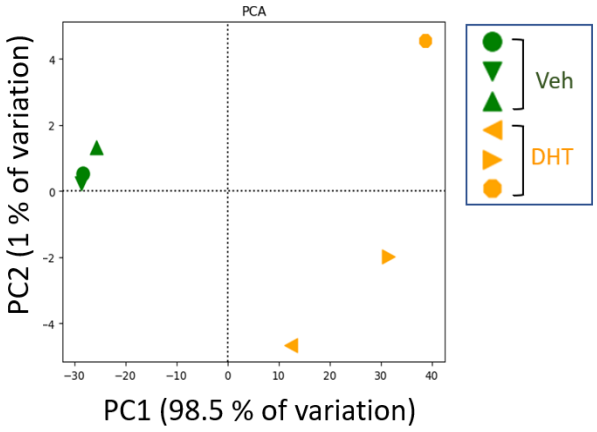
## MDA-MB-453 AR ChIP-Seq

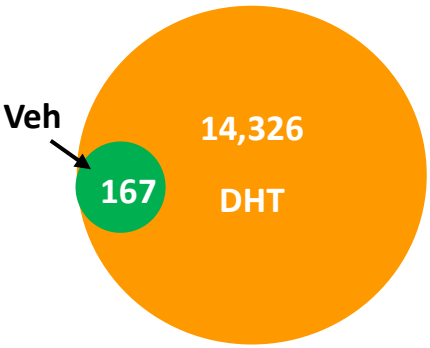



**B**

## MFM-223 AR ChIP-Seq




**C**

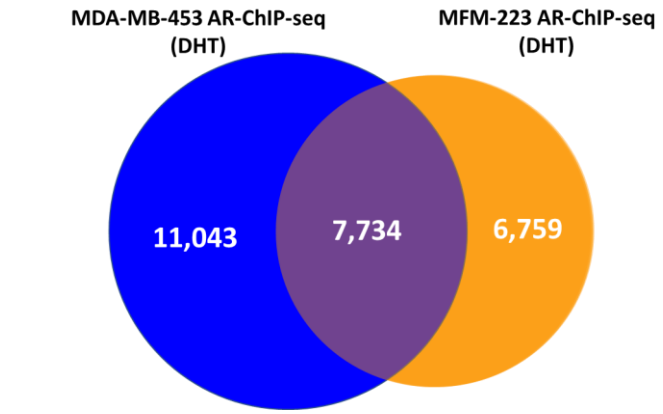


**Figure 3.5:** Genome-wide mapping of AR chromatin binding by ChIP-seq in MDA-MB-453 and MFM-223 cells. PCA plots and Venn diagrams displaying differences and similarities between replicate treatment samples and overlap of consensus AR cistromes between Vehicle (Veh) and DHT treatments in (**A)** MDA-MB-453 and (**B)** MFM-223 cells. **(C)** Venn diagram displaying overlap of the DHT-stimulated AR cistrome in MDA-MB-453 and MFM-223 cell lines.

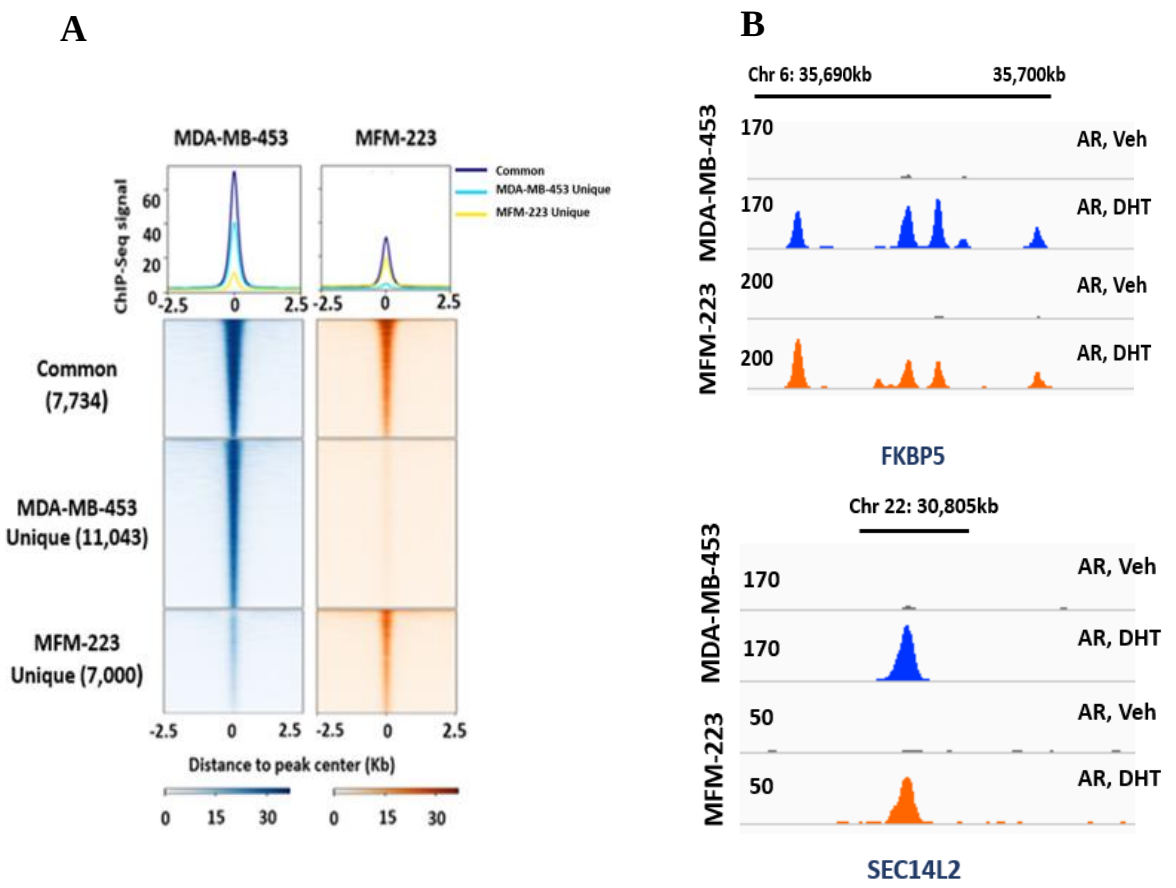



**Figure 3.6:** Comparison of AR ChIP-seq data in MDA-MB-453 versus MFM-223 cells. **(A)** Heatmaps and corresponding read density plots showing consensus ChIP-seq signals (fragments /kilobase pair/per million reads (FPKM)) and the number of common, MDA-MB-453 unique and MFM-223 unique AR binding sites with 10 nM DHT. **(B)** Genome browser images of AR-DNA binding sites associated with established AR target genes *FKBP5* and *SEC14L2*.

### 3.3.2.2 Genomic annotation and transcriptional regulation of AR binding sites

To examine whether unique and common AR-DNA binding sites correspond to the DHT-induced gene expression differences in MDA-MB-453 and MFM-223 cells, neighbouring genes associated with ARBS were identified using the CisGenome's annotation functions and integrated the results with the outcomes of the differential expression analysis. Approximately 65% of the MDA-MB-453 unique DHT-regulated (Log2 FC≥1, P <0.05) genes were associated with MDA-MB-453 unique ARBS. Examples of these genes are *FAR2, HAAO, ALDH1A3, BAG2, CLDN8, DDC, TOX3, EPHA4, CXCR4, MMP13,* and *PAX7*. Figure 3.7A shows IGV representative images of MDA-MB-453 unique ARBS at MDA-MB-453 unique DHT-regulated genes. However, the results were starkly different in MFM-223 cells, with less than 15% of the MFM-223 unique DHT-regulated genes exhibiting association with MFM-223 unique ARBS. These results were exemplified by visualisation of the ChIP-seq data using IGV software to show that AR was able to bind to chromatin at the same loci in both cell lines, but the associated genes was only regulated by DHT in MFM-223 cells. Interestingly, the tumour suppressor genes *ZBTB16* and *LRIG1* were among this set of genes. Figure 3.7B & 3.7C gives examples of AR-DNA binding sites at genes uniquely regulated by DHT in MFM-223 cells.

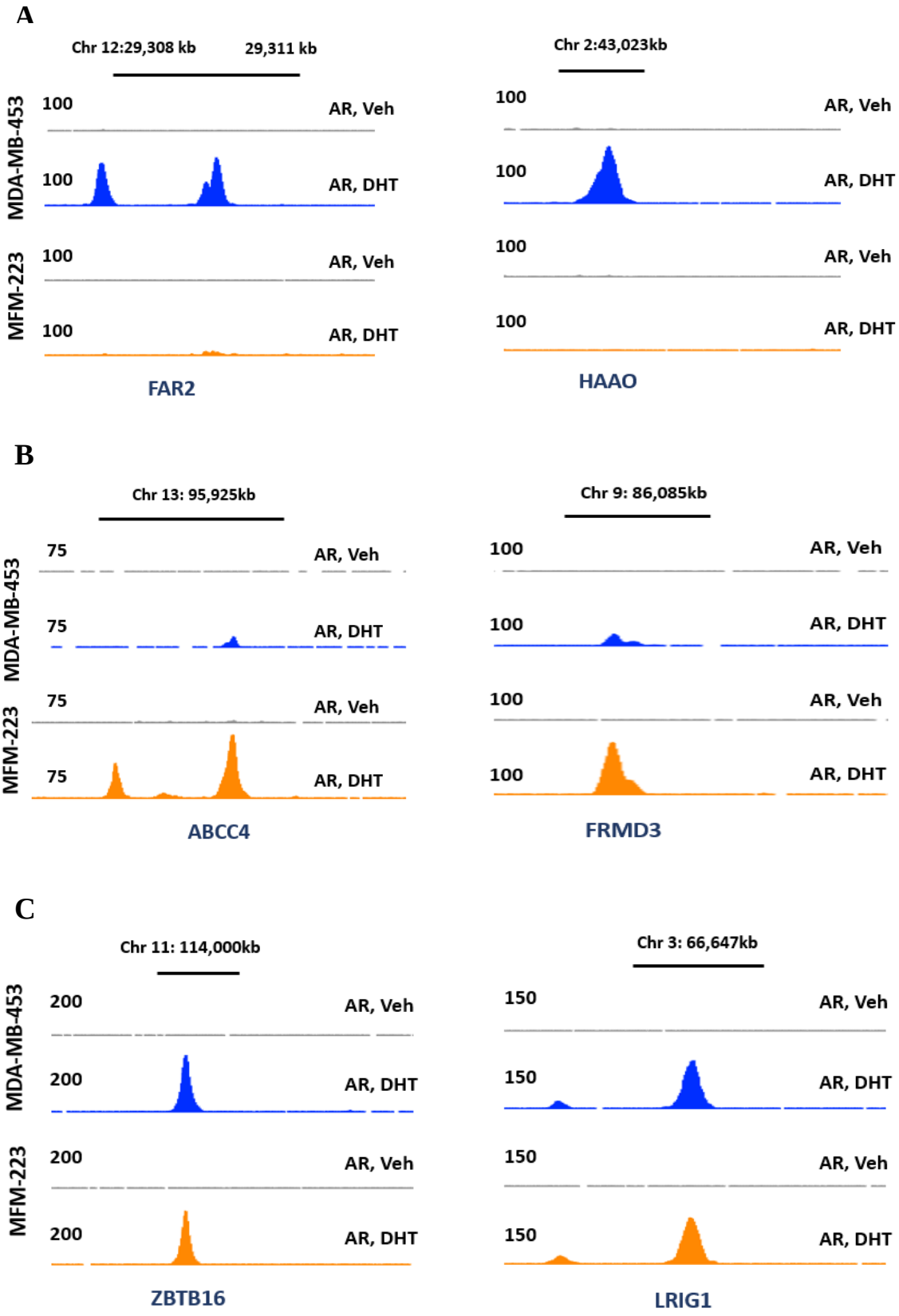

A
Chr 12:29,308 kb
29,311 kb
MDA-MB-453
MFM-223
100
AR, Veh
AR, DHT
FAR2
Chr 2:43,023kb
HAAO
B
Chr 13: 95,925kb
75
ABCC4
Chr 9: 86,085kb
FRMD3
C
Chr 11: 114,000kb
200
ZBTB16
Chr 3: 66,647kb
150
LRIG1

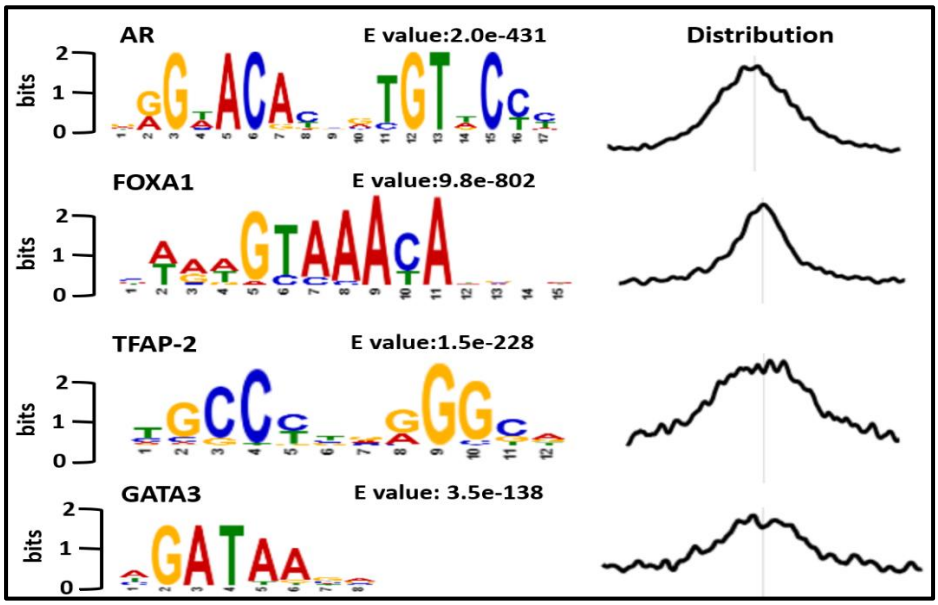
**Figure 3.7:** IGV images of AR binding sites in MDA-MB-453 and MFM-223 cells. **(A)** MDA-MB-453 unique AR binding sites associated with genes uniquely upregulated by DHT in MDA-MB-453 but not MFM-223 cells. **(B)** MFM-223 unique AR binding sites associated with genes uniquely upregulated by DHT in MFM-223 cells but not MDA-MB-453 cells. **(C)** AR binding sites present in both cell lines that are associated with tumour suppressor genes uniquely up-regulated by DHT in MFM-223 cells but not in MDA-MB-453 cells.

### 3.3.2.3 Motif analysis of the AR cistromes

Motif analysis of AR binding sites represents a starting point to identify candidate co-factors that may interact and facilitate or inhibit AR action. Motif analysis of AR cistromes representing MDA-MB-453 and MFM-223 cells treated with DHT was performed using the MEME tool (Machanick & Bailey 2011). As expected, we detected AR and FOXA1 motifs as the most highly enriched motifs in both cell lines (Figure 3.8). The function of FOXA1 as a pioneer factor that engages and reconfigures condensed chromatin to facilitate DNA binding of other transcription factors makes it an intimate companion to AR (Robinson, JL et al. 2011; Sahu et al. 2011). The AR and FOXA1 cistromes in MDA-MB-453 cells have been shown to have a very large overlap (Robinson, JL et al. 2011), but the FOXA1 cistrome in MFM-223 cells has not been performed yet. Other transcription factor motifs were also found to be commonly enriched in both cell lines, including motifs for GATA, SOX and IRX family members. More importantly, we found that the third most significantly enriched motif (after AR and FOXA1) in the AR cistrome of MDA-MB-453 cells was that for Transcription factor AP-2 (TFAP-2) (Figure 3.7). TFAP-2 is a family of sequence-specific DNA binding transcription factors that orchestrate a variety of processes including development, growth, and tissue differentiation (Ikram et al. 2016; Knight et al. 2005; Wenke & Bosserhoff 2010). TFAP-2 motifs were enriched in the AR cistrome of MDA-MB-453 cells but were not enriched in the AR cistrome of the MFM-223 cell line. To further explore disparities in the expression profiles of AR co-factors among these two cell lines, we utilized the RIME technique to study chromatin-associated AR interactome.

A

## MDA-MB-453




B

## MFM-223

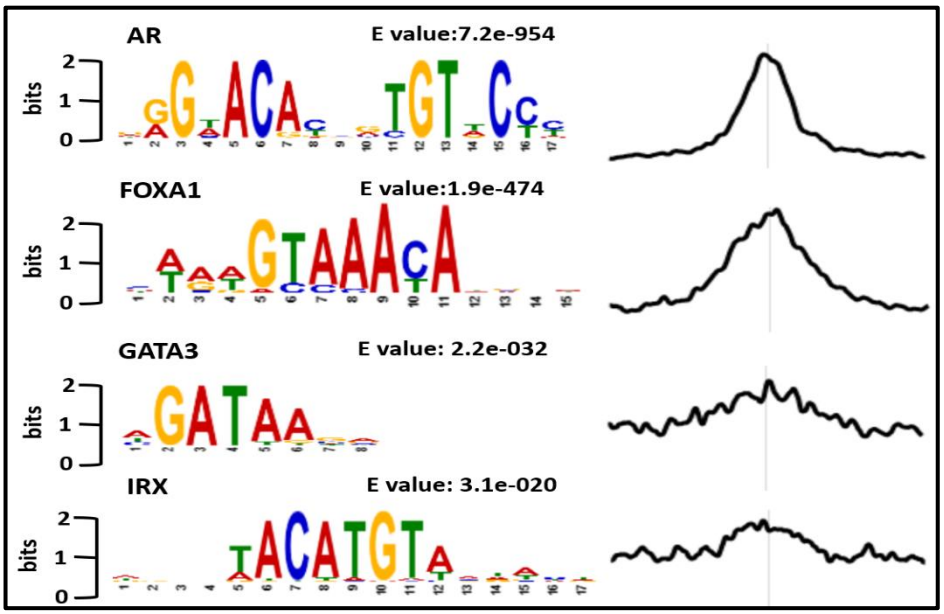


**Figure 3.8:** The top motif matrices predicted by motif analysis by MEME in **(A)** MDA-MB-453 cells and **(B)** MFM-223 cells. AR, FOXA1, GATA3, and IRX were enriched in both cell lines. TFAP-2 was found to be the third top motif matrices enriched in MDA-MB-453 cell line and was not enriched in the MFM-223 line. The motif distribution graph plots to the right show the position of significant motif matches within the input sequences. The vertical line in the center of the graph corresponds to the center of the sequences. E-value is the P- value multiplied by the number of motifs in the input data.

### 3.3.3 Endogenous AR interactome profiling in MDA-MB-453 and MFM-223 cells

To identify cooperating factors that are involved in the regulation of AR signalling in ERα-negative/AR-positive breast cancer cell lines, unpublished AR interactome data of MDA-MB-453 and MFM-223 cells generated using the RIME technique was analysed. The RIME experiment was performed by my supervisor, A/Prof Theresa Hickey, while she was working in the Carroll laboratory (Cancer Research UK, Cambridge Institute) when the RIME technique was first being developed. To define a high confidence interactome, only proteins that occurred in all independent biological replicates were considered and any protein that appeared in matching IgG controls was excluded. Using these criteria, 131 MDA-MB-453 specific, 81 MFM-223 specific, and 21 shared AR interacting proteins were identified. The AR itself was detected with high confidence in all AR-IP replicates in both cell lines, with 18 unique AR peptides routinely identified, which served as a positive control for experimental success. Within the shared AR interactor list, I identified SWI/SNF related proteins (SMCE1, SMRC2, SMRD2, and ARID1A), ELAVL1 which increases stability of mRNA, the transcription factor GATA3 (a known interactor of ERα) and other transcription factors including JUNB and TRPS1.

In the MDA-MB-453 cell line, the FOXA1 pioneer factor was identified as an AR interactor, consistent with previous studies highlighting the importance of FOXA1 for AR–chromatin interactions in this cell line (Robinson & Carroll 2012; Robinson, JL et al. 2011). Although FOXA1 motifs were enriched in the

AR cistrome of MFM223 cells, FOXA1 was not identified in the RIME data associated with this cell line. Several MDA-MB-453 specific AR interacting proteins were associated with chromatin organisation and RNA processing (Figure 3.9A). Other members of the MDA-MB-453 AR interactome were involved in DNA repair (PAXX, PNKP, PRMT1, and UBA1), metabolic processes (e.g., ALDH16A1, BOLA2, DLD, and COPG1) transcriptional repression (PHB2, CTBP2, and CTBP2) and general transcription (GTF2I, TLF3, and STAT1). Approximately 7 proteins of the MDA-MB-453 AR interacting partners were involved in cell cycle/growth (Figure 3.9A). Examples of cell cycle regulation/progression proteins are CCD68, LAP2A, and NUDC. Importantly, among the novel differential precipitating factors, we found TFAP-2β, a member of the TFAP-2 transcription factor family, which we demonstrated to be enriched at AR-DNA binding sites by motif analysis in MDA-MB-453 but not MFM-223 cells (section 3.3.2.3). The identified MFM-223 specific AR interactors were involved mainly in chromatin remodeling, DNA occupancy, RNA processing/splicing, and chaperone-mediated protein folding (Figure 3.9B). Other members of the MFM-223 AR interactome were involved in the regulation cell growth/death (ANXA7, and YWHAZ), response to stress (PRDX1 and PRDX2), as well as transcription repression (CBX5) (Figure 3.9B).

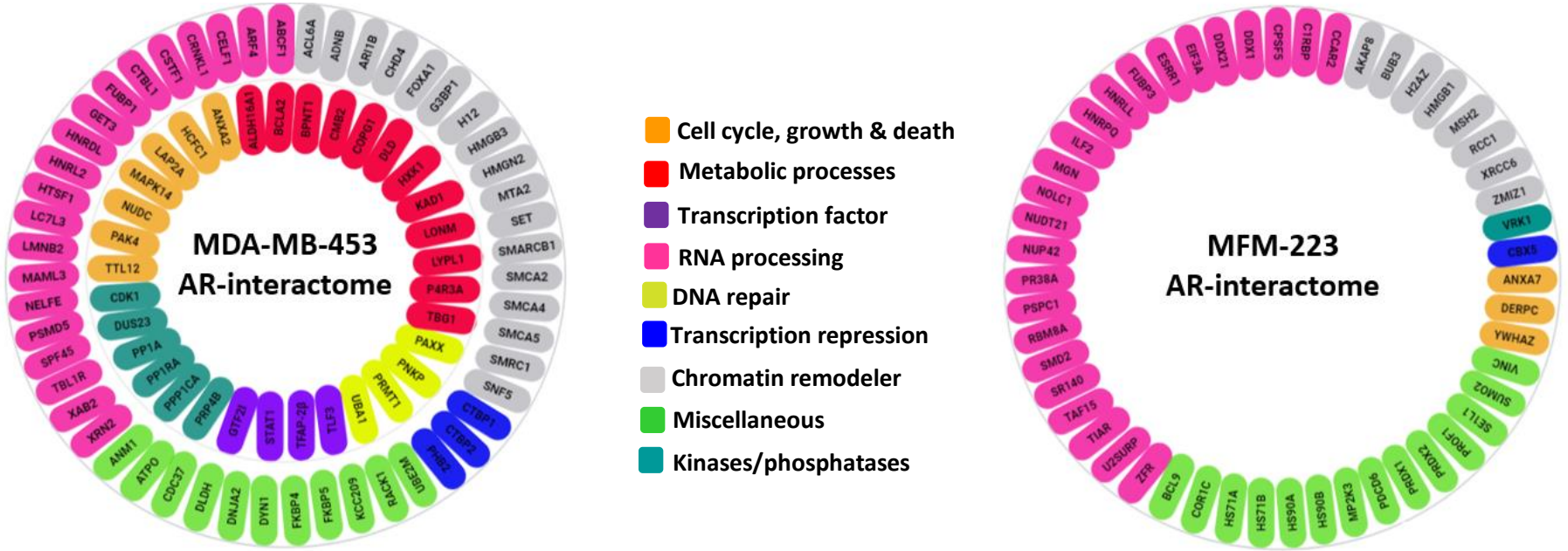



**Figure 3.9:** AR interacting proteins identified by RIME in MDA-MB-453 and MFM-223 cells. Unique AR co-regulators identified in **(A)** MDA-MB-453 & **(B)** MFM-223 cell lines were categorised according to their molecular function. The miscellaneous group contains proteins associated with chaperone-mediated protein folding, transport, homeostasis, and redox reaction. Metabolism group includes carbohydrate, lipid, and small molecules metabolism.

## 3.4 Discussion

How AR signalling can exert divergent proliferative effects in different models of ERα-negative breast cancer is not fully understood. This study aimed to define novel features of AR signalling that may underpin the mechanistic basis for divergent proliferative effects, in an effort to facilitate future development of biomarkers that may inform rational clinical AR target strategies for this disease sub-group.

When comparing the activated AR transcriptome of MDA-MB-453 with MFM-223 cells, we observed that DHT regulates distinct sets of genes that are associated with distinct biological functions (Figure 3.3 & 3.4). DHT significantly enhanced the expression of genes associated with tumour progression pathways in the MDA-MB-453 model, including lipid metabolism, development as well as cell survival and growth (Figure 3.3A). Expression of many lipid metabolism genes including *FAR2*, *ACSL4*, *ACSL1*, *PDSS1*, *ALDH1A3*, *PLCB2*, *PLCH2*, and *HSPG2* was also significantly and uniquely stimulated in MDA-MB-453 cells upon exposure to DHT. Lipid metabolism represents one of the key androgen-regulated processes in prostate cancer cells and alteration in lipid metabolism is a hallmark of prostate cancer (Barfeld et al. 2014; Butler, LM, Centenera & Swinnen 2016; Mah et al. 2019), whereby increased lipogenesis accompanied by overexpression of lipid metabolic genes are characteristic of primary and advanced disease (Chen, M et al. 2018; Iglesias-Gato et al. 2016; Zadra & Loda 2017). Therefore, as the similarities between molecular apocrine tumours and AR-positive prostate cancers that are also typically ER-negative are well described (Ni et al. 2011; Robinson, JL et

al. 2011), it is reasonable to argue that lipid metabolism pathways might also have a critical role in the growth of this disease sub-type and might differentiate AR signalling in molecular apocrine compared to a triple negative breast cancer sub-type. The ability of AR to promote growth of MDA-MB-453 cells could also be attributed to its ability to enhance transcription of development/ growth associated genes with purported oncogenic activity such as *EPHA2* (Kinch, Carles-Kinch & metastasis 2003; Song, W et al. 2017), *ISX* (Wang, S-N et al. 2016), *FGFR2* (Lei, Haipeng & Deng 2017), *TOX3* (Yu, Xin & Li 2015) and *WNT4* (Lei, Haipeng & Deng 2017). In MFM-223 cells, which are growth inhibited by AR signalling, DHT uniquely induced expression of genes with anti-tumour activity including *ZBTB16* and *LRIG1*. *ZBTB16* is an important transcriptional repressor, involved in various biological processes including cell death (Wei et al. 2018). Ectopic expression of *ZBTB16* inhibited proliferation, metastasis, and induced apoptosis of breast and prostate cancer cell lines (Cao, JingPing et al. 2013; He, J et al. 2020; Jiang, F & Wang 2004; Kikugawa et al. 2006). Conversely, inhibition of *ZBTB16* expression by siRNA or shRNA in LNCaP cells results in outgrowth (Hsieh et al. 2015; Jin et al. 2013). I show for the first time that *ZBTB16* expression is significantly stimulated by DHT (FC=29.27, p-value= 6.4E-21) in MFM-223 cell line but not the MDA-MB-453 cell line, which may play a role in mediating divergent growth effects. *LRIG1* is a negative regulator of growth factor signalling and a proposed tumour suppressor in several human cancers including breast, bladder, colon, cervical, and lung (Hedman & Henriksson 2007; Krig et al. 2011; Li, Q et al. 2019; Lindquist et al. 2014; Ljuslinder et al. 2007). The RNA-seq data in this chapter showed that treatment of MFM-223 cells with

DHT caused a high induction (FC=7, P-value=0.0) in mRNA levels of *LRIG1* but had no effect in MDA-MB-453 cells, suggesting that this gene may also play a role in mediating divergent growth effects of AR signalling in these models.

As only 6% of DHT down-regulated genes were shared between MDA-MB-453 and MFM-223 cells (Figure 3.2D), the unique DHT down-regulated genes and their functions in each cell line were investigated. Many cell-motility related genes demonstrated a reduction in mRNA expression in DHT-treated MDA-MB-453 cells (Figure 3.3B). More importantly, DHT impaired transcription of genes with proposed tumour suppression activity such as *DLC1* (Plaumann et al. 2003; Ullmannova-Benson et al. 2009) and *FBXW7* (Xu, L et al. 2019; Yang, L et al. 2015). *DLC1* is a *bona fide* tumour suppressor gene that is downregulated via genetic and epigenetic mechanisms in a variety of cancers, including breast cancer (Plaumann et al. 2003; Song, YF et al. 2006; Ullmannova-Benson et al. 2009). *DLC1* loss in cancer leads to constitutive activation of RhoA RhoGTPases and Cdc42 (Plaumann et al. 2003; Ullmannova-Benson et al. 2009). This results in increased cell proliferation, changes in cell morphology, and inhibition of apoptosis (Plaumann et al. 2003; Sekimata et al. 1999). *FBXW7* controls proteasome-mediated degradation of oncoproteins such as cyclin E, C-MYC, MCL1, and mTOR (Sailo et al. 2019; Yeh, C-H, Bellon & Nicot 2018). In contrast, the mRNA levels of genes involved in cell proliferation and development processes were decreased in MFM-223 cells treated with DHT (Figure 3.4B). Among these genes, we identified *LGR5* and *BCL2*. *LGR5* is a receptor for the R-spondin (RSPO), which, when bound, acts in cooperation with Wnt receptors to potentiate

Wnt/β-catenin signalling (Yang, L et al. 2015). *LGR5* promoted cell mobility, tumour formation, and epithelial-mesenchymal transition in breast cancer cells by activating Wnt/β-catenin signaling (Xu, L et al. 2019; Yang, L et al. 2015). *BCL2* contributes to cancer formation and progression by blocking apoptosis (Lindeman & Visvader 2013). Collectively, RNA-seq analysis provides compelling evidence that DHT enhances MDA-MB-453 cell proliferation by activating transcription of genes with oncogenic effects but inhibiting MFM-223 cell growth by increasing mRNA levels of tumour suppressor genes.

Comparing DEGs to AR binding sites revealed that 65% of genes uniquely regulated by DHT in the MDA-MB-453 cell line were associated with DHT-stimulated AR binding sites found only in this cell line, which suggests direct AR regulation of these molecular determinants. In contrast, a relatively small number of genes uniquely regulated by DHT in the MFM-223 line were associated with AR binding sites unique to this cell line. Instead, the peak annotation analysis showed that about 42% of these genes are associated with AR binding sites common to both lines, suggesting that the MDA-MB-453 cell line lacks the factors that required to activate these sites. These results highlight the importance of MDA-MB-453 unique AR binding sites in MDA-MB-453 and indicate that these sites could be the main mediator of differential proliferative effects of DHT.

As activity of the AR cistrome is modulated by a number of potential interaction factors, differences in the AR binding profile observed or their transcriptional activity might result from disparities in composition of the AR interactome. To test this hypothesis, I started with motif enrichment analysis of

the AR cistromes in MDA-MB-453 and MFM-223 cells. AR, FOXA1, and GATA motifs were enriched in the AR cistromes of both cell lines (Figure 3.8). FOXA1 acts as a cell-type specific pioneer factor that facilities recruitment and binding of ER and AR to their cis-regulatory elements across the genome (Lupien et al. 2008). Among all GATA family members, GATA3 is required for normal development of the mammary gland where it is estimated to be the most abundant transcription factor in luminal epithelial cells (Takaku, Grimm & Wade 2015). In breast cancer, GATA3 expression is highly correlated with ERα expression and with the luminal transcriptional program (Kong et al. 2011). Little is known about GATA3 and AR interaction in breast cancer, and there is an ongoing study by another student in our lab on GATA3 in ERα-negative/AR-positive breast cancer. The motif analysis revealed the enrichment of the TFAP-2 transcription factor motif in the MDA-MB-453 but not the MFM-223 AR cistrome (Figure 3.8). TFAP-2 was the third most significantly enriched motif (after AR and FOXA1) in the AR cistrome of MDA-MB-453 cells. The TFAP-2 transcription factors are required for normal development and morphogenesis of several organs including the breast (Pellikainen & Kosma 2007). This motif analysis suggested that one of the TFAP-2 family members could be a key differential AR interacting protein in these models.

The composition of AR protein complexes was investigated more broadly using an unbiased proteomic approach called RIME. Only 10% of all AR interacting partners were shared between MDA-MB-453 and MFM-223 models, indicating a large difference in the AR interactomes. Nevertheless, multiple chromatin remodeling, general transcription, and RNA

processing/splicing factors were precipitated with AR in both cell lines (Figure 3.9), and likely represent factors needed to facilitate the activity of transcription factors in a non-specific manner. These factors regulate transcriptional activity of AR via recognition of AREs and regulating pre-mRNA splicing of its target genes (Chmelar et al. 2007). Consistent with the motif analysis, GATA3 was identified in the MDA-MB-453 and MFM-223 AR interactomes. FOXA1 pioneer factor was identified as an AR interactor by AR RIME analysis and motif analysis of AR-DNA binding sites in MDA-MB-453 cells. However, in MFM-223 cell line, FOXA1 motifs were enriched in the AR cistrome but FOXA1 was not identified in the RIME. Other members of the AR interactome are involved in transcriptional repression such as CTBP2 in MDA-MB-453 and CBX5 in MFM-223. As a transcriptional corepressor, CTBP2 is known to repress tumour-suppressor genes and AR corepressors in prostate cancer cells, such as NCOR and RIP140, by binding with AR to the promoter and enhancers of these genes (Takayama et al. 2014). In contrast, CBX5 has been implicated in chromatin packing and epigenetic repression (Pongas et al. 2017). While the AR interacting proteins in MFM-223 cells are mainly involved in chromatin remodeling, DNA occupancy and RNA processing/splicing functions, the AR interaction partners in MDA-MB-453 are more diverse in the molecular functions they regulated. Factors involved in cell cycle regulation such as HCFC1, which facilitates G1 to S phase transition (Xiang et al. 2020), LAP2A, which stabilises Retinoblastoma (Rb) (Gesson, Vidak & Foisner 2014), and NUDC, which is involved in spindle formation during mitosis and in microtubule organization during cytokinesis (Chuang et al. 2013), were isolated from AR protein complexes uniquely in MDA-MB-453

cells. Consistent with previous studies that functionally link AR action with DNA damage repair (Bartek, Mistrik & Bartkova 2013; Polkinghorn et al. 2013), DNA repair proteins PAXX, PNKP, PRMT1 and UBA1 were also found in MDA-MB-453 cells. AR transcriptional activity is known to be modulated by kinases as well as phosphatases (Chen, S et al. 2009). Some of the identified MDA-MB-453 AR interacting proteins may regulate AR function by the means of phosphorylation or dephosphorylation. For example, CDK1 has been shown to stimulate AR activity through phosphorylation of serine 81 in the N-terminal domain (Chen, S et al. 2006; Skraškova et al. 2016). A study by Chen and colleagues showed that phosphorylation of AR by CDK1 at S81 *in vitro* is associated with prevention of AR degradation, thereby increasing AR stability and increasing AR protein expression (Chen, S et al. 2006).

Of notable significance, this chapter is the first to describe the interaction of AR with the TFAP-2β transcription factor in MDA-MB-453 but not MFM-223 cells. TFAP-2β is a member of the TFAP-2 family that was detected as significantly enriched by motif analysis of the AR cistrome in MDA-MB-453 cells (section 3.3.2.3). By activating its target genes, TFAP-2β plays important roles in regulating vertebrate development, cell growth, survival and differentiation (Ikram et al. 2016; Knight et al. 2005; Wenke & Bosserhoff 2010). TFAP-2β is thought to be oncogenic in various cancer entities, including rhabdomyosarcoma (Ebauer et al. 2007), lung adenocarcinoma (Fu, L et al. 2014), and thyroid cancer (Fu, X et al. 2019). Moreover, clinical studies using human breast tissues have reported nuclear TFAP-2 expression in benign breast epithelium in which both TFAP-2α and TFAP-2β have been located in

the ductal epithelium (Friedrichs et al. 2005; Hurst, Haffty & Williams). As a transcription factor involved in many different molecular signalling pathways and as a novel AR interacting partner in the context of AR oncogenic effects, we sought to investigate the role of TFAP-2β in ERα-negative/AR-positive breast cancers. Chapter four presents *in silico* validation and wet lab experiments conducted to test the hypothesis that TFAP-2β is a critical regulator of the molecular apocrine breast cancer growth and may be a determinant of whether AR signalling has oncogenic or tumour suppressive activity.

# CHAPTER FOUR

# TFAP-2B IS CRITICAL FOR THE GROWTH OF MOLECULAR APOCRINE BREAST CANCER CELLS

## 4.1 Introduction

TFAP-2β is a member of a sequence-specific DNA binding transcription factor family, which comprises five different but closely related proteins (TFAP-2α, -2β, -2γ, -2δ, -2ε) transcribed from different genes (*TFAP2A-E*), each having a molecular weight of approximately 50kDa (Eckert et al. 2005; Pellikainen & Kosma 2007). As a member of the TFAP-2 family, the TFAP-2β protein has a highly conserved helix-span-helix motif at the carboxyl terminus, which, together with a central basic region, mediates factor dimerisation and DNA binding (Eckert et al. 2005; Pellikainen & Kosma 2007). A third, less conserved region toward the amino terminus contains a short proline-rich sequence important for transactivation (Eckert et al. 2005). TFAP-2 proteins form homo- or heterodimers, bind to the consensus sequence 5'-GCCNNNGGC-3' and transactivate genes involved in a large spectrum of important biological functions including development, growth, differentiation and metabolism (Satoda et al. 2000; Wenke & Bosserhoff 2010). Functional missense mutations in TFAP-2β basic domain have been implicated in the human condition Char Syndrome (Satoda et al. 2000), an autosomal disorder characterised by abnormal heart, limb, and facial development (Satoda et al. 1999; Zhao, F et al. 2001). In mice, homozygous deletion of TFAP-2β has been reported to cause congenital polycystic kidney disease due to excessive

apoptosis of renal epithelial cells, ultimately resulting in terminal renal failure (Moser et al. 1997). Scientific studies have also revealed a diverse range of biological functions related to TFAP-2β (Hara et al. 2019; Ikram et al. 2016; Joost et al. 2019; Nordquist et al. 2009). Small interfering RNA-mediated knockdown of TFAP-2β in corneal endothelial cells decreased expression of the corneal endothelium–specific proteins type VIII collagen α2 (COL8A2) and zona pellucida glycoprotein 4 (ZP4), and suppressed cell proliferation (Hara et al. 2019). TFAP-2β is also involved in neuronal differentiation through up-regulation of the catecholamine biosynthesising enzyme genes *DBH* (dopamine beta-hydroxylase) and *TH* (tyrosine-hydroxylase), and down-regulation of *MYCN* and *REST*, master neuronal gene repressors (Ikram et al. 2016). TFAP-2β can regulate adipocyte metabolism by facilitating glucose uptake and lipid accumulation (Joost et al. 2019; Nordquist et al. 2009; Ugi et al. 2010). Since TFAP-2β is implicated as a mediator of these diverse biological functions, there has been interest in studying the role of this transcription factor in relation to cancer.

TFAP-2β has been shown to be oncogenic in various cancer types, including rhabdomyosarcoma (Ebauer et al. 2007), lung adenocarcinoma (Fu, L et al. 2014), and thyroid cancer (Fu, X et al. 2019). Little is known about the role of TFAP-2β in mammary gland development and breast disease, including breast cancer. Higher TFAP-2β levels have been shown to distinguish lobular from ductal breast carcinomas and siRNA-mediated knockdown of TFAP-2β diminished proliferation of lobular breast cancer cell lines *in vitro* (Raap et al. 2018), suggesting an oncogenic role in this disease sub-type. TFAP-2β is also strongly expressed in apocrine metaplasia, a common benign feature of the

aging human mammary gland (Raap et al. 2018). Using an unbiased proteomic approach (RIME), this study identified TFAP-2β as a novel AR interacting protein in MDA-MB-453 breast cancer cells (Chapter 3), a cell line commonly used as a model of molecular apocrine breast cancer (Doane et al. 2006; Farmer et al. 2005; Naderi & Hughes-Davies 2008; Ni et al. 2011; Robinson, JL et al. 2011). This chapter aimed to examine the biological role of TFAP-2β in the molecular apocrine sub-type and determine whether TFAP-2β is a critical determinant of AR oncogenic activity in molecular apocrine cells.

## 4.2 Methods

### 4.2.1 Analysis of *TFAP2B* expression in published gene expression datasets

Relative *TFAP2B* expression levels were assessed by analysing gene-expression microarray datasets derived from cases of breast cancer that formed part of the phase III clinical trial by the European Organisation for Research and Treatment of Cancer (EORTC; NCT00017095), a trial that tested whether p53 mutant tumours respond better to anthracycline-based chemotherapy (Bonnefoi, Hervé et al. 2011). Classification of breast tumours into luminal, basal, and molecular apocrine subtypes was performed using PAM50 classification of breast cancer as well as sub-type specific signature genes as described elsewhere (Farmer et al. 2005; Iggo 2018).

### 4.2.2 Plasmid construction and virus production

TFAP-2α (pDRM13) and TFAP-2β (pDRM14) vectors were generated from pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene, #12252) by Gibson assembly (Thermo Scientific). The structure of the plasmids is listed in Appendix 1C-D. A doxycycline-inducible TFAP-2β vector (pDRM23) was generated from pCW57.1 (Addgene #41393) by Gateway cloning (Invitrogene) from an entry vector (pDRM21) containing the TFAP-2β open reading frame (orf). The insert in pDRM21 was derived from an RZPD clone (IRATp970A0639D). A doxycycline-inducible empty vector (EV, pDRM51) was made by self-ligation of pCW57.1 cut with BsrGI restriction enzyme to serve as a negative control. The NLS-tomato entry vector (pDRM89) was constructed by Gateway cloning

by inserting an SV40 NLS-tagged tdTomato gene into the pJS64 plasmid, a Gateway destination vector based on the pRRLSIN.cPPT.PGK-GFP.WPRE backbone (Addgene #12252) containing the MND promoter (Halene et al. 1999) driving expression of the transgene and a PGK-hygroR cassette for selection. Lentiviral particles were produced by transfection of HEK 293T/17 cells with vector plasmid (pDRM13, pDRM14 or pDRM23) and packaging plasmids (psPAX2, Addgene, #12260; pMD2-G Addgene, #12259). Plasmid DNA was mixed with polyethylenimine (PEI MAX Transfection Grade Linear Polyethylenimine Hydrochloride MW 40,000, Polysciences GmbH, #24765-1), at a ratio of 1:3, allowed to form complexes for 15min at room temperature, then added to the HEK 293T/17 cells. The medium was changed to Opti-MEM (Gibco, #31985076) after 12hr of transfection. Conditioned medium containing viral particles was harvested after 48hr, concentrated 200-fold using Vivaspin 20 columns (GE Healthcare #28-9323-63) according to manufacturer's instructions, and frozen at -80°C.

MDA-MB-231 breast cancer cells (negative for TFAP-2α and TFAP-2β expression) were seeded in 6-well cell culture plates at a density of 3 x $10^5$ cells/well and infected with pDRM13 or pDRM14 viral particles at a multiplicity of infection of 1. MFM-223 breast cancer cells (negative for TFAP-2β expression) were seeded in 6-well cell culture plates at a density of 4 x $10^5$ cells/well and infected with pDRM23 viral particles at a multiplicity of infection of 1. After 48hr, the Opti-MEM medium was replaced with normal growth media, and antibiotic (5µg/ml blasticidin) was added to select for transfected cells. Cells were expanded under antibiotic selection for two passages then used for proliferation or western blot analysis (Appendix 3C).

### 4.2.3 Proliferation and apoptosis assays

MDA-MB-453 mKATE2-labelled cells were seeded at a density of 1.5 x $10^4$ cells/well in 96-well cell culture plates (CoStar) and reverse transfected with increasing concentrations (2.5, 5, and 10nM) of one of two distinct siRNAs (Integrated DNA technologies, # hs.Ri. TFAP2B.13.1 and hs.Ri. TFAP2B.13.7) specifically targeted to TFAP-2β (siAP-2β, Appendix 3B) using the Lipofectamine RNAiMax reagent (Thermo Scientific, #13778150) according to the manufacturer's instructions. Transfection of a non-specific siRNA (Qiagen AllStars Negative Control siRNA, Qiagen, #1027281) was used at a concentration of 10nM as a negative control. IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent (Essen Bioscience, #4440) was added to the culture media at a final concentration of 5µM to detect cell death.

MFM-223 tomato-labelled cells engineered to express TFAP-2β were seeded at a density of 7 x $10^3$ cells/well in 96-well cell culture plates (CoStar) and then treated with vehicle (EtOH) or 10nM DHT and 100ng doxycycline. Treatments were refreshed every 3 days. Plates were imaged over a period of 5-7 days, collecting four images per well every 3 hours with a 10x objective on the IncuCyte S3 (Essen Bioscience). The resultant images were analysed using the associated IncuCyte S3 software, adjusting the mask and filter settings for image analysis using a training set of 6 images (Appendix 3A).

### 4.2.4 Western blot

To confirm specificity of the C-6 (Santa Cruz, sc-390119) and PA5 (Thermo Scientific, # PA5-17348) anti-TFAP-2β antibodies, MDA-MB-231 cells engineered to express TFAP-2α or TFAP-2β were seeded in 6-well cell culture plates (CoStar) at a seeding density of 2 x $10^5$ cells/well and allowed to attach for 48hr. For silencing TFAP-2β, MDA-MB-453 cells were seeded at a density of 7.5 x $10^5$ cells/well in 6-well cell culture plates and transfected with siNC (10nM) or increasing concentrations (2.5, 5, and 10nM) of siAP-2β(a) or siAP-2β(b) using the Lipofectamine RNAiMax reagent for 72hr or 32hr, respectively (time points were selected based on the pre-determined potency of siAP-2β). Cells were harvested by scraping into ~150µl cold RIPA buffer, supplemented with 1x cOmplete Protease Inhibitor (Roche). Protein concentration was quantified by Pierce BCA protein assay (Thermo Scientific) before immunoblotting. Protein electrophoresis & immunoblotting were conducted as described in Chapter 2 section 2.2.5. Membranes were probed with the following primary antibodies: TFAP-2β (Thermo Scientific, # PA5-17348, 1:1000), TFAP-2α (Santa Cruz, SC-12726, 1:500), C-MYC (Cell Signaling Technology, #9402, 1:1000), AR (Santa Cruz, #SC-816, 1:1000), HER2 (DAKO, #A0485, 1:1000), and GAPDH (Millipore, #MAB374, 1:2000). Detection of primary antibodies was performed using HRP-conjugated anti-mouse (DAKO, #P0161, 1:1000) or anti-rabbit (DAKO, #P0448, 1:1000) secondary antibodies. Signals were visualized with Clarity Western ECL Substrate (Bio-Rad) as described by the manufacturer and imaged using ChemiDoc MP (Bio-Rad) imaging system.

### 4.2.5 RNA isolation and quantitative RT-PCR

MDA-MB-453 cells were seeded at a density of 7.5 x $10^5$ cells/well in 6-well cell culture plates and transfected with siNC (10nM) or increasing concentrations (2.5, 5, and 10nM) of siAP-2β(a) using the Lipofectamine RNAiMax reagent for 48hr. RNA was purified using Trizol (Sigma, #T9424) and DNAse treatment was performed using the TurboDNase kit (Thermo Scientific) following manufacturer's instructions. RNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Total RNA (1µg) was reverse transcribed into cDNA using an iScript Select cDNA Synthesis Kit (Bio-Rad, #1706691) following manufacturer's instructions in the presence of both oligo (dT) and random primers. Quantitative RT-PCR was conducted with iQ SYBR Green Supermix (Bio-Rad, #1708866) using a CFX384 Real-Time PCR Detection System (Bio-Rad). Primers and RT-PCR conditions are listed in Chapter 2 (Table 2.3). Relative gene expression was determined by the ΔΔCT method using GAPDH as an internal control for quantification analyses of gene targets.

### 4.2.6 Cell cycle analysis

MDA-MB-453 cells treated with siNC (10nM) or siAP-2β(a) (5 and 10nM) were washed once in PBS and centrifuged at 2700 rpm for 5min. Cell pellets were resuspended in 500µL PBS and slowly dropped into 4.5mL of pre-chilled 70% ethanol and incubated overnight at 4°C. The fixed cells were washed with PBS, then pelleted and resuspended in 1mL of PI solution containing 50µg/mL PI, 100µg/mL RNase A (Sigma, #R4642), 0.1% Triton X-100, and incubated

for 30min at RT. Cells were then analysed using FACS Canto II (BD Biosciences).

### 4.2.7 Immunohistochemistry (IHC)

Paraffin-embedded tissue sections (5µm) were baked onto adhesive microscope slides (TRAJAN) for 45min at 50°C before xylene deparaffinization and dehydration with 100% ethanol. Slides were incubated with 0.9% hydrogen peroxide (Chem-Supply, Australia) to quench endogenous peroxidase activity before subjected to heat-induced epitope retrieval using a Decloaking Chamber (BioCare-medical) and 10mM citrate buffer (pH 6.5). The slides were subsequently incubated for 1hr at RT with 5% goat serum followed by monoclonal mouse antibody to TFAP-2β (C-6, Santa Cruz, #sc-390119, 1:400); AR (AR (Abcam, # ab108341, 1:1000); and ERα (DAKO, #M7047, 1:300) in a humidified chamber at 4°C. Slides were washed two times with 1x PBS and incubated with Goat Anti-Mouse Immunoglobulins/Biotin (DAKO, #E0433, 1:400) or Goat Anti-Rabbit Immunoglobulins/Biotin (DAKO, #E0432, 1:400) secondary antibody for 1hr at RT, developed using 3-3′-diaminobenzidine chromogen (Sigma, #D9015), and counterstained with hematoxylin. Slides were scanned using a NanoZoomer 2.0HT Microscopy System (Hamamatsu).

### 4.2.8 Co-immunoprecipitation

MDA-MB-453 cells were seeded in 150mm tissue culture dishes (Corning) at a seeding density of 6 x $10^6$ cells/dish in DMEM media supplemented with 10% FBS and allowed to grow for 72hr. Two independent replicate experiments

were performed, representing consecutive passages of cells. Cells were cross-linked with 1%formaldehyde for 10min, quenched with glycine pH 7.5 (final concentration 0.2M), washed with ice-cold PBS, and harvested into ice-cold PBS containing protease inhibitors (1x PI Cocktail) using a cell scraper. For nuclear extraction, cell pellets were resuspended in lysis buffer-1 (LB1) followed by rotation mixing for 10 min at 4°C. Then, nuclei were pelleted and resuspended in LB2 buffer and rotated at 4°C for 5min. The samples were resuspended in LB3 buffer and sonicated for 10 cycles (30 seconds on, 30 seconds off) using a Diagenode Bioruptor. Lysates were immunoprecipitated overnight with 5µg TFAP-2β antibody (Santa Cruz, #SC-390119) bound to Protein G Dynabeads (Thermo Scientific, #10004D). The following day, beads were washed twice with wash buffer and bound protein was eluted by boiling beads in SDS sample buffer then analysed by Western blot using AR (Santa Cruz, #SC-816, 1:1000), TFAP-2β (Thermo Scientific, #PA5-17348, 1:1000), HRP-conjugated anti-rabbit (DAKO, #P0448, 1:1000), and mouse anti-rabbit IgG conformation specific (Cell signalling, # 5127, 1:1000).

### 4.2.9 Chromatin immunoprecipitation (ChIP) coupled with PCR (ChIP-PCR) or sequencing (ChIP-seq)

For TFAP-2β or AR ChIP, MDA-MB-453 cells were seeded at a density of 8 x $10^6$ into 150mm tissue culture dishes (3x dishes per treatment) in DMEM media supplemented with 5%(v/v) charcoal-stripped FBS (DCC-FBS). Cells were left to grow for 48hr and then treated with vehicle or DHT (10nM) for 4hr. For H3K27ac ChIP, MDA-MB-453 cells were cultured with 5nM siNC or siAP-2β(a) for 48hr under steroid-stripped conditions and then treated with

vehicle (EtOH) or DHT (10nM) for 4hr. Crosslinking, isolation of nuclear pellets and chromatin sonication were performed as per the co-immunoprecipitation protocol. Sonicated lysates were immunoprecipitated overnight with 5µg of mouse monoclonal TFAP-2β antibody (Santa Cruz, # SC-390119) bound to Protein G Dynabeads (Thermo Scientific, #10004D), 5µg of rabbit polyclonal AR antibody (Abcam, #ab108341) or 2µg rabbit polyclonal H3K27ac antibody (Abcam, # Ab4729) bound to Protein A Dynabeads (Thermo Scientific, #10002D). The following day, beads were washed six times with ice-cold RIPA buffer followed by one wash with cold TE buffer supplemented with 50mM NaCl to remove unbound DNA. Protein-DNA complexes were eluted from the beads using 200µL elution buffer. 150µL of the elution buffer was also added to the input sample and both the input and ChIP samples were incubated overnight at 65°C to reverse protein-DNA crosslinks. The following day, the supernatant was collected and diluted with 200µL elution buffer, ready for DNA purification. For DNA extraction, 8µL of 1mg/mL RNase A (Sigma, #R4642) was added to the samples and incubated at 37°C for 1hr. Proteins were digested by incubation with 20mg/mL Proteinase K for 2hr at 55°C. DNA was purified using 400µL phenol-chloroform-isoamyl alcohol (25:24:1). DNA precipitation was performed overnight at 80°C with 2µL glycogen and 800µL 75% isopropanol. DNA was pelleted at 14,000g for 30min, washed with 70% ethanol and air-dried. The pellets were resuspended in 20µL 10mM Tris-HCl pH 8.0. DNA concentration was measured using Qubit® 2.0 fluorometer (Invitrogen). For ChIP-seq, DNA was sequenced using an Illumina NextSeq 500 (High Output v2) with 75 bp

single-end reads to a depth of 20 million reads per sample. PCR was conducted as described in Section 4.2.5.

ChIP-seq data was analysed as described in Chapter 3 (section 3.2.2.2). In brief, FastQ files were quality checked using FastQC (Galaxy v0.72+galaxy1) and then trimmed using the default parameters of Filter by quality tool (Galaxy v1.0.2+galaxy0) to remove poor-quality reads and adapters. Trimmed reads were aligned to the hg19 genome assembly using Bowtie2 (Langmead et al. 2009). Poorly mapped (MAPQ < 10) and duplicate reads were removed using filter SAM/BAM tools (Galaxy v1.8+galaxy1). Peaks were called using MACS2 (default settings) against a pooled input sample. An overview of the number of the reads that passed through the quality control assessment steps, mapping efficiency, and number of called ChIP peaks for each sample are listed in Appendix 3D-E. Consensus cistromes were created using Bedtool intersect intervals (Galaxy v2.29.0) and combine overlapping-nearby intervals (Galaxy v2.29.2). Consensus cistromes included peaks called in at least two of the three independent replicates for a given factor and experimental condition. Differential analysis was performed using the edgeR package as previously described (Hickey, TE et al. 2021). ChIP-seq data were visualized on the genome using the Integrative Genomics Viewer (Robinson, JT et al. 2011); where an average enrichment signal was generated from all replicates by combining mapping files (bam) to a given condition using Merge bam Files (Galaxy Version 1.2.0), followed by conversion to bigwig format using bamCoverage tool (Galaxy Version 3.3.2.0.0). Venn diagrams were prepared using Galaxy Cistrome (Venn Diagram (version 1.0.0)). A principal component analysis (PCA) plot and heatmaps were generated from multiBigwigSummary

using Generate PCA plots (Galaxy v3.3.2.0.0) and plotHeatmap (Galaxy Version 3.3.2.0.1) tools, respectively. Gene ontology analysis was conducted using the default parameters of GREAT (Genomic Regions Enrichment of Annotations Tool) (McLean et al. 2010).

ChIP–PCR data were analysed by the percentage input method and further analysed as fold enrichment over negative control (NC) for DNA binding.

### 4.2.10 *In situ* proximity ligation assay (PLA)

For tumour tissues, the standard IHC protocol was followed until the protein blocking step. For 2D adherent cells, MDA-MB-453 cells were seeded on glass coverslips (20 x 20 mm) at a density of 3.5 x $10^5$ cells/well in 6 well tissue culture plates in DMEM media supplemented with 5% DCC-FBS. Following a 48hr incubation to allow cell adherence, cells were treated with vehicle (EtOH) or DHT (10nM) for 4hr and then fixed with 10% neutral buffered formalin for 10min at RT and permeabilized with 0.1% Triton X-100 in PBS for 1hr. Tumour tissues and cell line samples were protein blocked by adding 10% donkey serum in PBS (matching the host species of the labelled secondary antibody) and incubated for 30min at RT in a humidity chamber. The blocking solution was then replaced with a 1:400 dilution of rabbit anti-AR (LifeSpan Biosciences, #B3326) and 1:400 dilution of mouse anti-TFAP-2β (Santa Cruz, # SC-390119) antibodies and incubated overnight in a humidified chamber at 4°C. Secondary antibodies conjugated to unique DNA probes (PLA probe MINUS and PLA probe PLUS) were added for 1hr. PLA probes were ligated by incubating with ligase enzyme diluted 1:40 in ligation buffer for 30min at 37°C in a humidity chamber. Samples were washed three times with 1x wash

buffer A before incubation with polymerase enzyme (diluted 1:80 in amplification buffer) for 40min at 37°C in a humidity chamber. Following incubation, samples were washed twice for 10min in 1x wash buffer B and then washed once in 1:100 diluted wash buffer B for 1min. After that, samples were incubated with nuclear stain DAPI (1/1000 in PBS) for 1min, washed twice for 5min in PBS in the dark, and covered with DAKO mounting medium. Positive protein interaction signal was visualised as distinct fluorescent spot foci and was captured using Texas red and UV filters on the Olympus FV3000 confocal microscope (Adelaide Microscopy, The University of Adelaide).

### 4.2.11 Animal models and surgical procedures

Animal experiments involving the molecular apocrine patient-derived xenograft (PDX) breast cancer models were performed via collaboration (Prof. Elgene Lim, Connie Johnson Breast Cancer Research Laboratory, Garvan Institute for Medical Research, Sydney) using standard techniques as described (DeRose et al. 2011; Hickey, TE et al. 2021). In brief, pieces (~ $2mm^3$) of PDX tumours were placed into the fourth inguinal mammary fat pad of NSG mice (Australian BioResources). When tumours became palpable, they were measured twice weekly using electronic callipers to monitor growth, and volume was calculated using the formula (length x $width^2$)/2. Tumour-bearing mice were randomly assigned into vehicle or DHT treatment groups when tumour volume reached ~$200mm^3$ (n =4/arm). DHT pellets (60-day slow-release; 10mg, Innovative Research of America) were surgically implanted subcutaneously. Upon reaching endpoint, mice were euthanised, and primary tumours collected. After weighing, the tumour was fixed overnight at 4°C in

10% neutral-buffered formalin. Formalin-fixed samples were processed for IHC by the Garvan Institute Histology Core Facility.

### 4.2.12 Statistical analysis

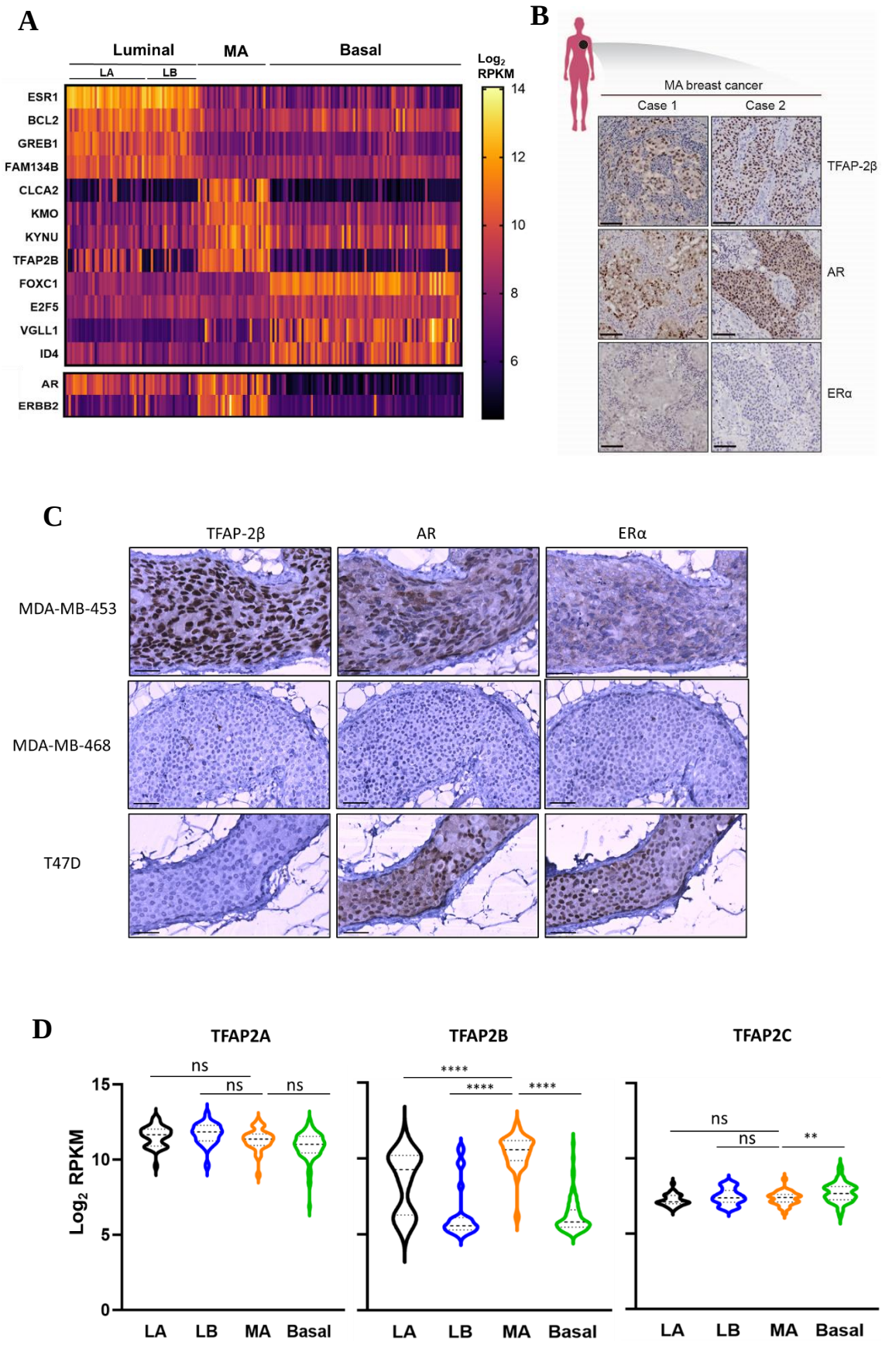
Statistics were performed using Graphpad Prism 9.0. Two-tailed Student t tests, or one-way or two-way ANOVA followed by Tukey post hoc multiple comparison tests were used as determined by the data. In the majority of cases, two independent experiments with three replicates per treatment condition were conducted. All data are expressed as the mean ± SEM. A value of $P < 0.05$ was considered statistically significant.

## 4.3 Results

### 4.3.1 Transcription factor AP-2β is highly expressed in molecular apocrine breast cancers.

Expression levels of *TFAP2B* were examined using microarray-based gene expression data generated from breast tumours (n=176) that represented a sub-group of the EORTC trial (Bonnefoi, Hervé et al. 2011). Luminal, molecular apocrine, and basal breast tumours were identified using PAM50 classification as well as subtype-specific signature genes as described (Farmer et al. 2005; Iggo 2018). Molecular apocrine tumours comprised all ERα-negative tumours outside the basal group that were positive for expression of AR and displayed apocrine histological features (e.g., abundant eosinophilic cytoplasm and prominent nucleoli). Relative expression of *AR* and *ERBB2* (commonly overexpressed in molecular apocrine tumours (Banneau et al. 2010; Farmer et al. 2005; Sanga et al. 2009)) is included in the clustered mRNA data presented in Figure 4.1A. Analysis of *TFAP2B* mRNA expression among the three breast cancer sub-types demonstrated that it was most highly enriched in molecular apocrine tumours (Figure 4.1A). While some luminal tumours were positive for this factor, *TFAP2B* clearly demarcated the molecular apocrine phenotype (Figure 4.1A) among the ERα-negative tumour subset. High expression of TFAP-2β was verified at the protein level via immunoreactive staining in representative primary molecular apocrine breast cancer tissues (Figure 4.1B) and tumours derived from breast cancer cell line mammary intraductal (MIND) xenografts (Figure 4.1C). As expected, high TFAP-2β protein levels were evident in the prototypic molecular apocrine breast cancer cell line model,

MDA-MB-453, but absent in cell line models representing luminal and basal breast cancer, T47D and MDA-MB-468, respectively (Figure 4.1C). Moreover, *TFAP2B* was the only TFAP-2 family member that discriminated molecular apocrine breast tumours from the other two subtypes of breast cancer (Figure 4.1D). Collectively, this data shows that expression of the TFAP-2β transcription factor is highly enriched in molecular apocrine breast cancers, indicating that this factor could play a major role in determining this disease context.


A
Luminal
MA
Basal
LA
LB
ESR1
BCL2
GREB1
FAM134B
CLCA2
KMO
KYNU
TFAP2B
FOXC1
E2F5
VGLL1
ID4
AR
ERBB2
Log2 RPKM
14
12
10
8
6
B
MA breast cancer
Case 1
Case 2
TFAP-2β
AR
ERα
C
TFAP-2β
AR
ERα
MDA-MB-453
MDA-MB-468
T47D
D
TFAP2A
TFAP2B
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Log2 RPKM
15
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LA
LB
MA
Basal

**Figure 4.1:** TFAP-2β expression in breast cancer. **(A)** mRNA expression of *TFAP2B* and breast cancer subtype signature genes in clinical breast tumours (n =34 luminal A (LA), n= 25 luminal B (LB), n=32 molecular apocrine (MA), and n= 85 basal) from the EORTC trial. **(B)** Representative images of TFAP-2β IHC staining in primary molecular apocrine breast cancer tissues. **(C)** TFAP-2β expression in cell line xenografts of molecular apocrine (MDA-MB-453), luminal (T47D), and basal (MDA-MB-468) subtypes of breast cancer. Scale bar = 100µm. **(D)** mRNA expression of *TFAP-2* family members in clinical breast tumours from the EORTC trial. ns $p>0.05$; *$p<0.05$; **$p<0.01$; ****$p<0.0001$ (One Way ANOVA).

### 4.3.2 TFAP-2β is required for growth and viability of molecular apocrine breast cancer cells.

To test whether TFAP-2β is required for growth and viability of molecular apocrine breast cancer cells, the effect of silencing TFAP-2β on proliferation and apoptosis of the MDA-MB-453 cell line was investigated. Cells were transfected with increasing concentrations (2.5, 5, and 10 nM) of one of two distinct siRNAs targeted to TFAP-2β (siAP-2β) or a non-target control (siNC, 10 nM). Knockdown of TFAP-2β expression significantly reduced proliferation (Figure 4.2A, Appendix 3C) and induced apoptosis, as shown by an increase in Caspase3/7 positivity and the appearance of a sub-G1 population in flow cytometry analysis (Figure 4.2B-D, Appendix 3C). siRNA-mediated knockdown of TFAP-2β did not affect TFAP-2α protein levels, indicating specificity of the siAP-2β and lack of compensation via increased expression of TFAP-2α (Figure 4.2E) . Importantly, the anti-cancer effect of silencing TFAP-2β was accompanied by a significant reduction in expression of key oncogenes, including C-MYC and HER2 (Figure 4.2 E-F). Since AR is the predominant sex steroid receptor in MDA-MB-453 cells and has been implicated as a driver of molecular apocrine breast cancer (Cochrane et al. 2014; Doane et al. 2006; Farmer et al. 2005; Naderi & Hughes-Davies 2008; Ni et al. 2011; Park, S et al. 2011; Robinson, JL et al. 2011), I examined whether loss of TFAP-2β expression affected AR protein levels in MDA-MB-453 cells. As shown in Figure 4.2E, silencing TFAP-2β did not affect AR protein levels. Collectively, these data provide novel evidence that TFAP-2β is critical for growth and viability of molecular apocrine breast cancer cells.

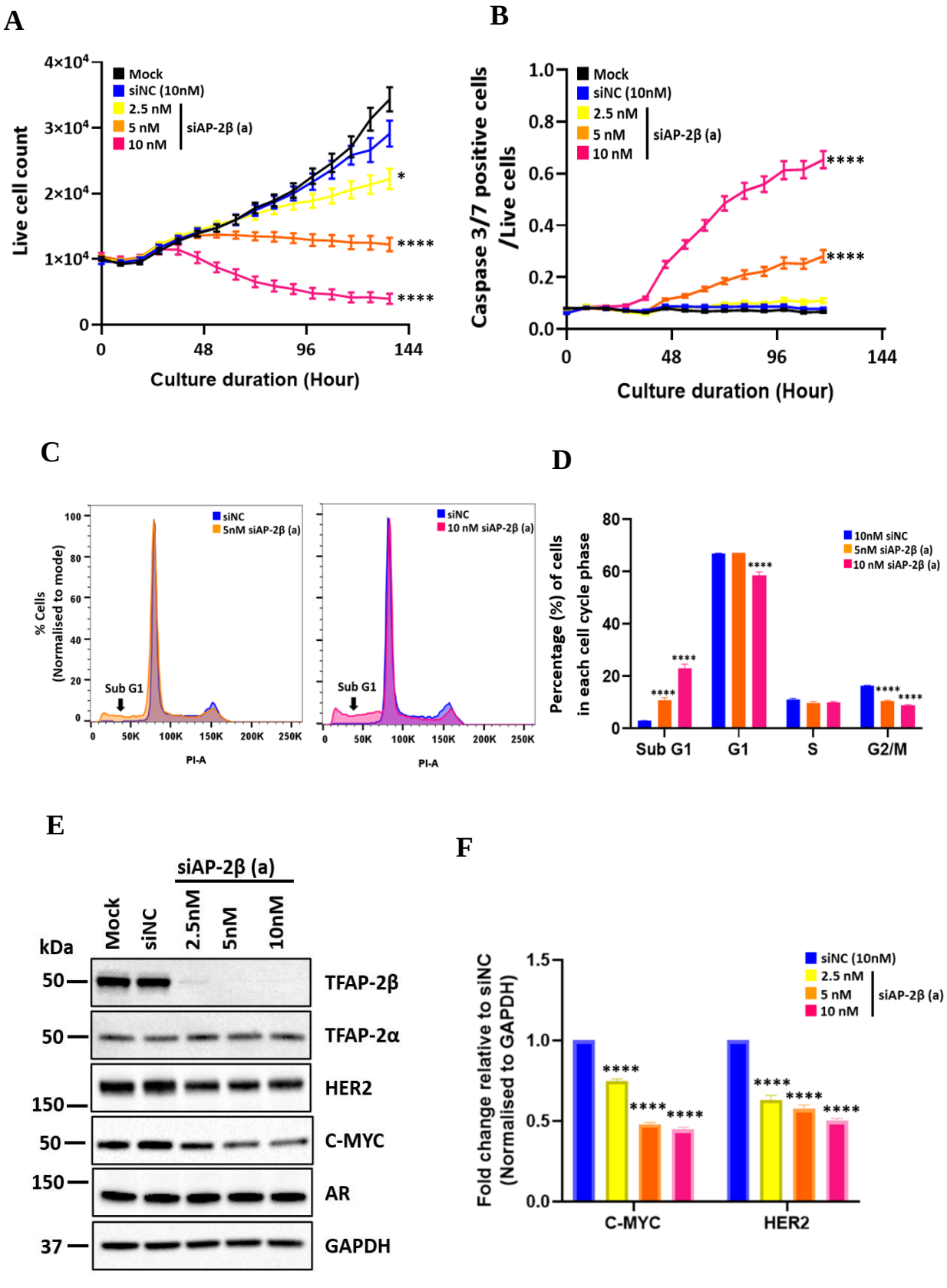

A
Live cell count
Mock
siNC (10nM)
2.5 nM
5 nM
10 nM
siAP-2β (a)
Culture duration (Hour)
B
Caspase 3/7 positive cells /Live cells
Culture duration (Hour)
C
% Cells (Normalised to mode)
siNC
5nM siAP-2β (a)
10 nM siAP-2β (a)
Sub G1
PI-A
D
Percentage (%) of cells in each cell cycle phase
10nM siNC
5nM siAP-2β (a)
10 nM siAP-2β (a)
Sub G1
G1
S
G2/M
E
siAP-2β (a)
Mock
siNC
2.5nM
5nM
10nM
kDa
TFAP-2β
TFAP-2α
HER2
C-MYC
AR
GAPDH
F
Fold change relative to siNC (Normalised to GAPDH)
siNC (10nM)
2.5 nM
5 nM
10 nM
siAP-2β (a)
C-MYC
HER2

**Figure 4.2:** The effect of TFAP-2β knockdown on proliferation, viability, and the expression of specific factors in MDA-MB-453 cells. **(A)** Growth curve showing a dose-dependent inhibition of MDA-MB-453 cell proliferation with siRNA targeted to TFAP-2β (siAP-2β) but no effect with a non-target control (siNC). **(B)** Death curve to accompany results in **(A)**, showing TFAP-2β knockdown increases Caspase 3/7-positive cells (as a dead-to live cells ratio) in a dose-dependent manner. All data represented as mean ± SEM of five replicate cell culture wells per condition and is a representative of two independent experiments. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, versus siNC (One Way ANOVA). **(C)** Cell cycle analysis representing data from MDA-MB-453 cells with 5nM (left panel) or 10nM (right panel) siAP-2β for 72hr. FlowJo software v10.7 was used for the analysis of data. FACS diagrams were normalised to the mode to depict the data in terms of % of max. The % of max denotes the number of cells in each bin (the numerical ranges for the parameter on the x axis) divided by the number of cells in the bin that contains the largest number of cells. FlowJo uses 256 bins, and each graph was scaled to the percentage of its maximum bin. **(D)** Bar graph from siNC or siAP-2β-treated MDA-MB-453 cells showing percentages of cells in different phases of the cell cycle. All data represented as mean ± SEM; ****p<0.0001, versus siNC (One Way ANOVA). **(E)** Representative immunoblots from MDA-MB-453 cells treated with or without siAP-2β for 72hr. GAPDH used as a loading control. **(F)** mRNA expression of *HER2* and C-*MYC* by RT-qPCR in MDA-MB-453 cells treated with siAP-2β for 48hr. All data represented as mean ± SEM of three replicate cell culture wells per condition and is a

representative of two independent experiments.; ****p<0.0001, versus siNC (One Way ANOVA).

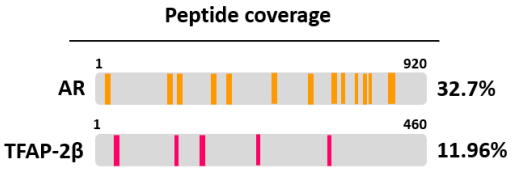
### 4.3.3 Activated AR interacts with TFAP-2β in MDA-MB-453 breast cancer cells.

Proteomic analysis of chromatin immunoprecipitated by AR revealed that TFAP-2β is part of the AR interactome in the MDA-MB-453 cell line but not in the MFM-223 cell line, a model of AR-positive/ TNBC breast cancer (Figure 4.3A-B). The presence of AR/TFAP-2β complexes was confirmed by co-immunoprecipitation (Figure 4.3C). To assess whether ligand activation of AR is required for AR/TFAP-2β interaction, we used a proximity ligation assay (PLA), which allows detection of endogenous protein-protein interactions with high sensitivity and specificity (Alam 2018). MDA-MB-453 cells were plated in media containing 5% steroid-stripped FBS and treated with either vehicle (EtOH) or 10nM DHT for 4h prior to PLA assessment. DHT significantly increased the number of nuclear foci indicative of an AR/TFAP-2β interaction (Figure 4.3D). Importantly, AR/TFAP-2β complexes were also detected in clinical molecular apocrine breast cancer tissues (Figure 4.3D).

Independent validation confirmed that TFAP-2β protein levels were highly expressed in MDA-MB-453 cells but were not detectable in MFM-223 cells (Figure 4.4A). To examine whether introducing TFAP-2β could alter AR signalling activity in the MFM-223 cell line, the effect of ectopic expression of TFAP-2β on DHT-mediated growth inhibition was tested. As shown in Figure 4.4 (B), expression of TFAP-2β did not alter basal proliferation of MFM-223 cells, but it blocked the DHT-induced growth inhibitory effect. Since knockdown of TFAP-2β significantly reduced HER2 and C-MYC expression in MDA-MB-453 cells (Figure 4.2 E-F), we hypothesized that overexpression

of TFAP-2β in MFM-223 cells may have facilitated AR-mediated expression of these factors. Surprisingly, treatment with DHT increased basal expression of HER2 and C-MYC in MFM-223 cells independent of TFAP-2β expression (Figure 4.4C). Interestingly, treatment with DHT further increased TFAP-2β levels in MFM-223 cells stimulated to overexpress this factor but had no effect in cells lacking TFAP-2β expression, suggesting an effect on protein stability. Collectively, this data suggests that TFAP-2β may interfere with the ability of AR to inhibit growth of ERα-negative breast cancer cells, but whether it actively promotes AR oncogenic activity in this context requires further investigation.

**A**

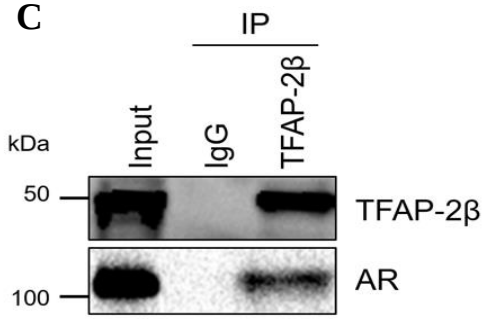



**B**

| TFAP-2β unique peptides | Query cover | Identities (%) | E value |
|---|---|---|---|
| SVTSLMMNK | 100% | 100% | 3.00E-04 |
| AVSEYLNR | 100% | 100% | 0.008 |
| AALPQLSGLDPR | 100% | 100% | 3.00E-06 |
| LVENVKYEDIYEDRHDGVPSHSSR | 100% | 100% | 3.00E-20 |
| QRQEVGSEAGSLLPQPR | 100% | 100% | 2.00E-11 |

```
  1 MHSPPRDQAA IMLWKLVENV KYEDIYEDRH DGVPSHSSRL SQLGSVSQGP YSSAPPLSHT
 61 PSSDFQPPYF PPPYQPLPYH QSQDPYSHVN DPYSLNPLHQ PQQHPWGQRQ RQEVGSEAGS
121 LLPQPRAALP QLSGLDPRRD YHSVRRPDVL LHSAHHGLDA GMGDSLSLHG LGHPGMEDVQ
181 SVEDANNSGM NLLDQSVIKK VPVPPKSVTS LMMNKDGFLG GMSVNTGEVF CSVPGRLSLL
241 SSTSKYKVTV GEVQRRLSPP ECLNASLLGG VLRRAKSKNG GRSLRERLEK IGLNLPAGRR
301 KAANVTLLTS LVEGEAVHLA RDFGYICETE FPAKAVSEYL NRQHTDPSDL HSRKNMLLAT
361 KQLCKEFTDL LAQDRTPIGN SRPSPILEPG IQSCLTHFSL ITHGFGAPAI CAALTALQNY
421 LTEALKGMDK MFLNNTTTNR HTSGEGPGSK TGDKEEKHRK
```

**C**




**D**

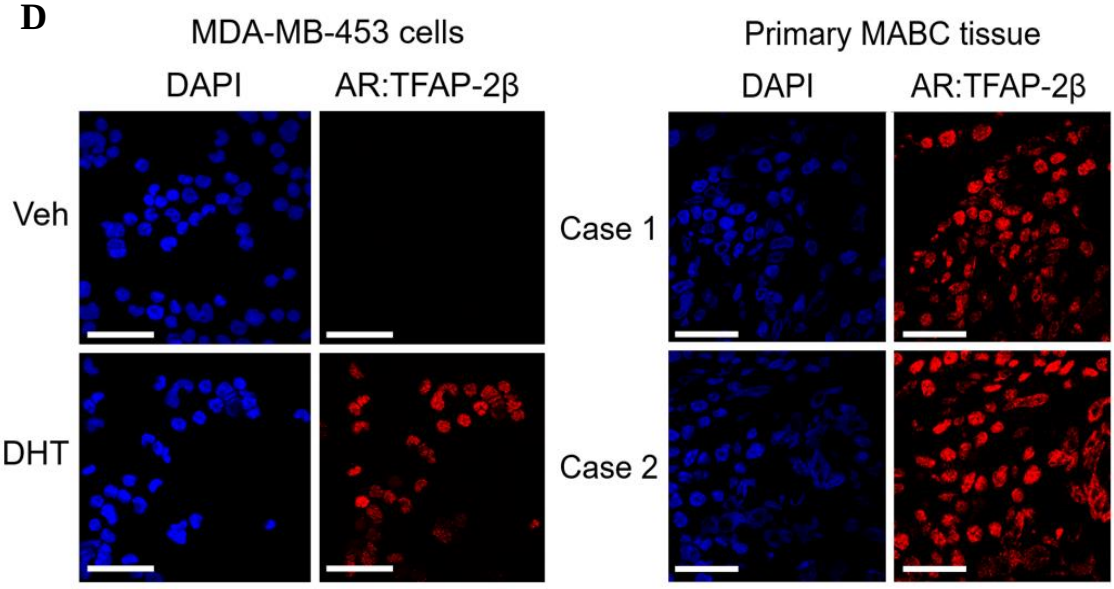


**Figure 4.3:** AR interacts with TFAP-2β in molecular apocrine breast cancers**. (A)** Peptide coverage of AR and TFAP-2β from AR RIME experiments in MDA-MB-453 cells. Orange and pink bars represent regions in which the peptides were identified; the combined sequence coverage of identified peptides is given as a percentage. **(B)** TFAP-2β unique peptides identified by RIME were tested by NCBI–Blast and five unique sequences with 100% query coverage & identities were verified (upper panel). TFAP2-β whole protein sequence was derived from NCBI and validated peptide sequences are shown (lower panel). **(C)** TFAP-2β co-immunoprecipitation (IP) validation of the AR: TFAP-2β interaction in MDA-MB-453 cells cultured in DMEM media supplemented with 10% FBS. **(D)** Proximity ligation assay experiments detecting co-localization of AR and TFAP-2β in MDA-MB-453 cells cultured under serum-deplete conditions then treated with either vehicle (Veh) or 10 nM DHT (left panels). PLA for AR and TFAP-2β in primary MABC tumours (right panels). Scale bars = 50 µm.

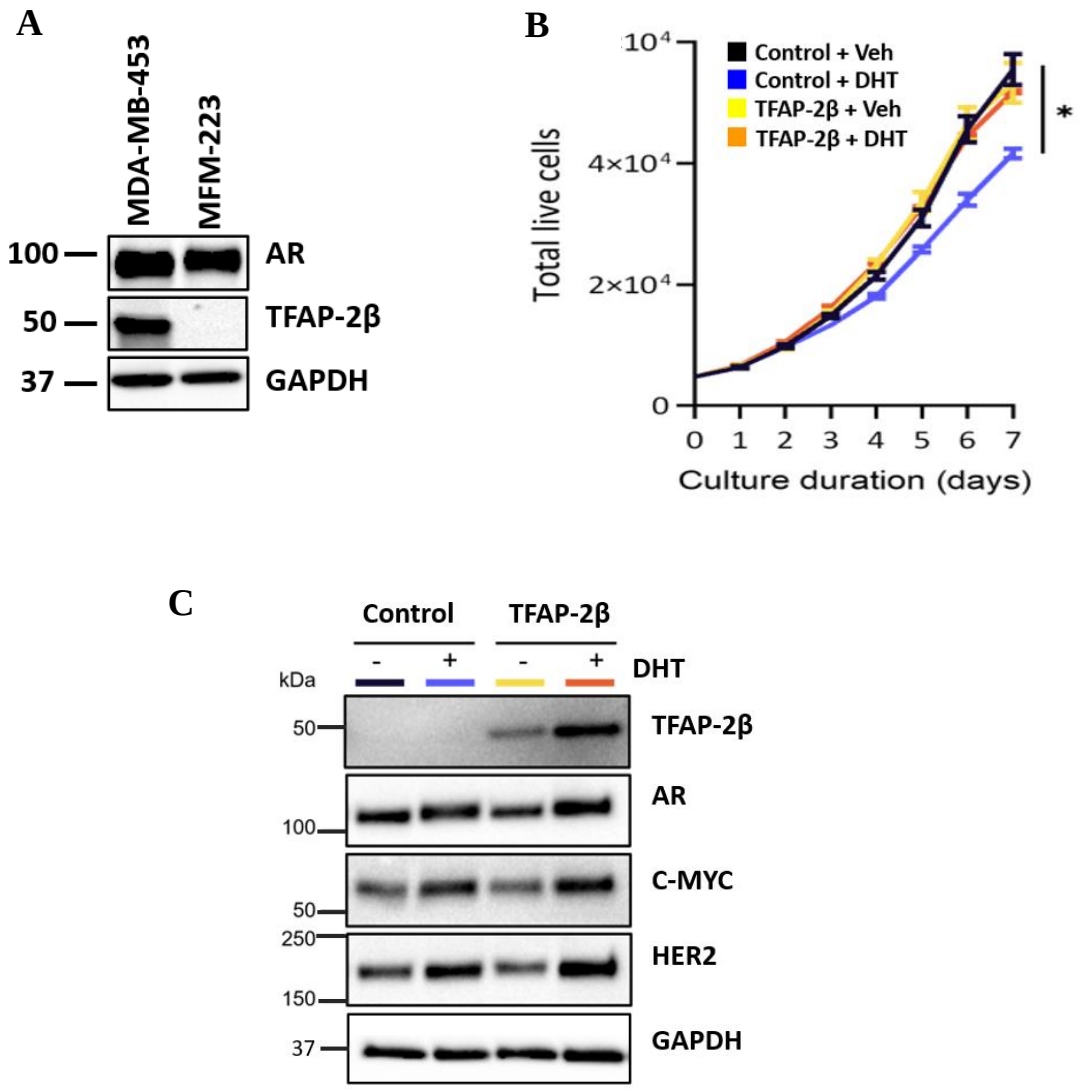



**Figure 4.4:** Lentivirus-mediated ectopic expression of TFAP-2β or empty vector (Control) constructs into the TFAP-2β negative MFM-223 TNBC cell line. **(A)** Western blot comparing TFAP-2β and AR protein levels in MDA-MB-453 and MFM-223 cell lines. **(B)** Growth curve data from MFM-223 cells engineered to ectopically express TFAP-2β then treated with either Vehicle (Veh) or 10 nM DHT. Data represents the mean ± SEM of three replicate wells, analysed using a two-way repeated measures ANOVA followed by Tukey's multiple comparisons test. *$p<0.05$. **(C)** End-point immunoblot results accompanying (B) collected after 5 days of culture.

### 4.3.4 Functional interplay between AR and TFAP-2β at the chromatin level in molecular apocrine breast cancer cells.

Since TFAP-2β interacts with AR in MDA-MB-453 cells and AR activity has been implicated as a driver of growth in this cell line, the TFAP-2β cistrome was characterized in the presence and absence of DHT to determine whether AR signalling alters TFAP-2β cis-acting targets. MDA-MB-453 cells were cultured under steroid-depleted conditions then treated 4 hours with vehicle (EtOH) or DHT prior to crosslinking. Consensus TFAP-2β chromatin binding events were generated for each treatment condition using ChIP-seq datasets derived from 3 consecutive passages of cells. As shown by the Venn diagram overlap and heat map analysis (Figure 4.5 A-B), DHT did not dramatically alter the TFAP-2β cistrome in MDA-MB-453 cells; 88% of TFAP-2β DNA binding events were shared under vehicle and DHT treatment conditions (Figure 4.5A). Moreover, our data showed that the consensus TFAP-2β was reduced by 5,315 peaks after treatment with DHT. Importantly, among the hormone-independent TFAP-2β binding sites, we identified loci at the enhancer of *ERBB2* and the promoter of *MYC* (Figure 4.5C). It is possible that loss of these binding sites following siRNA-mediated knockdown of TFAP-2β was responsible for reduced expression levels of these genes (as shown above in Figure 4.2D). Moreover, we identified TFAP-2β peaks at the promoter of other genes involved in cell cycle progression, including *WEE1* and *E2F2*, as well as the molecular apocrine signature genes *CLCA2* and *KMO* (Figure 4.5C). We hypothesized that knocking down TFAP-2β would alter transcriptional activity at TFAP-2β binding sites, so performed H3K27ac (a mark of transcriptional activation) ChIP-seq in MDA-MB-453 cells cultured

with siNC or 5nM siAP-2β for 48 hours. Globally, levels of H3K27ac were largely unchanged by knockdown of TFAP-2β (Appendix 3F). In total, 6,868 sites had a significantly lower enrichment of H3K27ac following knockdown of TFAP-2β (Appendix 3F). Gene ontology analysis of genes associated with these reduced H3K27ac ChIP-seq signals revealed enrichment for the Sterol regulatory element-binding proteins signalling pathway (SREBP, lipid metabolism pathway), chromatin remodelling, and cell cycle regulation (Appendix 3F). *MYC*, *WEE1* and *E2F2* were among the genes that showed a significant decrease in H3K27ac enrichment with siAP-2β (Figure 4.5C), consistent with their regulation by TFAP-2β.

As expected from previous results showing an interaction, DHT-induced activation of AR resulted in an association between the AR and TFAP-2β cistromes, whereby AR was recruited to 36% of all TFAP-2β DNA binding sites, representing 45% of the AR cistrome (Figure 4.7A, B). While we expected that this interaction might be important for AR recruitment and transcriptional activity at co-occupied cis-regulatory elements, this was not the case, as knocking down TFAP-2β with siAP-2β for 48hr reduced but did not prevent AR enrichment at representative loci in MDA-MB-453 cells (Figure 4.5C). Knockdown of TFAP-2β also had no significant effect on DHT-induced AR transactivation activity for associated genes (Figure 4.5C), indicating that TFAP-2β may facilitate AR binding but is not a critical determinant of AR DNA binding and transcriptional activity. Independent of AR signalling, siAP-2β significantly decreased expression of *CLCA2* (a molecular apocrine signature gene) and increased expression of *FAR2* (lipid metabolism gene) compared to siNC under vehicle (basal) conditions (Figure 4.5C).

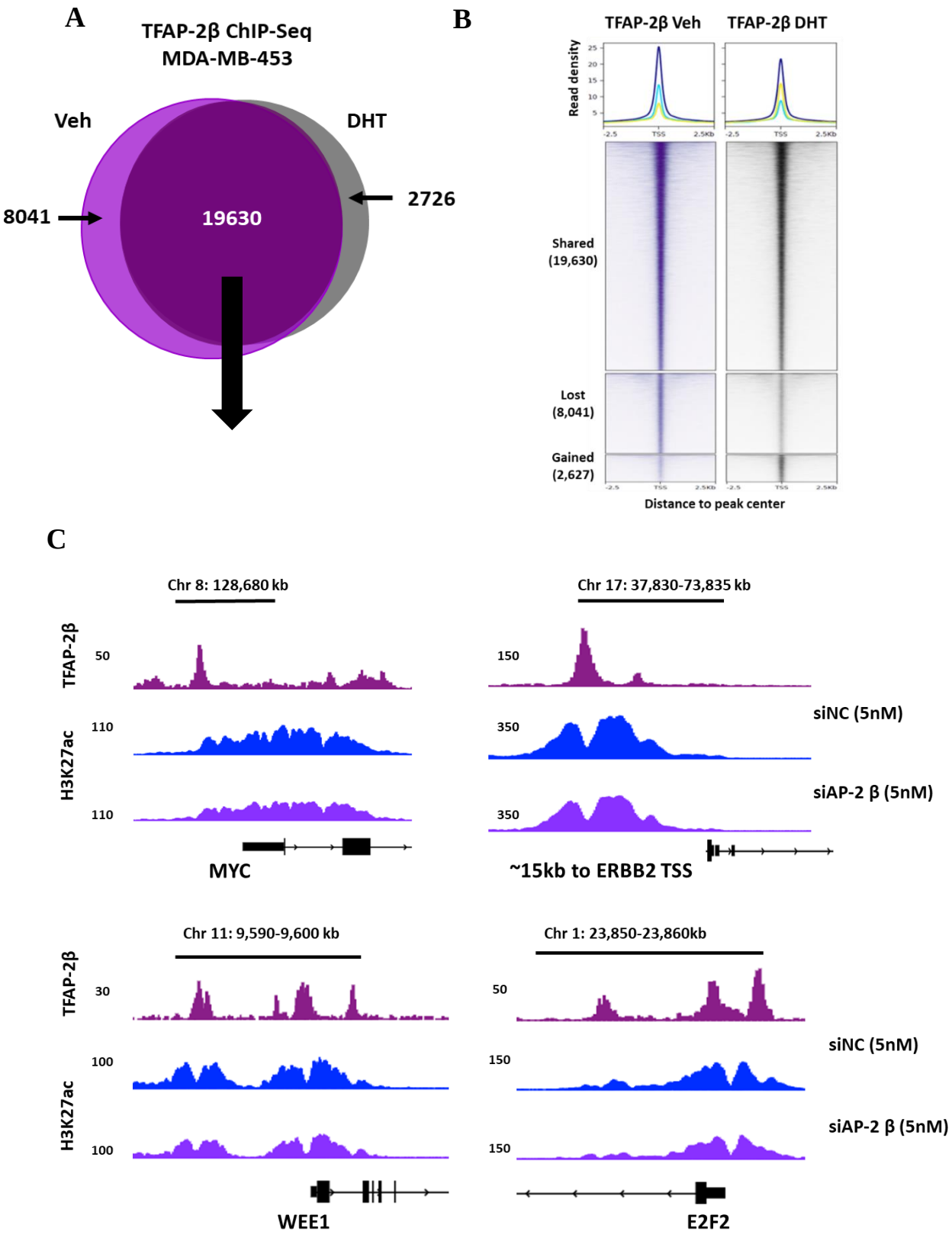

A
TFAP-2β ChIP-Seq
MDA-MB-453
Veh
DHT
8041
19630
2726
B
TFAP-2β Veh
TFAP-2β DHT
Read density
Shared
(19,630)
Lost
(8,041)
Gained
(2,627)
Distance to peak center
C
Chr 8: 128,680 kb
TFAP-2β
H3K27ac
MYC
Chr 17: 37,830-73,835 kb
siNC (5nM)
siAP-2 β (5nM)
~15kb to ERBB2 TSS
Chr 11: 9,590-9,600 kb
WEE1
Chr 1: 23,850-23,860kb
E2F2

**Figure 4.5:** Analysis of the TFAP-2β cistrome in MDA-MB-453 cells. **(A)** Venn diagram showing overlap of consensus TFAP-2β cistromes under vehicle control and DHT (10nM) treatment conditions. **(B)** Heatmaps and corresponding read density plots showing average consensus ChIP-seq signals (FPKM) and the number of shared, gained and lost TFAP-2β binding sites in MDA-MB-453 cells cultured in steroid-deplete conditions and treated for 4 hours with either Vehicle (Veh) or 10 nM DHT. **(C)** Genome browser images of TFAP-2β and H3K27ac chromatin occupancy near genes involved in cell growth. Treatment with 5nM siAP-2β for 48hr decreased H3K27ac enrichment at these sites.

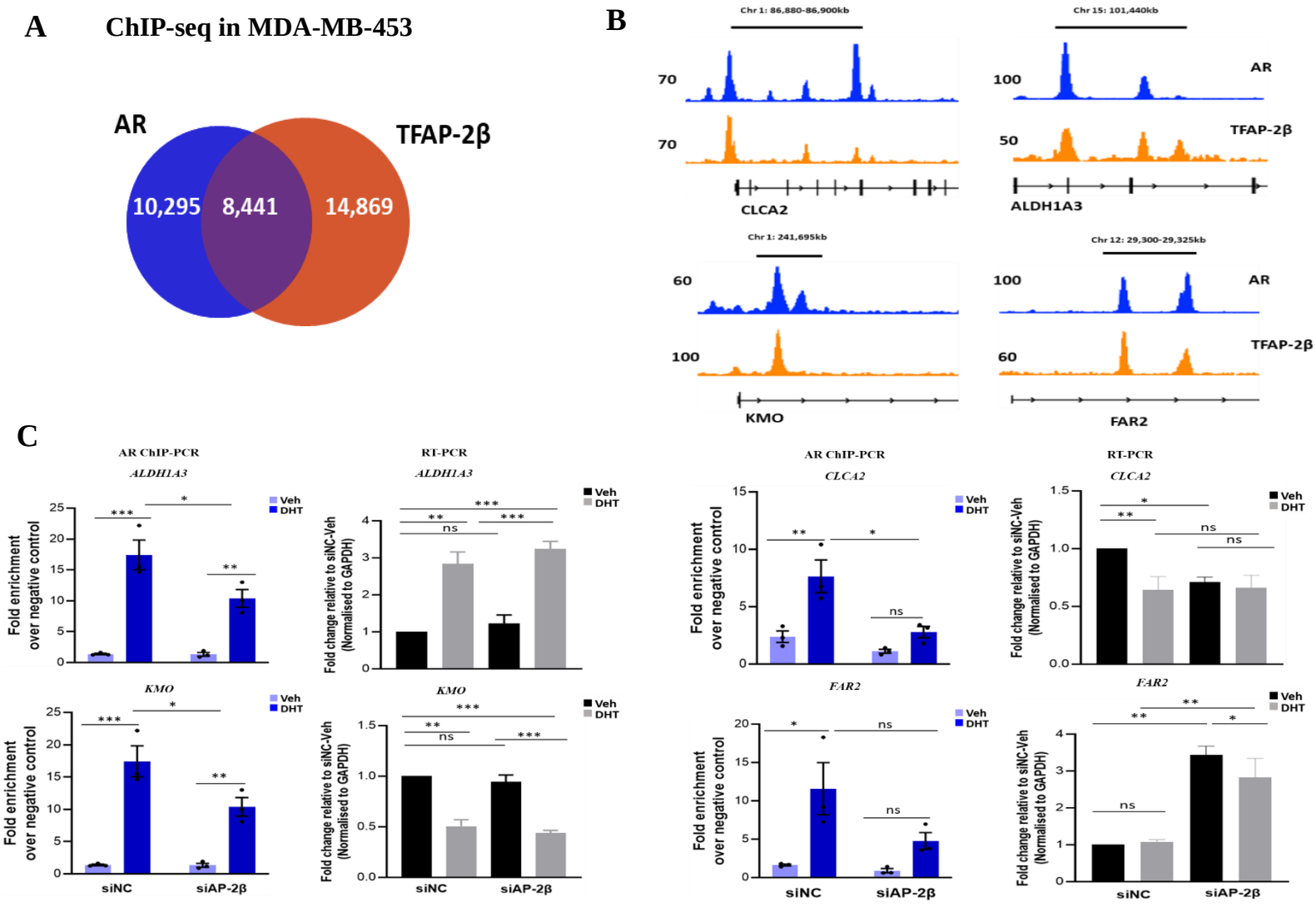

A
ChIP-seq in MDA-MB-453
AR
TFAP-2β
10,295
8,441
14,869
B
Chr 1: 86,880-86,900kb
Chr 15: 101,440kb
AR
TFAP-2β
CLCA2
ALDH1A3
Chr 1: 241,695kb
Chr 12: 29,300-29,325kb
KMO
FAR2
C
AR ChIP-PCR
RT-PCR
ALDH1A3
CLCA2
KMO
FAR2
Veh
DHT
Fold enrichment over negative control
Fold change relative to siNC-Veh (Normalised to GAPDH)
siNC
siAP-2β

**Figure 4.5:** Interaction of AR and TFAP-2β at the chromatin level. **(A)** Venn diagram showing overlap of consensus AR and TFAP-2β cistromes in MDA-MB-453 cells treated with 10 nM DHT for 4hr, showing that 45% of AR binding sites are co-occupied by TFAP-2β. **(B)** Representative genome browser images of TFAP-2β and AR shared DNA-binding sites associated with molecular apocrine breast cancer signature genes. **(C)** Representative examples of AR binding sites (assessed by ChIP-PCR) and associated genes (assessed by RT-PCR) showing that knockdown of TFAP-2β reduced but did not prevent AR enrichment at representative loci and did not significantly influence AR transcriptional activity. ChIP-seq, ChIP-PCR and RT-PCR experiments were conducted in MDA-MB-453 cells, cultured in serum-deplete conditions then treated for 4 hours with either Vehicle (Veh) or 10 nM DHT. ChIP-PCR data represented as mean ± SEM of three independent replicate experiments, representing consecutive passages of cells. RT-PCR data represented as mean ± SEM of three well replicates of two independent experiments. ns $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$, using One Way ANOVA.

### 4.3.5 Effect of DHT in patient-derived xenograft (PDX) models of molecular apocrine breast cancer

Activation of AR signalling with the AR agonist DHT has never been directly assessed in PDX models of molecular apocrine breast cancer. This study examined the effect of DHT on growth of three PDX models (CTPx3921, GAR 14-06A, and HCI-008) that were chosen for being ERα-negative, AR-positive and HER2-positive to specifically classify them as molecular apocrine tumours. Treatment with DHT (60-day slow-release; 10mg) significantly decreased CTPx3921 xenograft growth, but increased growth of GAR 14-06A tumours (Figure 4.6C), providing compelling pre-clinical evidence for differential growth effects of AR in the molecular apocrine breast cancer context. A slight decrease in the growth of HCI-008 tumours was observed with DHT but it was not significant (Fig.4.6C). High TFAP-2β levels were detected in these three molecular apocrine PDX models (Figure 4.6B), confirming findings from Figure 1 and the molecular apocrine status of these models. However, the high expression of TFAP-2β in all models suggest that levels of TFAP-2β do not predict response to DHT, consistent with findings from section 4.3.3 that TFAP-2β influences but is not a critical determinant of AR oncogenic effect in molecular apocrine breast cancer.

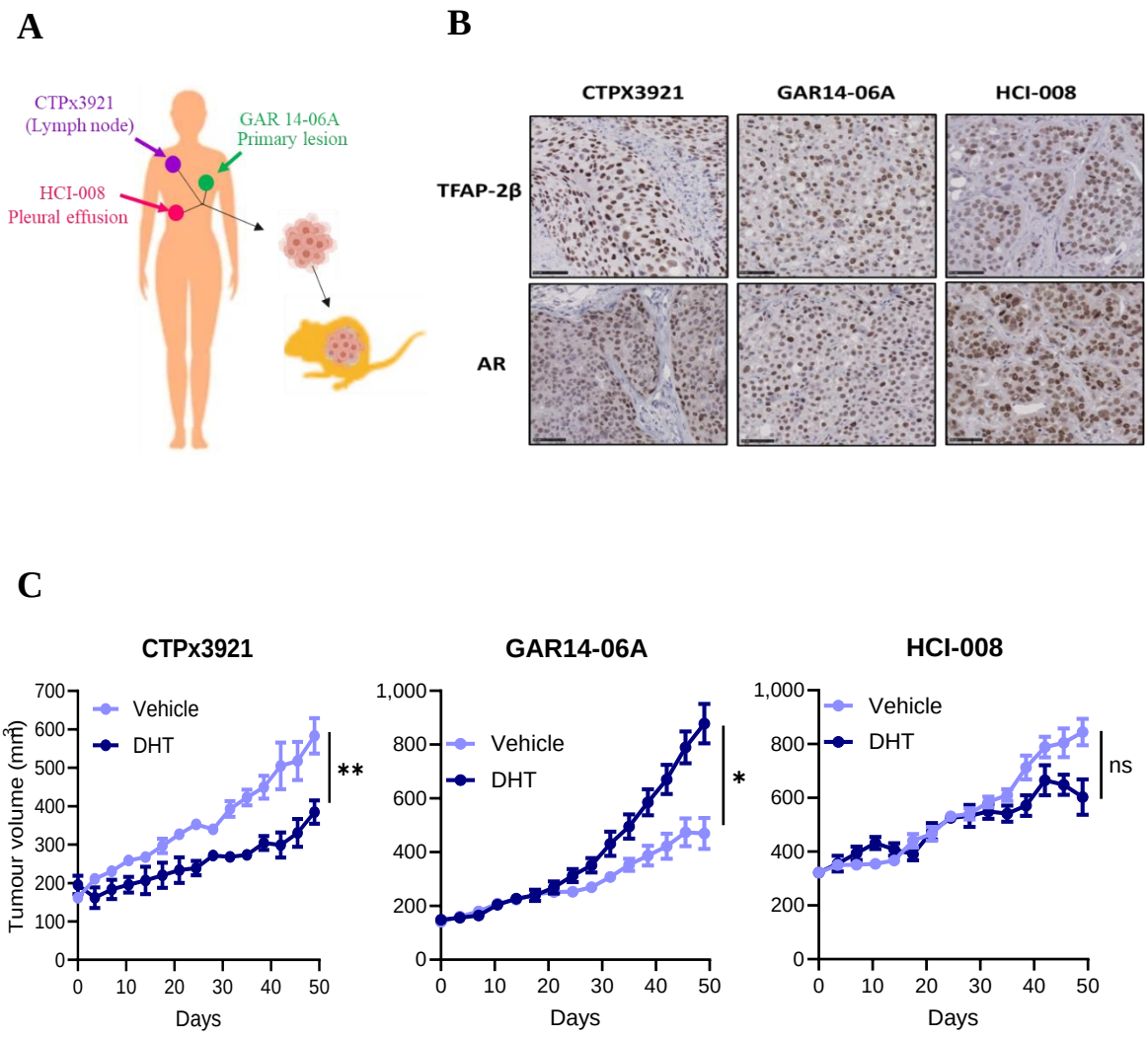



**Figure 4.6:** Differential growth effects of AR signalling in PDX models of molecular apocrine breast cancer. **(A)** PDX models derived from primary or metastatic deposits of clinical molecular apocrine breast cancers. **(B)** Representative IHC images of AR and TFAP-2β in molecular apocrine PDX models. Scale bars = 50 µm. **(C)** Tumour growth plots from three molecular apocrine PDX models treated *in vivo* with vehicle or DHT (60-day slow-release DHT pellets (10 mg). N= 4 for each treatment arm for each experiment. All data represented as mean ± SEM; ns $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$, using unpaired, one-tailed t-test.

## 4.4 Discussion

Members of the TFAP-2 family of transcription factors have diverse biological roles and have been implicated in cancer. Herein, the biological role of TFAP-2β in the molecular apocrine subtype of breast cancer was investigated. Using gene expression datasets and immunoreactive staining, we demonstrated high TFAP-2β expression in clinical molecular apocrine tumours and using a representative cell line (i.e., MDA-MB-453), showed that TFAP-2β was required to sustain proliferative capacity and cell viability. Although we reveal a novel interaction between TFAP-2β and AR, a putative driver of molecular apocrine breast cancer, we did not find evidence that this interaction was a critical determinant of oncogenic AR signalling. Hence, TFAP-2β is a transcription factor specifically enriched in molecular apocrine breast cancers that has a role in promoting cell viability and proliferative capacity, perhaps independent of AR signalling.

Within the context of ERα-negative breast cancers, TFAP-2β was almost exclusively expressed in molecular apocrine tumours, with no or very little expression in basal tumours. This remarkable difference in TFAP-2β expression could be due to differential expression of the HER2 oncogene. Overexpression of HER2, particularly via *ERBB2* amplification, commonly occurs in molecular apocrine breast cancers (Banneau et al. 2010; Farmer et al. 2005; Sanga et al. 2009). In molecular apocrine tumours without *ERBB2* amplification, *ERBB2* activating mutations are found in 9% of cases, suggesting that hyper-activation of the *ERBB2* pathway may be a universal feature of this subtype (Jiang, Y-Z et al. 2019). Indeed, high *ERBB2* expression was detected in the molecular apocrine tumours that were used in this study.

The higher expression of TFAP-2β in the context of HER2-enriched molecular apocrine disease compared to HER2-negative basal breast tumours suggests a direct association between TFAP-2β and HER2 expression. Using ChIP-seq analysis, this study showed that TFAP-2β binds to an enhancer region of HER2 and that knockdown of TFAP-2β reduced the H3K27ac signal at this locus. These genomic findings were consistent with reduced expression of HER2 at the gene and protein level in MDA-MB-453 cells following TFAP-2β knockdown. Based on motif analyses, previous studies have suggested the ability of a member of the TFAP-2 family to bind and activate the *ERBB2* promoter in breast cancer cells (Bosher et al. 1996; Bosher, Williams & Hurst 1995; Hollywood & Hurst 1993). However, those studies pre-dated identification of specific members of the TFAP-2 family and the ability to interrogate the genomic occupancy of a specific member using ChIP-seq or ChIP-PCR technology. Of notable significance, our study is the first to describe the TFAP-2β cistrome in any model system and to show that TFAP-2β does not bind the promoter of the HER2 gene, but it binds to an enhancer element.

Even though high expression of *TFAP2B* distinguished molecular apocrine tumours among other tumour sub-types, *TFAP2B* expression was also detected in some luminal tumours. Importantly, the TFAP-2β expression we saw in these luminal tumours is not due to the presence of lobular cancers because we excluded them from the analysis. Nearly all of the TFAP-2β positive luminal tumours fall into the luminal-A category. In contrast to luminal-B tumours, high differentiation status and low proliferation activity characterise the luminal A subtype (Sørlie et al. 2001; Yaşar et al. 2017; Yersal & Barutca

2014). Our data could indicate that TFAP-2β plays a role in promoting a higher degree of differentiation in this particular phenotype. The exclusive expression of *TFAP2B* in luminal-A tumours is of interest and needs further investigation to understand the mechanisms underlying this finding.

Our data also showed that MFM-223 cells lack the expression of TFAP-2β (Fig. 4.4A). While MFM-223 is known to have a high expression of AR that is comparable to MDA-MB-453 cells and accordingly Lehmann et al (2011) classified it as a luminal AR (another term for molecular apocrine) (Lehmann et al. 2011), our data indicate that AR expression alone is not sufficient for the identification of molecular apocrine cell lines and that TFAP-2β can be used as a marker for this subgroup. Since MFM-223 cells have a basal phenotype and lack ERBB2 amplification, we speculated that they lack expression of TFAP-2β. In contrast, MDA-MB-453 cells showed high expression of TFAP-2β (Figure 4.1C) .While some studies describe MDA-MB-453 cells as a triple-negative breast cancer cell line because they lack ERBB2 amplification (Espinosa Fernandez et al. 2020; Lehmann et al. 2011), other studies consider MDA-MB-453 as a model for an ERα/HER2-positive cell line due to high levels of HER2 expression and used it to study resistance to HER2-targeted therapies (Goel et al. 2016; Narayan et al. 2009). Higher expression of HER2 could be associated with high expression of TFAP-2β in this cell line model of molecular apocrine breast cancer.

Is the TFAP-2β transcription factor important for maintaining proliferation and viability of molecular apocrine cells? To answer this question, we examined the effect of silencing TFAP-2β using the prototypic molecular apocrine cell

line MDA-MB-453. Silencing TFAP-2β exerted a potent growth inhibitory effect and induced caspase-dependent apoptosis *in vitro*, accompanied by a reduction in mRNA and protein levels of key oncogenes including C-MYC and HER2. While the ability of TFAP-2β to regulate the expression of C-MYC in breast cancers specifically and cancers in general was not reported prior to this study, a few studies have examined the interaction of TFAP-2 with C-MYC in other biological contexts (Gaubatz et al. 1995; Moser et al. 1997). Using motif analysis, a study by Gaubatz and colleagues (1995) showed that MYC-response elements that transcriptionally regulate two MYC target genes, prothymosin-a and ornithine decarboxylase (ODC) from different organisms (e.g. human, rat, mouse and pig), contain a high-affinity binding site for a TFAP-2 factor (Gaubatz et al. 1995). The latter study also used electrophoretic mobility shift assay to show that recombinant TFAP-2 was able to recognise and bind the MYC-response elements from prothymosin-a and ODC, and the specificity of the interaction was verified using different competitors (Gaubatz et al. 1995). Mechanistically, the Gaubatz *et al* (1995) study found that TFAP-2 did not affect the expression of C-MYC in HeLa cells transfected with MYC response elements and then co-transfected with a TFAP-2 or C-MYC expression plasmid, but it inhibited C-MYC-induced transactivation by competing for DNA binding by C-MYC and by directly interacting with the C-MYC protein, which impaired DNA binding of the MYC/MAX complex (Gaubatz et al. 1995). A subsequent study provided further evidence, reporting that TFAP-2β knock-out in mice did not change C-MYC expression but it induced apoptotic cell death of renal epithelial cells via enhancement of C-MYC transactivation (Moser et al. 1997). Herein, we demonstrated that TFAP-

2β regulates C-MYC in molecular apocrine cells and this is likely due to a binding site at the promoter region as shown by the ChIP-seq data. Loss of TFAP-2β also impaired HER2 expression in MDA-MB-453 cells. HER2 signalling pathways have shown growth-promoting effects in MDA-MB-453 cells (Ni et al. 2011) and we know now that TFAP-2β regulates HER2 expression in the context of this disease and that is likely through association with the enhancer region as shown in ChIP-seq analysis. Moreover, analysis of the TFAP-2β cistrome in MDA-MB-453 cells showed TFAP-2β binding sites at the promoter of genes involved in the cell cycle including *WEE1* and *E2F2*. The WEE1 kinase is a key regulator of the G2 cell cycle checkpoint (Ghiasi et al. 2013). In response to DNA damage, WEE1 inhibits the activity of CDK1 through phosphorylation, resulting in cell cycle arrest to allow for DNA repair (Ghiasi et al. 2013). E2F2 is a member of the E2F transcription family of transcription factors, which regulates transcription of several genes involve in cell cycle (Johnson & Schneider-Broussard 1998). Enforced E2F2 expression significantly increased MCM4 (DNA replication licensing factor) and CCNE2 (regulates the transition from G1 to S phase) expression in SKOV3 and A2780 ovarian cancer cell lines (Xie, Li & Yang 2017). In this study, we showed that knocking down TFAP-2β reduced H3K27ac enrichment at these genes but did not completely quench the signal. This may be because we did not fully knockdown TFAP-2β or may be the influence of TFAP-2β is not best reflected by H3K27ac and it could have had a very different effect on some other histone markers.

Using several proteomic techniques to detect AR interacting proteins, we identified TFAP-2β as a candidate AR interacting factor. Activation of AR did

not majorly influence the TFAP-2β cistrome, but it induced an association between AR and TFAP-2β at 36% of TFAP-2β DNA-binding sites, supporting the fact that these two factors interact on chromatin. Another transcription factor, FOXA1, has been shown to be present at approximately 100% of AR binding sites in MDA-MB-453 cells (Robinson, JL et al. 2011), indicating that the interaction between AR and TFAP -2β is more selective. The data also suggests that TFAP-2β would have AR-independent effects. We were interested to see if AR/TFAP-2β complexes are critical for AR transcriptional activity and AR oncogenic effects in molecular apocrine breast cancer cells. Experiments in this study showed that knocking down TFAP-2β reduced but did not prevent the binding of AR to co-occupied loci. The reduced AR binding did not prevent transactivation of associated genes, suggesting that AR signalling is not reliant on TFAP-2β for transactivation capacity. However, we did not perform a complete knockdown of TFAP-2β for this experiment since complete silencing would have killed the cells, precluding a definitive conclusion. Alternatively, because the AR cistrome completely overlaps with the FOXA1 cistrome and it is clear that FOXA1 is a pioneer factor for AR binding in the MDA-MB-453 cell line, we deduced that AR is probably still bound to chromatin in the absence of TFAP-2β because FOXA1 is there.

For the first time, three PDX models of molecular apocrine breast cancer were used in this study to examine the effect of AR agonist DHT *in vivo*. PDX models were selected on the basis of having high AR, no ERα and HER2 amplification. Consistent with data from clinical molecular apocrine tumours, high expression of TFAP-2β was revealed in these PDX models. The response to DHT was different in all three molecular apocrine PDX models, a surprising

finding given the prevailing view that AR drives growth of this breast cancer sub-type. However, since all of these models are TFAP-2β positive, it is unlikely this factor is the key determinant of AR oncogenic activity, which aligns with our *in vitro* data.

In summary, this work provides preliminary evidence for the clinical significance of TFAP-2β in the molecular apocrine subtype of breast cancer. High expression of TFAP-2β characterises molecular apocrine breast cancer, indicating a potential for being a candidate marker of this disease. We have also shown that TFAP-2β is critical for proliferation and viability of a cell line model of this disease. This preclinical data provides the rationale for creating a selective inhibitor of TFAP-2β. Indeed, a previous study described the development of a small molecule inhibitor against TFAP-2α/γ (Hu et al. 2018), which highlights the potential to create anti-TFAP-2β drugs.

# CHAPTER FIVE

# A NOVEL AND HIGHLY SELECTIVE CDK9 INHIBITOR (D-11) EFFECTIVELY SUPPRESSES PROLIFERATION OF TRIPLE NEGATIVE BREAST CANCERS

## 5.1 Introduction

Triple negative breast cancers (TNBC) comprise a group of highly aggressive tumours that are negative for the three major diagnostic biomarkers of breast cancer: ERα, PR, and HER2 (Chen, J-Q & Russo 2009; Yersal & Barutca 2014). Therefore, patients with TNBC are insensitive to ERα and HER2 targeted therapies and are mainly clinically managed with cytotoxic chemotherapies, which are associated with a high rate of local and systemic relapse and death (Al-Mahmood et al. 2018; Dent et al. 2007). This highlights an important and unmet clinical need to develop targeted therapies for TNBC. One avenue of recent interest is to target cyclin dependent kinases (CDKs).

CDKs are serine/threonine protein kinases that activate their substrates upon binding to a regulatory cyclin subunit bound to the substrate, with subsequent phosphate transfer (Echalier et al. 2010). To date, twenty different CDKs have been discovered in mammalian cells (Malumbres & Barbacid 2005) and based on their functional role, are broadly categorised into two main groups: 1) cell cycle CDKs (CDK1-6) that control progression through various phases of the cell cycle by phosphorylating and activating cell cycle proteins (Hartwell & Kastan 1994; Lamb et al. 2013; Malumbres & Barbacid 2005), and 2) transcriptional CDKs (CDK7-9, 12 and 19) that control initiation and elongation of mRNA transcripts by phosphorylating and activating RNA

polymerase II (RNAPII) (Lim & Kaldis 2013; Malumbres & Barbacid 2005). Dysregulation in the expression or activity of CDKs has been associated with tumorigenesis and progression of multiple cancers, including breast cancer (Diaz-Padilla, Siu & Duran 2009; Ding et al. 2020; Romano 2013). Consequently, pharmacological inhibition of CDKs has been considered an attractive anti-cancer therapeutic strategy (Cicenas et al. 2014; Mayer 2015). Indeed, inhibition of CDK4/6 has shown therapeutic efficacy in both endocrine therapy naïve and resistant ERα-positive breast cancer and are now included as standard-of-care for metastatic disease (Finn et al. 2015; Finn et al. 2009; Herschkowitz et al. 2008; Turner et al. 2015). CDK4/6 inhibitors mainly act by blocking the phosphorylation of retinoblastoma protein (Rb), which arrests the cell cycle in G1 phase (Malumbres & Barbacid 2005). However, inhibition of CDK4/6 lacks therapeutic efficacy in TNBC since this type of cancer generally lacks Rb expression (Dean et al. 2012; Ribnikar, Volovat & Cardoso 2019; Witkiewicz et al. 2016). Hence, investigation of other CDK members as potential therapeutic targets for TNBC is warranted.

Although CDK4/6 inhibitors cannot be targeted for TNBC, preclinical studies have reported that targeting CDK9 using small interference RNA or pan-CDK inhibitors (e.g., Flavopiridol and Dinaciclib) have anti-proliferative effects in models of TNBC (Horiuchi et al. 2012; McLaughlin et al. 2017; Rajput et al. 2016). In complex with its cyclin partner cyclin T1, CDK9 forms the positive transcription elongation factor, P-TEFb (McLaughlin et al. 2017). Through phosphorylation of serine 2 (pSer2) in the heptapeptide repeat motif within the C-terminal domain (CTD) of RNAPII, P-TEFb releases RNAPII from a paused state to enable transcriptional elongation (Hsin, Manley & development 2012;

Malumbres & Barbacid 2005; Napolitano et al. 2000). Basal transcription (production of the basal level of transcripts) does not require phosphorylation of the RNAPII CTD (Serizawa, Conaway & Conaway 1993), but CDK9-mediated RNAPII phosphorylation increases transcriptional activity (Bacon & D'Orso 2019; Nekhai, Petukhov & Breuer 2014). This increased RNAPII activity facilitates sustained expression of short-lived mRNA transcripts that encode oncogenic and anti-apoptotic proteins such as MYC and MCL1, which are important oncogenic drivers of TNBC (Horiuchi et al. 2012; Wang, S & Fischer 2008). While targeting CDK9 has shown promise, clinical translation of a CDK9 targeted therapy has been hampered by poor selectivity, and subsequent toxicity, of existing CDK9 inhibitors (CDK9i) (Boffo et al. 2018; Mariaule & Belmont 2014; Sonawane et al. 2016). Therefore, we tested the pre-clinical efficacy and established the mechanism of action of a newly synthesised, highly selective CDK9i (D-11) in TNBC, with view to providing a new drug for clinical development.

## 5.2 Methods

### 5.2.1 Test compounds

The CDK9 inhibitors, D-11 and L-453, were provided by Professor Shudong Wang (Drug Discovery and Development Group, Cancer Research Institute, University of South Australia, Australia). For the *in vitro* assays, 10mM concentrated stock solutions of D-11 and L-453 was prepared in 100% DMSO and stored at -20°C and 4°C, respectively. Concentrated stocks were diluted in growth media as required on the day of treatment. For *in vivo* experiments, D-11 was freshly formulated in 0.1M sodium acetate buffer, pH 4.5 and gavaged daily at a concentration of 150-200mg/kg/day.

### 5.2.2 Kinase assay

The *in vitro* kinase assay (Kinome scan) was performed by Reaction Biology Corporation (Malvern, USA) as described previously (Tadesse et al. 2018). D-11 and L-453 were screened against 396 human kinases and enzyme activity was determined by percentage of remaining kinase activity in test samples compared to vehicle (DMSO) after subtraction of background (derived from control reactions containing inactive enzyme). The concentration required to produce half maximum inhibition (Ki) was calculated using the Cheng–Prusoff equation: Ki = IC50 / (1 + ([ATP]/Km (ATP))), where Km is the Michaelis constant. A kinome activity map was prepared using Kinome Mapper.

### 5.2.3 Cell lines

MDA-MB-453, MDA-MB-231 and MDA-MB-468 cell lines were obtained from the American Type Culture Collection (ATCC, USA) and maintained in DMEM-High glucose media, supplemented with 10% FBS, 2mM L-glutamine and 2mM sodium pyruvate. The MFM-223 cell line was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany) and maintained in EMEM supplemented with 10% FBS, 2 mM L-glutamine, and a 1x concentration of insulin–transferrin–sodium selenite (ITS) supplement. All cell lines were maintained at 37°C in a humidified incubator with 5% $CO_2$. Cell lines were routinely tested for mycoplasma infection using Stratagene MycoSensor qPCR kit (#302106) and cell line identity by short tandem repeat profiling (Cell Bank Australia).

### 5.2.4 Proliferation and apoptosis assays

MDA-MB-453, MDA-MB-468, and MDA-MB-231 cell lines were plated at a density of 4 x $10^3$ cells/well and MFM-223 at a density of 5 x $10^3$ cells/well in 96-well tissue culture plates (CoStar). Following a 24hr incubation period to allow cell adherence, the cell culture medium was removed and replaced with medium containing IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent (final concentration 5µM) and IncuCyte® NucLight Rapid Red Reagent for Live-Cell (diluted 1:2000). Concurrently, cells were treated with either vehicle (DMSO) or D-11 (150, 300, 600, and 1200nM doses). Plates were imaged over a period of 5 days, collecting four images per well every 3 hours with a 10x objective on the IncuCyte S3 (Essen Bioscience), with drug treatments refreshed every 72hr. The resultant images were analysed to determine the

number of live and dead cells using the associated IncuCyte S3 software, adjusting the mask and filter settings for image analysis using a small training set of images (6 images) from the vehicle and treated wells at early and late time points (Appendix 3A).

For proliferation assays with L-453 compound, MDA-MB-453, MDA-MB-468, and MDA-MB-231 cell lines were plated at a density of 3 x $10^4$ cells/well and MFM-223 at a density of 5 x $10^4$ cells/well in 12-well tissue culture plates (CoStar). Cells were left to grow for 24hr prior to treatment with either vehicle (DMSO) or L-453 (75, 150, 300, 600, and 1200nM doses). Treatments were refreshed every 3 days. Cells were quantified by haemocytometer counting using trypan blue dye.

### 5.2.5 Western blot

Cells were seeded in 6-well cell culture plates (CoStar) at a seeding density of $5x10^5$ cells/well and allowed to attach for 48hr. Cells were treated with vehicle or with 2-fold increasing concentrations of D-11 for 6hr, then harvested by scraping into RIPA buffer, supplemented with 1x cOmplete Protease Inhibitor (Roche) and 1x Halt Phosphatase Inhibitor (Thermo Scientific). Protein concentration was quantified by Pierce BCA protein assay (Thermo Scientific) before immunoblotting. Protein electrophoresis & immunoblotting were conducted as described in Chapter 2 section 2.2.5. Membranes were probed with the following primary antibodies: CDK9 (Cell Signaling Technology, #2316, 1:1000 dilution), RNAPII (Abcam, #Ab817, 1:1000 dilution), RNAPII CTD repeat YSPTSPS (p-RNAPII Ser2, Abcam, # ab193468, 1:5000 dilution), C-MYC (Cell Signaling Technology, #9402, 1:1000 dilution), MCL1 (Cell Signaling Technology, #5453, 1:1000 dilution) and GAPDH (Millipore, #MAB374, 1:2000 dilution). Detection of primary antibodies was performed using HRP-conjugated anti-mouse (DAKO, #P0161, 1:1000 dilution) or anti-rabbit (DAKO, #P0448, 1:1000 dilution) secondary antibodies. Signals were visualized with Clarity Western ECL Substrate (Bio-Rad) as described by the manufacturer and imaged using a ChemiDoc MP (Bio-Rad) imaging system.

### 5.2.6 RNA isolation and quantitative RT-PCR

Cells were treated with vehicle or D-11 (300 and 600nM) for 4hr prior to harvest with Trizol reagent (Sigma, #T9424). RNA was purified according to the Trizol protocol and DNase treatment was performed using the TurboDNase kit (Thermo Scientific) according to manufacturer's instructions. RNA was

quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Total RNA (1µg) was reverse transcribed into cDNA using an iScript Select cDNA Synthesis Kit (Bio-Rad, #1706691) in the presence of both oligo (dT) and random primers. Quantitative RT-PCR was conducted with iQ SYBR Green Supermix (Bio-Rad, #1708866) using a CFX384 Real-Time PCR Detection System (Bio-Rad). Relative gene expression was determined by the ΔΔCT method using GAPDH as an internal control for quantification analyses of gene targets.

### 5.2.7 Cell cycle analysis

Vehicle or D-11 (300 or 600nM) treated cells were washed once in 1x PBS and centrifuged at 2700 rpm for 5min. Cell pellets were resuspended in 500µL PBS and slowly dropped into 4.5mL of pre-chilled 70% ethanol and incubated overnight at 4°C. The fixed cells were washed with PBS, then pelleted and resuspended in 1mL of PI solution containing 50µg/mL PI, 100µg/mL RNase A (Sigma, #R4642), 0.1% Triton X-100, and incubated for 30min at room temperature. Stained cells were analysed using FACS Conto II (BD Biosciences).

### 5.2.8 *Ex vivo* explant culture of normal human breast tissues.

Normal breast tissues were collected from consenting women undergoing breast reduction surgery at the Flinders Medical Centre, Adelaide, South Australia (ethics approval number H-2015-175). Tissues were cultured *ex vivo* as previously described (Centenera et al. 2018; Dean et al. 2012). In brief, tissues were dissected into ~1 $mm^3$ pieces and randomly placed onto gelatin

dental sponges (Ethicon) pre-soaked in phenol-red-free RPMI 1640 media supplemented with 10% FBS, 2mM L-glutamine, 10µg/mL human recombinant insulin (Sigma, #I0516), 10µg/mL hydrocortisone (Sigma, #H0888), and 1x antimycotic-antibiotic (Sigma, #A5955) and incubated at 37°C with 5% $CO_2$. After 24hr, the medium was replaced with new culture medium supplemented with vehicle or D-11 (900nM & 2.7µM), followed by culture for a further 48hr. Four tissue pieces from each case were randomly allocated to each treatment on a single sponge. Cultured tissue pieces were fixed in 10% neutral-buffered formalin at 4°C overnight, dehydrated and embedded in paraffin wax for H&E and IHC analyses.

### 5.2.9 TNBC mammary intraductal (MIND) xenografts

Animal experiments were approved by the University of Adelaide Animal Research Ethics Committee (approval number M-2018-088). The mammary intraductal (MIND) xenografting technique was employed following previously described protocols (Richard et al. 2016; Sflomos et al. 2016). A total of 2 x $10^5$ MDA-MB-453 cells labelled with luciferase and dTomato fluorescent protein under puromycin resistance (LTP) were resuspended in 10µL normal growth media and injected into one of the fourth inguinal mammary ducts of NSG mice. Tumours were allowed to establish for 5 days, and then mice allocated by simple randomisation to receive vehicle (n =10) or D-11 (10 mice received 8 doses of 200mg/kg/day and then 7 doses of 150mg/kg/day due to toxicity concerns) for 15 consecutive days. The number of mice per treatment was determined based on previous *in vivo* studies (Hickey, TE et al. 2021), showing that a 20% reduction in tumour size

compared to vehicle can be achieved with 10 mice/treatment group ($p<0.05$; 80% power). Tumour growth was monitored using an IVIS Lumina X5 imaging system (PerkinElmer). IVIS imaging was conducted five days after injection and every two days after treatment. IVIS-Living images 64-bit software was used to quantify non-saturated bioluminescence in regions of interest (ROI) and bioluminescence was quantified as photons/second for each ROI.

At the end of the experiment, the injected mammary glands were harvested for IHC (see section 5.2.10). Spleen, liver, and lung tissues were collected and stained with H&E to examine the effect of D-11 on normal tissue histology (see section 5.2.9).

### 5.2.10 Histology and immunostaining

General procedures for H&E and IHC were performed as described previously in Chapter 2 Section 2.2.9. Ki-67 IHC was performed using a monoclonal mouse antibody (DAKO #M7240, 1:400 dilution) and a Goat Anti-Mouse Immunoglobulins/Biotin (DAKO, #E0433, 1:400 dilution) secondary antibody Slides were scanned using a NanoZoomer 2.0HT Microscopy System (Hamamatsu). Ki67 staining was quantified by manual counting of all fields containing breast epithelia, over multiple representative areas using a FIJI cell counter.

### 5.2.11 Statistical analysis

Statistics were performed using Graphpad Prism 9.0. Two-tailed Student t tests, or one-way ANOVA followed by Tukey post hoc multiple comparison

tests were used as determined by the data. In the majority of cases, two independent experiments with three technical replicates per treatment condition were conducted. The half-maximal inhibitory concentration (IC50) was determined from the generated concentration-response curves using a non-linear regression curve fit. All data are expressed as the mean ± SEM. A value of $P < 0.05$ was considered statistically significant.

## 5.3 Results

### 5.3.1 Biochemical characteristics of newly developed CDK9 inhibitors D-11 and L-453

The CDK9 inhibitors, D11-8 and L-453, was derived from the CDKI-73 parent compound (Lam et al. 2014) and synthesized by Professor Shudong Wang (Drug Discovery and Development Group, University of South Australia, Australia). D-11 and L-453 compounds were designed to have a suitable size that enable them to pass through the space that exists between CDK9 and cyclin T1 but does not enable it to reach the ATP binding site of other CDKs (see section 1.5.4.5). D-11 exhibited a Ki of 8nM against CDK9/cyclin T1 and displayed over 50-fold selectivity over CDK1, 2, 4, 6 and 7, (Figure 5.1A). While L-453 inhibited CDK9/cyclin T1 activity at low pM (100pM), it showed Ki of 177 and 238nM against CDK2/cyclin A and CDK7/cyclin H, respectively. Compared to other known CDK9 inhibitors, including Dinaciclib (Selleck chemicals) and CDKI-73 (obtained from S. Wang, Uni SA), both D-11 and L-453 showed superior selectivity towards CDK9 (Figure 5.1A). These results were further substantiated via kinome analysis in which D-11 and L-453 specificity were determined against a panel of 369 human kinases. At a screening concentration of 1µM, which is at least 125 times above the Ki measured in the CDK9 biochemical assay, TRKC, FLT3, KHS, MST, MINK, TNIK and NUAK were the only other kinases that displayed <10% residual activity by D-11, while CLK, DYRK, TRKC, FLT3 and TAO were affected to the same degree by L-453 (Figure 5.1 B).

**A**

**Kinase inhibition, $K_i$ (nM)**

| CDKs | D-11 | L-453 | CDKI-73 | Dinaciclib |
|---|---|---|---|---|
| CDK9 | 8 | 0.1 | 5.78 | 4 |
| CDK1 | 1,530 | 265 | 8.17 | 3 |
| CDK2 | 430 | 177 | 3.27 | 1 |
| CDK4 | 2,620 | NA | 8.18 | 68 |
| CDK6 | 1,540 | NA | 37.68 | 60 |
| CDK7 | 600 | 238 | 134.26 | 70 |

**B**

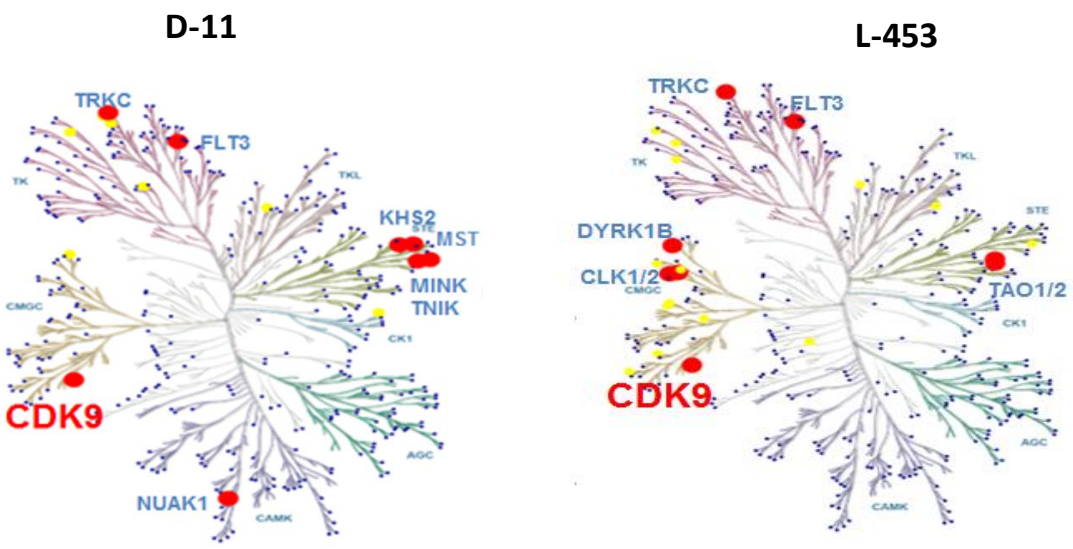



**Figure 5.1:** Kinase profiles of D-11 and L-453. **(A)** Comparison of Ki values for D-11, L-453 and non-selective CDK9 inhibitors Dinaciclib and CDKI-73. NA= not active **(B)** Phylogenetic tree representation of kinase targets of D-11 (left panel) and L-453 (right panel) profiled against 369 kinases. Red circles represent <10% residual activity, respectively.

### 5.3.2 Selective CDK9 inhibition effectively reduces *in vitro* proliferation of TNBC cell lines.

To determine the therapeutic potential of CDK9 as a therapeutic target for TNBC, we began by examining protein expression of CDK9 and its known targets, the proto-oncogene C-MYC, and the anti-apoptotic marker MCL1 in a panel of TNBC cell lines. Two CDK9 isoforms are expressed in mammalian tissues: a major 42 kDa protein ($CDK9_{42}$) and a minor 55 kDa ($CDK9_{55}$) protein (Liu, H & Rice 2000; Shore et al. 2003). Western blotting analysis showed that both CDK9 isoforms are expressed in TNBC cell lines (MDA-MB-453, MDA-MB-468, MFM-223, and MDA-MB-231) (Figure 5.2A). As expected, $CDK9_{42}$ was more highly expressed than $CDK9_{55}$ in all cell lines (Figure 5.2A). Therefore, in the rest of this study, we focused on the $CDK9_{42}$ isoform and herein refer to it as CDK9.

TNBC cell lines exhibited variable basal levels of CDK9 protein, whereby MDA-MB-453 cells had the highest and MDA-MB-468 cells the lowest level of expression (Figure 5.2A-B). Basal expression of C-MYC and MCL1 proteins also varied in the four TNBC cell lines. C-MYC protein was higher in MFM-223 cells compared to the other TNBC cell lines evaluated (Figure 5.2A-B). MDA-MB-231 cells also exhibited relatively high C-MYC expression compared to the MDA-MB-453 and MDA-MB-468 cell lines (Figure 5.2A-B). MCL1 was expressed in all TNBC cell lines assessed, with relatively higher expression in MFM-223 cells (Figure 2A-B). Hence, CDK9 and its key targets were present in all TNBC models assessed, suggesting that targeting this factor could have broad application in this disease context.

Next, we assessed the anti-proliferative efficacy of targeting CDK9 in TNBC cell lines *in vitro*. Cells were treated with 2-fold increasing concentrations of D-11 (150, 300, 600 and 1200nM) and cell nuclei counted over a period of five days. A time and dose-dependent inhibition of proliferation occurred in all TNBC cell lines in response to D-11 (Figure 2.5C-F). The results indicate that D-11 had cell line specific effects, whereby some cell lines were more sensitive to D-11 than others. The MDA-MB-453 cell line was most sensitive; 300nM D-11 incurred a 33% growth inhibition (evident after 72 hr of starting treatment) and the $IC_{50}$ was 281nM at 120hr (Figure 2.5C). In contrast, MDA-MB-231 and MFM-223 cells required 600nM of D-11 to inhibit proliferation at 72hr and their $IC_{50}$ values were 658 and 737nM at 120hr, respectively (Figure 2.5 E-F). Although MDA-MB-453 cells may be more sensitive to CDK9 inhibition due to higher expression of CDK9, MDA-MB-468 cells have relatively low CDK9 expression and a relatively high sensitivity to D-11 with $IC_{50}$ of 342nM at 120 hr (Figure 2.5D), suggesting that the basal level of CDK9 alone is not a good indicator of therapeutic efficacy. Results of the effect of another CDK9 inhibitor L-453 on the proliferation of TNBC cell lines are shown in the appendix 4. While this study started off with two compounds, D-11 was chosen for further study because it evoked the greatest effect in inhibiting TNBC cells growth.

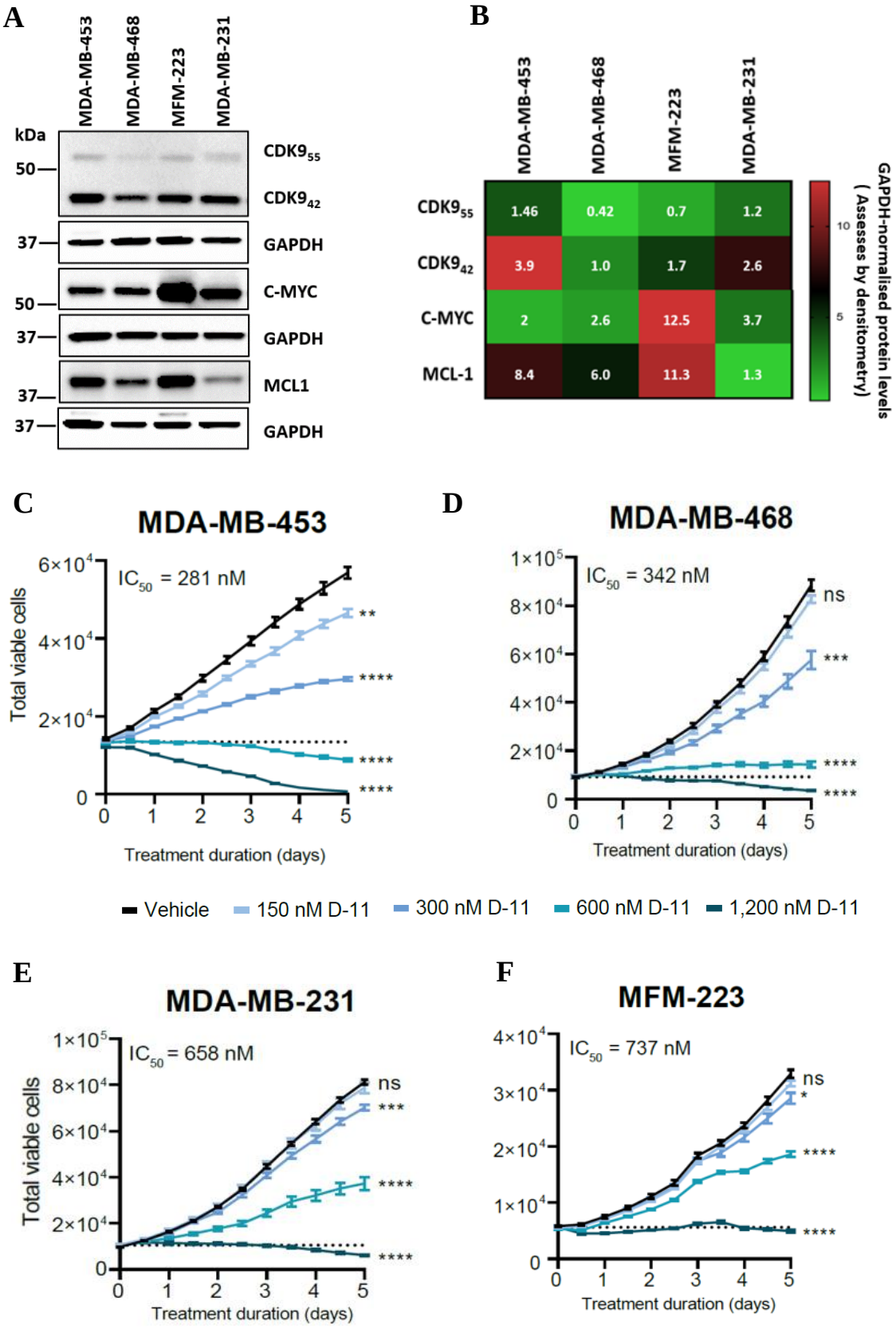
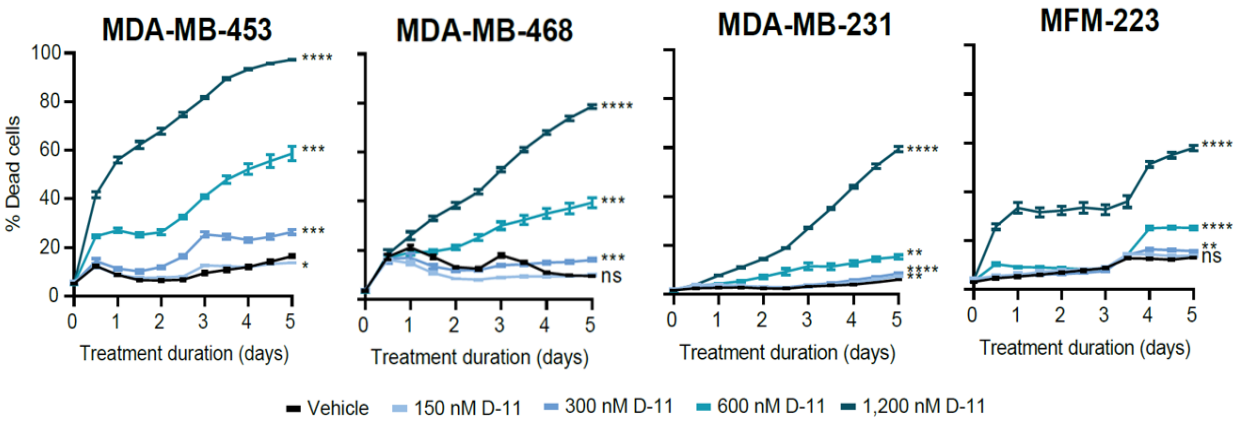

A
MDA-MB-453
MDA-MB-468
MFM-223
MDA-MB-231
kDa
50
CDK9$_{55}$
CDK9$_{42}$
37
GAPDH
50
C-MYC
37
GAPDH
37
MCL1
37
GAPDH
B
MDA-MB-453
MDA-MB-468
MFM-223
MDA-MB-231
CDK9$_{55}$ 1.46 0.42 0.7 1.2
CDK9$_{42}$ 3.9 1.0 1.7 2.6
C-MYC 2 2.6 12.5 3.7
MCL-1 8.4 6.0 11.3 1.3
10
5
GAPDH-normalised protein levels
( Assesses by densitometry)
C
MDA-MB-453
IC$_{50}$ = 281 nM
Total viable cells
Treatment duration (days)
D
MDA-MB-468
IC$_{50}$ = 342 nM
ns
***
****
****
Treatment duration (days)
Vehicle
150 nM D-11
300 nM D-11
600 nM D-11
1,200 nM D-11
E
MDA-MB-231
IC$_{50}$ = 658 nM
Total viable cells
ns
***
****
****
Treatment duration (days)
F
MFM-223
IC$_{50}$ = 737 nM
ns
*
****
****
Treatment duration (days)

**Figure 5.2:** D-11 reduces *in vitro* proliferation of TNBC cell lines. **(A)** Protein expression of CDK9 and its known targets C-MYC and MCL1 in TNBC cell lines by western blot. GAPDH shown as a loading control. **(B)** Heat map showing expression of the GAPDH-normalised protein levels as assessed by densitometry of CDK9, C-MYC & MCL1 in TNBC cell lines. **(C-F)** Proliferation of TNBC cells measured by live cell count using the Incucyte S3 at varying concentrations of D-11. Data represents mean ± SD of three replicate cell culture wells per condition and is a representative of two independent experiments. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (One Way ANOVA).

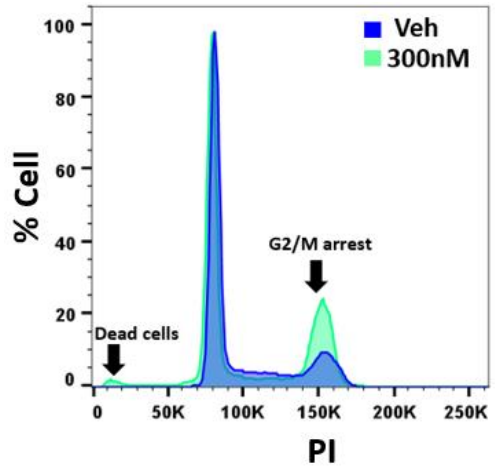
### 5.3.3 D-11 has cytotoxic effects in TNBC cells by inducing apoptosis and G2/M cell cycle arrest.

To examine whether the observed growth inhibitory effects of D-11 were accompanied by cell death, apoptotic cells were quantified using the Incucyte Caspase-3/7 green dye. Since there were significant growth inhibitory effects of D-11, a dead/live cell ratio was calculated to account for fewer cells present in wells with higher drug doses. D-11 significantly induced apoptosis in all of the TNBC cell lines tested (Figure 5.3A). At a dose of 600nM, a more dramatic induction of apoptosis ($p$<0.0001) was observed in MDA-MB-453 and MDA-MB-468 cells compared to the MDA-MB-231 cell line (Figure 5.3A). A higher dose of 1200nM was required to induce apoptosis in MFM-223 cells (Figure 5.3A). Moreover, a G2/M cell cycle arrest was observed upon treatment with 300nM D-11 in MDA-MB-453 and 600nM D-11 in MDA-MB-468 and MFM-223 for 72hr, while this occurred with only 600nM D-11 at 120hr in MDA-MB-231 cells (Figure 5.3B-E). Collectively, these results provide evidence that D-11 has growth inhibitory effects through a dual mechanism: inducing apoptosis and cell cycle arrest.

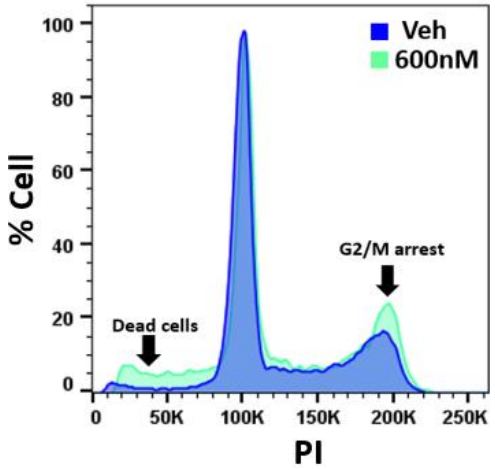
**A**



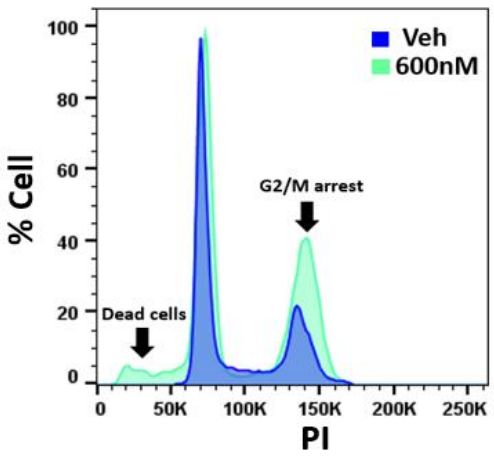

**B** **MDA-MB-453**




**C** **MDA-MB-468**




**D** **MDA-MB-231**




**E** **MFM-223**

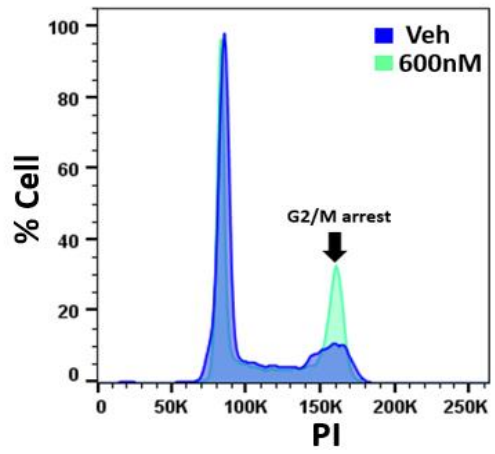
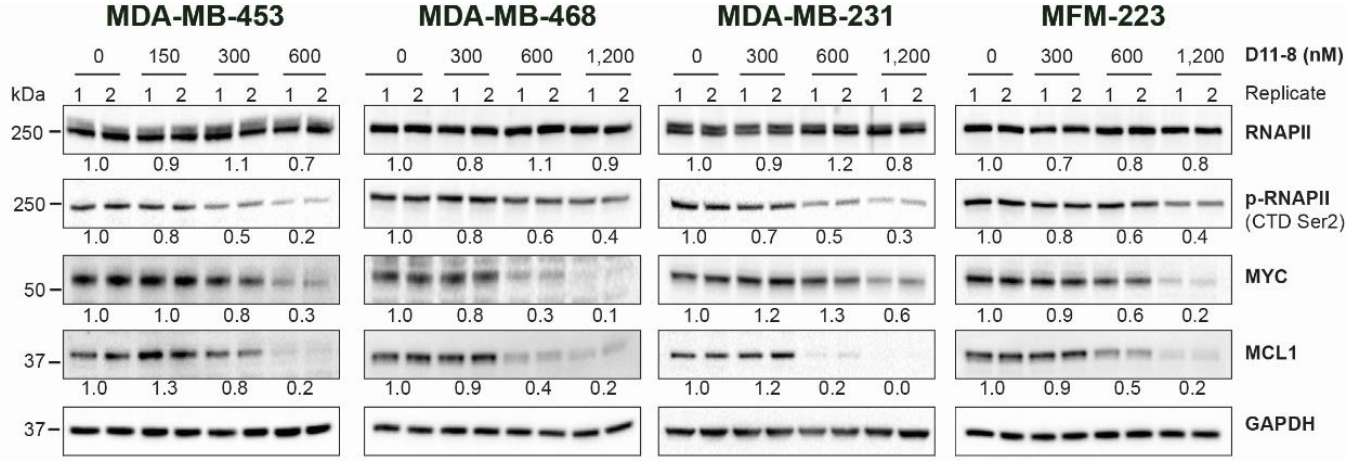


**Figure 5.3:** D-11 induces apoptosis & G2/M cell cycle arrest in TNBC cells. **(A)** Effect of D-11 on cell death measured using the Incucyte Caspase-3/7 green Dye. Data represents mean ± SD of three replicate cell culture wells per condition and is a representative of two independent experiments. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (One-Way ANOVA). **(B)** Cell cycle analysis after exposure to D-11 for 72hr in MDA-MB-453, MDA-MB-468 & MFM-223 and 120hr in MDA-MB-231 cells. Results are representative of two independent experiments with three replicates per condition.

### 5.3.4 D-11 reduces phosphorylation of RNAPII CTD and reduces expression of C-MYC and MCL1

To confirm that the anti-proliferative and cell cycle effects of D-11 in TNBC cell lines were due to targeted inhibition of CDK9, expression levels of CDK9 downstream targets were examined, including phosphorylated Ser2-RNAPII (pSer2-RNAPII), C-MYC, and MCL1 in TNBC cells following treatment with D-11 (300 and 600nM) for 4hr (mRNA expression) or 6hr (protein analysis). As expected, D-11 significantly reduced Ser2-RNAPII phosphorylation in TNBC cell lines without affecting total RNAPII (Figure 5.4). In MDA-MB-453 cells, the most sensitive to D-11-induced growth inhibition, phosphorylation of RNAPII was reduced by approximately 70% after a 6hr treatment with 600nM D-11 (Figure 5.4A), and this effect was accompanied by a 70% reduction in mRNA and protein levels of C-MYC and MCL1 (Figure 5.4B). Treating MDA-MB-468 and MFM-223 cells with 600nM D-11 induced a 30-40% inhibition of pSer2-RNAPII (Figure 5.4A), which coincided with a significant inhibition of C-MYC and MCL1 protein and mRNA levels (Figure 5.4B). In MDA-MB-231 cells, MCL1 expression was almost abrogated by 600nM D-11 (Figure 5.4B), but a higher dose of 1200nM was required to cause a decrease in C-MYC expression (Figure 5.4B). Together, these data clearly demonstrate the ability of D-11 to specifically target CDK9 activity and likely represent a key means by which D-11 reduces proliferation and viability of TNBC cells.

**A**




**B**

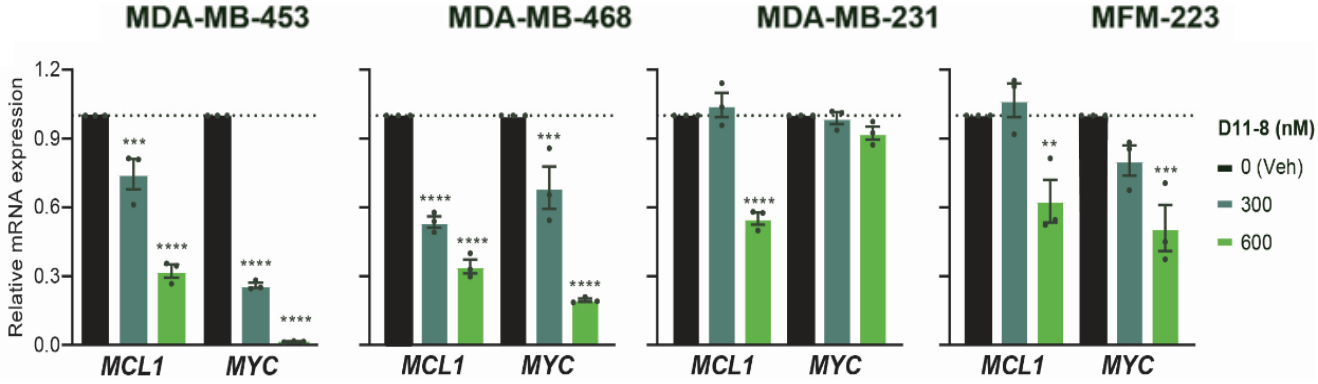



**Figure 5.4:** Targeting CDK9 activity using D-11 reduces phosphorylation of RNAPII and expression of C-MYC and MCL1 in TNBC cells in vitro. **(A)** Western blot analysis showing effects of D-11 on total RNAPII, p-RNAPII, C-MYC & MCL1 in TNBC cell lines after 6hr of treatment. GAPDH was used as a loading control. Blots are representative of 2 independent experiments. **(B)** RT-qPCR displaying the relative mRNA levels of C-MYC and MCL1 after 4hr treatment with D-11. GAPDH was used as a reference gene. Data represented as mean ± SD; **p<0.01; ***p<0.001 (One-Way ANOVA).

### 5.3.5 *In vivo* anti-tumour efficacy of D-11 in TNBC models

Since selective targeting of CDK9 effectively suppressed proliferation of TNBC cell lines *in vitro*, we established mammary intraductal (MIND) xenografts using the MDA-MB-453 breast cancer cell line to validate the effect of D-11 *in vivo*. MIND technique, in which cells are injected directly into the mouse milk ducts, has gains over the mammary fat pad technique, as it mimics the original breast tumour microenvironment (Richard et al. 2016; Sflomos et al. 2016). NSG mice were injected unilaterally with MDA-MB-453 cells, and after 5 days were allocated to treatment with vehicle or D-11 using simple randomisation. This was the first time of testing D-11 *in vivo*, so tolerated doses were estimated based on *in vivo* data of a parent compound (CDKi-73, (Rahaman, Yu, et al. 2019)). Mice received 200mg/kg/day of D-11 for eight days by daily oral gavage. After that, the D-11 dose was reduced to 150mg/kg/day because one mouse became sick and there was concern that the cause might be D-11 toxicity. However, it was subsequently determined that the problem was technical (due to gavage damage), not due to inherent toxicity of the compound. As shown in Figure 5.5A-C, D-11 significantly reduced tumor growth of MDA-MB-453 MIND xenografts. No apparent behavioral or body weight changes were observed in treated mice, and there was no effect of D-11 on normal histology of liver and spleen tissues (Figure 5.5B), indicating that it was well tolerated and exerted no obvious harm to the animals. Collectively, these striking *in vivo* effects of D-11 strongly support the efficacy and tolerability of selective CDK9 inhibition in TNBC.

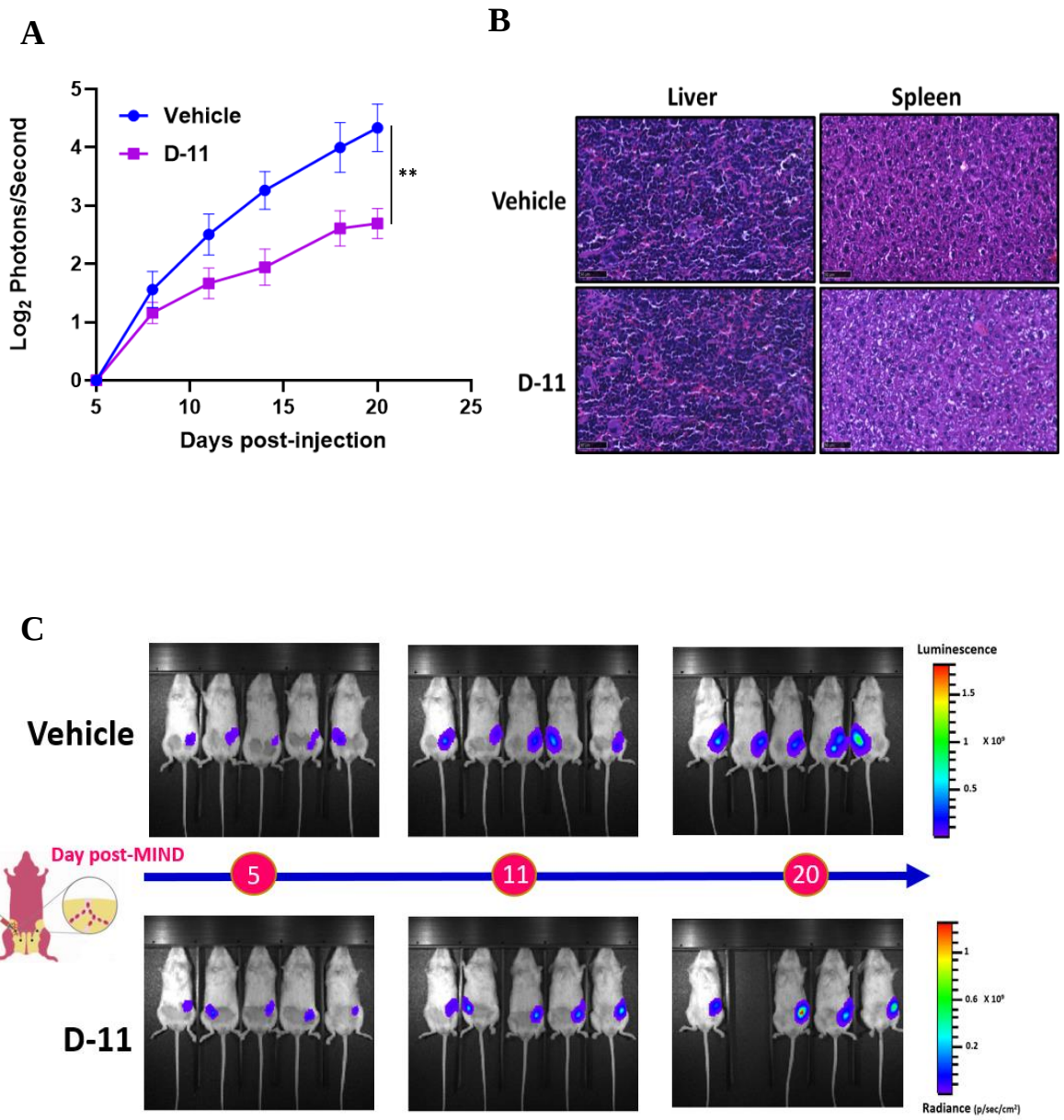

A
B
C
Vehicle
D-11
Log2 Photons/Second
Days post-injection
**
Liver
Spleen
Vehicle
D-11
Luminescence
X 10⁹
Day post-MIND
5
11
20
Radiance (p/sec/cm²)

**Figure 5.5:** *In vivo* antitumor efficacy of D-11 on TNBC MIND xenograft tumours. **(A)** Growth curve showing inhibition of MDA-MB-453 MIND xenograft tumor growth with D-11 as measured by in vivo bioluminescence (mean total flux [photons/s] ± SEM) (logarithmic scale). MDA-MB-453 MIND xenograft tumors treated 15 days with vehicle (n= 9) or D-11 (n=7). Four animals were excluded from the study due to technical reasons ( e.g. problems with oral gavage and cell injection). **p<0.01 (Two-tailed Student t tests). **(B)** Representative H&E images of liver and spleen tissues from endpoint vehicle and D-11 treated MDA-MB-453 MIND xenograft tumors. Scale bar depicts 50 μm**. (C)** Representative images of the *in vivo* bioluminescence signals (flux [photons/s] ) 5,11 and 20 days post MIND in each treatment group.

### 5.3.6 D-11 does not affect proliferation and histology of patient-derived explants of normal human breast tissues treated *ex vivo.*

CDK9 activity is not considered necessary to sustain homeostasis of normal cells, while cancer cells can rely on it to sustain expression of key oncogenic drivers (Falco, Giordano & therapy 2002; Serizawa, Conaway & Conaway 1993). To `test lack of toxicity in normal human breast tissues, the effect of D-11 was assessed using explants of reduction mammoplasty tissue samples (n=4 independent cases) treated *ex vivo* as previously described (Dean et al. 2012). This technique has been shown to sustain the tissue architecture, viability, cellular complexity and hormone responsiveness of normal human breast tissues (Centenera et al. 2018; Hickey, TE et al. 2021) and is thereby more clinically relevant than the testing of non-transformed breast epithelial cell lines in 2-dimensional culture (e.g. MCF10A). Briefly, surgically resected breast tissue specimens were dissected into 1 $mm^3$ pieces and placed on top of a gelatine sponge partly submerged in growth culture media, to which treatments were added (Figure 5.6A). Explants were treated with vehicle or 3-fold increasing doses (900 & 2700 nM) of D-11 for 48 hr and harvested for assessment of Ki-67 positivity and histological features. Compared to vehicle-treated explants, D-11 had no significant effect on Ki-67 positivity and histology of normal breast tissue explants (Figure 5.6 B-C). The absence of a toxic effect of D-11 in normal mammary cells is consistent with previous studies showing that the kinase function of CDK9 is not required for basal transcription (Scafe et al. 1990; Serizawa, Conaway & Conaway 1993).

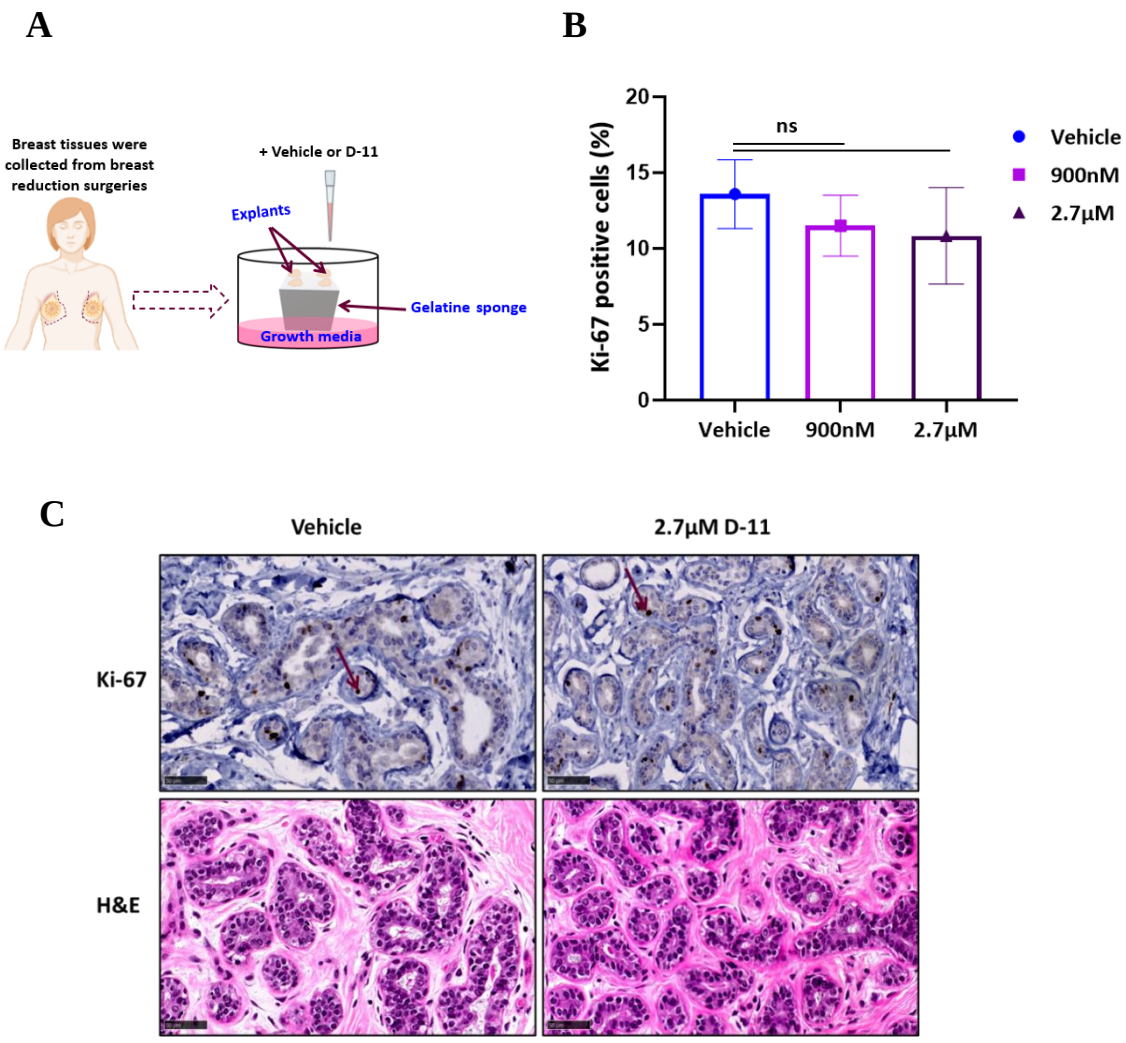



**Figure 5.6:** D-11 has no effect on proliferation and histology of normal human breast tissues**. (A)** Schematic showing *ex vivo* culture of normal breast tissue explants. **(B)** Quantification of Ki-67 positivity in normal breast tissues (n = 4 independent cases) treated with Vehicle or D-11 for 48hr. Data represented as mean ± SEM; ns p>0.05 (Two-tailed Student t tests). **(C)** Representative images of Ki-67 IHC and H&E staining in normal breast tissues treated with Vehicle or 2.7µM D-11 for 48hr. Brown arrows demonstrate examples of Ki-67 positive cells. Scale bar depicts 50 µm.

## 5.4 Discussion

A potential therapeutic strategy to treat TNBC is to impair the sustained expression of oncogenes and pro-survival factors that drive growth of this aggressive subtype of breast cancer. This could be achieved by inhibiting CDK9 activity, which impedes RNAPII-mediated transcription of short-lived oncogenic gene transcripts. In the current pre-clinical study, we demonstrate the anti-tumour efficacy of a novel, potent, and highly selective CDK9 inhibitor (D-11) in the context of TNBC using a panel of cell lines to provide *in vitro* evidence and a cell line mammary intraductal (MIND) xenograft model to provide *in vivo* evidence. The non-toxic nature of D-11 was demonstrated via lack of effect on several mouse tissues in vivo and human breast tissues ex vivo. Collectively, this data supports advancement of D-11 to clinical trials involving TNBC.

Using cell-free assays for biochemical analysis, we showed that D-11 inhibits CDK9 kinase activity at a low Ki value (8nM) and displayed remarkable selectivity for CDK9 over other CDKs as well as other protein kinases in the kinome (Figure 5.1). D-11 suppressed proliferation and triggered apoptosis in TNBC cells *in vitro* and these effects are ascribed to the reduction of p-RNAPII, C-MYC & MCL1 levels, indicative of targeted CDK9 inhibition. In surviving cells, D-11 induced a cell cycle block at the G2/M phase, consistent with previous data which reported that silencing CDK9, but not CDK1 or CDK2, reduced cyclin B1 protein levels in MDA-MB-231 and BT549 breast cancer cell lines, leading to G2/M cell cycle arrest (Rajput et al. 2016). Moreover, the data presented in our study highlight that some TNBC cell lines are more responsive to CDK9 inhibition than others. Initially, it was postulated

that variation in response may be due to the difference in endogenous CDK9 levels, but our findings showed that the basal protein level of CDK9 alone is not sufficient to predict response to CDK9 inhibition in the context of the TNBC models used (Figure 5.2 A-B). A study conducted by Brisard and colleagues (2018) reported that TNBC cell lines with high mRNA levels of CDK9 (e.g., MDA-MB-231, MDA-MB-436, and MDA-MB-453) exhibited significantly higher sensitivity to atuveciclib (another newly developed CDK9 inhibitor with $IC_{50}$=13nM) as compared to a panel of low-CDK9 expressing cell lines (e.g., HCC1937, MDA-MB-157, and HCC3153) (Brisard et al. 2018). Interestingly, atuveciclib $IC_{50}$ at 96h in MDA-MB-231 cells (identified as the cell line with the highest CDK9 mRNA expression based on the latter study) was 1.32 μM and was 0.63 μM in MDA-MB-436 cells, which express lower levels of CDK9 compared to MDA-MB-231. Similar observations were noticed in low CDK9 expressing cell lines, where 4μM of atuveciclib was used to inhibit HCC1937 cell line proliferation compared to 2.2 μM for the MDA-MB-157 cell line (which has the lowest CDK9 expression), supporting our conclusion that the CDK9 level alone did not predict response to CDK9 inhibition. Therefore, we examined the protein levels of CDK9 targets in an attempt to see if expression of these targets associated with response to D-11 and the data showed that the less responsive cell lines had higher levels of C-MYC. These observations suggest that the presence of amplification and/or overexpression of the downstream CDK9 target C-MYC could make TNBC cells less sensitive to selective CDK9 inhibition. However, further experiments such as analysis of CDK9i-induced changes to the p-RNAPII genome-wide chromatin binding profile and associated transcriptome in TNBC cells treated

with/without D-11 are needed to develop a biomarker signature of treatment response.

The anti-tumour effect of D-11 was also investigated *in vivo* with MDA-MB-453 mammary intraductal (MIND) xenografts. Our study is the first to use the MIND technique, in which cells are injected directly into the mouse milk ducts, to examine the efficacy of targeting CDK9 in breast cancer *in vivo*. Conventional mammary fat pad xenografting was used in previous studies to test transcriptional CDK inhibitors in breast cancer (Rajput et al. 2016; Sun, B et al. 2020; Wang, Yubao et al. 2015). The MIND technique has advantages over the mammary fat pad technique, as it better mimics the breast tumour microenvironment where cancers normally develop (Richard et al. 2016; Sflomos et al. 2016). The outcomes of our study showed that oral administration of D-11 significantly inhibited MDA-MB-453 MIND tumour growth, was well tolerated and did not affect histology of normal tissues. Key future experiments that will further clarify therapeutic efficacy of D-11 could include testing D-11 *in vivo* using TNBC patient-derived xenograft (PDX) models and using the MIND model to assess the effect of D-11 on metastasis, as this model offers opportunities to study breast cancer progression (transition from in situ to invasive disease and metastasis to relevant sites).

Taken altogether, this chapter demonstrates that selective CDK9 inhibition is an attractive therapeutic strategy for treatment of patients with TNBC, and that the novel and highly selective CDK9 inhibitor D-11 is a promising candidate for clinical development.

# CHAPTER 6

# DISCUSSION

## 6.1 General discussion

Treatment options for ERα-negative breast cancers are largely limited to chemotherapy due to the lack of effective, clinically proven targeted therapies. The studies presented in this thesis examined three potential therapeutic targets for ERα-negative breast cancer: the androgen receptor (AR), transcription factor AP-2β (TFAP-2β) and cyclin-dependent kinase 9 (CDK9). The major findings of the thesis are discussed below.

### 6.1.1. Genomic parameters of AR signalling associated with proliferative versus anti-proliferative effects in ERα-negative breast cancers.

The AR is expressed in approximately 20-50% of ERα and PR negative breast cancers (Liu, YX, Zhang & Tang 2018; McNamara, K et al. 2013; Niemeier et al. 2010; Xu, M et al. 2020). Although there is strong interest in targeting AR for treatment of breast cancer, the role AR signalling plays in different breast cancer contexts is controversial, which restricts clinical implementation of currently available AR-targeted therapies. Recently, this controversy was resolved in the context of ERα-positive breast cancer, in which AR was shown to be a tumour suppressor and use of an AR agonist, rather than an AR antagonist, the rational therapeutic strategy (Hickey, TE et al. 2021). However, the controversy remains in the context of ERα-negative breast cancers and is

complicated by the fact this disease context is comprised of many different molecular sub-types. To inform this controversy, the first part of this thesis (Chapter 3) aimed to define genomic parameters of AR signalling activity associated with proliferative versus anti-proliferative cellular responses in the ERα-negative breast cancer context, with the view to understanding the mechanistic basis of oncogenic versus anti-oncogenic potential. Two representative ERα-negative/AR-positive cell lines (MDA-MB-453 and MFM-223) were investigated chosen for having comparable levels of AR expression but differing proliferative responses to AR activation with the endogenous ligand, DHT. Expression profiles of DHT-regulated genes were analysed in MDA-MB-453 (growth stimulated by DHT) and MFM-223 (growth inhibited by DHT) cell lines using next-generation sequencing (NGS) techniques, to determine whether differential expression of AR target genes could underpin differential proliferative responses. The potent androgen DHT stimulated the transcription of genes that mediate growth, development, and metabolism in MDA-MB-453 cells (Chapter 3, Figure 3.3). Many of these genes have been reported to have a growth-stimulating effect in several types of cancer. Examples are *ISX* (Hsu et al. 2013; Wang, S-N et al. 2016), *EPHA2 (Brantley-Sieders et al. 2008; Song, W et al. 2017)*, *TOX3* (Dittmer et al. 2011; Seksenyan et al. 2015), *SGK1* (Lang et al. 2010; Shanmugam et al. 2007), *CAMKK1* (Brzozowski & Skelding 2019; Karacosta et al. 2012; Massie et al. 2011), and *WNT4* (Ni et al. 2011; Yang, D et al. 2020). Moreover, Gene Ontology analysis revealed up-regulation of several lipid metabolism genes upon treatment with DHT in MDA-MB-453 cells. Dysregulation of lipid metabolism pathways as a means of promoting cancer growth has been linked

to the progression of several types of cancer including colorectal, prostate, breast, and ovarian cancer (Butler, LM, Centenera & Swinnen 2016; Marino et al. 2020; Tania, Khan & Song 2010; Yeh, C-S et al. 2006). Increased lipid metabolism is thought to contribute to cancer cell proliferation in part by providing building materials for cell membrane synthesis needed for cell duplication, supplying energy, and increasing the synthesis of second messenger lipid molecules that mediate tumorigenesis pathways (Butler, L et al. 2020; Huang, C & Freter 2015). Hence, in addition to up-regulation of classic growth-stimulatory pathways, dysregulation of lipid metabolism pathways might be a critical mediator of DHT-induced proliferation in MDA-MB-453 cells. In MFM-223 cells, DHT induced expression of known tumour suppressor genes, including *ZBTB16*, *Nkx 3.1*, and *LRIG1* (Cao, JingPing et al. 2013; Hedman & Henriksson 2007; Kikugawa et al. 2006; Krig et al. 2011; Li, Q et al. 2019; Lindquist et al. 2014; Ljuslinder et al. 2007), consistent with the anti-proliferative effect of DHT in this cell line. In addition, DHT reduced expression of genes involved in cell survival or with purported oncogenic activity (e.g., *LGR5*, *TM4SF18*, and *BCL2*) in MFM-223 cells (Chapter 3, section 3.3.1.3), another potential means of growth inhibition. Using a diverse, clinically relevant panel of cell-line and patient-derived models of ERα-positive breast cancer, a study recently published by my host laboratory (Hickey, TE et al. 2021) showed that AR activation has antitumor activity in multiple contexts of ERα-positive breast cancer. Mechanistically, they found that agonist activation of AR altered the genomic distribution of ERα and essential co-activators, resulting in repression of cell cycle and pro-survival genes, including *BCL2*, and upregulation of known tumour suppressors,

including *ZBTB16* (Hickey, TE et al. 2021). Importantly, these gene regulatory events (down-regulation of *BCL2*, up-regulation of *ZBTB16*) were recapitulated by AR signalling in MFM-223, a cell line that lacks ERα expression, suggesting that AR regulation of these genes can be independent of ERα signalling. The inability of DHT to stimulate a similar transcriptional program in MDA-MB-453 cells may explain the lack of anti-proliferative effects. Interestingly, genome-wide mapping of AR cis-regulatory binding sites in MDA-MB-453 cells showed that lack of AR enrichment at genomic loci associated with tumour suppressor genes exclusively upregulated by DHT in MFM-223 cells (Chapter3, Figure 3.7) did not explain the inability of AR to enhance transcription of these genes in MDA-MB-453 cells. This finding suggested that disparities in recruitment of AR co-regulatory proteins (nuclear factors that work with AR to regulate its transcriptional capacity) to these loci may be involved. To identify potential candidates, an unbiased proteomic technique called RIME was performed to identify and compare the AR interactomes in the two cell line models. Indeed, analysis of cooperating factors that are potentially involved in the regulation of AR signalling in MDA-MB-453 and MFM-223 cells based on the RIME data showed that only 10% of the precipitated proteins were shared between the two cell lines, indicating a large discrepancy in the AR interactomes. Among the shared AR interacting proteins are SWI/SNF (chromatin remodelling complex) related proteins (SMCE1, SMRC2, SMRD2, and ARID1A), known to interact with AR in the context of prostate cancer (Chmelar et al. 2007) and are required for AR to reorganize chromatin upon activation (Marshall et al. 2003). It makes sense that these factors are common AR interacting proteins in the two breast cancer

cell lines as well as in prostate cancer cells because recruitment of chromatin remodellers is a standard feature of steroid receptor mediated transcriptional activity (Achinger-Kawecka et al. 2020; Orlando et al. 2019; Senapati, Kumari & Heemers 2020). The AR interacting proteins that were specific for MDA-MB-453 cells included factors involved in cell cycle regulation (e.g., HCFC1, LAP2A, and NUDC), DNA repair (PAXX, PNKP, PRMT1, and UBA1), and transcription factors (e.g., TFAP-2β and TLF3). The MFM-223 specific AR partners were categorised into chromatin remodeling (e.g., HMGB1 H2AZ, and RCC1), RNA processing (e.g., EIF3A, CCAR2, and FUBP3), cell growth/death (ANXA7, and YWHAZ), response to stress (PRDX1 and PRDX2), as well as transcription repression (CBX5). The observed differences in the AR interactomes could feasibly be attributed to the mutational status of AR in MDA-MB-453 cells; the *AR* gene in MDA-MB-453 cells contains a G-T transversion in exon 7 (Moore et al. 2012b). In contrast, the AR gene is not mutated in MFM-223 cells (Magklara, Brown & Diamandis 2002). The MDA-MB-453 *AR* mutation results in an AR protein variant consisting of a glutamine to histidine substitution at amino acid 865 (Q865H) of the ligand binding domain (Moore et al. 2012b). Molecular modelling of the mutated AR protein showed conformational changes likely to affect the structure of the ligand binding pocket (Moore et al. 2012b), which may lead to the recruitment of a distinct set of AR co-regulators. Another possibility is that the gene/protein expression of some of these co-regulators could be different between MDA-MB-453 and MFM-223 cell lines, leading to differential recruitment to AR. The latter suggestion could be supported by the results of Transcription factor AP-2β (TFAP-2β). The TFAP-2β transcription factor is known to regulate

many important biological processes and was detected as an AR co-regulatory protein in MDA-MB-453 cells but not the MFM-223 cells. Independent validation showed that TFAP-2β protein levels were highly expressed in MDA-MB-453 cells but were not detectable in MFM-223 cells. These data inspired the hypothesis that TFAP-2β may be a critical determinant of AR oncogenic action in ERα-negative breast cancers, which was explored in Chapter 4.

### 6.1.2 TFAP-2β is critical for growth of molecular apocrine breast cancer cells.

Identification of TFAP-2β as an AR interacting protein in MDA-MB-453 cells and not MFM-223 cells stirred further investigation of this factor in the context of all breast cancers and then in the specific context of ERα-negative disease, the theme of my thesis. Examining *TFAP2B* expression levels in a cohort of 176 clinical breast cancers showed strong enrichment in tumours classified as molecular apocrine breast cancers. Our demonstration that TFAP-2β expression is also high in MDA-MB-453 cells but not in other AR-positive cell line models of ERα-negative breast cancer, is consistent with the fact that the MDA-MB-453 cell line is commonly considered and utilized as the prototypic model of the molecular apocrine disease sub-type (Doane et al. 2006; Farmer et al. 2005; Ni et al. 2011). As a sequence-specific DNA-binding transcription factor, TFAP-2β regulates the expression of genes with roles in important biological processes such as development and cell survival (Moser et al. 1997; Satoda et al. 2000; Wenke & Bosserhoff 2010). Therefore, we aimed to investigate the biological role of TFAP-2β in the context of molecular apocrine

disease before specifically investigating its potential role as a regulator of oncogenic AR signalling. Experimental knockdown of TFAP-2β strongly suppressed proliferation and induced apoptosis in MDA-MB-453 cells. An oncogenic effect of TFAP-2β has been demonstrated in several cancer types (Fu, L et al. 2014; Fu, X et al. 2019; Hara et al. 2019). For example, silencing TFAP-2β inhibited cell growth and induced apoptosis in lung adenocarcinoma models *in vitro* and *in vivo*, whereas TFAP-2β overexpression promoted cell growth (Fu, L et al. 2014). The observed regulation of cell growth by TFAP-2β in the latter study was accompanied by modulation of cancer-associated pathways including stimulation of ERK/p38 and VEGF/PEDF-dependent signalling (Fu, L et al. 2014). In our study, we showed that ectopic expression of TFAP-2β in MFM-233 cells did not stimulate growth of this cell line, suggesting that this effect is context specific.

Other pre-clinical data reported that TFAP-2β mediates thyroid cancer cell proliferation and migration via the COX-2 signaling pathway (Fu, X et al. 2019). Herein, we showed that TFAP-2β promotes growth and viability of MDA-MB-453 cells by maintaining expression of C-MYC and HER2 oncogenes. Moreover, genome-wide mapping of the TFAP-2β cistrome showed TFAP-2β binding sites at the enhancer region of HER2 and in the promoter region of C-MYC and other genes involved in cell cycle progression. Consistent with this finding, siRNA-mediated knockdown of TFAP-2β reduced enrichment of H3K27ac (a mark of transcriptional activation) at loci associated with these genes, including *WEE1*, a G2/M checkpoint regulator protein (Ghiasi et al. 2013), *E2F2*, a transcriptional regulator of G1/S target genes (Johnson & Schneider-Broussard 1998), and *DKC1*, another transcriptional

regulator of G1 target genes (Miao et al. 2019). However, examining global changes in H3K27ac at the ChIP-seq level did not capture the full effect of TFAP-2β knockdown on genomic changes, and thus experiments investigating TFAP-2β gene regulation by RNA-seq to capture global gene expression changes with siRNA-mediated inhibition of TFAP-2β are in progress.

As mentioned above, we hypothesized that TFAP-2β might be a mediator of oncogenic AR signalling in the context of ERα-negative breast cancer. Using several proteomic techniques, we identified and validated the interaction of AR with TFAP-2β in MDA-MB-453 cells but not in MFM-223 cells (Chapter 4, Figure 4.3). These results were explained by showing that TFAP-2β is highly expressed in MDA-MB-453 cells and is not detectable in MFM-223 cells. As we were interested in identifying AR genomic activity that differentiates proliferative from the anti-proliferative androgenic effects, we further examined TFAP-2β/AR interplay. Cistrome analysis showed a substantial co-localization of these two factors (at about 45% of loci) when AR was stimulated by DHT, supporting the results of our proteomic data and showing that these two transcription factors may have interactive as well as independent signalling events and physiological effects. Subsequent investigation showed that TFAP-2β knockdown reduced but did not prevent AR enrichment at representative co-occupied loci in MDA-MB-453 cells and had no significant effect on DHT-induced AR transactivation activity for associated genes. While this data indicates that TFAP-2β is not a critical determinant of AR binding or transactional capacity, the results are not definitive because the full effect of silencing TFAP-2β may have been masked because we did not perform a complete knockdown due to the toxic effect on cells. However, a previous

study has reported that the pioneer factor FOXA1 co-occupies ~ 100% of the AR cistrome in MDA-MB-453 cells (Robinson, JL et al. 2011), indicating a more global influence compared to TFAP-2β. Our data indicate that a subset of FOXA1-AR binding sites is co-occupied by TFAP-2β, so that TFAP-2β silencing may not have a strong impact on AR binding at these co-occupied loci because the chromatin was opened due to the presence of FOXA1.

PDX models are now considered the gold standard for modelling breast cancer and to date, no one has examined the effect of AR signalling on models that represent molecular apocrine breast cancer. Three PDX models selected on the basis of being ERα-negative and having high AR expression and HER2 amplification were treated *in vivo* with DHT to shed critical light on the fact that even within the context of molecular apocrine PDXs, AR does not have a universal oncogenic effect. Interestingly, immunoreactive staining of TFAP-2β showed high expression of TFAP-2β in all of the tested molecular apocrine PDX models, supporting our conclusion that TFAP-2β plays a major role in determining this disease subtype and also showing that expression of TFAP-2β alone does not determine response to androgen in this disease context.

While the current study provides preliminary evidence for the clinical significance of TFAP-2β in molecular apocrine breast cancer, all *in vitro* data presented here were generated using one representative cell line (i.e., MDA-MB-453). Whether this result could be extrapolated to clinical molecular apocrine breast cancer remains unknown. We tried to address this limitation by investigating another representative molecular apocrine cell line model (HCC-202) but experiments with this cell line were hampered by failure to

successfully revive old frozen stocks provided by a collaborator and delays to receiving new stocks from ATCC due to COVID-19. Future studies will test whether HCC-202 cells are also dependent on TFAP-2β for growth and survival. This limitation could be further addressed by future studies with the PDX models of molecular apocrine breast cancer we characterised, which all have high expression of TFAP-2β. These studies could involve genetic knockdown of TFAP-2β or treatment with an inhibitor. While a specific inhibitor targeting TFAP-2β is still not available, there is a small molecule inhibitor previously used to target TFAP-2α / TFAP-2γ (Hu et al. 2018) and from our experience in developing a selective inhibitor of CDK9 (Chapter 5), it may be possible to modify this inhibitor to be specific for TFAP-2β. Our study provides a rational for creating a selective inhibitor of TFAP-2β. Moreover, as the finding of this work showed that TFAP-2β is not the main determinant of AR action in this disease, further studies are required to identify biomarkers that can be used in patient selection to predict what type of AR target therapy (i.e., agonist or antagonist) would be appropriate. In the context of molecular apocrine breast cancers, a key future experiment is to interrogate AR signalling at the molecular level using our panel of PDX models of this disease sub-type that we have shown to have differential growth responses to DHT. Comparison of genomic factors that mediate AR signalling in more clinically relevant models such as molecular apocrine PDXs, with those of cell line models may facilitate the identification of biomarkers of androgen response in this disease context and avoid cell line biases, for example, the MDA-MB-453 cell line contains a functional mutation in the LBD of AR specific to this cell line.

### 6.1.3 A new highly selective CDK9 inhibitor, D-11, effectively suppresses growth of triple negative breast cancer (TNBC)

CDK9 inhibition is an attractive therapeutic strategy for TNBC tumours, as they are addicted to continuous transcription of short half-life oncogene and pro-survival factors (Brisard et al. 2018; Rajput et al. 2016). Although several inhibitors (e.g., Dinaciclib and Flavopiridol) have shown high potency against CDK9 activity, the lack of strong selectivity for CDK9 has limited their clinical utility due to off-target side effects (Aklilu et al. 2003; Boffo et al. 2018; Criscitiello et al. 2014; Parry et al. 2010). Therefore, developing highly selective CDK9 inhibitors is important to advance the field and clinical implementation of this targeting strategy for TNBC or other relevant cancers. Here, we report development and pre-clinical efficacy of a novel and highly selective CDK9 inhibitor, D-11, which exhibited high potency against CDK9 and displayed excellent selectivity over 369 human kinases. D-11 effectively suppressed the proliferation of TNBC cell line models *in vitro*. Through inhibition of RNAPII phosphorylation at Ser-2, D-11 decreased levels of the anti-apoptotic protein MCL1 and induced Caspase-dependent apoptosis in TNBC cells. These data are consistent with previous studies that have associated CDK9 inhibition with MCL1 depletion and subsequent induction of tumour cell death (Cidado et al. 2020; O'Reilly et al. 2018; Rahaman, Yu, et al. 2019; Tong et al. 2019). In addition, our study demonstrated that D-11 impairs proliferation of TNBC cells by diminishing protein levels of C-MYC and arresting cells in the G2/M phase. C-MYC regulates expression of a variety of

genes involved in cell cycle progression, including genes encoding CDK1 and cyclin B1, which mediate cell cycle progression from the G2/M phase, (Albihn, Johnsen & Henriksson 2010; Dang, CVJM & biology 1999). Consistent with our findings, a previous study by Yang and colleagues reported that silencing C-MYC suppressed cyclin B1 expression in leukemia cells (Yang, Y et al. 2018). These data indicate that G2/M cell cycle arrest induced by D-11 could be a result of down-regulation of the C-MYC target gene cyclin B1.

The anti-tumour efficacy of D-11 was also observed *in vivo* using mammary intraductal (MIND) xenografting of a TNBC cell line. Our study is the first to test *in vivo* efficacy of D-11 but also to use this more clinically relevant xenografting technique to examine the effect of CDK9 inhibition *in vivo*. The MIND technique is preferred over mammary fat-pad xenografting because it establishes a tumour where breast cancers naturally develop (within the mammary ducts) and retains the capacity to recapitulate stages of disease progression (in situ, invasive, metastatic) (Richard et al. 2016; Sflomos et al. 2016). Oral administration of D-11 resulted in a significant inhibition in tumour growth ($p < 0.01$) without causing body weight loss and without affecting histology of normal tissues such as the liver and spleen. In contrast to its cytotoxic effect against cancer cells, D-11 also showed no toxicity on histology of normal human breast tissues that were cultured and treated *ex vivo* as explants, an important pre-clinical model that sustains tissue architecture, viability, and cellular complexity (Centenera et al. 2018; Flores, Taylor & Clinics 2015; Hickey, TE et al. 2021). These observations demonstrate the potential safety of D-11 and support its selectively for CDK9. To further clarify the relevance of selective CDK9 inhibition in TNBC and to provide

more compelling evidence that D-11 is a promising drug for clinical development, examining D-11's effect *in vivo* using TNBC PDX would be a key future experiment for clinical translation.

## 6.2 Conclusion

The findings of this project expand current knowledge about the action of AR signalling in ERα-negative breast cancer and provide a descriptive investigation of the molecular factors associated with its oncogenic versus anti-oncogenic potential that can serve as an important resource for generating and testing new hypotheses. Moreover, this project provides preclinical evidence for novel therapeutic targets in ERα-negative breast cancer. TFAP-2β is highly expressed in molecular apocrine breast cancer tumours and is critical for the growth and viability of a cell line model of this disease sub-type. Our study presented TFAP-2β as a growth-promoting factor and a potential treatment strategy in molecular apocrine disease and highlighted the importance of identifying inhibitors of this factor. Targeting CDK9 with a novel, potent, and highly selective inhibitor is an effective treatment strategy for ERα-negative breast tumours. The new CDK9 inhibitor D-11 has marked anti-cancer efficacy *in vitro* and *in vivo*. With its high oral bioactivity along with an appreciable safety profile, D-11 offers a very exciting prospect as a clinical development candidate for treating women with triple negative breast cancer.

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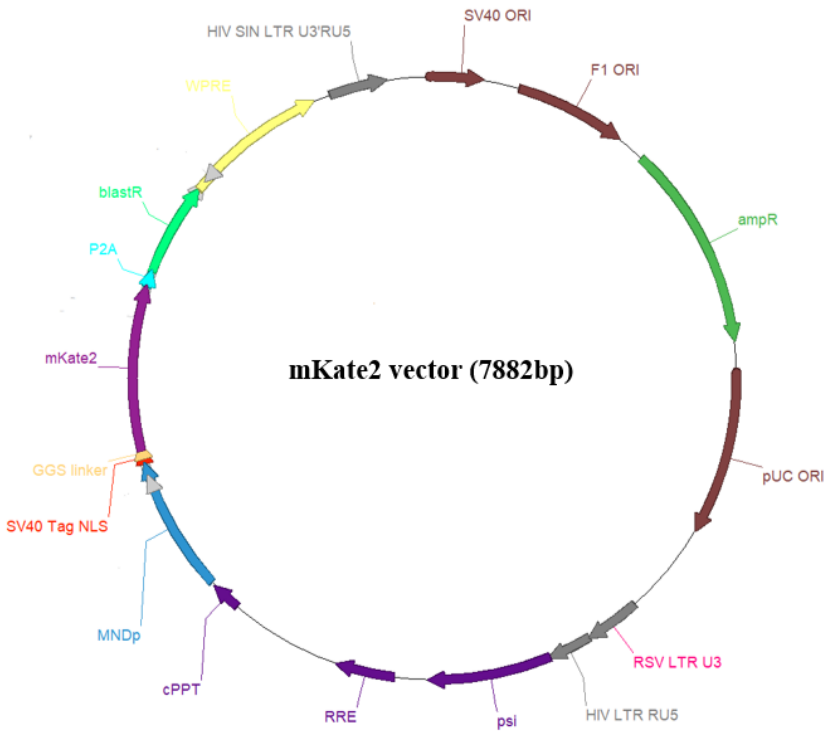
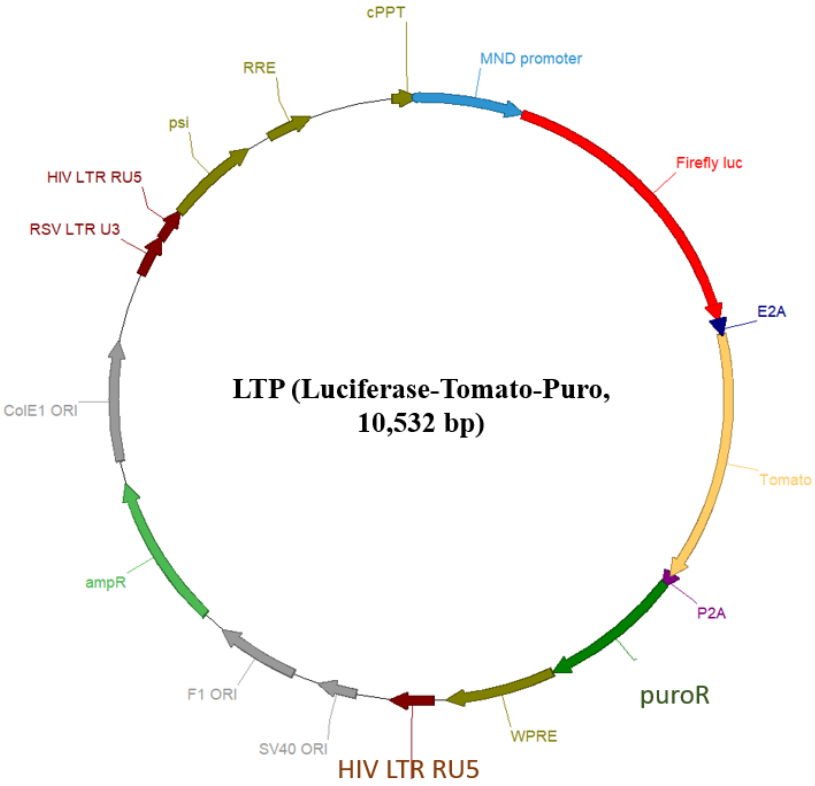
# APPENDIX (1)

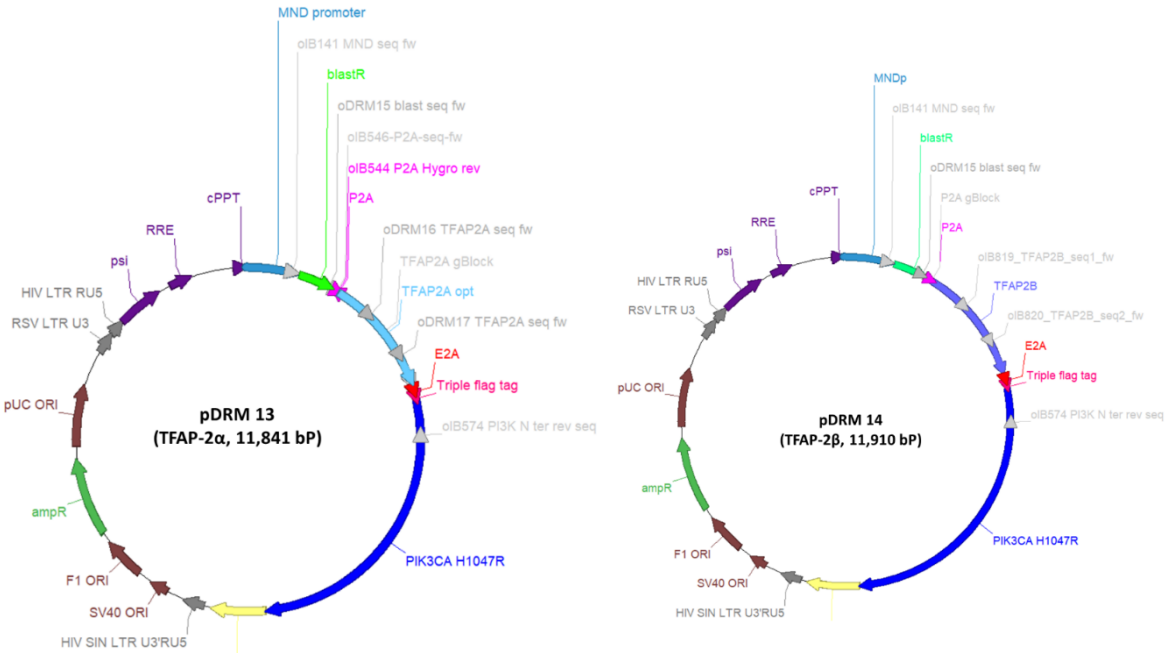
## Plasmid constructs



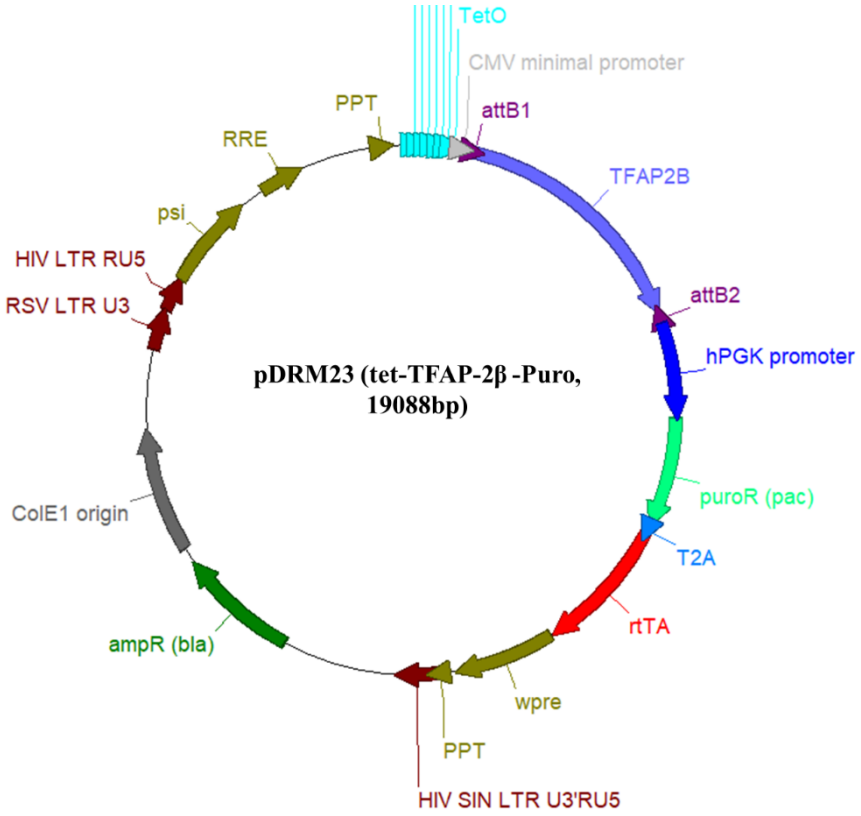

**Appendix 1A:** Map of mKate2 plasmid. mKate2 plasmid was prepared by Prof. Richard Iggo by ligation of mKate 2 sequence (693bp) into pJS137 basic vector.




**Appendix 1B:** Map of LTP plasmid. LTP plasmid was derived from pRRL-MND-GFP (Addgene, #36247) by replacing the GFP insert with a firefly Luciferase-E2A-tdTomato-T2A-Puro cassette.




**Appendix 1C:** Map of TFAP-2α and TFAP-2β plasmids. Plasmids were derived from pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene, #12252) using Gibson assembly.




**Appendix 1D:** Map of tet-TFAP-2β plasmids. Doxycycline-inducible TFAP-2β vector (pDRM23) was generated from pCW57.1 (Addgene #41393) by Gateway cloning (Invitrogene) from an entry vector containing the TFAP-2β open reading frame (orf).

# APPENDIX (2)

# AR RNA-seq and ChIP-seq in ERα-/AR+ cell lines

**Table 2A:** Common DHT up-regulated genes in MDA-MB-453 and MFM-223 cell lines.

| Ensembl gene ID | Gene symbol | MDA-MB-453 | | MFM-223 | |
|---|---|---|---|---|---|
| | | FC | p-value | FC | p-value |
| ENSG00000132142 | ACACA | 1.54930830 | 0.003643008 | 1.962977602 | 6.66E-16 |
| ENSG00000155893 | ACPL2 | 1.57319551 | 0.005032503 | 2.031462973 | 5.77E-15 |
| ENSG00000143199 | ADCY10 | 2.64671725 | 0.035150762 | 2.569958563 | 0.012046592 |
| ENSG00000150594 | ADRA2A | 7.97068569 | 0 | 2.630504506 | 0.000175059 |
| ENSG00000121057 | AKAP1 | 1.76228945 | 0.000253703 | 1.529244503 | 2.46E-07 |
| ENSG00000179841 | AKAP5 | 2.26434562 | 4.19E-06 | 1.75080106 | 3.69E-08 |
| ENSG00000116337 | AMPD2 | 1.67674713 | 0.00092076 | 1.543486089 | 5.70E-07 |
| ENSG00000165272 | AQP3 | 16.5941171 | 0 | 2.715839098 | 5.38E-05 |
| ENSG00000047648 | ARHGAP6 | 2.32230975 | 1.54E-06 | 2.492723778 | 0 |
| ENSG00000114790 | ARHGEF26 | 1.50161506 | 0.009455329 | 1.861935896 | 8.08E-14 |
| ENSG00000183111 | ARHGEF37 | 1.62619576 | 0.001801174 | 1.571240408 | 3.57E-07 |
| ENSG00000160862 | AZGP1 | 1.92989916 | 1.95E-05 | 1.890118221 | 6.55E-07 |
| ENSG00000214313 | AZGP1P1 | 3.94583335 | 0 | 3.001392647 | 6.20E-11 |
| ENSG00000113916 | BCL6 | 1.88621609 | 6.49E-05 | 1.60840286 | 2.07E-05 |
| ENSG00000107815 | C10orf2 | 1.83348908 | 0.00013962 | 1.505589429 | 2.71E-06 |
| ENSG00000182795 | C1orf116 | 9.44545292 | 6.43841E-12 | 3.89568152 | 2.8017E-16 |
| ENSG00000187889 | C1orf168 | 2.61711788 | 0.001596461 | 1.878678201 | 1.51E-13 |
| ENSG00000181744 | C3orf58 | 1.56384625 | 0.005161988 | 1.564446415 | 7.60E-07 |
| ENSG00000105879 | CBLL1 | 1.60907415 | 0.003807358 | 2.43735679 | 2.01E-10 |
| ENSG00000110104 | CCDC86 | 2.46332104 | 1.21E-08 | 1.528903236 | 7.29E-06 |
| ENSG00000111666 | CHPT1 | 3.11101356 | 6.98E-13 | 1.581644734 | 9.15E-08 |
| ENSG00000174469 | CNTNAP2 | 2.31352734 | 6.53E-08 | 1.8002665 | 2.15E-12 |
| ENSG00000005469 | CROT | 3.10415186 | 5.13E-09 | 1.747539059 | 1.02E-11 |
| ENSG00000101441 | CST4 | 4.92083303 | 0.035326788 | 1.867938175 | 0.013473705 |
| ENSG00000171954 | CYP4F22 | 9.61657439 | 5.59E-05 | 3.210659845 | 0.01227653 |
| ENSG00000186526 | CYP4F8 | 59.6631906 | 0 | 13.24234328 | 0 |
| ENSG00000102780 | DGKH | 1.56185747 | 0.006451761 | 1.564756041 | 0.00697099 |
| ENSG00000116133 | DHCR24 | 1.67336482 | 0.001008527 | 1.613123669 | 3.78E-09 |

| | | | | | |
|---|---|---|---|---|---|
| ENSG00000137976 | DNASE2B | 4.94106208 | 1.54E-06 | 2.941899426 | 4.78E-05 |
| ENSG00000128512 | DOCK4 | 1.70134589 | 0.000846856 | 4.231370492 | 6.41E-11 |
| ENSG00000158050 | DUSP2 | 2.18554893 | 1.70E-06 | 1.701970583 | 3.07E-08 |
| ENSG00000145088 | EAF2 | 1.80397111 | 0.003693614 | 1.777603583 | 8.85E-09 |
| ENSG00000135373 | EHF | 1.95239492 | 1.20E-05 | 1.508092057 | 0.002530622 |
| ENSG00000149218 | ENDOD1 | 1.88859850 | 0.000149669 | 2.754202834 | 0 |
| ENSG00000145569 | FAM105A | 3.17524376 | 1.30E-10 | 6.240225868 | 0 |
| ENSG00000096060 | FKBP5 | 4.14547218 | 6.64182E-11 | 3.296009 | 9.65666E-15 |
| ENSG00000111816 | FRK | 3.77557783 | 0.011001938 | 1.575328143 | 0.01808232 |
| ENSG00000172159 | FRMD3 | 8.1128943 | 1.07E-07 | 2.689795615 | 1.31E-08 |
| ENSG00000116717 | GADD45A | 1.69640104 | 0.000946689 | 1.668119554 | 7.71E-09 |
| ENSG00000099860 | GADD45B | 1.71887687 | 0.000795294 | 1.656246064 | 3.03E-05 |
| ENSG00000135821 | GLUL | 1.70563310 | 0.001130029 | 1.816466439 | 3.54E-13 |
| ENSG00000146535 | GNA12 | 1.84440606 | 5.51E-05 | 1.678553412 | 2.65E-10 |
| ENSG00000138641 | HERC3 | 1.95543014 | 1.20E-05 | 1.700848463 | 0.000362568 |
| ENSG00000100644 | HIF1A | 1.53321459 | 0.005388702 | 1.637905886 | 3.31E-05 |
| ENSG00000125430 | HS3ST3B1 | 2.24435692 | 0.000211439 | 1.949457 | 1.90E-05 |
| ENSG00000145703 | IQGAP2 | 2.88158052 | 2.83E-11 | 4.849193546 | 0 |
| ENSG00000177508 | IRX3 | 2.21012894 | 2.92E-07 | 2.238061361 | 2.16E-08 |
| ENSG00000177606 | JUN | 1.82528263 | 0.00012674 | 1.513985227 | 2.42E-06 |
| ENSG00000178695 | KCTD12 | 2.16723262 | 0.001138853 | 3.362106071 | 3.16E-08 |
| ENSG00000127663 | KDM4B | 2.08412368 | 2.66E-06 | 2.037094301 | 0 |
| ENSG00000102554 | KLF5 | 1.85370315 | 0.000411496 | 2.163470023 | 3.33E-16 |
| ENSG00000124743 | KLHL31 | 3.13770321 | 0.013000129 | 1.929752132 | 0.010681415 |
| ENSG00000138795 | LEF1 | 2.04916218 | 0.007492439 | 1.690393963 | 0.042001843 |
| ENSG00000113594 | LIFR | 2.864589 | 0.000129388 | 1.552406336 | 0.008977533 |
| ENSG00000171517 | LPAR3 | 3.3277719 | 0.005748094 | 10.08532713 | 1.81E-08 |
| ENSG00000079691 | LRRC16A | 2.74045622 | 1.69E-10 | 1.787534752 | 9.81E-07 |
| ENSG00000204103 | MAFB | 1.99333217 | 0.000671161 | 1.50935327 | 9.81E-06 |
| ENSG00000172175 | MALT1 | 1.56126794 | 0.004447891 | 2.399247353 | 0 |
| ENSG00000131844 | MCCC2 | 1.60208995 | 0.002013681 | 1.853007737 | 5.88E-14 |
| ENSG00000166391 | MOGAT2 | 5.53765204 | 1.34E-05 | 5.48788837 | 3.36E-10 |
| ENSG00000143158 | MPC2 | 1.99337491 | 7.83E-06 | 1.570848897 | 5.38E-08 |
| ENSG00000196091 | MYBPC1 | 63.0307981 | 0 | 4.723398333 | 7.66E-08 |
| ENSG00000136997 | MYC | 3.67025796 | 1.11E-16 | 1.756900166 | 3.03E-05 |
| ENSG00000005810 | MYCBP2 | 1.61924376 | 0.002654789 | 1.743754646 | 0.002275898 |
| ENSG00000229644 | NAMPTL | 1.78133380 | 0.02556212 | 1.553184516 | 0.040893481 |
| ENSG00000100906 | NFKBIA | 3.53990344 | 2.20E-06 | 1.512224923 | 2.74E-06 |
| ENSG00000198435 | NRARP | 1.97626208 | 1.11E-05 | 1.761083429 | 3.73E-05 |
| ENSG00000169116 | PARM1 | 2.24056498 | 3.54E-07 | 2.179843596 | 2.21E-11 |
| ENSG00000179094 | PER1 | 1.80896392 | 0.00023487 | 2.942630637 | 7.77E-16 |
| ENSG00000123836 | PFKFB2 | 1.65206954 | 0.001506376 | 1.914014192 | 2.77E-13 |
| ENSG00000241878 | PISD | 2.10804734 | 1.68E-06 | 1.619990324 | 7.60E-08 |
| ENSG00000170927 | PKHD1 | 2.51750881 | 0.001990599 | 7.02470649 | 1.89E-11 |
| ENSG00000125630 | POLR1B | 1.60282537 | 0.002272145 | 1.563571638 | 1.36E-07 |
| ENSG00000155846 | PPARGC1B | 2.19447714 | 8.13E-06 | 1.954702623 | 1.99E-06 |

| | | | | | |
|---|---|---|---|---|---|
| ENSG00000085377 | PREP | 2.47378173 | 3.81E-07 | 1.615088365 | 1.29E-08 |
| ENSG00000131791 | PRKAB2 | 1.79862228 | 0.00022834 | 1.666133938 | 1.78E-05 |
| ENSG00000150687 | PRSS23 | 1.60452403 | 0.002211462 | 2.086964791 | 2.90E-08 |
| ENSG00000156675 | RAB11FIP1 | 2.03159453 | 4.92E-06 | 1.894745784 | 9.39E-11 |
| ENSG00000164188 | RANBP3L | 18.5221481 | 1.25300E-11 | 16.36094124 | 5.56776E-18 |
| ENSG00000100302 | RASD2 | 3.72759255 | 0.049620006 | 1.56017092 | 0.017346732 |
| ENSG00000111961 | SASH1 | 1.52314963 | 0.005802305 | 1.967749583 | 5.09E-06 |
| ENSG00000100003 | SEC14L2 | 52.7691783 | 1.18446E-13 | 5.290915021 | 2.11620E-16 |
| ENSG00000174705 | SH3PXD2B | 1.77494095 | 0.001083022 | 2.149331528 | 1.24E-06 |
| ENSG00000142178 | SIK1 | 2.43883252 | 3.11E-06 | 1.511856519 | 3.13E-06 |
| ENSG00000163406 | SLC15A2 | 2.19647344 | 0.008799525 | 1.585633617 | 3.49E-05 |
| ENSG00000108932 | SLC16A6 | 2.18537148 | 4.08E-07 | 2.088967028 | 9.11E-10 |
| ENSG00000197208 | SLC22A4 | 1.53661894 | 0.014743124 | 2.047084088 | 0.017529513 |
| ENSG00000197375 | SLC22A5 | 1.54540730 | 0.005245623 | 1.779704126 | 3.39E-11 |
| ENSG00000155850 | SLC26A2 | 2.03272792 | 0.003878015 | 2.30156232 | 1.95E-09 |
| ENSG00000091138 | SLC26A3 | 11.2563002 | 6.36E-09 | 8.721099322 | 0 |
| ENSG00000212588 | SNORA26 | 2.10513955 | 0.032047452 | 3.092683638 | 0.029528089 |
| ENSG00000198142 | SOWAHC | 1.51042259 | 0.008565055 | 1.885831802 | 4.41E-14 |
| ENSG00000139351 | SYCP3 | 2.00351017 | 0.007373518 | 1.575516141 | 0.010317472 |
| ENSG00000141384 | TAF4B | 3.33981727 | 2.75E-10 | 1.620475894 | 1.21E-07 |
| ENSG00000198650 | TAT | 2.26766307 | 5.24E-06 | 2.293465992 | 0.001061457 |
| ENSG00000167291 | TBC1D16 | 1.84042165 | 5.01E-05 | 1.611580792 | 1.83E-05 |
| ENSG00000090447 | TFAP4 | 1.64791209 | 0.001944944 | 1.578344206 | 7.56E-07 |
| ENSG00000042832 | TG | 2.21893251 | 0.021289033 | 3.911816447 | 6.74E-06 |
| ENSG00000249242 | TMEM150C | 2.58170825 | 0.000352122 | 1.627825473 | 0.004433852 |
| ENSG00000157600 | TMEM164 | 1.67129966 | 0.000761814 | 1.549158523 | 2.72E-07 |
| ENSG00000179104 | TMTC2 | 2.00353108 | 9.08E-06 | 2.390760724 | 9.90E-13 |
| ENSG00000109814 | UGDH | 1.77651936 | 0.000213616 | 2.405049811 | 0 |
| ENSG00000213759 | UGT2B11 | 139.984007 | 0 | 27.34309596 | 0 |
| ENSG00000135226 | UGT2B28 | 32.099369 | 1.12183E-08 | 14.21662551 | 2.34450E-07 |
| ENSG00000169884 | WNT10B | 1.84242252 | 0.001162088 | 1.593431267 | 0.012519392 |
| ENSG00000188064 | WNT7B | 4.64794852 | 0 | 1.836853797 | 1.32E-10 |
| ENSG00000113645 | WWC1 | 2.03895404 | 3.72E-06 | 1.933281086 | 7.89E-14 |
| ENSG00000047644 | WWC3 | 2.85301189 | 1.64E-11 | 2.299163257 | 4.38E-10 |
| ENSG00000198740 | ZNF652 | 1.81913455 | 0.000105432 | 1.589266322 | 0.003213479 |
| ENSG00000183779 | ZNF703 | 1.51121351 | 0.008802198 | 1.513742752 | 6.84E-07 |

**Table 2B:** Common DHT down-regulated genes in MDA-MB-453 and MFM-223 cell lines.

| Ensembl gene ID | Gene symbol | MDA-MB-453 | | MFM-223 | |
|---|---|---|---|---|---|
| | | FC | p-value | FC | p-value |
| ENSG00000139211 | AMIGO2 | 0.471174647 | 1.26E-06 | 0.33035501 | 0 |
| ENSG00000169083 | AR | 0.538193762 | 6.08E-05 | 0.59594927 | 1.02E-05 |
| ENSG00000102048 | ASB9 | 0.484062903 | 0.032143436 | 0.41791152 | 0.000605009 |
| ENSG00000180347 | CCDC129 | 0 | 0.017578627 | 0.20553627 | 0.04119962 |
| ENSG00000197599 | CCDC154 | 0.37540699 | 0.021690816 | 0.56385417 | 0.000388605 |
| ENSG00000163823 | CCR1 | 0.197445904 | 0.038244651 | 0.31298030 | 0.038593696 |
| ENSG00000175264 | CHST1 | 0.433237474 | 9.50E-08 | 0.17322701 | 1.26E-07 |
| ENSG00000077063 | CTTNBP2 | 0.38528894 | 0.000952371 | 0.40091238 | 0 |
| ENSG00000121966 | CXCR4 | 0.362292072 | 9.42E-06 | 0.17714627 | 0 |
| ENSG00000153071 | DAB2 | 0.345190367 | 0.000321009 | 0.40530817 | 8.28E-06 |
| ENSG00000171617 | ENC1 | 0.554977665 | 0.00022448 | 0.33520464 | 0 |
| ENSG00000091831 | ESR1 | 0.304409448 | 0.018632583 | 0.56346681 | 2.49E-05 |
| ENSG00000159784 | FAM131B | 0 | 0.017250037 | 0.58787891 | 0.038464954 |
| ENSG00000185112 | FAM43A | 0.567327323 | 0.000492306 | 0.56182214 | 1.82E-06 |
| ENSG00000162645 | GBP2 | 0.438738495 | 1.84E-05 | 0.48416342 | 0 |
| ENSG00000117009 | KMO | 0.347940775 | 2.76E-11 | 0.30154472 | 0.002119881 |
| ENSG00000108244 | KRT23 | 0.276203258 | 3.88E-09 | 0.54480202 | 5.53E-07 |
| ENSG00000212766 | LINC0027 | 0.151333246 | 0.00532552 | 0.57284453 | 3.38E-05 |
| ENSG00000153714 | LURAP1L | 0.591364913 | 0.002954293 | 0.50553609 | 7.52E-12 |
| ENSG00000178573 | MAF | 0.456576366 | 3.49E-05 | 0.39778311 | 0.013983257 |
| ENSG00000144063 | MALL | 0.260259638 | 0.037183871 | 0.56774638 | 6.22E-05 |
| ENSG00000122584 | NXPH1 | 0.331040753 | 0.021589538 | 0.46888825 | 0.003740489 |
| ENSG00000179715 | PCED1B | 0.592220844 | 0.004058584 | 0.54589347 | 2.47E-06 |
| ENSG00000184588 | PDE4B | 0.475303352 | 2.47E-06 | 0.34234393 | 8.60E-10 |
| ENSG00000086717 | PPEF1 | 0.36058859 | 0.002044024 | 0.54749317 | 0.001742634 |
| ENSG00000128045 | RASL11B | 0.539492162 | 0.00037267 | 0.48494931 | 0.00722323 |
| ENSG00000112246 | SIM1 | 0 | 0.035281118 | 0.44492123 | 0.007234161 |
| ENSG00000116991 | SIPA1L2 | 0.583455529 | 0.000486588 | 0.49290527 | 1.41E-10 |
| ENSG00000243244 | STON1 | 0.331097304 | 0.011945442 | 0.46744381 | 6.66E-16 |
| ENSG00000067715 | SYT1 | 0.120542341 | 0.029658818 | 0.52825936 | 0.006064981 |
| ENSG00000137501 | SYTL2 | 0.584500813 | 0.014080706 | 0.54707167 | 1.68E-11 |
| ENSG00000073282 | TP63 | 0.171369783 | 0 | 0.55858652 | 9.88E-05 |
| ENSG00000188001 | TPRG1 | 0.257075082 | 2.66E-10 | 0.53238956 | 1.81E-05 |
| ENSG00000038427 | VCAN | 0.377796831 | 0.000309025 | 0.45946620 | 1.51E-07 |
| ENSG00000134258 | VTCN1 | 0.589920878 | 0.014100951 | 0.51019568 | 0.000289626 |
| ENSG00000115596 | WNT6 | 0 | 0.047759347 | 0.30297769 | 1.73E-07 |
| ENSG00000158125 | XDH | 0.346436245 | 6.43E-08 | 0.50138935 | 2.42E-05 |

**Table 2C:** GO analysis of genes up-regulated in MDA-MB-453 in response to DHT

| Ensembl gene ID | Gene symbol | FC | p-value |
|---|---|---|---|
| Lipid and steroid metabolism | | | |
| ENSG00000186204 | CYP4F12 | 11.29847623 | 0.00148 |
| ENSG00000196620 | UGT2B15 | 6.852113975 | 0.025572 |
| ENSG00000137841 | PLCB2 | 4.789161047 | 2.31E-05 |
| ENSG00000215009 | ACSM4 | 4.096730518 | 0.032372 |
| ENSG00000171903 | CYP4F11 | 3.453236418 | 0.030478 |
| ENSG00000184254 | ALDH1A3 | 3.336076817 | 0.002098 |
| ENSG00000180616 | SSTR2 | 3.314596609 | 0.043959 |
| ENSG00000064763 | FAR2 | 2.372752424 | 3.20E-06 |
| ENSG00000176387 | HSD11B2 | 2.307855786 | 3.16E-07 |
| ENSG00000155380 | SLC16A1 | 2.237470805 | 3.90E-06 |
| ENSG00000068366 | ACSL4 | 2.230804542 | 1.57E-05 |
| ENSG00000149527 | PLCH2 | 2.200475337 | 0.015289 |
| ENSG00000142798 | HSPG2 | 1.992454438 | 0.001598 |
| ENSG00000165914 | TTC7B | 1.976457087 | 0.020896 |
| ENSG00000005249 | PRKAR2B | 1.854497244 | 0.000912 |
| ENSG00000136826 | KLF4 | 1.793416267 | 0.00023 |
| ENSG00000163659 | TIPARP | 1.774055202 | 0.000243 |
| ENSG00000137124 | ALDH1B1 | 1.752036847 | 0.000418 |
| ENSG00000095637 | SORBS1 | 1.746265876 | 0.022469 |
| ENSG00000179477 | ALOX12B | 1.736814273 | 0.012048 |
| ENSG00000162409 | PRKAA2 | 1.719414107 | 0.000613 |
| ENSG00000152270 | PDE3B | 1.719106133 | 0.009149 |
| ENSG00000162139 | NEU3 | 1.707558114 | 0.000625 |
| ENSG00000069667 | RORA | 1.668606742 | 0.00202 |
| ENSG00000060971 | ACAA1 | 1.654766404 | 0.001194 |
| ENSG00000166261 | ZNF202 | 1.576397017 | 0.004351 |
| ENSG00000001630 | CYP51A1 | 1.575518615 | 0.011111 |
| ENSG00000168118 | RAB4A | 1.556500713 | 0.00437 |
| ENSG00000156804 | FBXO32 | 1.544733587 | 0.005102 |
| ENSG00000158470 | B4GALT5 | 1.531618539 | 0.005212 |
| ENSG00000120833 | SOCS2 | 1.530637678 | 0.005858 |
| ENSG00000151726 | ACSL1 | 1.526910654 | 0.005322 |
| ENSG00000148459 | PDSS1 | 1.52047039 | 0.009912 |
| ENSG00000084676 | NCOA1 | 1.513640736 | 0.007494 |
| ENSG00000184886 | PIGW | 1.502449243 | 0.009868 |
| Carbohydrate, RNA, or small molecules metabolism | | | |
| ENSG00000162882 | HAAO | 3.833677042 | 0.012874 |
| ENSG00000132437 | DDC | 2.922265229 | 4.07E-08 |
| ENSG00000211448 | DIO2 | 2.916671575 | 0.009179 |
| ENSG00000133424 | LARGE | 2.165574202 | 5.83E-07 |

| | | | |
|---|---|---|---|
| ENSG00000065989 | PDE4A | 1.814179654 | 0.016966 |
| ENSG00000124357 | NAGK | 1.755051672 | 0.000267 |
| ENSG00000247626 | MARS2 | 1.708388402 | 0.00071 |
| ENSG00000123009 | NME2P1 | 1.673724911 | 0.017106 |
| ENSG00000162174 | ASRGL1 | 1.66404022 | 0.009369 |
| ENSG00000112541 | PDE10A | 1.643067235 | 0.047395 |
| ENSG00000171004 | HS6ST2 | 1.605527612 | 0.002243 |
| ENSG00000185818 | NAT8L | 1.604394102 | 0.018098 |
| ENSG00000103222 | ABCC1 | 1.60346301 | 0.00267 |
| ENSG00000147224 | PRPS1 | 1.555132923 | 0.004636 |
| ENSG00000168282 | MGAT2 | 1.504005709 | 0.031181 |
| Development | | | |
| ENSG00000175329 | ISX | 16.98644844 | 2.25E-12 |
| ENSG00000138311 | ZNF365 | 7.615307453 | 2.72E-12 |
| ENSG00000117148 | ACTL8 | 3.81358398 | 0.000668 |
| ENSG00000179111 | HES7 | 3.541165881 | 8.70E-05 |
| ENSG00000163884 | KLF15 | 3.435058132 | 1.39E-10 |
| ENSG00000198812 | LRRC10 | 3.172554735 | 0.042075 |
| ENSG00000005073 | HOXA11 | 3.045195236 | 0.006563 |
| ENSG00000128606 | LRRC17 | 2.844360398 | 0.047881 |
| ENSG00000181418 | DDN | 2.1832350143 | 1.00E-07 |
| ENSG00000154734 | ADAMTS1 | 2.788841121 | 9.78E-11 |
| ENSG00000144218 | AFF3 | 2.742996312 | 1.61E-08 |
| ENSG00000027869 | SH2D2A | 2.507451966 | 0.000129 |
| ENSG00000166833 | NAV2 | 2.408425851 | 4.08E-06 |
| ENSG00000117013 | KCNQ4 | 2.2903795 | 3.49E-05 |
| ENSG00000043039 | BARX2 | 2.220582083 | 0.011631 |
| ENSG00000204335 | SP5 | 2.211365742 | 0.031964 |
| ENSG00000166068 | SPRED1 | 1.94836328 | 1.52E-05 |
| ENSG00000142627 | EPHA2 | 1.910921643 | 0.000197 |
| ENSG00000187210 | GCNT1 | 1.888984057 | 0.001337 |
| ENSG00000104998 | IL27RA | 1.790499839 | 0.003786 |
| ENSG00000164442 | CITED2 | 1.78822987 | 0.037307 |
| ENSG00000179041 | RRS1 | 1.785078772 | 0.000192 |
| ENSG00000166197 | NOLC1 | 1.78506511 | 0.000155 |
| ENSG00000120254 | MTHFD1L | 1.771780655 | 0.000316 |
| ENSG00000253293 | HOXA10 | 1.731867885 | 0.033346 |
| ENSG00000015133 | CCDC88C | 1.725847281 | 0.000618 |
| ENSG00000101384 | JAG1 | 1.714762713 | 0.000552 |
| ENSG00000145220 | LYAR | 1.69390364 | 0.000776 |
| ENSG00000176171 | BNIP3 | 1.684580549 | 0.002039 |
| ENSG00000066468 | FGFR2 | 1.618614325 | 0.018231 |
| ENSG00000133816 | MICAL2 | 1.655069307 | 0.015757 |
| ENSG00000172458 | IL17D | 1.65357437 | 0.011307 |
| ENSG00000123572 | NRK | 1.633050311 | 0.001524 |
| ENSG00000107731 | UNC5B | 1.619729065 | 0.001739 |

| | | | |
|---|---|---|---|
| ENSG00000177283 | FZD8 | 1.61648453 | 0.005014 |
| ENSG00000159216 | RUNX1 | 1.612041124 | 0.002038 |
| ENSG00000091127 | PUS7 | 1.609290622 | 0.003 |
| ENSG00000115318 | LOXL3 | 1.599386611 | 0.010691 |
| ENSG00000115758 | ODC1 | 1.589324114 | 0.002652 |
| ENSG00000033867 | SLC4A7 | 1.585988788 | 0.003748 |
| ENSG00000112578 | BYSL | 1.563360078 | 0.01025 |
| ENSG00000160712 | IL6R | 1.561482828 | 0.007081 |
| ENSG00000128059 | PPAT | 1.561419154 | 0.004327 |
| ENSG00000105835 | NAMPT | 1.556106619 | 0.00583 |
| ENSG00000136068 | FLNB | 1.550254856 | 0.003498 |
| ENSG00000163251 | FZD5 | 1.531333743 | 0.022149 |
| ENSG00000083307 | GRHL2 | 1.513602229 | 0.006714 |
| ENSG00000136205 | TNS3 | 1.51328749 | 0.008395 |
| ENSG00000057294 | PKP2 | 1.509345763 | 0.042056 |
| ENSG00000168234 | TTC39C | 1.501861728 | 0.017602 |
| Growth or cell signalling | | | |
| ENSG00000124140 | SLC12A5 | 7.079760201 | 0.039799 |
| ENSG00000118160 | SLC8A2 | 4.634768249 | 0.039592 |
| ENSG00000120162 | MOB3B | 3.533618072 | 0.005469 |
| ENSG00000143507 | DUSP10 | 3.355452682 | 4.16E-14 |
| ENSG00000124743 | KLHL31 | 3.137703217 | 0.013 |
| ENSG00000111666 | CHPT1 | 3.111013563 | 6.98E-13 |
| ENSG00000131711 | MAP1B | 2.485615134 | 0.000114 |
| ENSG00000118515 | SGK1 | 2.425439788 | 0.002339 |
| ENSG00000127863 | TNFRSF19 | 2.39644025 | 3.46E-05 |
| ENSG00000158050 | DUSP2 | 2.185548938 | 1.70E-06 |
| ENSG00000113739 | STC2 | 2.138432242 | 2.14E-06 |
| ENSG00000125931 | CITED1 | 2.13084488 | 0.000595 |
| ENSG00000101695 | RNF125 | 2.107823575 | 0.000712 |
| ENSG00000138795 | LEF1 | 2.049162182 | 0.007492 |
| ENSG00000113645 | WWC1 | 2.038954048 | 3.72E-06 |
| ENSG00000120899 | PTK2B | 2.030820446 | 1.17E-05 |
| ENSG00000178607 | ERN1 | 2.024484801 | 2.03E-05 |
| ENSG00000162552 | WNT4 | 2.016264669 | 0.017307 |
| ENSG00000135333 | EPHA7 | 2.013460906 | 0.000319 |
| ENSG00000198915 | RASGEF1A | 1.908842172 | 0.019618 |
| ENSG00000118965 | WDR35 | 1.894844207 | 8.39E-05 |
| ENSG00000146376 | ARHGAP18 | 1.872828878 | 8.54E-05 |
| ENSG00000165732 | DDX21 | 1.81428836 | 0.000103 |
| ENSG00000017797 | RALBP1 | 1.789178839 | 0.000163 |
| ENSG00000104369 | JPH1 | 1.72779145 | 0.000559 |
| ENSG00000170345 | FOS | 1.684710549 | 0.036036 |
| ENSG00000149089 | APIP | 1.635251949 | 0.002569 |
| ENSG00000106785 | TRIM14 | 1.625044711 | 0.001563 |
| ENSG00000111845 | PAK1IP1 | 1.539082341 | 0.005211 |

**Table 2D:** Biological processes associated with DHT down-regulated genes in MDA-MB-453 cells

| Ensembl gene ID | Gene symbol | FC | p-value |
|---|---|---|---|
| Cell motility, adhesion, or extracellular structure | | | |
| ENSG00000065618 | COL17A1 | 0 | 0.029081 |
| ENSG00000189056 | RELN | 0 | 0.018671 |
| ENSG00000102837 | OLFM4 | 0 | 0.015949 |
| ENSG00000134757 | DSG3 | 0 | 0.011299 |
| ENSG00000132205 | EMILIN2 | 0 | 0.040211 |
| ENSG00000160791 | CCR5 | 0 | 0.001599 |
| ENSG00000081041 | CXCL2 | 0 | 0.03163 |
| ENSG00000211890 | IGHA2 | 0 | 0.019882 |
| ENSG00000211895 | IGHA1 | 0.056928 | 0.014989 |
| ENSG00000175899 | A2M | 0.098361 | 0.044878 |
| ENSG00000137673 | MMP7 | 0.105178 | 0.033412 |
| ENSG00000255604 | VTN | 0.119487 | 0.041232 |
| ENSG00000166670 | MMP10 | 0.127361 | 0.012948 |
| ENSG00000173432 | SAA1 | 0.152622 | 0.017424 |
| ENSG00000101335 | MYL9 | 0.166702 | 0.003006 |
| ENSG00000152268 | SPON1 | 0.20093 | 0.049172 |
| ENSG00000120217 | CD274 | 0.231999 | 0.041468 |
| ENSG00000080573 | COL5A3 | 0.233182 | 0.033674 |
| ENSG00000130300 | PLVAP | 0.240275 | 0.040161 |
| ENSG00000152583 | SPARCL1 | 0.252878 | 0.015347 |
| ENSG00000147065 | MSN | 0.265894 | 0.010835 |
| ENSG00000121807 | CCR2 | 0.276147 | 0.023726 |
| ENSG00000146555 | SDK1 | 0.279631 | 0.031706 |
| ENSG00000148702 | HABP2 | 0.285999 | 1.23E-09 |
| ENSG00000143520 | FLG2 | 0.305903 | 0.026992 |
| ENSG00000091986 | CCDC80 | 0.309876 | 0.038893 |
| ENSG00000113140 | SPARC | 0.311592 | 0.021068 |
| ENSG00000120896 | SORBS3 | 0.326719 | 0.044562 |
| ENSG00000142156 | COL6A1 | 0.329797 | 0.01876 |
| ENSG00000140945 | CDH13 | 0.329891 | 0.005766 |
| ENSG00000138829 | FBN2 | 0.338722 | 1.73E-07 |
| ENSG00000139329 | LUM | 0.338983 | 0.008498 |
| ENSG00000171119 | NRTN | 0.361854 | 0.030735 |
| ENSG00000011465 | DCN | 0.369724 | 0.005076 |
| ENSG00000148926 | ADM | 0.378075 | 0.025102 |
| ENSG00000163359 | COL6A3 | 0.383347 | 0.039275 |
| ENSG00000185499 | MUC1 | 0.386519 | 0.014089 |
| ENSG00000148948 | LRRC4C | 0.392653 | 0.003831 |
| ENSG00000163638 | ADAMTS9 | 0.404246 | 0.019345 |

| | | | |
|---|---|---|---|
| ENSG00000146197 | SCUBE3 | 0.420484 | 0.000807 |
| ENSG00000116141 | MARK1 | 0.424395 | 5.09E-05 |
| ENSG00000144824 | PHLDB2 | 0.435167 | 3.17E-06 |
| ENSG00000116106 | EPHA4 | 0.437109 | 5.84E-07 |
| ENSG00000164692 | COL1A2 | 0.445433 | 0.048807 |
| ENSG00000106541 | AGR2 | 0.447506 | 0.021201 |
| ENSG00000118785 | SPP1 | 0.455873 | 0.04846 |
| ENSG00000161638 | ITGA5 | 0.458459 | 0.000395 |
| ENSG00000140092 | FBLN5 | 0.462107 | 0.024721 |
| ENSG00000150672 | DLG2 | 0.483081 | 0.0019 |
| ENSG00000163536 | SERPINI1 | 0.487389 | 0.04999 |
| ENSG00000125966 | MMP24 | 0.493843 | 0.034174 |
| ENSG00000166106 | ADAMTS15 | 0.500032 | 0.026533 |
| ENSG00000076706 | MCAM | 0.504521 | 0.035845 |
| ENSG00000143546 | S100A8 | 0.510126 | 0.021648 |
| ENSG00000172638 | EFEMP2 | 0.522717 | 0.015174 |
| ENSG00000140859 | KIFC3 | 0.525971 | 0.04516 |
| ENSG00000106571 | GLI3 | 0.537247 | 8.86E-05 |
| ENSG00000157227 | MMP14 | 0.547407 | 0.011157 |
| ENSG00000129038 | LOXL1 | 0.549336 | 0.006255 |
| ENSG00000076716 | GPC4 | 0.549964 | 0.00024 |
| ENSG00000078401 | EDN1 | 0.562732 | 0.001351 |
| ENSG00000137699 | TRIM29 | 0.573743 | 0.029525 |
| ENSG00000138772 | ANXA3 | 0.573972 | 0.005354 |
| ENSG00000041982 | TNC | 0.57471 | 0.000328 |
| ENSG00000119535 | CSF3R | 0.582702 | 0.04323 |
| ENSG00000170577 | SIX2 | 0.584349 | 0.002824 |
| ENSG00000171444 | MCC | 0.59113 | 0.000698 |
| ENSG00000151914 | DST | 0.593161 | 0.000586 |
| Sex differentiation | | | |
| ENSG00000157404 | KIT | 0 | 0.001431 |
| ENSG00000138207 | RBP4 | 0 | 0.03071 |
| ENSG00000081051 | AFP | 0.155244 | 1.64E-08 |
| ENSG00000134853 | PDGFRA | 0.311845 | 0.018887 |
| ENSG00000104332 | SFRP1 | 0.361703 | 0.001642 |
| ENSG00000244588 | RAD21L1 | 0.555058 | 0.010347 |
| ENSG00000125398 | SOX9 | 0.56725 | 0.000673 |
| ENSG00000132130 | LHX1 | 0.599839 | 0.00593 |
| Regulation of cell growth or death | | | |
| ENSG00000164741 | DLC1 | 0 | 0.020456 |
| ENSG00000136160 | EDNRB | 0 | 0.043352 |
| ENSG00000118971 | CCND2 | 0.091317 | 0.014621 |
| ENSG00000010671 | BTK | 0.153937 | 0.042683 |
| ENSG00000070808 | CAMK2A | 0.206164 | 0.028214 |
| ENSG00000177398 | UMODL1 | 0.357873 | 7.12E-09 |
| ENSG00000104081 | BMF | 0.412629 | 0.010613 |

| | | | |
|---|---|---|---|
| ENSG00000157613 | CREB3L1 | 0.435743 | 5.11E-07 |
| ENSG00000009709 | PAX7 | 0.478992 | 0.028605 |
| ENSG00000121858 | TNFSF10 | 0.541056 | 0.000164 |
| ENSG00000109670 | FBXW7 | 0.54639 | 9.84E-05 |
| ENSG00000124762 | CDKN1A | 0.549035 | 0.000263 |
| ENSG00000035664 | DAPK2 | 0.580864 | 0.001622 |

**Table 2E:** GO biological pathways associated with MFM-223 DHT up-regulated genes

| Ensembl gene ID | Gene symbol | FC | p-value |
|---|---|---|---|
| Regulation of transmembrane or ion transport | | | |
| ENSG00000139209 | SLC38A4 | 11.30529 | 0.009143 |
| ENSG00000163380 | LMOD3 | 6.209539 | 8.88E-16 |
| ENSG00000080618 | CPB2 | 6.096618 | 0.016526 |
| ENSG00000018625 | ATP1A2 | 5.344711 | 5.59E-10 |
| ENSG00000147257 | GPC3 | 4.855859 | 0.00779 |
| ENSG00000125257 | ABCC4 | 4.79848 | 9.71E-14 |
| ENSG00000184408 | KCND2 | 4.382823 | 0.010199 |
| ENSG00000166828 | SCNN1G | 4.154343 | 0.001087 |
| ENSG00000143473 | KCNH1 | 3.97686 | 0.014758 |
| ENSG00000149295 | DRD2 | 3.265144 | 0 |
| ENSG00000082482 | KCNK2 | 3.167954 | 0.020869 |
| ENSG00000138741 | TRPC3 | 3.103477 | 0.003382 |
| ENSG00000143318 | CASQ1 | 2.987924 | 1.41E-14 |
| ENSG00000123643 | SLC36A1 | 2.504679 | 2.12E-10 |
| ENSG00000132681 | ATP1A4 | 2.456964 | 5.60E-10 |
| ENSG00000144285 | SCN1A | 2.410777 | 1.61E-05 |
| ENSG00000173210 | ABLIM3 | 2.262304 | 0.046962 |
| ENSG00000106688 | SLC1A1 | 1.907325 | 5.45E-09 |
| ENSG00000112041 | TULP1 | 1.886675 | 0.045893 |
| ENSG00000026559 | KCNG1 | 1.886335 | 4.70E-10 |
| ENSG00000084628 | NKAIN1 | 1.834272 | 0.013169 |
| ENSG00000116396 | KCNC4 | 1.828824 | 0.003081 |
| ENSG00000168214 | RBPJ | 1.721868 | 7.54E-08 |
| ENSG00000126016 | AMOT | 1.686845 | 0.002559 |
| ENSG00000154309 | DISP1 | 1.663872 | 0.001331 |
| ENSG00000149177 | PTPRJ | 1.612114 | 1.39E-08 |
| ENSG00000187486 | KCNJ11 | 1.609128 | 1.94E-07 |
| ENSG00000136040 | PLXNC1 | 1.601103 | 0.00942 |
| ENSG00000182324 | KCNJ14 | 1.589181 | 0.03406 |
| ENSG00000165548 | TMEM63C | 1.582025 | 0.000622 |
| ENSG00000123607 | TTC21B | 1.556649 | 2.61E-07 |
| ENSG00000117586 | TNFSF4 | 1.537133 | 0.007957 |

| | | | |
|---|---|---|---|
| ENSG00000144136 | SLC20A1 | 1.530727 | 3.80E-07 |
| Cell death | | | |
| ENSG00000109906 | ZBTB16 | 29.2781610 | 6.4662908E-21 |
| ENSG00000144749 | LRIG1 | 6.9595 | 0 |
| ENSG00000116741 | RGS2 | 6.524396 | 0 |
| ENSG00000164120 | HPGD | 5.950249 | 0 |
| ENSG00000167034 | NKX3-1 | 2.798494 | 7.22E-15 |
| ENSG00000118503 | TNFAIP3 | 2.447703 | 1.85E-06 |
| ENSG00000162734 | PEA15 | 2.323361 | 0 |
| ENSG00000176720 | BOK | 2.258723 | 0.014772 |
| ENSG00000146674 | IGFBP3 | 2.211972 | 3.91E-08 |
| ENSG00000064393 | HIPK2 | 1.797362 | 0.001531 |
| ENSG00000140538 | NTRK3 | 1.771558 | 0.012716 |
| ENSG00000135116 | HRK | 1.641136 | 0.023305 |
| ENSG00000130222 | GADD45G | 1.585375 | 0.033787 |
| ENSG00000168209 | DDIT4 | 1.522963 | 2.03E-07 |
| ENSG00000124664 | SPDEF | 1.507525 | 4.67E-07 |
| Response to hormones | | | |
| ENSG00000131482 | G6PC | Inf | 0.001605 |
| ENSG00000070886 | EPHA8 | 4.352906 | 0.006751 |
| ENSG00000166147 | FBN1 | 2.827927 | 2.33E-14 |
| ENSG00000124225 | PMEPA1 | 2.734252 | 0 |
| ENSG00000153162 | BMP6 | 2.409597 | 0.001848 |
| ENSG00000112293 | GPLD1 | 2.1688 | 9.69E-05 |
| ENSG00000127955 | GNAI1 | 2.164146 | 2.54E-05 |
| ENSG00000084207 | GSTP1 | 2.078986 | 0.049464 |
| ENSG00000125820 | NKX2-2 | 1.872614 | 0.011729 |
| ENSG00000140538 | NTRK3 | 1.771558 | 0.012716 |
| ENSG00000149212 | SESN3 | 1.732872 | 2.71E-05 |
| ENSG00000126368 | NR1D1 | 1.613607 | 0.007678 |
| ENSG00000160999 | SH2B2 | 1.608908 | 0.020263 |
| ENSG00000119138 | KLF9 | 1.553361 | 1.69E-05 |
| ENSG00000171345 | KRT19 | 1.511443 | 4.78E-06 |
| Adhesion | | | |
| ENSG00000120332 | TNN | Inf | 0.033456 |
| ENSG00000117090 | SLAMF1 | Inf | 0.002694 |
| ENSG00000182636 | NDN | Inf | 0.019755 |
| ENSG00000179399 | GPC5 | Inf | 0.032426 |
| ENSG00000248383 | PCDHAC1 | Inf | 0.029648 |
| ENSG00000103647 | CORO2B | 7.538193 | 0.000599 |
| ENSG00000128218 | VPREB3 | 2.898465 | 0.027351 |
| ENSG00000173391 | OLR1 | 2.893267 | 1.81E-09 |
| ENSG00000075618 | FSCN1 | 2.732786 | 1.31E-06 |
| ENSG00000117155 | SSX2IP | 2.519799 | 0 |
| ENSG00000163638 | ADAMTS9 | 2.231762 | 0.026749 |
| ENSG00000137809 | ITGA11 | 2.153337 | 0.008607 |

| | | | |
|---|---|---|---|
| ENSG00000149294 | NCAM1 | 2.140775 | 5.35E-06 |
| ENSG00000124721 | DNAH8 | 2.114258 | 0.000948 |
| ENSG00000170873 | MTSS1 | 1.91149 | 4.00E-15 |
| ENSG00000023902 | PLEKHO1 | 1.897235 | 4.15E-08 |
| ENSG00000162729 | IGSF8 | 1.769201 | 3.44E-09 |
| ENSG00000133121 | STARD13 | 1.638316 | 4.05E-05 |
| ENSG00000106852 | LHX6 | 1.535011 | 0.000322 |
| ENSG00000162849 | KIF26B | 1.515107 | 0.00038 |
| ENSG00000053747 | LAMA3 | 1.501032 | 0.046309 |

**Table 2F:** GO biological processes of DHT down-regulated genes in MFM-223 cells

| Ensembl gene ID | Gene symbol | FC | p-value |
|---|---|---|---|
| Homeostatic process | | | |
| ENSG00000160883 | HK3 | 0.158131 | 0.042946 |
| ENSG00000163464 | CXCR1 | 0.254358 | 1.79E-07 |
| ENSG00000118729 | CASQ2 | 0.273788 | 0.003166 |
| ENSG00000105929 | ATP6V0A4 | 0.323451 | 0.020668 |
| ENSG00000104321 | TRPA1 | 0.359585 | 5.06E-08 |
| ENSG00000185745 | IFIT1 | 0.372191 | 0.003263 |
| ENSG00000149596 | JPH2 | 0.416853 | 0.007092 |
| ENSG00000158055 | GRHL3 | 0.455516 | 3.08E-10 |
| ENSG00000165029 | ABCA1 | 0.497136 | 0.007483 |
| ENSG00000143153 | ATP1B1 | 0.5163 | 6.56E-13 |
| ENSG00000118432 | CNR1 | 0.521622 | 1.71E-05 |
| ENSG00000050628 | PTGER3 | 0.528846 | 2.23E-10 |
| ENSG00000023171 | GRAMD1B | 0.552507 | 0.014104 |
| ENSG00000171992 | SYNPO | 0.554048 | 0.026481 |
| ENSG00000116701 | NCF2 | 0.55722 | 0.000106 |
| ENSG00000134489 | HRH4 | 0.559335 | 0.038385 |
| ENSG00000160183 | TMPRSS3 | 0.572814 | 0.004362 |
| Development | | | |
| ENSG00000170819 | BFSP2 | 0 | 0.019912 |
| ENSG00000171431 | KRT20 | 0.065582 | 0.011373 |
| ENSG00000159307 | SCUBE1 | 0.115028 | 0.002501 |
| ENSG00000139445 | FOXN4 | 0.177594 | 0.000374 |
| ENSG00000164093 | PITX2 | 0.203694 | 0.018669 |
| ENSG00000171587 | DSCAM | 0.271705 | 0.013973 |
| ENSG00000179520 | SLC17A8 | 0.29233 | 0.011191 |
| ENSG00000110876 | SELPLG | 0.294518 | 0.046485 |
| ENSG00000101265 | RASSF2 | 0.335381 | 5.63E-13 |
| ENSG00000162891 | IL20 | 0.361448 | 0.010907 |
| ENSG00000085276 | MECOM | 0.380544 | 9.42E-11 |

| | | | |
|---|---|---|---|
| ENSG00000163207 | IVL | 0.387175 | 0.01111 |
| ENSG00000006611 | USH1C | 0.393003 | 0.000862 |
| ENSG00000075223 | SEMA3C | 0.420552 | 1.88E-10 |
| ENSG00000113262 | GRM6 | 0.424882 | 0.041047 |
| ENSG00000167601 | AXL | 0.4513 | 0.034799 |
| ENSG00000181291 | TMEM132E | 0.453828 | 0.039378 |
| ENSG00000095585 | BLNK | 0.477283 | 2.00E-15 |
| ENSG00000171243 | SOSTDC1 | 0.489361 | 0.003318 |
| ENSG00000089692 | LAG3 | 0.496164 | 0.037682 |
| ENSG00000134256 | CD101 | 0.499421 | 0.002275 |
| ENSG00000083857 | FAT1 | 0.502946 | 0.013436 |
| ENSG00000144821 | MYH15 | 0.512367 | 0.026756 |
| ENSG00000166527 | CLEC4D | 0.55608 | 0.026184 |
| ENSG00000125730 | C3 | 0.569549 | 0.001001 |
| ENSG00000176788 | BASP1 | 0.58235 | 2.58E-08 |
| ENSG00000150893 | FREM2 | 0.59069 | 0.015254 |
| Proliferation | | | |
| ENSG00000137440 | FGFBP1 | 0 | 0.008424 |
| ENSG00000206557 | TRIM71 | 0 | 0.024306 |
| ENSG00000139292 | LGR5 | 0.0316 | 0.000252 |
| ENSG00000064300 | NGFR | 0.066346 | 0.034876 |
| ENSG00000122641 | INHBA | 0.368664 | 0.02877 |
| ENSG00000182533 | CAV3 | 0.372131 | 0.026081 |
| ENSG00000064989 | CALCRL | 0.375284 | 0.000798 |
| ENSG00000163762 | TM4SF18 | 0.387838 | 8.24E-07 |
| ENSG00000106278 | PTPRZ1 | 0.423705 | 0.008775 |
| ENSG00000110492 | MDK | 0.477215 | 0.027137 |
| ENSG00000166333 | ILK | 0.480452 | 0.009005 |
| ENSG00000118523 | CTGF | 0.558853 | 8.66E-08 |
| ENSG00000171791 | BCL2 | 0.566371 | 2.47E-10 |

**Table 2G:** Statistics of AR ChIP-seq analysis.

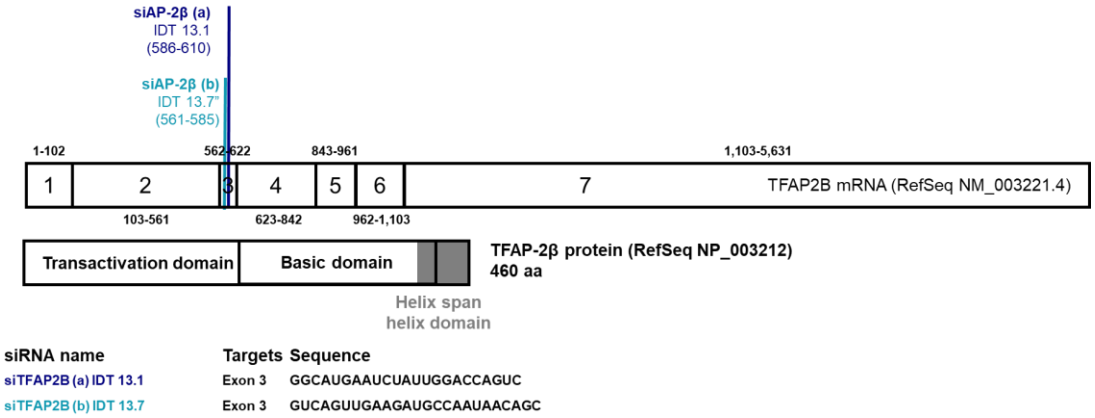
| Cell line | Treatment | Replicate | Trimmed reads | Uniquely mapped reads | Un/Multi mapped reads | Unique aligned rate (%) | AR Peaks |
|---|---|---|---|---|---|---|---|
| **MDA-MB-453** | **Vehicle (EtOH)** | 1 | 24,670,557 | 18,525,351 | 6,145,206 | 75 | 67 |
| | | 2 | 22,508,937 | 16,886,198 | 5,622,739 | 75 | 33 |
| | | 3 | 23,066,473 | 17,251,151 | 5,815,322 | 74 | 20 |
| | **DHT (10nM)** | 1 | 23,173,996 | 17,879,708 | 5,294,288 | 77 | 15,612 |
| | | 2 | 22,949,494 | 18,145,749 | 4,803,745 | 79 | 17,942 |
| | | 3 | 23,629,638 | 18,255,961 | 5,373,677 | 77 | 26,162 |
| | **Pooled input** | | 15,748,748 | 12,288,971 | 3,459,777 | 78 | |
| **MFM-223** | **Vehicle (EtOH)** | 1 | 22,245,909 | 16,681,820 | 5,564,089 | 75 | 159 |
| | | 2 | 21,741,731 | 16,341,733 | 5,399,998 | 75 | 113 |
| | | 3 | 22,510,571 | 16,924,370 | 5,586,201 | 75 | 418 |
| | **DHT (10nM)** | 1 | 19,893,313 | 15,258,535 | 4,634,778 | 76 | 8,951 |
| | | 2 | 21,718,681 | 16,621,578 | 5,097,103 | 76 | 15,512 |
| | | 3 | 23,066,473 | 17,251,151 | 5,815,323 | 74 | 20,224 |
| | **Pooled input** | | 21,129,147 | 15,932,892 | 5,196,255 | 75 | |

# APPENDIX (3)

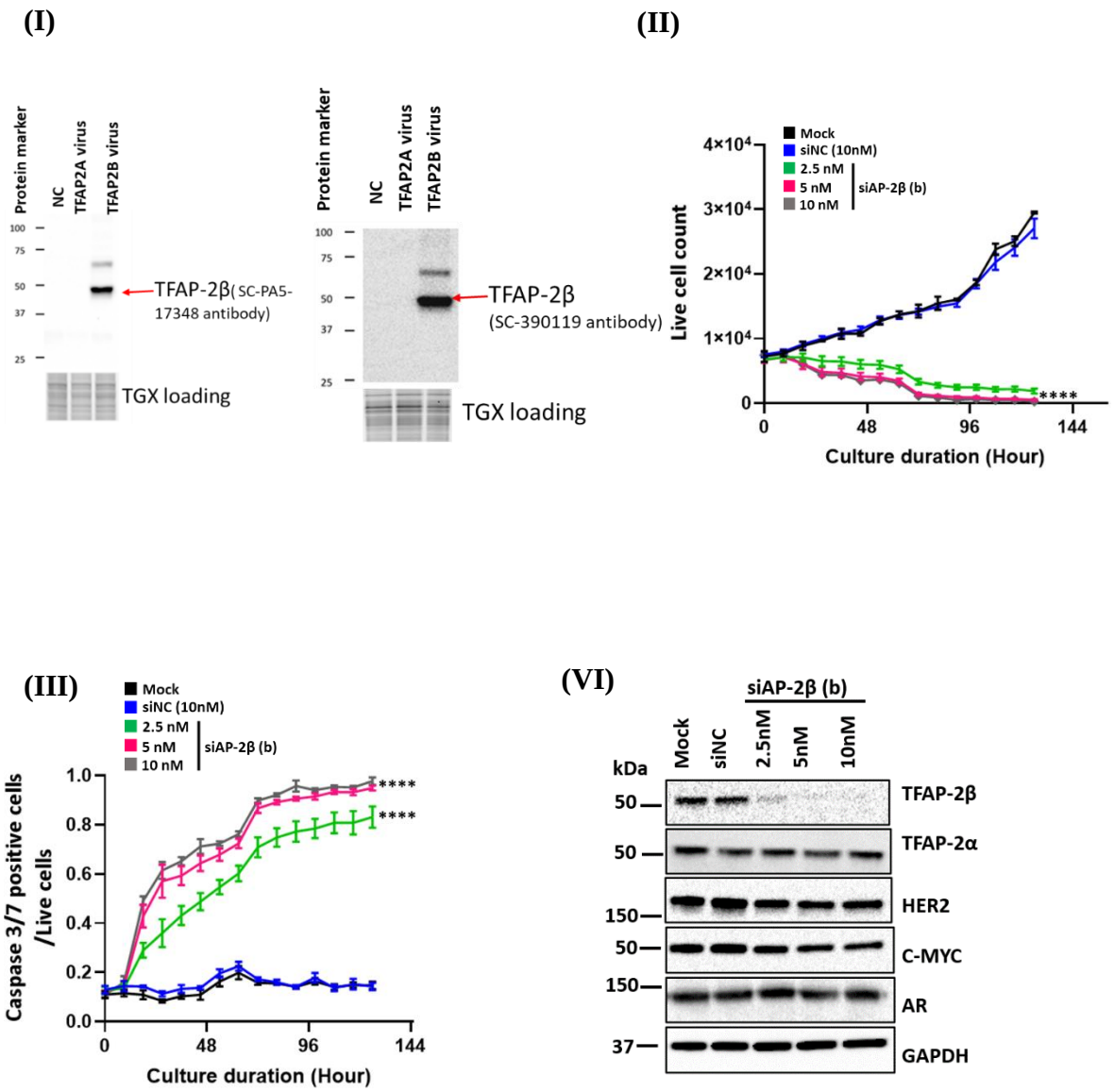
# Incucyte analysis parameters and supplementary data of TFAP-2β study

**Table 3A:** Parameters of Incucyte basic analyser image analysis.

| Cell line | Target | Exposure | Channel | Segmentation | Edge sensitivity | Min. Area ($\mu m^2$) | Max. Area ($\mu m^2$) |
|---|---|---|---|---|---|---|---|
| D-11 proliferation effect in TNBC cells | | | | | | | |
| MDA-MB-453 | NucLight Rapid Red | 400 | Red | 0.2 | 0 | 50 | - |
| | Caspase 3/7 | 300 | Green | 2.5 | -20 | 20 | 1000 |
| MDA-MB-468 | NucLight Rapid Red | 400 | Red | 0.2 | -30 | 50 | 3000 |
| | Caspase 3/7 | 300 | Green | 1 | -40 | 10 | - |
| MFM-223 | NucLight Rapid Red | 400 | Red | 0.2 | -10 | 20 | - |
| | Caspase 3/7 | 300 | Green | 0.5 | -30 | 20 | - |
| MDA-MB-231 | NucLight Rapid Red | 400 | Red | 0.3 | -20 | 10 | 1500 |
| | Caspase 3/7 | 300 | Green | 0.6 | -45 | 20 | 800 |
| siAP-2β (a) proliferation assay | | | | | | | |
| MDA-MB-453 | mKate2 | 400 | Red | 0.5 | -20 | 100 | 1000 |
| | Caspase 3/7 | 300 | Green | 5 | -60 | 50 | 1000 |
| siAP-2β (b) proliferation assay | | | | | | | |
| MDA-MB-453 | mKate2 | 400 | Red | 2 | 5 | 150 | 1000 |
| | Caspase 3/7 | 300 | Green | 5 | 30 | 150 | 1000 |




**Appendix 3B:** Relative location of siAP-2β target sites and sequences**.**




**Appendix 3C:** Confirm the anti-cancer effects of silencing TFAP-β using a second siAP-2β. **(I)** Immunoblotting of MDA-MB-231 cells transfected with TFAP2A, or TFAP2B to test specificity of TFAP-2β antibodies. NC represents non-transfected MDA-MB-231 cells. **(II)** Western blot showing TFAP-2β protein expression in MDA-MB-453 and MFM-223 cells. (**III**) Growth curve showing a dose-dependent inhibition of MDA-MB-453 cell proliferation with siAP-2β (b). **(IV)** Death curve to accompany results in (A), showing TFAP-2β(b) knockdown increases Caspase 3/7-positive cells (as a dead to live cells

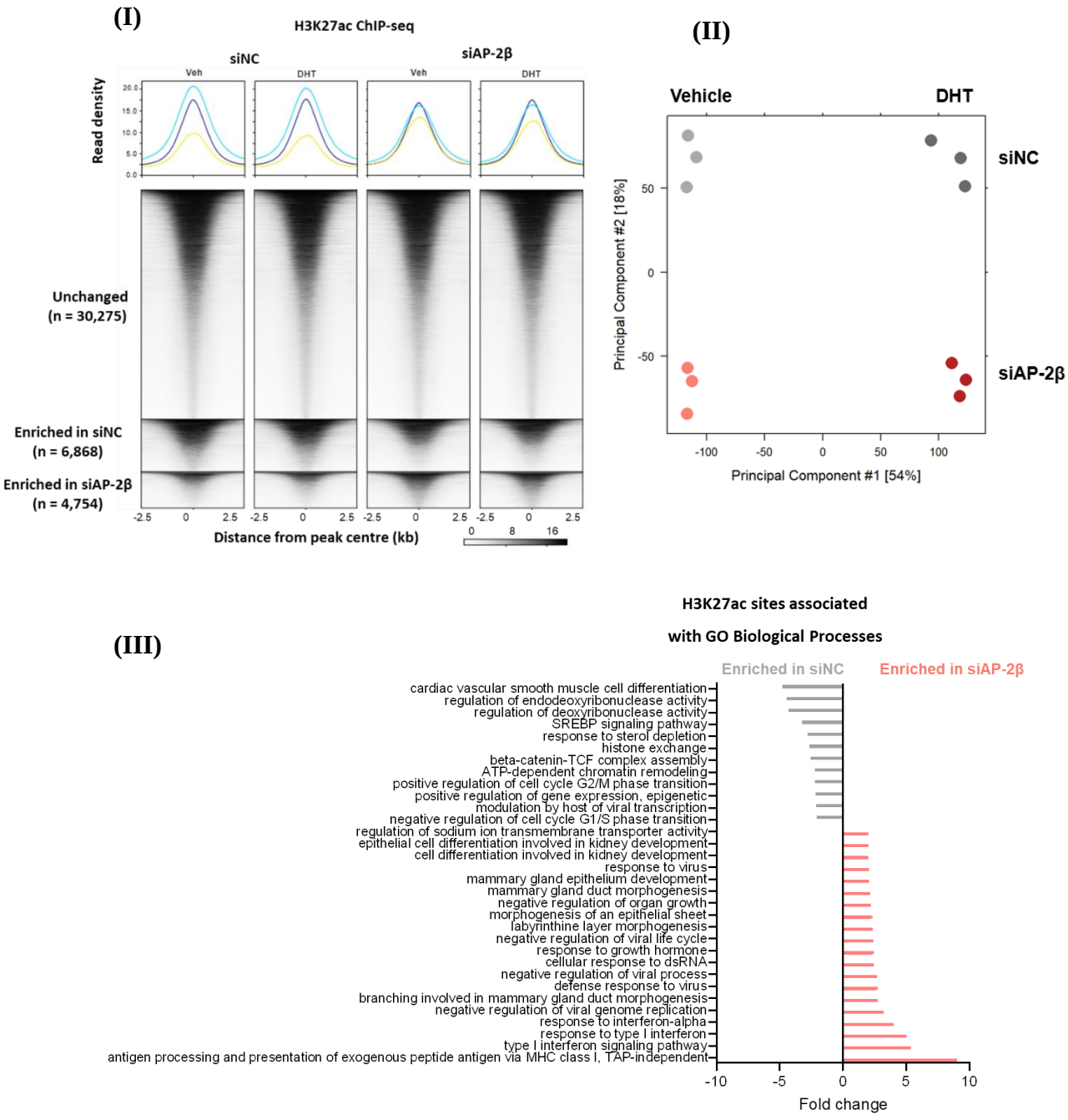
ratio) in a dose-dependent manner. All data represented as mean ± SEM; *p<0.05; **p<0.01; ***p<0.001; ****p<0.000, versus siNC (One Way ANOVA). **(V)** Immunoblotting showing decrease in C-MYC and HER2 expression in MDA-MB-453 cells treated with siAP-2β for 32hr.

**Table 3D**: High throughput sequencing of TFAP-2β ChIP.

| Treatment | Replicate | Trimmed reads | Uniquely mapped reads | Un/Multi mapped reads | Unique aligned rate (%) | TFAP-2β peaks |
|---|---|---|---|---|---|---|
| **Vehicle (EtOH)** | 1 | 27,633,239 | 21,001,033 | 6,632,206 | 76.0 | 26,084 |
| | 2 | 28,414,833 | 21,628,889 | 6,785,944 | 76.1 | 32,931 |
| | 3 | 29,435,241 | 22,356,807 | 7,078,434 | 76.0 | 26,612 |
| **DHT (10nM)** | 1 | 27,195,060 | 20,571,210 | 6,623,850 | 75.6 | 19,302 |
| | 2 | 25,861,959 | 19,683,755 | 6,178,204 | 76.1 | 26,399 |
| | 3 | 27,674,199 | 20,955,863 | 6,718,336 | 75.7 | 24,671 |
| **Pooled input** | | 28,865,500 | 21,783,262 | 7,082,238 | 75.5 | |

**Table 3E** High throughput sequencing of H3K27ac ChIP.

| Knockdown | Treatment | Replicate | Trimmed reads | Uniquely mapped reads | Un/Multi mapped reads | Unique aligned rate (%) | H3K27ac peaks |
|---|---|---|---|---|---|---|---|
| **siNC (5nM)** | **Vehicle (EtOH)** | 1 | 26,932,749 | 21,647,255 | 5,285,494 | 80.38 | 40,556 |
| | | 2 | 27,295,875 | 21,929,687 | 5,366,188 | 80.34 | 41,696 |
| | | 3 | 24,955,658 | 20,234,035 | 4,721,623 | 81.08 | 44,044 |
| | **DHT (10nM)** | 1 | 27,522,924 | 22,047,186 | 5,475,738 | 80.10 | 41,198 |
| | | 2 | 24,893,260 | 20,160,565 | 4,732,695 | 80.99 | 43,857 |
| | | 3 | 27,783,473 | 22,389,409 | 5,394,064 | 80.59 | 42,230 |
| **siAP-2β (5nM)** | **Vehicle (EtOH)** | 1 | 27,047,591 | 21,546,474 | 5,501,117 | 79.66 | 39,802 |
| | | 2 | 27,356,953 | 22,194,577 | 5,162,376 | 81.13 | 45,366 |
| | | 3 | 29,112,102 | 23,323,630 | 5,788,472 | 80.12 | 42,353 |
| | **DHT (10nM)** | 1 | 27,849,232 | 22,325,523 | 5,525,602 | 80.17 | 41,403 |
| | | 2 | 29,137,762 | 23,618,178 | 5,519,584 | 81.06 | 45,638 |
| | | 3 | 26,327,941 | 21,071,676 | 5,256,265 | 80.04 | 40,906 |
| **Pooled input** | | | 30,713,468 | 23,128,702 | 7,584,766 | 75.30 | |

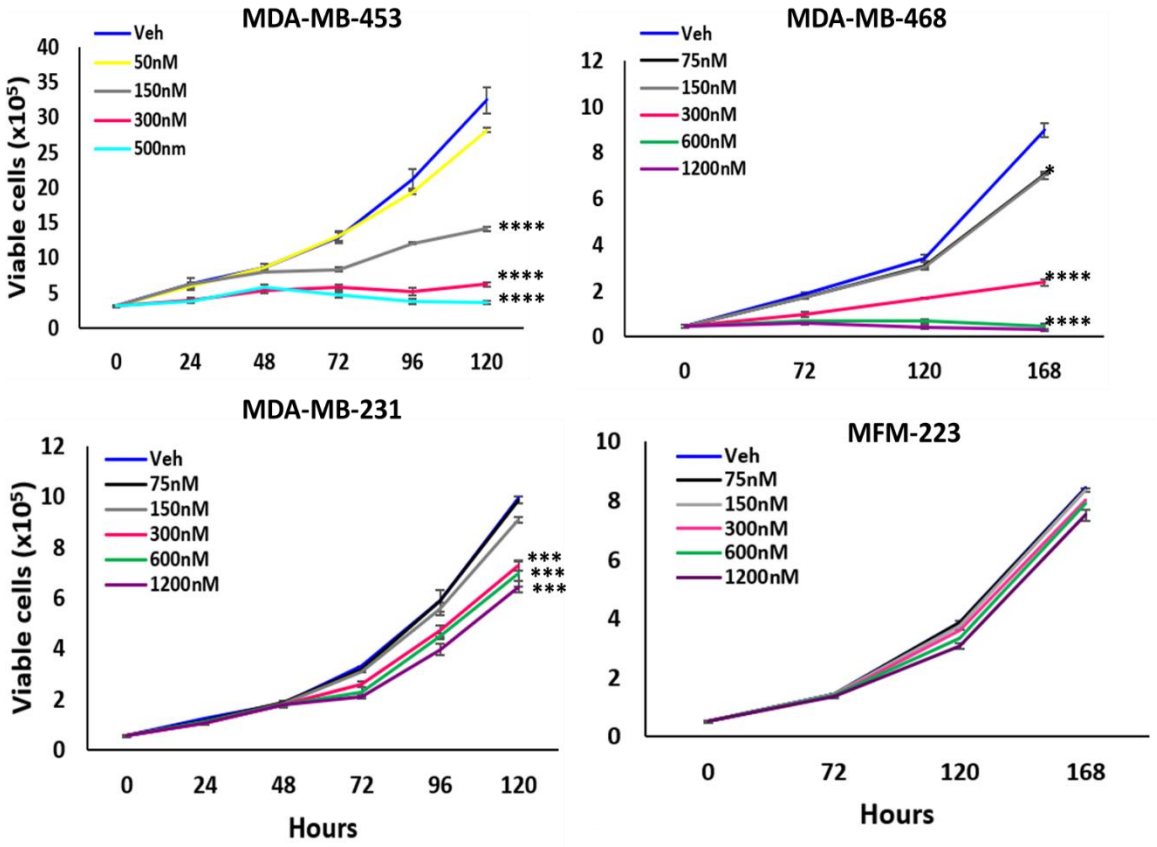



**Appendix 3F:** The effect of TFAP-2β knockdown on H3K27ac cistrome in MDA-MB-453 cells. (I) Heatmaps and corresponding read density plots showing consensus ChIP-seq signals and the number of unchanged, siNC enriched and siAP-2β enriched peaks in MDA-MB-453 cells treated with 5nM siAP-2β for 48 hr and then treated with DHT for 4hr. (II) PCA plot displaying differences and similarities in H3K27ac cistromes between replicate treatment

samples and treatment conditions in MDA-MB-453 cells. (III) Differentially enriched H3K27ac binding sites were analysed using GREAT to determine their associations with GO Biological Processes.

# APPENDIX (4)

## Effect of L-453 compound on proliferation of TNBC cell lines




**Appendix 4:** Proliferation of TNBC cells measured by live cell count at varying concentrations of L-453. Data represents mean ± SEM of three replicate cell culture wells per condition and is a representative of two independent experiments. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (One Way ANOVA).