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Integrative analysis of the SRC-1 transcriptional networks promoting endocrine resistance in breast cancer

Alacoque L. Browne

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3-6-2018

Integrative analysis of the SRC-1 transcriptional networks promoting endocrine resistance in breast cancer.

Alacoque Browne
Integrative analysis of the SRC-1-regulated transcriptional networks promoting endocrine resistance in breast cancer

Alacoque L. Browne B.Sc. (Hons), M.Sc.

A thesis presented to the Royal College of Surgeons in Ireland, St. Stephens Green,

Dublin 2

Submitted for Doctor of Philosophy

Endocrine Oncology Research Group
Royal College of Surgeons in Ireland

Supervisor: Assoc. Professor Leonie Young
Head of Department: Professor Arnold Hill

February 2018
Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Doctor of Philosophy is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed _____________________________________________________

Student Number ______________________________________________

Date _______________________________________________________


Acknowledgements

I am very thankful to Assoc. Prof. Leonie Young for giving me the opportunity to complete this PhD.

To everyone in the lab, you have all been so helpful and kind throughout my time here and I am so grateful for all the science ramblings and laughs that we have had. If I write personalised thank-you’s to you all here, it would be longer than the thesis proper so I best keep it short and sweet. To Fiona, Sinead, Marie, Laura, Jean, Ailis, Christopher, Damir, Brendan, Sara, Ricardo, Elspeth, Ben, Stuart, Azlena, Azie, Rachel, Karen, Siobhan, Nicola and Preeti - you all made this PhD a much more enjoyable experience. You are the reason I look fondly upon my time here. In particular, thanks to Elspeth and Nicola, for the Guinness and Taytos that were often needed to take our minds off the PhD. To Brendan and Sara, thank you for all your help across this final hurdle.

To all of my friends, in particular, Aisling, for your continued friendship despite my hermit PhD life.

To my amazing family, Bernadette, Adrian, Anne Marie and Gerard (GB), for all the support, love and encouragement you have given me over the past four years, I wouldn’t have completed the PhD without you.

To Stefan, for always putting a smile on my face.
Dedication

This thesis is dedicated to my parents, Adrian and Bernadette, for their boundless support, encouragement and love.
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<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>AD</td>
<td>Activation domain</td>
</tr>
<tr>
<td>AF</td>
<td>Activation function</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>AMBIC</td>
<td>Ammonium bicarbonate</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Tissue Type Collection</td>
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<tr>
<td>BCA</td>
<td>Bicinchinic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDS</td>
<td>Charcoal dextrane stripped</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating tumour cell</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>E₁</td>
<td>Oestrone</td>
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<td>E₂</td>
<td>Oestradiol</td>
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<td>E₃</td>
<td>Oestriol</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
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<td>ER</td>
<td>Oestrogen receptor</td>
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<tr>
<td>ERE</td>
<td>Oestrogen response element</td>
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<td>ESR1</td>
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</tr>
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<td>ESR2</td>
<td>Oestrogen receptor 2</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>GFR</td>
<td>Growth factor receptor</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophin-releasing hormone</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase activity</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>HER</td>
<td>Human epidermal growth factor receptor</td>
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<tr>
<td>ICI</td>
<td>Imperial Chemical Industries</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>LB</td>
<td>Lysis buffer</td>
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<td>LBD</td>
<td>Ligand binding domain</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essentials medium</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal to epithelial transition</td>
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<tr>
<td>METABRIC</td>
<td>Molecular Taxonomy of Breast Cancer International Consortium</td>
</tr>
<tr>
<td>MFE</td>
<td>Mammosphere forming efficiency</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinases</td>
</tr>
<tr>
<td>MMTV-PyMT</td>
<td>Mouse mammary tumour virus-polyoma middle T</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>Experimental number</td>
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<tr>
<td>NCOA</td>
<td>Nuclear receptor coactivator</td>
</tr>
<tr>
<td>NCRI</td>
<td>National Cancer Registry of Ireland</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>NRID</td>
<td>Nuclear receptor interacting domain</td>
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<tr>
<td>p</td>
<td>p-value</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient derived xenograft</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PhI</td>
<td>Phosphatase inhibitor</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Pol</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PRF</td>
<td>Phenol red free</td>
</tr>
<tr>
<td>PWM</td>
<td>Position weight matrix</td>
</tr>
<tr>
<td>qPCR</td>
<td>semi-quantitative PCR</td>
</tr>
<tr>
<td>RIME</td>
<td>Rapid immunoprecipitation of mass endogenous proteins</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective ER down-regulators</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective ER modulators</td>
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<tr>
<td>SRC</td>
<td>Steroid receptor coactivator</td>
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<tr>
<td>TBS</td>
<td>Tris buffered sulphate</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
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<tr>
<td>TN</td>
<td>Triple negative</td>
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<tr>
<td>TSS</td>
<td>Transcriptional start site</td>
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Summary

Tamoxifen has been the gold standard treatment of oestrogen receptor positive breast cancer for over 40 years. While its use has significantly improved the outlook for breast cancer patients, up to 40% of patients relapse while on therapy. Steroid receptor co-activator 1 (SRC-1) is a master regulatory protein which is over-expressed in breast cancer. It is associated with high grade tumours, disease recurrence and is an independent predictor of poor disease-free survival. SRC-1 interacts with nuclear receptors and other transcription factors to initiate transcriptional networks and regulate downstream genes which enable the cell to evade therapy. Here, a top-down approach to map out the transcriptional network, regulated by SRC-1 in endocrine resistant breast cancer was taken. RNA-sequencing was performed to identify the transcriptional targets of SRC-1. Molecular characterization identified E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 as SRC-1-regulated transcription factors/chromatin remodellers. Rapid immunoprecipitation of mass endogenous proteins was employed to uncover SRC-1 interacting partners. STAT1 was confirmed as an SRC-1-interacting transcription factor in endocrine resistant breast cancer. Extended analysis of the SRC-1 effector target gene network revealed genes important in activating cell cycle, proliferation and pathways in cancer. Concerted activity of the SRC-1-mediated network is responsible for driving the highly migratory and proliferative phenotypes of endocrine resistant breast cancer. Moreover, they play a significant role in regulating the undifferentiated tumour population. Upon clinical investigation, high expression of this SRC-1-regulated network is predictive of poor disease-free survival in a tamoxifen-treated patient population. This study provides important insight into a SRC-1-STAT1 complex initiating a transcriptional cascade and regulating key genes involved in endocrine resistance.
1. Introduction
1.1. Cancer

Cancer is the second leading cause of global morbidity and mortality [1]. It is responsible for 1 in 6 deaths worldwide and its occurrence is estimated to increase by 70% in the next two decades [1]. Cancer is a general term for an extensive group of diseases that may affect any part of the body [2]. Despite the heterogeneity of all cancers, they have a common defining feature; the uncontrolled proliferation and inappropriate survival of defective cells [3]. Oncogenic transformation is manifested by many genetic alterations that contribute to the conversion of a normal cell into its malignant derivative [4]. Several additional features have been described by Hanahan and Weinberg as the hallmarks of cancer; ten biological capabilities and enabling characteristics attained by the cell in its conversion from healthy to malignant [5]. These hallmarks include (Figure 1-1):

- Sustained proliferative signalling
- Evading growth suppressors
- Avoiding immune destruction
- Enabling replicative immortality
- Tumour-promoting inflammation
- Activating invasion and metastasis
- Inducing angiogenesis
- Genome instability and mutation
- Resisting cell death
- Deregulating cellular energetics

While these hallmarks represent the common cellular derailments in cancer, they do not explain why such alterations occur in the first place. This was addressed in a notable publication by Tomasetti and Vogelstein providing insight into the origin of cancer. They proposed that the variation of cancer risk in different tissues is largely attributable to their varying rates of inherent stem-cell activity, which normally maintain tissue homeostasis [6]. They identified an almost linear correlation between the number of stem cell divisions in a tissue and lifetime risk of developing cancer. Their study further suggests that only one third of cancers arise from inherited predispositions or environmental factors. Instead the majority of cancers are due to ‘bad luck’ with acquired mutations during the replication of DNA in healthy stem cells.
The biological capabilities, proposed by Hanahan and Weinberg as the hallmarks of cancer, that are common to all cancers. Image modified from Hanahan and Weinberg, 2011 [5].

1.2. Breast cancer

1.2.1. Incidence and risk factors

Breast cancer is the most common invasive cancer diagnosed in women. It occurs in 1 in 10 Irish women and ranks 2nd as a cause of invasive cancer-related mortality [7]. Annually, there are an estimated 3,000 newly diagnosed cases and almost 700 deaths [7]. The incidence of breast cancer is increasing, yet despite this, the mortality rate has been reduced due to heightened awareness, effective screening and advancements in treatment [8]. While there is no means to predict the development of sporadic breast cancer, considerable epidemiological and etiological studies have established risk factors. Besides older age, various reproductive factors such as early menarche, late menopause, nuliparity, late age at first pregnancy, and short duration of life breast-feeding, all increase the risk associated with developing breast cancer [9-13]. Moreover, lifestyle-associated risk factors for the development of breast cancer include excess weight, alcohol consumption, smoking and exogenous hormones (oral contraceptive pill / hormone replacement therapy) [14-18]. Among the strongest risk factors are dense breast tissue, lobular carcinoma in situ and atypical hyperplasia, previous breast cancer, family history and inherited genetic mutations.
Gene mutations conferring proliferative advantage in a tumour are described as ‘driver’ mutations [20]. Inherited germ-line mutations in the tumour suppressor genes BRCA1/2 increase the risk of developing breast cancer by 60 - 85% [21]. Moreover, there are rare, cancer pre-disposing syndromes that associate with a moderate to high risk of developing breast cancer. These encompass syndromes caused by gene mutations in tumour protein 53 (TP53- Li-Fraumeni syndrome), phosphatase and tensin homolog gene (PTEN – Cowman syndrome) and cadherin 1 (CDH1 - familial lobular breast cancer) [22]. Many driver mutations are suspected to be acquired in somatic cells over a patient’s lifetime. For example, gene mutations in DNA repair proteins such as CHEK2, BRIP1, ATM and PALB2 further carry moderate risk of development of breast cancer [23]. Genome-wide association studies (GWAS) have significantly advanced our knowledge of genetic susceptibility in breast cancer. GWAS on over 120,000 women identified 15 novel low risk genes. Inclusion of these new risk loci in polygenic risk scores may allow the identification of individuals at low or high risk, thereby improving breast cancer screening and prevention [24]. Whole genome sequencing of breast cancer samples revealed the 10 most commonly mutated genes in breast cancer to be TP53, PIK3CA, MYC, CCND1, PTEN, ERBB2, ZNF703/FGFR1 locus, GATA3, RB1 and MAP3K1 [20]. Mutation of these 10 genes accounts for 62 % of drivers in breast cancer. Identification of these drivers provides a comprehensive understanding of both the source and the consequences of somatic mutations in breast cancer [20]. Interestingly, interactions between genetic and environmental risk factors have been shown to further increase risk of breast cancer; pointing towards alterations in the epi-genome [25-27].

1.2.2. Classification and subtypes

Breast cancer is mainly classified by clinic-pathological features; histological subtype, hormone receptor and human epidermal growth factor 2 (HER2) status, tumour grade, stage and nodal involvement [28]. However, over fifteen years ago, our understanding of breast cancer was revolutionised with the publication of data by Perou et al., which defined the first molecular classification system for breast cancer [29]. This gene expression profiling defined four intrinsic breast cancer subtypes.
Pursuant to this, additional gene expression studies established the 7 principal subclasses of breast cancer recognised today [30-32]. These subtypes have been designated luminal A, luminal B, HER2-enriched, claudin-low, basal-like, triple-negative (TN), and normal breast-like. In a more recent study by the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC), 10 intrinsic molecular breast cancer subtypes were defined, resulting in the most comprehensive gene classification of breast cancer to date [33].

Approximately 70% of all breast cancers are luminal in subtype [34]. Luminal A breast tumours are characterised as having a high expression of the oestrogen receptor (ER) and ER-related genes (e.g. progesterone receptor (PR)) and low expression of proliferative genes like ki67. This subtype has the best clinical prognosis in comparison to other classified subtypes [34]. Luminal B tumours have lower ER and PR, in addition to higher expression of proliferation/cell cycle genes. Increased expression of growth factor (GF) receptor genes, including HER2, means that luminal B breast cancer has a poorer prognosis than luminal A subtype [35].

Approximately 15% – 20% of breast cancer cases are HER2-enriched (HER2+) as defined by either increased HER2 gene copy number or HER2 protein over-expression. HER2+ breast cancers have increased proliferation and pro-survival signalling and as such they have a poor prognosis [34, 36].

The basal-like subgroup of breast tumours account for 15% - 20% of breast cancers and is associated with a poorer clinical outcome [37, 38]. These tumours can be further distinguished into basal, TN, claudin-low and normal-like. Both TN and basal carcinomas lack of ER, PR and HER2 expression, however, basal tumours have a distinct gene expression signature characterised by basal markers; cytokeratin 5/6, 14 or 17 and/or the expression of epidermal growth factor receptors (EGFRs) [39]. Claudin-low breast tumours represent a relatively rare subtype and are characterised by a low expression of genes involved in cellular tight junctions and adhesion. These tumours are closely linked to an undifferentiated phenotype and have a poor prognosis [31, 34]. Normal-like breast cancers are poorly characterised; they lack hormone receptor expression, exhibit features of adipose tissue and non-epithelial cells, and have an intermediate prognosis [40].
The molecular features as identified by each distinct subtype are utilised clinically to guide diagnosis, prognosis and treatment regime [41].

1.2.3. Oestrogen receptor signalling

As almost two thirds of breast cancers express ER and rely on its signalling for survival, understanding its mechanism of action is fundamental to the treatment of this disease. Decades of research have been invested in deciphering its signalling pathways. Oestrogens are a family of naturally occurring steroid hormones that exert vast physiological effects but specifically are vital for the female oestrous cycle. 17β-oestradiol (E2) is the most potent of the naturally occurring oestrogens, followed by oestrone (E1) and oestriol (E3) [42]. Oestrogens have two homologous receptors, ERα (referred to as ER from here throughout the text) and ERβ, which arise from different genes (ESR1 and ESR2). However, it is signalling via ERα that appears to be responsible for promoting tumour growth and survival [43]. ER is a nuclear receptor (NR) which is a ligand-inducible transcription factor (TF) which functions by both genomic and non-genomic signalling.

Classical genomic signalling begins with binding of oestrogen to the ER in the cytosol. This causes ER dissociation from an inhibitory complex (heat-shock proteins), homo/heterodimerisation and translocation to the nucleus [44]. Here, it binds to specific sequences of DNA called oestrogen response elements (EREs; GGTCAnnnTGACC) and recruits co-activators to exert its genomic regulatory potential [45]. Non-classical genomic ER signalling involves ER tethering to other DNA-bound TFs such as AP-1 or SP-1 to induce gene expression [46]. Non-genomic ER signalling comprises of cytoplasmic and membrane-bound ER which influence gene expression through signal transduction pathways [47]. Non-genomic signalling does not involve direct transcription of ER target genes but instead exerts its effects via various growth factor receptors (GFR) and cytoplasmic kinases. Pathways activated by non-genomic ER signalling include phosphoinositide 3-kinase (PI3K)/AKT, mitogen activated protein kinase (MAPK), protein kinase A/C (PKA/PKC) and cyclic AMP (cAMP).
Both the genomic and non-genomic ER signalling pathways can converge in complementary and even synergistic interactions. This pathway integration is facilitated through cell signal transduction pathways and ‘cross-talk’ with GFs [48, 49]. Insight into the ER mechanism of driving tumour growth continues to allow for accurate clinical intervention.

1.2.4. Treatment

Treatment options for breast cancer are decided between a multi-disciplinary clinical team and the patient. Even at the time of primary therapy, breast cancer is considered to be a systemic disease [50]. Therefore, in addition to loco-regional treatment (radiotherapy, surgery), adjuvant systemic treatment is frequently administered alongside. The overall prognosis of breast cancer is improving all the time due to advances in such adjuvant therapy. The type of adjuvant treatment applied is decided based on obligatory factors such as hormone receptor, HER2 and menopausal status [41]. Furthermore, for adjuvant chemotherapy, factors such as tumour size/grade, nodal status and age must be considered [41]. Endocrine therapy is given in patients with ≥1 % ER expression [41, 51].

1.2.4.1. Endocrine therapy

The menopausal status of the patient primarily determines the choice of endocrine therapy administered [41]. There are three classes of agents employed for the treatment of ER positive breast cancer; selective oestrogen receptor modulators (SERMs), selective oestrogen receptor down-regulators (SERDs) and aromatase inhibitors (AIs). All are targeted at reducing ER mediated tumour growth by alternate mechanisms of action [52].

1.2.4.2. SERMs

SERMs are non-steroidal oestrogen analogues classed as competitive inhibitors of oestrogen/ER binding [53]. The most prominent SERM today is tamoxifen (ICI 46474). Starting out as a failed contraceptive, the use of tamoxifen in the
treatment of breast cancer was not immediately hailed as a revolution [54]. Despite its approval for use in the U.K. and the U.S. in the early- and mid-1970’s respectively, preliminary studies found that a year of tamoxifen treatment was not effective in enhancing survival in ER+ patients compared to ER- patients [55]. While this data was later disproved, subsequent clinical investigations found that a longer duration of tamoxifen treatment proved to be a success in treating ER+ breast cancer [56].

Tamoxifen is administered orally as the prodrug, tamoxifen citrate, and undergoes extensive metabolism by cytochrome p450 enzymes in the gut and liver [57]. The activated, hydroxylated metabolites include 4-hydroxytamoxifen (4-OHT) and N-desmethyltamoxifen. These can be further converted to endoxifen, and are 100-fold more potent than the parent, tamoxifen [58]. Competitive binding of tamoxifen metabolites to the ER induce a conformational change in ER. While ER receptor dimerization and nuclear transfer still occurs, the conformational change in the ligand binding domain (LBD) occludes the co-activator binding necessary for ligand-dependent transcription of ER target genes [59]. The use of tamoxifen has decreased a woman’s 10-year risk of recurrence by approximately 50% and the risk of death by almost 30% [60]. However, as their name indicates, SERMs exert selective modulation of the oestrogen receptor; either by agonistic or antagonistic means, and tamoxifen can exert tissue selective agonist effects [61]. Often side effects and risk factors associated with these agonist properties include menopausal symptoms, an increased risk of developing endometrial cancer and thromboembolism [62]. As a result, attention has been moved to other ER-targeted agents, with different mechanisms of action lacking the aforementioned agonist properties i.e. SERDS and AIs.

1.2.4.3. SERDs

SERDs including fulvestrant (ICI 182780) are classified as pure steroidal anti-oestrogens [63]. Fulvestrant binds to the ER with an affinity 100-fold greater than tamoxifen [64]. In binding to the ER, fulvestrant blocks receptor dimerization, promotes nuclear export and degradation, thereby inhibiting ER signalling [65]. Unlike tamoxifen, the conformational change induced by fulvestrant binding, fully disrupts ER signalling and as such is almost exclusively an ER antagonist [66]. This provides an
advantage over tamoxifen as it lacks oestrogenic properties associated with increased risk of endometrial cancer. A limiting factor of fulvestrant however, is the practicality of its administration via intramuscular injection [67]. Initial studies of low-dose fulvestrant did not demonstrate any major clinical significance over other anti-oestrogens [68, 69]. However, more recent studies using increased doses of fulvestrant have proven successful [70, 71]. While combination therapy of fulvestrant with other endocrine therapies have not been impactful, its combination with targeted therapies like cyclin-dependent kinase (CDK) 4/6 inhibitors (e.g. palbociclib) or phosphatidylinositol 3-kinase (PI3K) inhibitors (e.g. pictilisib) have shown promising results [67].

1.2.4.4. AIs

AIs are classified as both steroidal and non-steroidal agents that function by inhibiting the production of circulating oestrogens by targeting the enzyme required for their conversion from androgens [72]. Steroidal AIs ( exemestane) bind covalently and irreversibly to the aromatase enzyme. In contrast, non-steroidal AIs (letrozole and anastrozole) bind non-covalently and are reversible [73]. Recent studies have shown AIs to have superior efficacy in the treatment of postmenopausal women compared to tamoxifen and therefore AIs have become the standard of care adjuvant treatment for postmenopausal breast cancer patients [74]. For AI to be effective in the treatment of premenopausal breast cancer patients, it needs to be given in combination with gonadotropin-releasing hormone (GnRH) agonists or an oophorectomy, otherwise the reduced oestrogen levels feedback to stimulate the hypothalamus–pituitary–ovary axis resulting in the production of more oestrogens [72]. As such, a recent clinical trial demonstrated significant survival benefit for AI versus tamoxifen, in combination with ovarian suppression, in a premenopausal setting [75].

1.3. Endocrine resistance in breast cancer

Tamoxifen, the first successful molecular target for breast cancer has become a cornerstone in breast cancer patient care [54]. This was followed by additional
molecular targeting agents such as SERDs and AIs. However, it is now observed that the efficacy of endocrine therapies is greatly opposed by the advent of endocrine resistance in the tumour [76]. Almost 50% of ER+ breast tumours eventually relapse and develop distant metastases despite endocrine interventions [77-79]. The majority of deaths from breast cancer are not caused by the primary tumour itself, rather they are due to tumour progression, in the form of local or distant metastases, which are ultimately resistant to therapy [80, 81]. As such, there is an urgency to understand the mechanisms underlying why some tumours respond to endocrine treatment while others do not.

Resistance can be classified based on its time of occurrence. Some breast tumours display no response to first line therapies and represent intrinsic resistance. In contrast, acquired resistance is depicted by a tumour initially responding well but the efficacy is temporary, resulting in recurrence of the tumour. There is a high degree of complexity underlying endocrine resistance of which, several mechanisms have been noted. Some of the most important mechanisms are detailed in this section.

1.3.1. Intrinsic resistance

Intrinsic endocrine resistance implies that by the time the tumour is diagnosed, it is already ‘hard-wired’ to be resistant to endocrine therapy. Likewise, some tumours are very heterogeneous and harbour very small clusters of ER positive cells among a background of ER negative cells. The immediate eradication of these ER positive cells would overall not affect the otherwise resistant tumour where ER negative cells already dominated [82].

Altered metabolism of tamoxifen is the next more recently documented mechanism for intrinsic resistance. As discussed previously, tamoxifen is metabolised by hepatic enzymes into 4-hydroxytamoxifen and N-desmethyltamoxifen which are converted into the most abundant and biologically active metabolite, endoxifen [83]. However, approximately 8% of women carry inactive alleles of the cytochrome p450 enzyme CYP2D6, which is required for the metabolism of tamoxifen [84]. In these inactive alleles, there may be a gene polymorphism or deletion; producing a protein
with either no enzymatic activity, or no protein expression, respectively. As such, there is no conversion of tamoxifen to an active metabolite and these women will be non-responsive to treatment [85]. Therefore, these tumours are intrinsically resistant to tamoxifen and require alternative endocrine therapy, either SERD or AI. However, a promising recent study indicated that direct administration of the tamoxifen metabolite, endoxifen, was effective in reducing tumour activity in endocrine-refractory metastatic breast cancer irrespective of CYP2D6 metabolism [86].

1.3.2. Acquired resistance

In contrast to intrinsic resistance, acquired resistance occurs through tumour adaptation to endocrine treatment. Such tumours respond well to endocrine therapy initially, however, after a seemingly successful period of treatment, the tumour utilises a mechanism, or multiple mechanisms, through which it can continue to propagate despite ongoing treatment [87]. There are many, and varied, mechanisms through which a breast tumour acquires resistance; some of which are detailed below.

1.3.2.1. Oestrogen receptor expression

Oestrogen receptor switching in tumour progression from endocrine sensitive (ER+) to resistant (ER-) is relatively uncommon, occurring in approximately 10% of cases [88]. The majority of ER+ primary tumours that progress to recurrent resistant disease remain ER+. However, in 30% of ER+ recurrences there is a loss of PR expression, indicative of non-genomic ER signalling [88-90]. The preservation of ER signalling, even in an altered mode enables second-line therapies with SERDs or AIs to be successful in a subset of these recurrent ER+ tamoxifen-resistant patients. [87].

1.3.2.2. Oestrogen receptor mutations

Recent studies identified up to 20% of metastatic breast tumours harbour recurrent ESR1 gain-of-function mutations [91]. The hotspot in which most of these mutations are clustered is the LBD [92]. As such, they lead to a constitutively active ER
protein and ligand-independent activity. This could explain resistance to AI therapy and partially account for decreased sensitivity to tamoxifen. Fulvestrant is a potentially next in line therapy for those who have failed on alternative anti-oestrogens due to a constitutively active ER [67]. Two of the most frequently observed mutations are Y537S and D538G [93]. They lead to a conformational change in the LBD stabilisation in an active conformation which enables co-activator binding. Furthermore, Y537S and D538G mutations lead to reduced affinity for tamoxifen [94]. Various studies investigated ESR1 mutations in circulating tumour DNA as a means of developing a non-invasive, reliable method for detecting the association between mutations and response to endocrine treatment. The prevalence of ESR1 mutations in these studies was in a range of 25% - 40% [91]. A key finding was the association of ESR1 mutations with prior AI treatment suggesting a survival advantage for these mutated cells in oestrogen-deprived settings over other anti-oestrogens. However, the clinical relevance of ESR1 mutations is yet to be fully substantiated as these studies were carried out retrospectively and on relatively small sample patient numbers [91, 92, 94].

1.3.2.3. Growth factor pathway crosstalk

The signalling of GF receptors (GFR) has been broadly implicated in altering response to endocrine treatment in breast cancer [52, 76, 90]. Activated GFRs induce multiple downstream signals converging on kinases which phosphorylate the ER at various sites, including Ser118 and Ser167 [72, 95]. This generates a persistently active receptor driving endocrine resistance [52]. The ER-target gene amphiregulin is a cognate ligand for EGFR which can further set up a short circuit loop for maintained cell proliferation in the face of endocrine therapy [96]. Furthermore, tamoxifen treatment has been shown to be agonistic in membrane-bound ER; stimulating ER-HER2 crosstalk [97]. This crosstalk in turn phosphorylates and activates nuclear ER. Positive feed-back loops of molecular crosstalk between ER and GFRs are therefore accountable for cancer cell proliferation that involves both genomic and non-genomic ER signalling. While switching to an AI or SERD could be used to treat this type of tamoxifen resistance, increased activity of GF pathways is a mechanism of AI
resistance. In exemestane resistant breast cancer cells, high levels of amphiregulin were observed to promote cell proliferation via EGFR/MAPK [96]. Frequently, at this stage in such tumours, the cells have also become resistant to SERDs. This is a result of increased GFR signalling activating various TFs, independently of ER, to induce genes capable of promoting cell proliferation [98]. Overall, these pathways are capable of intricate crosstalk, playing central roles in allowing breast cancer cells to circumvent multiple endocrine therapies.

1.3.2.4. Cell cycle regulators

The loss of cell cycle regulation is a hallmark of cancer [5]. Proteins involved in cell proliferation and apoptosis are important mediators of acquired endocrine resistance in breast cancer [76, 99-102]. Importantly, the over-expression of positive regulators of the cell cycle, accompanied by down-regulation of negative regulators, may result in decreased sensitivity to anti-oestrogen therapies. Over-expression of cyclin D1, cyclin E1 and MYC induce endocrine resistance by activating CDKs vital for cell cycle progression through G1 phase [76]. Furthermore, their increased expression relieves the inhibitory effects of the tumour suppressor, retinoblastoma protein (Rb), and the cell cycle brakes, p21 and p27. Many mitogenic pathways (PI3K/Akt, MAPKs and STATs) converge on D-type cyclins to set the cell cycle cascade in motion [103]. This again demonstrates the multiple mechanisms of endocrine resistance interacting to promote tumour progression. Additionally, transcriptional regulators of cell cycle may be deregulated; MYC is over-expressed in 40 % of breast cancers and has been seen to trans-activate CDKs and cyclin genes while repressing p21 and p27 expression [104, 105]. Likewise, cyclins D1 and E1 are over-expressed in almost 50% and 40% of primary breast cancers, respectively [106]. All three cell cycle activators, cyclin E1, D1 and MYC, are associated with endocrine resistance and poor clinical outcome [101, 107-109].
1.3.2.5. Oestrogen receptor co-regulators

It is well-defined that the binding of ligand to ER induces a conformational change in the receptor which exposes surface area for the recruitment of accessory factors i.e. co-regulators [59]. These co-regulators control gene expression by modifying the transcriptional machinery required for the efficient ER target gene transcription [110]. Co-regulators may be expressed in the nucleus at rate-limiting levels, therefore alterations in their expression can change ER signalling [111]. The cellular context and bound ER-ligand structure is vital for co-regulator function [112]. Therefore, the balance between agonistic and antagonistic activities of SERMs can be skewed by an over-expression of co-activators or a down-regulation of co-repressors [113]. Tamoxifen predominantly acts as an ER antagonist in breast cancer cells thereby impeding its activity and decreasing tumour cell growth. However, increased co-activator expression in breast cancer cells promotes agonistic activity of tamoxifen [113]. Cancer cells can then grow and proliferate due to enhanced ER transcriptional activity irrespective of treatment. Similarly, decreased expression of the transcriptional co-repressors, SMRT and NCoR, correlate with tamoxifen resistance in both experimental and clinical samples of breast cancer [114, 115]. Furthermore, phosphorylation and methylation of ER by its co-regulators, CARM1 and PRMT1, respectively, mediates ligand-independent ER activation thereby promoting tamoxifen resistance in breast cancer [116, 117].

Extensive studies have been carried out with respect to the p160 NR co-activator family and endocrine resistance. The p160 family is composed of SRC-1 (NCOA1), SRC-2 (NCOA2, TIF2, GRIP1) and AIB1 (NCOA3, p/CIP, SRC-3). While much research has investigated the role of SRC-1 and AIB1 in breast cancer, the role of SRC-2 in breast cancer is less clear [118]. However, some studies have shown SRC-2 expression does correlate with cyclin D1 and cellular proliferation in breast cancer cells [115, 119].

Over-expression of AIB1 is associated with in vitro and clinical tamoxifen resistance. Patients with high AIB1 expression, receiving tamoxifen treatment, have poor clinical outcome [120]. Similarly, over-expression of SRC-1 is significantly associated with an resistance to endocrine treatment and disease recurrence [121].
Multiple studies have demonstrated that tamoxifen increases the expression of SRC-1 [122, 123]. This could set up a vicious cycle wherein SRC-1 potentiates the agonist effects of tamoxifen and tamoxifen increases the expression of SRC-1 to boost this effect. Not only is SRC-1’s over-expression considered as a mechanism of endocrine resistance but it is implicated in multiple other means of endocrine resistance. SRC-1 was identified as interacting with D538G-mutated ER, irrespective of the presence or absence of ligand [124]. Furthermore, SRC-1 is intertwined with GF signalling. In the presence of tamoxifen, protein kinase A induces phosphorylation of ER at Ser305, altering its conformation and inducing GF-mediated endocrine resistance [125]. SRC-1 can also bind between this altered ER orientation and its ligand-dependent domain, recruiting general transcription factors required for elevated transcription in the presence of tamoxifen [125]. Normal breast tissue has low expression of SRC-1 protein however its expression is elevated in breast cancer [126]. Multiple clinical studies observed SRC-1 over-expression in 19% - 36% of various subtypes of primary breast [126-129]. SRC-1 expression positively correlates with larger tumour size, higher tumour grade and HER2 positive status [126, 127]. SRC-1 serves as an independent predictor of disease recurrence [126]. Furthermore, its expression is identified in 92% of recurrent breast tumours [130]. The first in vivo SRC-1-null mouse model (SRC-1-/-;MMTV-PyMT) demonstrated that SRC-1 promotes undifferentiated mammary tumours, enhanced intravasation and metastasis. They also show SRC-1 is not required for breast tumour initiation or growth [131]. These findings were confirmed in another SRC-1 knockout mouse model (SRC-1;MMTV-neu) in which SRC-1 was demonstrated to repress the expression of CDKN1A [132]. CDKN1A (p21) is an important negative regulator of the cell cycle [133]. Both animal models used to investigate the effects of SRC-1 were ER negative, SRC-1 may be responsible for regulation of gene expression in an oestrogen independent setting. In addition to SRC-1’s tumour promoting role in breast cancer, it has also been found to be involved in melanoma, prostate, endometrial and thyroid cancers [134-137].
1.4. Transcription factors

1.4.1. Control of gene expression

The establishment and maintenance of a cell’s state is attributable to gene expression programmes which are tightly controlled by thousands of transcription-orientated proteins [138]. Over half a century ago, pioneering work by Jacob and Monad established the key principal of transcriptional control; that gene expression could be regulated by a group of trans-acting factors by binding to control elements of DNA sequences [139]. After many years of extensive research, we now know that gene expression is more complex and the consensus model for gene expression involves the recruitment of various chromatin remodellers, TFs, their co-factors, and general transcription machinery [140]. Stemming from the human genome project there are an estimated 19,000 protein-coding genes and 2,000 TFs, indicating there is one TF per 9 - 10 protein-coding genes [141, 142]. As not all 19,000 protein-coding genes are expressed at once in a cell, TFs control their spatial and temporal expression providing the cell with its individual identity and ability to respond to environmental cues [140]. TFs, like proteins involved in signal transduction, do not require high expression in the cell; the effect of a single TF molecule can result in multiple mRNA copies from the target gene [142]. While some TFs are ubiquitously expressed in the cell and are involved in ‘house-keeping’ gene expression, others respond only to internal or external signalling [143]. In addition, some TFs are considered master regulators as their expression is sufficient to completely regulate cellular identity (e.g. OCT4, SOX2, KLF-4, and MYC in induced pluripotent stem cells, ASCL1 in neuronal cells or GATA3 in breast cells) [144-146].

Transcriptional regulation requires a dynamic interplay consisting of TFs, their co-factors, the associated general transcriptional machinery, chromatin and its remodellers. In brief, TFs recruit co-factors which induce reorganisation of the local nucleosome structure, thereby facilitating successful TF interaction with genomic regulatory elements [147]. The complex of co-factors can then interact directly with general TFs and RNA polymerase II (Pol-II), forming the pre-initiation complex [148]. With the DNA now unwound, Pol-II breaks its contact with the promoter-bound TF complex and transcribes 20 - 50bp of DNA downstream of the transcription start site.
(TSS) [149]. Pol-II can then enter productive elongation, transcribing through the gene until termination when the Pol-II complex is removed and the RNA is released and can be used for protein synthesis [150].

The transcriptional regulatory system plays a vital role in how cells recognise and respond appropriately to diverse internal and external stimuli. Alterations in the activity of TFs can be viewed positively in terms of phenotypic diversity and evolutionary adaption. While this regulatory flexibility allows cells to adapt and thrive in changing environments and survive harsh conditions, it is also utilised by tumour cells for malignant progression.

1.4.2. Transcription factor dysregulation in tumour progression

TF deregulation is a pervasive theme in tumourigenesis [143]. Multiple layers of altered transcriptional regulation contribute to tumour progression. TFs are often subject to amplification, deletion or functional mutation. Moreover, misexpression of cell fate regulators or co-factors alters the function of TFs. As such, gene expression changes that drive malignant transformation can be activated. As TFs can contribute to each stage of tumourigenesis and the metastatic cascade, they can become the factors on which the tumour cell relies to progress [151].

1.4.2.1. Transcription factors in cell fate regulation

The generation of differentiated adult cells relies on precise spatio-temporal expression of lineage-specific TFs [143]. Analogous to the haematopoietic system, a cellular hierarchy exists in the mammary gland determining its heterogeneous cell population [152]. Misregulation of this hierarchy can lead to the acquisition of metastatic characteristics [153]. GATA3 is considered one of the master regulators in determining a mature luminal lineage, from luminal progenitor to ductal or alveolar cell [146]. In cancer, GATA3 is found to be lost upon tumour progression [154] and its forced expression reintroduces differentiation and luminal characteristics in primary breast tumours [154]. Likewise, NOTCH1 plays a critical role in mammary lineage-specific differentiation from common progenitor to luminal progenitor [155, 156].
Aberrant NOTCH1 signalling promotes aggressive breast carcinomas and is associated with the stem-like (CD44+/CD24-) population and metastases in breast cancer [157, 158]. Furthermore, NOTCH4 signalling appears to drive breast cancer stem cell (CSC) activity and anti-oestrogen resistance [159, 160]. The transcriptional profiling of breast cancer subtypes lead to the suggestion that the distinct breast cell lineages were the origins for the different subtypes [152]. Well differentiated luminal cells are thought to form luminal a/b subtypes, less differentiated luminal progenitor cells initiate basal breast cancers while the undifferentiated mammary stem cell reflects the mesenchymal features of claudin-low breast cancer [161]. As the degree of differentiation of a tumour cell inversely correlates an aggressive nature, TFs altering the differentiation status of a tumour would impact on patient prognosis [162, 163].

1.4.2.2. Transcription factors in tumour proliferation

The ability of cancer cells to sustain chronic proliferation is debatably the linchpin of a tumour. It is well documented that normal cells are under tightly regulated cell cycle conditions to control proliferation and thereby maintain tissue homeostasis [164, 165]. This is achieved by careful release of growth stimulating factors which prompt entry into the cell cycle followed by comprehensive checkpoint regulatory networks. In a tumour cell, however, this system is frequently deregulated and the cell no longer relies on external stimuli, rather it becomes self-sufficient in driving proliferation [5]. p53 is a tumour suppressor TF that mediates various physiological functions enabling cells to avoid the accumulation of genomic alterations [166]. p53 is mutated in 50% of all tumours and is one the most frequent genetic alterations in breast carcinomas [20, 167]. The gain-of-function p53 mutant not only leads to unrestrained tumour cell proliferation, but also provides a resistance to apoptosis [168]. The mutant p53 can amplify other TFs such as SP1 and ETS1 which additionally play a role in tumour cell proliferation [169]. On the other-hand, when the p53 gene is intact and functioning as a tumour suppressor in the cancer cell, TFs including AP-1 can bind to its promoter and prevent its transcription [170].

Other TFs that have a significant impact driving aberrant cell division include MYC, NFκB, AP1, ER and mutated RB [105, 171-176]. Such TFs not only impact
proliferation control pathways but also have profound impact on other cell functions and tumour progression. \textit{In vivo} hyper proliferation is often associated with tumour growth outstripping the blood supply resulting in hypoxic regions in the tumour. microenvironment [177]. Homeostatic response to alleviate such a hypoxic effect results in a profound impact on the cellular transcriptome [178]. The TF HIF1\(\alpha\) can help overcome such conditions [179]. Another way in which cancer cells circumvent hypoxia is via migration and metastases [180].

1.4.2.3. Transcription factors in migration and invasion.

As an early step in cancer metastasis, tumour cells must attain the ability to disseminate from solid tumour mass and invade the neighbouring stroma towards the circulation [181]. The acquisition of such invasive and motile properties is broadly attributable to the process of epithelial-to-mesenchymal transition (EMT) [182]. This pivotal step involves the dissolution of cell-to-cell junctions and loss of apico-basolateral polarity with a concurrent gain in mesenchymal features. The molecular reprogramming during EMT is orchestrated by a network of master TFs from the ZEB, SNAIL and TWIST families. SNAIL1, SLUG, TWIST1, ZEB1 and ZEB2 repress E-cadherin expression and induce the expression of N-cadherin; an event known as ‘cadherin switching’ [183]. In addition to altering their adhesive repertoire, cancer cells utilise TFs to dramatically change their cytoskeleton and form cellular protrusions to promote migration and invasion. MYC induces RhoA expression [184] which is responsible for ROCK control of the contractility of a cell [183] while RAC1 supports the formation of lamellipodia and filopodia; structures important in cellular motility [185, 186]. The ETS family TFs, ETS1 and ETS2 promote the regulation of matrix metalloproteinases (MMP), MMP1 and MMP9, to facilitate invasion through extracellular matrix (ECM) [187]. While EMT is difficult to define in human tumours, epithelial plasticity promoting migration and invasion is associated with tumour progression and treatment resistance [188, 189].
1.4.2.4. Transcription factors involved in survival in the circulation

Once a tumour cell intravasates into the nearby blood or lymphatic vasculature, it is met with numerous survival challenges. Detachment-induced programmed cell death or anoikis, mechanical destruction due to the sheer force of the blood circulation, together with immune surveillance are some of the intrinsic attributes in place to prevent the movement of such cells [190]. The potential metastatic cell is therefore surrounded by a hostile environment and as such, the vast majority of circulating tumour cells (CTCs) perish in the circulatory system [191]. While in the minority, some CTCs are adept at increasing the probability of their own survival before reaching a host organ. The aggregation of platelets around the CTC not only provides a guard against the immune system but also shields from the high force of the circulating blood [192]. Meanwhile, within the CTC, the TF, STAT1, induces expression of the immune inhibitory signalling protein, PD-L1, providing the tumour cell with the means to avoid immune surveillance [193]. Moreover, expression of the neurotrophic receptor, TRKB, promotes anoikis resistance and is under the transcriptional control of HIF1α [194, 195].

1.4.2.5. Transcription factors in colonisation

Metastasis is a highly inefficient process with only 0.018% of the tumour cell population successfully forming macrometastasis [196]. This is in part due to the obstacles alluded to previously. Yet despite these impedances, once tumour cells have established metastatic lesions in distant organs, they prove extremely durable and current treatments frequently fail to eliminate them [197]. The first step in colonisation involves the CTCs crossing the endothelial barrier. This is facilitated in part by expression of CD44 on the CTC which binds to E-selectin on the endothelial cell [198]. It has been proposed that the next step in colonisation is the reversal of EMT which facilitated the previous stages of metastasis. This is an event called mesenchymal to epithelial transition (MET) [199]. MET involves the reacquisition of epithelial cell features. It is not known whether this event is due to MET signals or a lack of EMT signalling [181]. This event is thought to occur as tumours at metastatic sites often display differentiated features corresponding to the primary tumour.
Nevertheless, the TF KLF4 is known to bind directly to the E-cadherin promoter and activate its expression inducing MET [200]. More specifically, the TF NFIC was found to modulate MET in breast cancer cells by the activation of KLF4 [201]. It has been postulated that the reduced proliferation associated with an EMT state must be arrested prior to colonisation [202]. In terms of organ-specific colonisation, the TF RUNX2 was identified as promoting colonisation of the bone by promoting factors important in osteolysis [203]. Moreover, the ID TF family members, ID1 and ID3, were found to support circulating breast cells colonisation in the lung [204].

1.4.3. The Oestrogen Receptor

One of the most important TFs in breast cancer is the ER [205]. ER target genes include cyclin D1 and MYC [206]. Cyclin D1 expression promotes cell cycle progression through G1 to S phase by the activation of CDK4 [207]. Cyclin D1 is a key cell cycle regulatory protein that promotes growth of tamoxifen resistant breast cancer cells [208]. Furthermore, MYC is a highly influential oncogenic TF that regulates up to 15% of human genes including those involved in cell growth, proliferation, apoptosis, differentiation and metabolism [209]. Both cyclin D1 and MYC are over-expressed in breast cancer and confer resistance to endocrine treatment [210, 211].

Originally, it was thought that ER was a stand-alone TF and as such the receptor-ligand complex was the exclusive determinant of its activity. However, it has become apparent that the ER itself is not the crux of dysregulated TF activity promoting tumour progression and endocrine resistance. Rather the ER is the focal point of convergence for transcriptional co-regulators which modulate general transcription machinery and induce chromatin remodelling, essential for transcriptional activation. As such, it is the co-regulators that determine the activity of receptor-ligand complex.
1.4.4. SRC-1 modulation of transcription factors

In addition to its pioneering role as a NR co-activator, SRC-1 can regulate non-steroidal TFs in its modulation of endocrine resistance. The TF ETS2 relies on SRC-1 co-activation to induce expression of MYC and MMP9 [212, 213]. As mentioned previously, MYC is a highly dominant oncogene involved in important cellular pathways. MMP9 is involved in cancer cellular invasion and its expression in breast cancer is associated with poor prognosis [214]. SRC-1 expression inversely correlates with the EMT marker, E-cadherin [215]. This is due to its interaction with the TF PEA3 which activates the EMT TF, TWIST [215]. TWIST is known to transcriptionally repress E-cadherin expression, and its high expression correlates with invasive breast carcinomas [216]. Furthermore, SRC-1 assists the migratory and invasive metastatic activity of α-integrin-5 via its interactions with AP-1 [217]. α-integrin-5 expression is associated with poor prognosis in breast cancer patients [218]. The aberrant expression of SRC-1 allows for disturbed transcriptional activation of key genes that promote tumour progression and metastases.
1.5. Hypothesis

SRC-1, through a series of complex transcriptional networks, regulates genes that enable the emergence of a steroid-independent/endocrine resistant phenotype (Figure 1-2).

Figure 1-2. Model of proposed hypothesis. (A) In the presence of tamoxifen, SRC-1 interacts with newly identified co-regulator TFs to (B) promote expression of downstream TFs. (C) These TFs promote cellular phenotypes necessary for endocrine resistance and tumour progression such as migratory potential, anoikis resistance, stem-ness and proliferation in the presence of tamoxifen.
1.6. Aims

i. Identify the SRC-1 regulated transcriptome and interacting partners in endocrine resistant breast cancer.

ii. Investigate the role of SRC-1 regulated TFs in tumour progression.

iii. Elucidate the clinical relevance of the SRC-1-centric network

iv. Investigate the effector target genes of the greater SRC-1 regulated network.

**Figure 1-3. Experimental approach** RNA-sequencing of LY2 cells will identify SRC-1 target genes. Bioinformatics, gene expression and ChIP analysis will establish SRC-1 transcription factors. Rapid immunoprecipitation of mass endogenous proteins of SRC-1 in LY2 cells will identify SRC-1-interacting proteins. Bioinformatics, protein biochemistry and ChIP analysis will identify SRC-1 transcription factor partners. Gene expression and ChIP analysis will determine the transcriptional complex regulating SRC-1-regulated transcription factors. Multiple functional assays will determine the role of SRC-1 regulated transcription factors in mediating an endocrine resistant phenotype. Bioinformatics and PDX investigations will assess the clinical relevance of SRC-1-regulated network. Finally, bioinformatics analysis will establish the extended SRC-1 gene network.
2. Materials and methods
2.1. Cell culture

2.1.1. Cell culture environment
Cell culture was performed using aseptic techniques in a sterile laminar airflow cabinet (HeraSafe H512). Cells were maintained in a humid incubator in 5% CO₂ atmosphere at 37 °C (HeraCell 150).

2.1.2. Routine cell culture
Breast cancer cell lines were cultured in T-75 cm² filtered flasks (Sarstedt, Nümbrecht, Germany). Cells were passaged every 3 - 4 days, at a confluency of 70 - 80 %, by washing twice with 6 mL phosphate-buffered saline (PBS; Oxoid Limited, Hampshire, UK) followed by incubation with 2 mL 0.05 % Trypsin/0.02 % EDTA (Sigma-Aldrich, MO, USA) at 37 °C for 5 minutes. Once the cells had dissociated, the trypsin was quenched with 8 mL of normal serum-containing cell culture media; the cell suspension was transferred to a 15 mL falcon tube (Greiner Bio-One, PA, USA) and centrifuged at 201 x g for 3 minutes. The supernatant was discarded; the cell pellet was re-suspended in cell culture media and seeded into a T-75 cm² flask at an appropriate experimental density. Experiments were carried out between 10 passages of culture.

2.1.3. Cell counting
For experiments requiring a specific seeding density, a haemocytometer (Blaubrand Neubauer, Germany) was used to manually count cells. During subculture, 20 µL of total cell suspension was diluted with 20 µL of 0.4 % Trypan Blue exclusion dye (Sigma-Aldrich). 10 µL was gently pipetted between the coverslip and haemocytomter allowing capillary action to draw the cell suspension into each chamber. The number of live cells in each of the corner squares (as marked by ‘X’ in) in both chambers were counted. The numbers of cells in both chambers were averaged and total cells per mL were calculated as follows:

\[
\text{Total cells per mL} = \frac{\text{total cells counted}}{\text{number of corner squares counted}} \times \text{dilution factor} \times 10^4
\]
2.1.4. Breast cancer cell lines

2.1.4.1. MCF-7 cell line

Mammary adenocarcinoma cells from a pleural effusion were used to generate the MCF-7 cell line. MCF-7 cells retain many characteristics of well differentiated mammary epithelium. They express ER, PR, androgen receptor (AR) and glucocorticoid receptor (GR), and are Ki67 low with no amplification of HER2. MCF-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Minimum Essential Medium Eagle (MEM; Sigma-Aldrich) supplemented with 10 % foetal bovine serum (FBS; Sigma-Aldrich) and 2mM L-Glutamine (LG; Sigma-Aldrich). MCF-7 cells were utilised as a hormone / endocrine therapy responsive cell line model and are classed as luminal A.

2.1.4.2. LY2 cell line

The LY2 cell line was a kind gift from Robert Clarke (Georgetown University, USA). LY2 cells are a stable variant of MCF-7 cells, generated by exposure to increasing doses of a potent anti-oestrogen (LY 117018) [219]. LY2 cells express ER (albeit lower expression than the parental MCF-7 cell line) and AR with no detectable PR, higher Ki67 and no HER2 amplification [220]. LY2 cells were cultured in phenol red free MEM (PRF-MEM) supplemented with 10 % charcoal:dextran stripped FBS (CDS-FBS), 2mM LG and 4-OHT 10^{-8}M. LY2 cells were utilised as an endocrine therapy resistant cell line model and are classed as luminal B.
2.1.4.3. LY2 shSRC-1 cell line
LY2 SRC-1 knock-down cells were previously created in the lab, using MISSION® (Sigma) short hairpin RNA (shRNA) technology directed against SRC-1. The pLKO.1-puro-CMV-tGFP plasmid (Sigma) was transfected with the virus packaging vectors and lentivirus particles produced were transduced into LY2 cells. A control shRNA cell line was also created, LY2 shNT. Both the LY2 shSRC-1 and LY2 shNT cells were maintained in PRF-MEM supplemented with 10 % CDS-FBS, 2mM LG, 4-OHT 10⁻⁸ M and puromycin (125 µg/ml) for antibiotic selection.

2.1.4.4. Tam-R cell line
An additional tamoxifen resistant cell line, Tam-Rs were also used in this study and were created previously in the lab. The cells were generated from MCF-7 cells by long-term (6 months) culture in tamoxifen at 10⁻⁶ M in PRF-MEM supplemented with 10 % CDS-FBS and 2mM LG. Once determined resistant to tamoxifen, the cells were maintained in at 10⁻⁷ M tamoxifen.

2.1.4.5. Let-R cell line
Previously in the lab aromatase over-expressing (Aro) cells were created by transfecting an aromatase, CYP19 over-expression plasmid (pcDNA-DEST47; Invitrogen) into MCF-7 cells. Long-term treatment of Aro cells with letrozole (Novartis, Basel, Switzerland) was used to create an AI resistant cell line (Let-R). Let-R cells were cultured in PRF-MEM supplemented with 10 % CDS-FBS, 2mM LG, androstenedione (10⁻⁹ M) Sigma Aldrich), letrozole (10⁻⁶ M, Novartis) and G418 (200 µg/mL; Sigma-Aldrich).

2.1.5. Endocrine treatments
For experiments requiring endocrine treatment, cells were deprived of steroids by twice washing with 1X PBS followed by maintenance in PRF-MEM supplemented with 3 % CDS-FBS and 2mM LG for 72 hours. Treatments were as follows: LY2s 4-OHT: 1 x
10^{-7} \text{ M, TamRs 4-OHT} \ 1 \times 10^{-6}\text{M, 17\beta-estradiol:} \ 1 \times 10^{-8}\text{M, vehicle: ethanol 0.01\%.}

Duration of treatment was dependent on the experiment: ChIP/ IP 45 minutes, RNA extraction 8 hrs, protein extraction 48 hrs.

2.2. Cellular transfections

2.2.1. Transient siRNA knockdown of gene expression

48 hrs prior to transfection, 1.5 \times 10^5 cells were seeded into each well of a 6 well plate (Greiner Bio-One) in PRF-MEM supplemented with 3 \% CDS-FBS and 2mM LG. For transient transfection, 5 \muL of transfection reagent, Lipofectamine 2000 (Invitrogen), was mixed with 245 \muL of Opti-MEM reduced serum media (Gibco, Life Technologies) and incubated for 5 minutes at room temperature. Each siRNA was diluted to its working concentration (Table 2.1) in a total volume of 250 \muL Opti-MEM and incubated for 5 minutes at room temperature. The two solutions were then mixed together and incubated for 20 minutes at room temperature. Cells were washed twice with 1X PBS and subsequently 500 \muL of the Lipofectamine/siRNA solution was added directly to the cells. The plate was gently rock back and forth to ensure full coverage of the transfection solution. 1.5 mL of Opti-MEM was added onto the cells and the plate was incubated for 5 hours at 37 \degree \text{C in the 5\% CO}_2 \text{ incubator. Following incubation, the transfection mix was removed and steroid depleted cell culture media was added. 24 hours later, treatments were added where required. For siRNA studies, Smartpool siRNA products were employed. The sequences provided for each siRNA in the smartpool were input in BLAST to ensure direct transcript targeting with no off-target effects} [221].
Table 2.1. List of siRNA

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<th>Gene of interest</th>
<th>siRNA code / Source</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
</tr>
</thead>
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<td>50 µM</td>
<td>40 nM</td>
</tr>
<tr>
<td>ERα</td>
<td>AM16708_s4824 Ambion</td>
<td>50 µM</td>
<td>40 nM</td>
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<td>50 nM</td>
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<td>4392420_s44663 Ambion</td>
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<td>25 nM</td>
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<td>TRPS1</td>
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<td>Neg. Control</td>
<td>D-001810-10-05 Dharmacon</td>
<td>50 µM</td>
<td>25 nM / 50 nM</td>
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</table>

2.3. Gene expression analysis

2.3.1. RNA extraction and quantification

Total RNA was extracted from cell pellets using the RNeasy kit (Qiagen). All steps were carried out at room temperature as per manufacturer’s instructions as follows. 350 µL of RLT buffer containing 1 % β-mercaptoethanol was added to the cell pellet and vortexed for 10 seconds. One volume of 70 % (v/v) ethanol (Sigma-Aldrich) was added to the cells, pipette mixed and the solution, was added to an RNeasy spin column in a 2 mL tube. This was centrifuged at ≥ 8,000 x g for 15 seconds and the flow-through was discarded. The membrane was washed with 350 µL RW1 buffer, centrifuged at ≥ 8,000 x g for 15 seconds and the flow-through was discarded. 80 µL of DNase in RDD buffer (Qiagen) was added to the spin column membrane and incubated for 15 minutes. The column was washed again with 350 µL RW1 buffer, centrifuged at ≥ 8,000 x g for 15 seconds and the flow-through was discarded. 500 µL of RPE buffer was added, the column was centrifuged at ≥ 8,000 x g for 15 seconds and the flow-through was discarded. A further 500 µL RPE buffer was added and the column was centrifuged at ≥ 8,000 x g for 2 minutes. The 2 mL collection tube and the flow-through were discarded, the RNeasy spin column was placed in a clean 2 mL collection tube and centrifuged at top speed for 1 minute. The RNeasy spin column was placed in a clean 1.5 mL Eppendorf, 30 µL of RNase-free H2O was added directly to the membrane,
incubated for 1 minute and centrifuged at ≥ 8,000 x g for 1 minute. The eluted RNA was then pipetted back onto the spin column membrane, incubated for 1 minute and centrifuged at ≥ 8,000 x g for 1 minute. The concentration and quality of the RNA was quantified (3 measurements per sample) on a NanoDrop spectrophotometer 2000c (ThermoFisher Scientific) and stored at – 80 °C until ready for use.

2.3.2. Reverse transcription PCR (RT-PCR) and cDNA synthesis
The SuperScript® III First-Strand Synthesis System (Invitrogen) was used to convert 1 µg of RNA to complementary DNA (cDNA). 1 µg of RNA was added to 0.5 µL dNTP (10 mM), 0.5 µL random hexamers (50 ng/µL) and made to a final volume of 10 µL with nuclease-free H₂O (Qiagen). Using a SimpliAmp Thermal Cycler (BioSciences) the sample was heated at 65 °C for 5 minutes then incubated on ice for 1 minute. 10 µL of cDNA master mix (2 µL 10x RT buffer, 4 µL MgCl₂ [25 mM], 2 µL DTT [0.1 M], 1 µL RNaseOUT [40 U/µl], 0.5 µL Superscript III RT [200 U/µl] and 0.5 µL nuclease-free H₂O) was added to each sample and mixed by pipetting. The sample was then placed in the Thermal Cycler and heated to 25 °C for 10 minutes followed by 50 °C for 50 minutes and 85 °C for 5 minutes. For each RT-PCR performed, a control with no Superscript III enzyme was included (negative RT control). cDNA was stored at – 20 °C until ready for semi-quantitative PCR (qPCR) analysis.

2.3.3. qPCR
Experiments were run on a LightCycler 2.0 (Roche, Switzerland) with cycling conditions as per Table 2.2. 2 µL of a 1:10 DNA template dilution was added to 18 µL qPCR master mix (10 µL SYBR Green [Invitrogen], 6 µL nuclease-free H₂O (Qiagen), 1 µL forward primer [10 nM, Sigma-Aldrich], 1 µL reverse primer [10 nM, Sigma-Aldrich]). The master mix and DNA template reaction was prepared in a 20 µL LightCycler Capillary (Roche). The list of all primers used for mRNA and ChIP analysis can be found in Table 2.3 and Table 2.4, respectively.
Table 2.2. SYBR Green Thermocycling Conditions

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</tr>
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<td></td>
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<td></td>
<td></td>
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<td></td>
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Table 2.3. mRNA qPCR Primer Sequences

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<td>ERα</td>
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</tr>
<tr>
<td>STAT1</td>
<td>CAAGGTGGCCAGGTATCTC</td>
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<tr>
<td>E2F7</td>
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</tr>
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Table 2.4. ChIP qPCR Primer Sequences

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<td>DEK</td>
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2.4. Protein biochemistry

2.4.1. Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic protein was extracted from cell pellets using the NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific). All centrifugation steps were performed at 16,000 x g and 4 °C. All vortexing was at highest speed. Cell pellets were slowly defrosted on ice (and maintained on ice for the duration of incubation steps). Cell pellets were re-suspended in 99 µL CER I supplemented with 1 µL 1X protease inhibitor (PI; Sigma-Aldrich), vortexed for 15 seconds and incubated for 10 minutes. 5.5 µL ice-cold CER II was added to the pellet, vortexed for 5 seconds, incubated on ice for 1 minute and centrifuged for 5 minutes. The cytoplasmic fraction (supernatant) was transferred to a clean Eppendorf. The remaining pellet was re-suspended in 39.6 µL NER supplemented with 0.4 µL PI and vortexed for 15 seconds, every 10 minutes, for a total of 40 minutes. The sample was vortexed for 15 seconds and centrifuged for 10 minutes. The nuclear fraction was transferred to a clean Eppendorf and pellet discarded. Protein extracts were stored at –80 °C.
2.4.2. Protein quantification

The concentration of protein samples was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, ThermoFisher Scientific). A set of 8 albumin standards ranging from 0 - 1,400 µg/mL were prepared and 25 µL of each was pipetted in triplicate into wells of a 96 well plate (Greiner Bio-One). 25 µL of a 1:20 dilution of protein samples (lysate : H₂O) was pipetted in duplicate into the 96 well plate. BCA working reagent was prepared by mixing 50 parts reagent A with 1 part reagent B and 200 µL of this was pipetted into each well containing albumin standard or protein lysate. The plate was covered with aluminium foil, mixed on a plate mixer for 30 seconds and incubated at 37 °C for 30 minutes. The plate was then allowed to reach room temperature and the absorbance was measured at 562 nM using a Wallac spectrometer (PerkinElmer). All standards and samples were averaged. The absorbance measurement of the Blank standard (0 µg/mL) was subtracted from the absorbance measurement of the standards and samples. A standard curve was created by plotting the standards against their known concentration in µg/mL. Using Excel, a linear regression line was plotted through the standard points and used to calculate the concentration of unknown protein lysate.

2.4.3. Co-Immunoprecipitation

A T75 cm² flask of 50 % confluent LY2 cells were steroid depleted for 72 hours followed by treatment with 4-OHT 10⁻⁷ M for 45 minutes. Cells were harvested and lysed in 80 µL of lysis buffer (Appendix I) supplemented with PI. 35 µL of Protein A DynaBeads (LifeTechnologies) were pipetted into a 2 mL round-bottomed Eppendorf and placed on a DynaMag2 magnetic stand (LifeTechnologies) on ice. The supernatant was removed, the beads were washed 3 times with 1 mL of RIPA lysis buffer (Appendix I), supplemented with PI and phosphatase inhibitors (PhI; Sigma) and rotated for 5 minutes at 4 °C. Beads were then blocked in 1 mL of 1X PBS with 5 mg/mL BSA (PBS/BSA) and blocking solution removed. Antibody (Table 2.5) was added to the beads (IgG controls were included), total volume brought to 200 µL with 1X PBS and samples were rotated at 4 °C for 2 hours. Beads were washed 3 times on a rotator (5 minutes each) with 1 mL of RIPA lysis buffer. 400 µg of protein lysate was added to
the washed beads, brought to a total volume of 700 μL with PBS/BSA and rotated overnight at 4 °C. Following overnight incubation, beads were washed twice on a rotator (5 minutes each) with RIPA lysis buffer and once with PBS/BSA (10 minutes). The beads were re-suspended in 100 μL PBS/BSA and transferred to a clean Eppendorf. The supernatant was removed and 50 μL of RIPA lysis buffer supplemented with PI and Phl was added. 6X loading dye was added to each sample, beads were re-suspended and incubated at 70 °C for 10 minutes. The supernatant was collected using the magnetic stand; transferred to a clean Eppendorf and beads were discarded. Samples were stored at –20 °C until analysed by Western blotting.

Table 2.5. IP Antibody details

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Company / Code</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>SRC-1</td>
<td>SantaCruz_sc8995</td>
<td>5 μg</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>STAT1</td>
<td>Cell Signalling_9172</td>
<td>1 μg</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>IgG</td>
<td>Diagenode_10002D</td>
<td>1 μg / 5 μg</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

2.4.4. Western blotting

For Western blot experiments, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) gels were made as per Table 2.6. Electrophoresis was carried out using the ATTO electrophoresis system (ATTO, Tokyo, Japan). The resolving gel was poured into the gel cast plates and allowed to polymerize under a layer of isopropyl alcohol for approximately 30 minutes. Once solidified, the layer of isopropyl alcohol was removed and the stacking gel was poured on top. A 1.5mm 10-well comb was inserted into the stacking gel and allowed to set for a further 15 minutes.
Table 2.6. Gel Preparation for SDS-PAGE

<table>
<thead>
<tr>
<th>Reagent</th>
<th>10% Resolving Gel (mL)</th>
<th>5% Stacking Gel (mL)</th>
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<tr>
<td>dH₂O</td>
<td>4.0</td>
<td>2.1</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>3.3</td>
<td>0.5</td>
</tr>
<tr>
<td>1 M Tris pH 8.8</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>1.5 M Tris pH 6.8</td>
<td>-</td>
<td>0.38</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1</td>
<td>0.03</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
<td>0.003</td>
</tr>
</tbody>
</table>

30% Acrylamide/bis-acrylamide (Sigma), 1M Tris (Appendix I), 1.5M Tris (Appendix I), Sodium Dodecyl Sulphate (SDS; Sigma), Ammonium Persulfate (APS; Sigma), N,N′,N′-Tetramethylethylenediamine (TEMED; Sigma)

40 μg of protein was added to 6X loading dye, brought to a total volume of 20 μL with lysis buffer (Appendix ) and boiled at 95 °C for 6 minutes. The gel was placed into an ATTO gel electrophoresis tank filled with 1X running buffer (Appendix I). The samples were loaded into the wells alongside 10 μL of a Multi-Colour Broad Range Protein Ladder (ThermoScientific). The gel was run at 90 volts (V) for 20 minutes and then at a constant voltage of 127 V for approximately 2.5 hours. The separated proteins were transferred from the gel to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). 10 sheets of Whatman filter paper (GE Healthcare) and 1 piece of nitrocellulose membrane were soaked in 1X semi-dry transfer buffer (Appendix I). Within the semi-dry transfer unit (ATTO) a 'gel sandwich' was created using 5 sheets of filter paper, the nitrocellulose membrane, the gel and 5 sheets of filter paper with the membrane orientated near the anode and the gel orientated near the cathode. The unit was then set to 250 mA and run for 1.5 hours. The membrane was immersed in Ponceau S red solution (Sigma Aldrich) to ensure complete protein transfer. The membrane was blocked in 5 mL of 5% blocking reagent (antibody dependent see Table 2.7) in tris-buffered saline (TBS; Sigma) (Appendix I) containing 1% Tween 20 (TBS-T; Sigma) and incubated for 1 hour at room temperature. The membrane was rotated at 4 °C overnight in primary antibody (Table 2.7). Following primary antibody incubation, the membrane was washed 4 X 7 minutes in 1X TBS-T. The membrane was transferred into a suitable secondary antibody (Table 2.7) and incubated for 1 hour at room temperature. Following secondary antibody incubation, the membrane was washed 4
X 7 minutes in 1X TBS-T. ECL chemiluminescent (Thermoscientific) was added to the membrane and the proteins were visualised using the Amersham Imaging system (GE Healthcare).

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Company</th>
<th>Source</th>
<th>Blocking Reagent</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>MW</th>
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<tr>
<td>SRC-1</td>
<td>SantaCruz</td>
<td>Rabbit polyclonal</td>
<td>5% milk</td>
<td>1:200</td>
<td>1:2000</td>
<td>160 kDa</td>
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<tr>
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<td>CellSignalling</td>
<td>Rabbit polyclonal</td>
<td>5% BSA</td>
<td>1:1000</td>
<td>1:2000</td>
<td>91 kDa</td>
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</tbody>
</table>

2.5. Patient-derived xenografts (PDX)

PDX mouse experiments were performed, by D. Vareslija, S. Purcell and S. Cocchiglia, in accordance with the ECC directive 2010/63/EU and were reviewed and approved by the Research Ethics Committee under license from the Health Product Regulatory Authority of Ireland. PDX models were generated from three ER positive, tamoxifen-treated patient samples; one endocrine therapy sensitive primary sample and two endocrine therapy resistant metastatic samples (liver and lung). PDXs were established by orthotopic implantation in Nod-SCID mice (Charles Rivers, MA, USA) supplemented with 17β-oestradiol pellets (Innovative Research of America). Upon harvesting, tumours were formalin fixed and paraffin embedded.

2.6. Immunohistochemistry (IHC)

For IHC studies, one slide per PDX sample, as described in section 2.5, was examined for each antibody. Slides were heated at 65 °C for 8 hours to dissolve excess paraffin wax. The samples were de-parrafinised by two (3 minute) passages in xylene (Lennox) and then rehydrated by two passages (3 minutes each) through 100 % IMS and one passage (3 minutes) through 70 % IMS. The samples were then immersed in 1X PBS for 5 minutes. Heat-mediated antigen retrieval was carried out by placing the samples in 10 mM sodium citrate (Sigma) pH 6.0 and heating at high power in a domestic microwave for 8 minutes. Samples were allowed to cool to room
temperature for 20 minutes and were washed twice (5 minutes each) in 1X TBS-T. A liquid blocker pen (Sigma) was used to mark around the sample. Endogenous peroxidase activity was quenched by applying Dako Peroxidase Block (Dako EnVision+ System [Biocompare, CA, USA]) for 5 minutes followed by a 5 minute wash with dH$_2$O. Samples were incubated with primary antibodies (Table 2.8) in antibody diluent (0.05mol/l Tris-HCL buffer pH 7.2-7.6 containing 1% BSA) for one hour at room temperature. A negative control (IgG) was included with each staining.

Following primary antibody incubation, slides were washed 3 X 5 minutes in TBS-T. Samples were then incubated for 30 minutes at room temperature with a horseradish peroxidase labelled polymer (Dako EnVision+ System), then washed 3 times (5 minutes each) in TBS-T. Next, 3,3’-diaminobenzidine + substrate-chromogen (Dako EnVision+ System) was applied for a minimum of 2 minutes and the reaction was quenched by dH$_2$O. The nuclear fraction of the sample was counter-stained using haematoxylin (Sigma) for 3 minutes followed by a 5 minute wash in tap H$_2$O. The samples were then dehydrated by passage though 70 % IMS (3 minutes) and twice through 100 % IMS (3 minutes each). Samples were cleared by two washes in xylene (3 minutes each) and allowed to dry in the fumehood. Finally, the slides were mounted using DPX mountant (Sigma) and covered with a cover slip (VWR, PA, USA). Slides were allowed to dry and stored at room temperature. A minimum of 5 locations per specimen were imaged on a light microscope at 10X, 20X and 40X.
Table 2.8. Immunohistochemistry Antibody details

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Company</th>
<th>Source</th>
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<tr>
<td>SRC-1</td>
<td>SantaCruz_sc8995</td>
<td>Rabbit polyclonal</td>
<td>Sodium Citrate</td>
<td>1:35</td>
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<tr>
<td>STAT1</td>
<td>CellSignalling_9172</td>
<td>Rabbit polyclonal</td>
<td>Sodium Citrate</td>
<td>1:750</td>
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<tr>
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<td>Rabbit polyclonal</td>
<td>Sodium Citrate</td>
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<td>NFIA</td>
<td>Abcam_ab41851</td>
<td>Rabbit polyclonal</td>
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<tr>
<td>SMAD2</td>
<td>CellSignalling_3108</td>
<td>Rabbit monoclonal</td>
<td>Sodium Citrate</td>
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<tr>
<td>ASCL1</td>
<td>BD Biosciences BD556604</td>
<td>Mouse monoclonal</td>
<td>Sodium Citrate</td>
<td>1:12</td>
</tr>
</tbody>
</table>

2.7. Chromatin immunoprecipitation

**Day 1:** 50 μL of Protein A DynaBeads were added to a 2 mL round-bottomed Eppendorf and placed on a magnetic stand on ice. Cells were washed 4 times in PBS/BSA. Final wash was removed and the beads were re-suspended in 350 μL PBS/BSA. Antibody (Table 2.9) was added to the bead suspension and rotated at 4 °C overnight. For each antibody source, an IgG control was included.

**Day 2:** Following 72 hours steroid depletion, LY2 cells were treated with 4-OHT 10^{-7} M for 45 minutes. (Note: 1 x 15 cm^2 80 % confluent (Sarstedt) petri dish was used per antibody). Next, the media was removed, cells were washed with 1X PBS and cross-linked with 15 mL 1 % formaldehyde (Sigma) in serum free PRF-MEM supplemented with 1 % LG (warmed to 37 °C) and incubated at 37 °C for 10 minutes. Crosslinking was stopped by adding 1.5 mL of 1 M glycine (Fisher) to the dish and incubated for 2 minutes at room temperature. The media was removed and the cells were washed with ice-cold 1X PBS. PBS was removed, plates were placed on ice, 1 mL 1X PBS supplemented with cOmplete, mini PI cocktail (Roche; 1 tablet per 10 mL) was added to each plate and cells were scraped (Sarstedt) into a 2 mL round-bottomed Eppendorf. Cells were then centrifuged at 2000 x g for 3 minutes at 4 °C. The supernatant was removed; cell pellet re-suspended in 1 mL Lysis Buffer 1 (Appendix I) supplemented with PI (1 tablet per 10 mL), rotated at 4 °C for 10 minutes and
centrifuged at 2000 x g for 5 minutes. The supernatant was discarded; cell pellet was re-suspended in 1 mL Lysis Buffer 2 (Appendix I) supplemented with PI (1 tablet per 10 mL), rotated at 4 °C for 5 minutes and centrifuged at 2000 x g for 5 minutes. The supernatant was discarded; cell pellet was re-suspended in 600 μL Lysis Buffer 3 (Appendix I) and samples were sonicated using a Diagenode Biorupter for 20 cycles of 30 seconds ON and 60 seconds OFF. 60 μL of 10 % Triton-X-100 (Sigma) was added to the sonicated lysate and this was centrifuged at 20,000 x g for 10 minutes at 4 °C. DNA was quantified on Nanodrop spectrophotometer 2000c. Samples were adjusted to 500 μg of DNA in a final volume of 1 mL Lysis Buffer 3. 40 μL was removed from each sample as input control and stored at – 80 °C. Antibody-bound beads were retrieved from 4 °C and washed 3 times in PBS/BSA using the magnetic stand. Final supernatant was removed and the beads were re-suspended in 200 μL 1X PBS/BSA. This was added to each lysate sample and was rotated overnight at 4 °C.

**Day 3:** Using the magnetic stand on ice, supernatant was removed from beads. 1 mL of freshly prepared RIPA buffer (Appendix I) was added and the beads were re-suspended and washed 7 X 1 minute. The final supernatant was removed and beads were re-suspended and washed in 1X TE buffer (Appendix I). Supernatant was removed and beads were centrifuged at 1000 x g for 3 minutes. Excess TE buffer was removed and 200 μL of Elution Buffer (Appendix I) was added to the beads. 200 μL of Elution Buffer was also added to the input control. Samples were gently vortexed and incubated in at water bath at 65 °C for a maximum of 16 hours.

**Day 4:** Samples were removed from the water bath and quickly centrifuged to pellet the beads. The supernatant was transferred to a clean 1.5 mL Eppendorf. 150 μL Proteinase K mix (Appendix I) was added to each sample, gently vortexed and incubated at 37 °C for 2 hours. DNA was extracted using 300 μL of phenol chloroform: isoamyl alcohol (24:1) (Sigma Aldrich). Samples were pipette-mixed until an emulsion formed and centrifuged at 12,000 x g for 3 minutes at room temperature. The upper (aqueous) phase was transferred to a clean 1.5 mL Eppendorf; twice the volume (~ 700 μL) of ice-cold 99.89 % (v/v) ethanol was added and the samples were incubated at – 80 °C for a minimum of 30 minutes. Samples were then centrifuged a 20,000 x g for 20 minutes at 4 °C. The supernatant was removed; the pellet was gently washed
with 500 μL of ice-cold 70 % (v/v) ethanol and then centrifuged at 20,000 x g for 5 minutes. All ethanol was removed and the pellet was air-dried at 37 °C for 15 minutes or until all ethanol had evaporated. Each DNA pellet was carefully re-suspended in 30 μL nuclease-free H₂O and stored at –20 °C.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Company_Code</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRC-1</td>
<td>SantaCruz_ sc8995</td>
<td>Rabbit polyclonal</td>
<td>5 μg</td>
</tr>
<tr>
<td>STAT1</td>
<td>CellSignalling_9172</td>
<td>Rabbit polyclonal</td>
<td>1 μg</td>
</tr>
<tr>
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<td>Rabbit polyclonal</td>
<td>5 μg</td>
</tr>
<tr>
<td>IgG</td>
<td>Diagenode_10002D</td>
<td>Rabbit</td>
<td>1 μg / 5 μg</td>
</tr>
</tbody>
</table>

**2.8. ChIP re-ChIP**

ChIP re-ChIP follows the ChIP protocol above to Day 3, with the addition of control samples for re-ChIP (Figure 2-2). Additionally, ChIP re-ChIP requires larger quantities of starting material (3 x 15 cm² 80 % confluent plates per antibody and [Table 2.10]). Following the first overnight immunoprecipitation, the bead-protein-DNA complex was washed 3 times with ChIP washing buffer (Appendix I) and 2 times with TE buffer. The protein-DNA complex was then eluted by adding 75 μL of Re-ChIP buffer (Appendix I) and incubating the samples at 37 °C for 30 minutes. The beads were collected by centrifuging at 600 x g for 3 minutes. The supernatant was isolated and diluted 1:20 (to a final volume of 1.5 mL) in ChIP dilution buffer (Appendix I) supplemented with 50 μg BSA and 1X PI. The second immunoprecipitation was the performed by adding the appropriate antibodies (Table 2.10) against the protein of interest and the relevant controls (Figure 2-2) and incubated overnight rotating at 4°C.
Figure 2-2. Flow through diagram of ChIP-reChIP. LY2 cells were treated with tamoxifen $10^{-7}$ M for 45 minutes, cross-linked, lysed and sonicated. 1st immunoprecipitation was carried out with 3x antibody A (SRC-1), 2x IgG control and 3x antibody B (STAT1). The complex was washed, eluted and a reChIP with relevant antibodies as above performed. This was washed, the complex was reverse cross-linked and the DNA isolated. qPCR quantification of occupancy at transcription factor promoters was assessed.

Following overnight incubation, the antibody bound protein-DNA complexes were recovered by adding 50 μL of protein A Dynabeads and incubated for 90 minutes rotating at 4 °C. The beads were pelleted by centrifugation at 600 x g for 1 minute and the supernatant discarded. The bead-protein-DNA complex was washed 7 times with 1 mL RIPA buffer, re-suspended in 1 mL 1X TE buffer and mixed gently. The beads were captured and TE buffer discarded. Beads were then centrifuged at 900 x g for 3 minutes and any excess buffer removed without disturbing the bead pellet. 200 μL of elution buffer was added to the beads, samples were gently mixed and reverse cross-linked by overnight incubation in a water bath at 65 °C. Following reverse cross-linking;
DNA was extracted as per section 2.5. Occupancy and co-occupancy of proteins was analysed by qPCR.

Table 2.10. Sequential ChIP Antibody Details

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<th>Target Protein</th>
<th>Company Code</th>
<th>Source</th>
<th>Primary Antibody</th>
</tr>
</thead>
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<tr>
<td>SRC-1</td>
<td>SantaCruz_sc8995</td>
<td>Rabbit polyclonal</td>
<td>15 μg</td>
</tr>
<tr>
<td>STAT1</td>
<td>CellSignalling_917</td>
<td>Rabbit polyclonal</td>
<td>3 μg</td>
</tr>
<tr>
<td>IgG</td>
<td>Diagenode_10002</td>
<td>Rabbit</td>
<td>3 μg / 15 μg</td>
</tr>
</tbody>
</table>

2.9. Functional assays

All functional assays were set up 24 hours post transient transfection.

2.9.1. Cellular viability

Transfected LY2 cells were trypsinised, counted and seeded in triplicate into a 96 well plate at a density of 1 x 10^3 cells per well in 150 μL of media (PRF-MEM supplemented with 3 % CDS-FBS, 1 % LG and 4-OHT 10^-7 M) and incubated at 37 °C for 5 days. To calculate autofluorescence, 150 μL of media alone was also included. Following incubation, 30 μL of MTS reagent (MyBio, Ireland) was added to each sample and cells were further incubated for 4 hours at 37 °C, wrapped in aluminium foil to protect the cells from light. The plate was then gently mixed on a plate shaker for 1 minute and the absorbance was recorded at 495 nM using a Wallacspectrometer (PerkinElmer, MA, USA) plate reader. Background absorbance was subtracted from the sample absorbance.

2.9.2. Cellular migration

Using the Cellomics® Cell Motility Kit, the appropriate number of wells in a 96-well plate were coated with calf-skin collagen (BioData Corporation, PA, USA) and incubated for 1 hour at room temperature. The wells were washed with 100 μL of 1X PBS and coated with 75 μL of blue fluorescent microsphere beads. The plate was
incubated in the dark at 37°C for 1 hour. Transfected LY2 cells were trypsinized, re-suspended in PRF-MEM (supplemented with 10 % CDS-FBS and 1 % LG), counted and a cell suspension of 1 x 10^4 cells / mL was prepared. Cell treatment was prepared at twice the concentration (i.e. 4-OHT at 2 x 10^{-7} M). The wells were washed five times with 200 μL of 1X Wash Buffer (Cellomics® Cell Motility Kit). The last wash was aspirated off and 50 μL of the cell suspension was added to each well (i.e. 500 cells per well). 50 μL of the appropriate treatment was then added to each well to give the final treatment concentration (i.e. 4-OHT 1 x 10^{-7} M). The plate was incubated in the dark at 37 °C for 24 hours. Cells were fixed in 200 μL of warm 5.5 % formaldehyde solution (Sigma) and incubated in a fume hood for 1 hour at room temperature. The fixation solution was aspirated and the plate washed three times with 100 μL of 1X Wash Buffer. Cells were then permeabilised by incubation in 100 μL of 1X Permeabilisation Buffer (Cellomics® Cell Motility Kit) for 15 minutes. Permeabilisation buffer was then aspirated off and 100 μL of rhodamine phallodin staining solution (Cellomics® Cell Motility Kit) was added for 30 minutes. The staining solution was aspirated and wells washed three times with 100 μL 1X Wash Buffer. The final wash was not aspirated and the plate was sealed with parafilm (Sigma). Cell tracks were examined on a light microscope using DAPI and TRITC filters on CellSens Entry software (Olympus). Sealed plates were stored in the dark at 4 °C.

2.9.3. 3D assays

2.9.3.1. Anchorage Independent Growth

0.6 % agarose (Promega) was melted at 65 °C in PRF-MEM (supplemented with 10 % CDS-FBS and 2mM LG). 2 mL of this solution was pipetted into each well of a 6 well plate and allowed to solidify for 30 minutes at room temperature. Once solidified, 2 x 10^8 of transfected LY2 cells were counted, added to 2 mL pre-warmed 0.3 % agarose media (PRF-MEM supplemented with 10 % CDS-FBS and 2mM LG and 4-OHT 10^{-7} M) and plated on top of the 0.6 % agarose layer of and allowed to solidify. 300 μL of media (PRF-MEM supplemented with 10 % CDS-FBS and 1 % LG and 4-OHT 10^{-7} M) was added as a top layer. Samples were plated in duplicate, refed with 300 μL of media every 3
days and incubated for a total of 14 days. On day 15, colonies were stained with 400 μL of p-iodonitrotetrazolium chloride (1 mg/mL) (Sigma Aldrich) for 24 hours at 37 °C with 5 % CO₂, wrapped in aluminium foil then stored in the fridge for 24 hours before counting. Using a graticule, colonies over 100 μM in diameter were counted at 8 different points per well under a light microscope (Olympus).

2.9.3.2. Mammosphere Formation

Mammosphere protocol was obtained from Shaw et al. [222]. Transfected LY2 cells were trypsinised and centrifuged at 580 x g for 2 minutes. The supernatant was removed and the pellet re-suspended in 3 mL of ice-cold 1X PBS. Cells were passed through a 25 G needle and syringe three times to generate a single cell suspension. 2 mL of mammosphere media (DMEM-F12 phenol red free [Life Technologies] supplemented with 1 X B27 [Life Technologies], human epidermal growth factor [hEGF.20 ng / mL, Sigma] and 4-OHT 10⁻⁷ M), was added to each well of an ultra-low adherence 6 well plate (Corning, MA, USA). Cells were seeded, in duplicate, at a density of 5,000 cells per well. Plates were incubated for 5 days at 37 °C with 5 % CO₂ and undisturbed. Mammospheres greater than 50 μM in diameter were counted using a light microscope (Olympus) and mammosphere forming efficiency (MFE) was calculated as follows: (# of mammospheres per well /# of cells seeded per well) x 100. These spheres are noted as the 1ˢᵗ generation.

To passage mammospheres, the media containing mammospheres from each well was pipetted into a 15 mL falcon tube. The wells were washed with 2 mL of 1X PBS to ensure no loss of mammospheres and added to the collected media. Mammospheres were centrifuged at 115 x g for 5 minutes. The supernatant was carefully discarded, the pellet re-suspended in 300 μL of 0.5 % Trypsin/0.2 % EDTA to disaggregate the mammospheres and incubated at 37 °C for 3 minutes. Cells were passed through a 25 G needle and syringe to produce a single cell suspension. The trypsin was quenched with 600 μL PRF-MEM (supplemented with 10 % CDS-FBS and 2mM LG) and cells were centrifuged at 580 x g for 5 minutes. The supernatant was carefully removed and the pellet was re-suspended in 150 μL of ice-cold 1X PBS. Cell suspension was confirmed as containing single, viable cells and counted. The entire cell suspension was plated
into 2 mL mammosphere media in an ultra-low adherence 6 well plate (at a density of 5,000 cells / well). Plates were again incubated for 5 days. The number of mammospheres greater than 50 µM in diameter were counted using a light microscope. MFE was calculated once again as above. To assess the self-renewal ability of the cells: the # of mammospheres formed in the 2nd generation was divided by the # of mammospheres formed in the 1st generation.

2.9.3.3. Acini

Growth factor-reduced matrigel basement membrane matrix (Corning) was thawed slowly on ice, chamber slides (LabTek, PA, USA) and p200 pipette tips were chilled. 40 µL of liquid matrigel was pipetted onto a pre-chilled chamber slide and allowed to solidify by incubation at 37 ºC for 15 minutes. During the incubation time, transfected LY2 cells were pelleted and re-suspended in acini assay media (PRF-MEM supplemented with 10 % CDS-FBS, 1 % LG, 4-OHT 10-7 M) and pipette-mixed until a single cell suspension was achieved. The suspension was confirmed as single cell and all viable cells were counted. The cell suspension was adjusted using assay media to a concentration of 25,000 cells / mL. Assay media containing 4 % matrigel was added to the cell suspension in a 1 : 1 ratio. 400 µL of this suspension was added onto the solidified matrigel; corresponding to 5,000 cells per well in a media containing 2 % matrigel. Cells were incubated in a humid 5% CO2 atmosphere at 37 ºC for 21 days and refed with assay media with 2 % matrigel every 4 days. On day 22, acini were fixed in 4 % paraformaldehyde (Sigma) pH 7.2 at room temperature for 15 minutes. Next, the acini were permeabilised by 0.5 % Triton X 100 for 10 minutes a 4 °C and blocked in 10 % goat serum (Invitrogen) in 1X PBS for 1 hour at room temperature in a humid chamber. F-actin was stained using Alexa Fluor 594 (1:200 ThermoFisher) for 20 minutes at room temperature under aluminium foil. Nuclei were counter-stained using 4′,6-diamidino-2-phenylindole (DAPI, 5 ng / mL, Sigma) for 5 minutes at room temperature under aluminium foil. The chamber slide was then separated and a drop of DAKO fluorescent mounting media (DAKO) and a coverslip were added. Slides were allowed to dry overnight (in the dark) at room temperature and then sealed with varnish. Once dry, slides were imaged using a Carl Zeiss LSM 710 NLO microscope.
equipped with a Chameleon tunable laser. Protocol adopted from Debnath et al., 2003 [223].

2.10. Flow cytometry

Prior to flow cytometry analysis, transfected LY2 cells were cultured in the presence of tamoxifen for 48 hours. Each flow cytometry experiment required $1 - 5 \times 10^5$ cells per sample and the following experimental controls: an unstained sample for auto-fluorescence and a single stain control for each fluorophore analysed. Fluorescence activated cell sorting (FACS) buffer was prepared by adding 2 % FBS to 1X PBS. Cells were transferred to a new 1.5 mL Eppendorf, washed with FACS buffer and kept on ice. Cells were then stained with PE-conjugated CD24 (1:25; BD Biosciences) and FITC-conjugated-CD44 (1:6; BD Biosciences) in a 100 µL reaction and incubated for 30 minutes on ice, in the dark. Following staining, cells were washed twice with 500 µL FACS buffer. After the second wash, the supernatant was carefully removed, the pellet was re-suspended in 500 µL FACS buffer and cells were transferred to a 5 mL round-bottomed Falcon tube (Falcon). Cells were placed on ice in the dark until analysis on the BD FACS Canto II. Immediately prior to analysis, cells were filtered through 70 micron cell strainer (BD Biosciences) in order to achieve a single cell suspension. Results were analysed using FlowJo Software (V10.1). Gating was first setup using the unstained control. Firstly, any necessary adjustments to forward scatter and side scatter were implemented to ensure cells were correctly positioned for fluorescent analysis. Any necessary compensation for overlap in the fluorophores was next performed. Live cells were identified and gated; any doublet cells were excluded. Quadrants for positive cells were drawn based on unstained control cells. Protein expression was analysed in terms of cell population.

2.11. Rapid immunoprecipitation of mass endogenous proteins (RIME)

RIME was carried out in collaboration with Dr. A. Redmond from Cancer Research UK, Cambridge Institute. 4 x 15 cm² plates of 80 % confluent LY2 cells were used per
sample. Three technical replicates of each cell line were used for the experimental samples while 2 technical replicates were used for IgG controls.

Day 1: RIME was carried out as per day 1 of ChIP protocol (section 2.7) with the following adaptations; 100 µL of Protein A Dynabeads were used per sample as was a higher concentration of antibody was used for each protein of interest, 10 µg SRC-1 antibody and 10 µg IgG control. Day 2 of RIME was carried out as per day 2 of ChIP (section 2.5) with the following adaptions. Following 72 hours steroid depletion, LY2 cells were treated with 4-OHT $10^{-7}$ M for 45 minutes. Treatment was removed, cells were washed twice in 1X PBS and were cross-linked for 6 minutes with 10 mL of 1% formaldehyde (Polysciences, PA, USA) in serum-free PRF-MEM supplemented with 1% LG. Cross-linking was quenched by adding 1 mL of 2.5M glycine. The cells were washed twice with ice-cold 1X PBS and then harvested by scraping in 1 mL of 1X PBS supplemented with cOmplete, mini PI cocktail (Roche; 1 tablet per 10 mL) into a 2 mL round-bottomed Eppendorf. Samples were centrifuged for 10 minutes at 1,500 x g.

Nuclear extraction, was carried out as per section 2.7 using 300 µL LB3 was used prior to sonication. Samples were sonicated using a Branson sonicator (s450) at the following settings: 10 cycles of 15 % amplification 1 second on and 1 second off for 60 seconds. 30 µL of 10% Triton X-100 was added to the sonicated lysate. Samples were pooled as required and centrifuged at 20,000 x g for 10 minutes at 4 °C.

Supernatant was transferred to a fresh 2 mL Eppendorf and diluted to a final volume of 1 mL with LB3 (+PI). The bead/antibody complex was washed as per the ChIP protocol (section 2.7) and culminated with cell lysate/bead/antibody complex being incubated overnight rotating at 4 °C. Following overnight incubation, the lysate/bead/antibody complex was washed 10 times with 1mL RIPA buffer (Appendix I) and twice with fresh, chilled 100 mM ammonium bicarbonate (AMBIC) solution. After the first AMBIC wash, the suspension was transferred to clean 2 mL Eppendorf tube. After removing second AMBIC wash buffer, the sample was centrifuged at 900 x g for 3 minutes at 4 °C and all residual liquid was removed. From this point on, the protocol was performed out by A. Redmond as per methodology found in Mohammad et al., 2016 [224].
2.12. Bioinformatic analyses

2.12.1. RNA-sequencing
RNA-sequencing had previously been performed on tamoxifen treated LY2 shSRC-1 and LY2 shNT RNA cells were sent for RNA-sequencing. Bioinformatic analysis was done by Dr. A. Fagan as follows. Using default parameters, TopHat v2.0.9 [225] was used to align the reads from samples to the hg19 genome. The reads were quantified into gene counts using HT-Seq v0.6.0 [226]. The counts were loaded into the R and were processed by the edgeR package [227] from Bioconductor [228]. Differential expression analysis was carried out using the exact negative binomial test. Genes were corrected for multiple testing using Benjamini-Hochberg. A gene was considered to be differentially expressed if it exhibited a log2 fold change of greater than 1.2 and if the gene had an adjusted p-value less than 0.01. The differentially expressed gene lists were filtered for potential transcription factors or chromatin remodelers using a list of 1,988 transcription factors/chromatin remodelers from the FANTOM consortium [229]. The TFs were then further refined based on having a known PWM (identified using HOCOMOCO [230]) and/ or a known tumorigenic role.

2.12.2. RIME data analysis, visualisation and motif enrichment
Raw data was pre-processed by Dr. A. Redmond as per Mohammad et al. [224, 231] methodology. Visualisation was performed by N. Cosgrove using R, the msarc package and Adobe Illustrator. The list of significantly enriched SRC-1 cofactors identified from RIME in LY2 cells were investigated in several TF databases to identify those proteins that are known TFs. This was carried out by N. Cosgrove using MotifDB in R (v3.3.2) [232]. TFs that had an identified PWM were brought forward and input into FIMO (v4.11.2) [233]. FIMO was used to scan the PWM of SRC-1 cofactors against the DNA sequences of the TFs identified from SRC-1 RNA-sequencing relevant to this study. The area 10 kB upstream of the TSS was scanned. A motif was considered to be significantly enriched if it had a p value < 0.001.
2.12.3. Transcription factor effector target network analysis

Network analysis was performed with the assistance of N. Cosgrove and Dr. S. Charmsaz. The Cytoscape (v.3.4.0) plugin, iRegulon (v.1.3 Build ID: 1024), was used to query the cisTargetDB for direct downstream effector targets of SRC-1-regulated TFs [234-236]. Meta-regulons for each TF were obtained using an occurrence count threshold of 5 and nodes number of 200. The result was then merged using set union to remove duplicate genes and set intersection in Cytoscape to find those genes common to all TFs relevant to this study. Genes common to two or more TFs from the iRegulon network analysis were input into to ClueGO (v2.3.2) using Cytoscape, for functional enrichment analysis against the Gene Ontology Biological Process and KEGG Pathway databases (settings: p < 0.05; min #Genes = 10, min 2 % Genes, GO Tree Interval min level 1, max level 3, Leading Group Term based on %Genes / Term vs Cluster) [237]. The regulon of each TF was cross-referenced with the differentially expressed genes from LY2 shSRC-1 RNA-sequencing to identify SRC-1’s regulation of effector target genes. DAVID (v6.8) was then used to identify significant upregulated and down-regulated pathways associated with SRC-1’s regulation of effector target genes using default parameters [238].

2.12.4. Gene clustering and heatmap generation

Previously in the lab, RNA-sequencing was carried out on nine ER positive patient samples; three primary breast tumours from patients with good response to therapy, 3 primary breast tumours with poor response to endocrine therapy and their matched local recurrences. Alignment was performed (by Dr. A. Fagan) using Tophat2 to the GrCH37 (v.75) transcriptome assembly from Ensembl [239]. RNA-sequencing counts were calculated using HTSeq (v.0.6.1). The next steps were performed by N. Cosgrove. Raw count data was imported into R (v.3.3.2) where rows with a total count less than 1 were removed and values were rlog transformed using DESeq (v.1.14.1) [240] in order to stabilize variance across the mean of the samples. Expression values were visualised using default parameters (average cluster linkage based on euclidean distance) in the online tool, Heatmapper [241].
2.12.5. Pam50 and Kaplan-Meier survival analysis

Pam50 subtyping was carried out by Dr. A.Fagan using the TCGA data in R as described in McBryan et al. [242]. Kaplan-Meier survival analysis was generated using ER positive, endocrine treated patients in KM plotter web application and ER positive, tamoxifen treated patients in the BreastMark web application [243, 244].

2.12.6. Analysis of ICI182780 microarray and SRC-1, ER ChIP-Sequencing datasets

In-house microarray data of ICI 182780 treated LY2 cells was analysed for differential expression of SRC-1-regulated TFs (accession number E-MTAB-1870) [245]. In-house ChIP-sequencing data of SRC-1 and ER in tamoxifen treated LY2 cells was probed for binding peaks 500bp upstream of SRC-1 regulated TFs (Accession numbers; GSE28987 and E-MTAB-1865, respectively) using UCSC genome browser [245-247].

2.13. Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 5 software (La Jolla, CA, USA). Data are shown as mean ± standard error of the mean (SEM), with a minimum three replicates per experiment. Exact ‘n’ numbers for each graph are reported in the figure legends. Significance was determined using unpaired, two-tailed, Students T-Test. p values <0.05 were considered significant and are denoted by ‘*’.
3. Investigating the SRC-1 regulated transcriptome in endocrine resistant breast cancer
3.1. Introduction

As discussed in the General Introduction, the relative expression of co-regulatory proteins can promote acquired endocrine resistance in breast cancer and are therefore associated with tumour progression. One such co-regulatory protein is the ER co-activator, SRC-1; the first identified nuclear receptor (NR) co-regulator [248]. As SRC-1 has no known DNA-binding domain it does not bind directly to DNA to regulate gene expression [249]. Instead it utilises multiple, versatile structural domains to execute efficient gene transcription via its interaction with DNA-binding TFs and enzymes to facilitate this process (Figure 3-1). SRC-1 has three main structural domains: N-Terminal, nuclear receptor interaction domain (NRID) and C-Terminal, each with well described roles. First, the N-terminal contains SRC-1’s binding motif; a basic helix-loop-helix-Per/Ah receptor nuclear translocation/Sim (bHLH/PAS) motif that is important for nuclear translocation [250]. It also contains the transactivation domain 3 (AD3), which allows for the recruitment of secondary co-activators [251]. Second, the NRID has 3 central LXXLL motifs which facilitate the direct interaction of SRC-1 with NRs [252]. Finally, the C-terminal contains the transactivation domains, AD1 and AD2. AD1 recruits the histone acetyltransferase p300 and the cAMP response element binding protein (CREB)-binding protein (CBP) [253-255]. AD2 recruits histone methyl transferases (HMTs) such as protein arginine N-methyltransferase 1 (PRMT1) and co-activator-associated arginine methyltransferase 1 (CARM1) important for chromatin remodelling [256-258]. The large complexes formed between SRC-1 and its co-factors typically contain 6 - 10 stably associated proteins with many additional less stably-bound proteins [259]. These large co-activator complexes not only act as enzymes for chromatin remodelling, but also as a bridge between the DNA-binding TFs and the general transcription machinery, in order to activate gene transcription.
Figure 3-1. Molecular structure of SRC-1 and its mediation of NRs. SRC-1 has three structural domains; its C-terminal has a basic helix-loop-helix-Per/Ah receptor nuclear translocation/Sim (bHLH/PAS) motif and AD3 domain important in recruiting the chromatin remodellers, SWI/SNF. SRC-1 interacts with NRs via 3 LXXLL motifs in its NRID domain. AD1 and AD2 in its N-terminal recruits CBP/p300, PRMT1 and CARM1 to restructure the chromatin. The large co-factor complex facilitates interaction with the general transcription factors (GTFs) to activate transcription. Figure adapted and modified from Xu et al., 2001.

The concept of a master gene was first proposed in 1969 by Britten and Davidson. They theorised that a subgroup of ‘master genes’ existed which regulated the expression of subservient genes, designated ‘producer genes’ [260]. In the modern era, we have come to know these as ‘effector genes’. Co-activators, including SRC-1, fit the criteria of a master gene/regulator. It has the capacity to bind across different families of transcription factors, in multiple tissue types and regulate the expression of various genes inducing a myriad of physiological actions. As master regulators influence the transcriptional activity of several TFs, they bring about vast genome-wide effects on gene networks and therefore have the ability to significantly contribute to a broad spectrum of physiological diseases. Some of the non-NR TFs that SRC-1 has been shown to interact with include HOXC11, PEA3, AP1, ETS2, and MYB [128, 213, 215, 217, 247]. These TFs promote distinct aspects of tumour progression by activation of effector genes including S100β, TWIST, ITGA5, MMP9 and ADAM22, [128, 213, 215, 217, 247].

In summary, SRC-1 is considered as an important modulator of endocrine resistance in breast cancer. It is a master regulator that mediates pro-tumourigenic effects by co-activation of a range of TFs. As previously described, dysregulated TF
signalling promotes multiple aspects of tumour progression. Moreover, dysregulated TFs often become the core dependencies on which cancer cells rely to promote their survival. Therefore, this study set out to perform comprehensive network analysis of SRC-1 where data generated from SRC-1 RNA-sequencing and ChIP-sequencing alongside SRC-1 RIME data was systematically mined for well characterised TFs using a suite of bioinformatics tools. This approach facilitates the comprehensive understanding of SRC-1, its interacting TFs and downstream targets with their role in driving endocrine resistance in breast cancer.

3.2. Aim
Identify the SRC 1 regulated transcriptome and interacting partners in endocrine resistant breast cancer.
3.3. Results

3.3.1. The SRC-1 transcriptome in an endocrine resistant breast cancer model

To define the SRC-1-regulated transcriptome in a cell line model of endocrine resistant breast cancer, RNA-sequencing was carried out in tamoxifen-treated LY2 cells with stable knockdown of SRC-1 expression and a non-targeting control. 3,226 genes were established as differentially expressed based on SRC-1 expression (shSRC-1 vs shNT; log2FC ≥ 1.2; adj. p-value < 0.01). Of these, 1,731 genes were identified as being positively regulated by the presence of SRC-1 and 1,495 genes were down-regulated by SRC-1 expression. As SRC-1 is primarily known as an activator of gene expression, the genes positively regulated by SRC-1 were prioritised (Appendix I). The list of positively regulated genes was stratified for the presence of TFs and chromatin remodelers using the FANTOM consortium database which identified 153 TFs and/or chromatin remodelers. This list was further refined using HOCOMOCO to identify TFs with a known position weight matrix (PWMs) (n=37) in order to determine probabilistic TF binding [261]. These 37 TFs/chromatin remodelers represent well characterised, SRC-1 targets important for investigation as downstream mediators of endocrine resistance. In addition to these 37, a further three TFs/chromatin remodelers were included in the primary panel based on a characterised role in tumourigenesis (DEK, SMARCA1 and TRPS1) (Figure 3-2). Detailed information on the 40 TF / chromatin remodeler structure and function can be found in Appendix I Table 8.4.
Figure 3-2. Identification of SRC-1 regulated transcription factors. RNA-sequencing was carried out on LY2 shNT and LY2 shSRC-1 cells that had been steroid depleted for 72 hours and treated with tamoxifen $10^{-7}$ M for 8 hours. 1,731 genes were found to be positively enriched by SRC-1 ($\log_{2} FC \geq 1.2$; adj. p-value < 0.01). The FANTOM consortium tool was utilised to identify 153 TFs/chromatin remodelers. HOCOMOCO identified 37 of these TFs to have well characterised PWMs. 3 additional TFs/chromatin remodelers were included based on characterised tumour promoting properties.
3.3.2. SRC-1 transcriptional target validation

To identify biologically and measurably robust SRC-1 regulated TFs/chromatin remodelers (herein shortened to SRC-1-regulated TFs), a set of complementary SRC-1 knockdown experiments were designed. As per the original RNA-sequencing experiments, LY2 shNT and shSRC-1 cells were steroid depleted for 72 hours followed by 8 hours of tamoxifen treatment. Of the 40 TFs, 14 were selected for validation. The effect of SRC-1 knockdown on the transcript of 14 target genes was investigated by qPCR. SRC-1 mRNA expression was observed to be reduced by approximately 50%. The effect of this resulted in a significant decrease in E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1, TRPS1 and FOSL1 mRNA. However, EPAS1, TEAD1, ELK4, EGR1, KLF4, and TFAP2A mRNA was not consistent with the RNA-sequencing (Figure 3-3A).

To test these findings by means of an alternative method, a siRNA against SRC-1 was transfected into endocrine resistant LY2 cells that had been steroid depleted 72 hours and tamoxifen treated for 8 hours. In this model, SRC-1 mRNA was decreased by more than 70%. E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 mRNA expression followed that of SRC-1 and was significantly decreased. However, EPAS1, TEAD1, ELK4, EGR1, KLF4, TFAP2A and FOSL1 were not consistent with RNA-sequencing (Figure 3-3B). For functional investigation, the genes that were taken forward had to have the same direction of differential expression in RNA-sequencing and both downstream qPCR analyses. Moreover, gene expression fold change, as assessed by qPCR must be greater than 1.2. Therefore, E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 (as highlighted by the green box; Figure 3-3) were brought forward for functional validation as mediators of endocrine resistance and its associated phenotypes.
Figure 3-3. Validation of SRC-1-regulated transcription factors. (A) LY2 shSRC-1 and shNT cells were steroid depleted for 72 hours followed by 8 hour tamoxifen $1 \times 10^{-7}$ M treatment. RNA was extracted from the cells and qPCR analysis was performed. (B) LY2 cells were steroid depleted for 72 hours and transiently transfected with siSRC-1 or siScramble. 24 hours post transfection; cells were treated with tamoxifen $1 \times 10^{-7}$ M for 8 hours. For both (A) and (B), relative quantification was assessed by the ΔΔCT method in comparison to siScramble control with β-actin as a housekeeping gene. Results are expressed as the mean of three independent replicates and error bars represent the S.E.M. *p<0.05 in comparison to shNT or siScramble. The genes taken forward are highlighted by the green box.
3.3.3. Direct SRC-1 regulation of transcription factors

Having observed SRC-1 expression was capable of altering downstream TF expression, whether this was a direct regulation was yet unknown. Using previously generated ChIP-sequencing data, the genome binding of SRC-1 in vehicle and tamoxifen treated LY2 cells was first queried [247]. The immediate upstream area (≤500bp) of the TFs first exon was considered the proximal promoter region. The presence of a CpG island was further used as an indicator of true promoter status [262]. SRC-1 binding peaks in addition to CpG islands were observed in the promoter region of all TFs. To ensure that these TFs were direct targets of SRC-1-associated regulatory machinery, primers were designed within the binding peak regions. ChIP of SRC-1-associated DNA was then carried out in tamoxifen-treated LY2 cells and analysed by qPCR. This demonstrated all seven candidate TFs had recruitment of SRC-1 over IgG control at their promoters (Figure 3-4B). These ChIP analyses along with the original mRNA findings confirm that E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 are directly regulated at the transcript level by SRC-1-regulated transcriptional complexes.
Figure 3-4. Direct SRC-1 regulation of transcription factors. (A) UCSC genome browser images of SRC-1 binding peaks at E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 promoters identified from ChIP-sequencing of SRC-1 in untreated and tamoxifen treated LY2 cells. (B) ChIP-sequencing results were validated by ChIP-qPCR of SRC-1 in tamoxifen 1 x 10^{-7} M treated LY2 cells. Data represents the average of four independent replicates with SRC-1 recruitment relative to IgG recruitment and error bars representing S.E.M. *p<0.05.
3.3.4. SRC-1 cofactors in endocrine resistance

RIME was carried out in tamoxifen treated LY2 cells with the aim of identifying SRC-1 cofactors in a cell line model of breast cancer endocrine resistance. This experiment was performed in collaboration with Dr. A. Redmond (Cambridge, Cancer Research, U.K.). RIME for endogenous SRC-1 was performed in triplicate while an IgG control was performed in duplicate. LY2 cells were deprived of steroid for 72 hours then transcription was induced by the brief addition of tamoxifen for 45 minutes. Only SRC-1-associated cofactors which occurred in all three replicates, and were not present in IgG control, were considered. This resulted in 148 SRC-1-associated proteins with SRC-1 itself being one of most confident proteins identified (Figure 3-5). Functional annotation of these proteins was carried out using DAVID. Of the 148 SRC-1 interacting proteins identified, 146 had a main molecular function of binding; mainly, protein and DNA/RNA binding. While 65 were additionally involved in enzymatic activity.
Figure 3-5. SRC-1 cofactors in endocrine resistant breast cancer. RIME analysis of SRC-1 co-factors in LY2 cells was visualised as an MS arc plot. SRC-1-associated proteins were clustered and coloured based on molecular function identified from DAVID. Blue arc represents the hierarchical ‘binding’ term, while the yellow arc represents hierarchical ‘enzymatic’ activity.
3.3.5. Identification of SRC-1 cofactor transcription factors

While SRC-1 is considered a master regulator of gene expression, it does not have a known DNA-binding domain and therefore does not bind directly to the DNA of its target genes. Rather, SRC-1 recruits additional transcriptional machinery in order to regulate gene expression. Therefore, to identify potential TFs which may be utilised by SRC-1 to execute transcriptional activation of target genes, the list of SRC-1 interacting proteins identified from SRC-1 RIME in LY2 cells was probed for TFs. Using MotifDB in R, the 148 SRC-1 interacting proteins were searched against multiple TF databases to characterise those proteins which are known TFs. 27 TFs were identified. TFs were stratified based on PWMs to identify genes that bind to the promoter of SRC-1 target genes identified in section 3.3.3. Of the 27 TFs, 9 had defined PWMs. Using the programme FIMO, the DNA sequence 10 kbp upstream of the transcriptional start site (TSS) was scanned for the occurrence of motifs of any of the nine SRC-1 interacting TFs (Figure 3-6A). 3 TFs, H2AFZ, STAT1 and H1FX, were found to have PWMs in the promoter/enhancer regions of several of SRC-1-regulated TFs. Only STAT1 had binding motif present upstream of all seven SRC-1-regulated TFs (Figure 3-6B).
Figure 3-6. SRC-1-interacting transcription factors. (A) Flow-through diagram of the selection process for SRC-1-interacting TFs with known PWMs. 148 SRC-1 interacting proteins were characterised for known TFs, 27 TFs were identified of which 9 had defined PWMs which were input to FIMO (B) The sequence-based motif analysis tool, FIMO, was used to scan the 10 kB DNA sequence upstream of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 for probable PWM binding of H2AFZ (purple), STAT1 (blue) and H1FX (yellow). PWM graphic adapted from Davies et al., 2007 [263]
3.3.6. SRC-1-STAT1 protein interaction validation

To validate the results observed from SRC-1 RIME in LY2 cells, a non-crossed-linked Co-IP was performed. To replicate the experimental conditions of RIME, LY2 cells were steroid depleted for 72 hours then treated with tamoxifen for 45 minutes. SRC-1 was immunoprecipitated from LY2 cells and STAT1 protein expression was analysed by western blot. The reverse Co-IP was performed by immunoprecipitating STAT1 from tamoxifen treated LY2 cells and examining SRC-1 protein expression by western blot. Confirmation of a successful IP was performed by blotting for the relevant IP antibody (Figure 3-7A and B).

![Image](image.png)

**Figure 3-7. STAT1-SRC-1 protein interactions.** (A) LY2 cells were steroid depleted for 72 hours then treated with tamoxifen $1 \times 10^{-7}$ M for 45 minutes. SRC-1 was immunoprecipitated and pull down was confirmed by western blot analysis. Interaction of STAT1 with SRC-1 was confirmed by probing the SRC-1 IP for STAT1 protein expression. (B) The reverse IP was carried out whereby STAT1 was immunoprecipitated first from LY2 cells and confirmed by western blot analysis. Interaction of SRC-1 with STAT1 was confirmed by probing the STAT1 IP for SRC-1 protein expression. IgG was used as non-specific binding control. IPs and western blot images represent three independent experiments.
3.4. Discussion

Here, a comprehensive set of TFs upregulated by SRC-1 expression in an endocrine resistant breast cancer setting were identified. Building on these findings, additional RIME analysis characterised the DNA-binding partners of SRC-1 facilitating this regulation. These data provide a comprehensive description of SRC-1’s regulatory network mediating the endocrine resistant phenotype.

SRC-1 is one of the master regulators of the endocrine resistant state and RNA-sequencing of endocrine resistant LY2 cells showed that SRC-1 is responsible for the differential expression of 3,226 genes. 1,731 genes were established as positively regulated by SRC-1, of which 153 were classed as TFs. Therefore, a substantial number of transcriptional regulators are in turn controlled by SRC-1 expression. A bioinformatics pipeline paired with literature review defined 40 of these 153 TFs as likely to generate insight into the SRC-1 regulatory network that drives endocrine resistance. Many tumour cells are considered to be ‘addicted’ to transcriptional programmes that activate pathways required for their survival [151]. Therefore, identifying these core transcriptional programmes would provide deeper understanding into the mechanisms driving endocrine resistance.

Identifying SRC-1-regulated TFs that were biologically and technically robust was important as discordance between RNA-sequencing and subsequent qPCR gene expression assays is not uncommon [264]. A representative subset of 14 TFs were brought forward for downstream analysis. Of these 14, seven (E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1) were carried forward due to their robust differential expression.

Next, direct regulation of these TFs by SRC-1 was established by utilising in-house ChIP-seqencing data and further by ChIP-qPCR. Significant recruitment of SRC-1 to each TF promoter suggests a direct regulation by SRC-1 rather than a knock-on downstream transcriptional effect.

As SRC-1 does not have a known DNA binding domain (DBD), the TFs it employs to regulate gene expression in endocrine resistant breast cancer was investigated. 148 SRC-1 interacting partners were identified. Upon functional annotation of the SRC-1 interacting partners, binding and catalytic activity were the two most enriched
molecular functions. This outcome was not unexpected as SRC-1 is known to complex with TFs which are involved in nucleic acid binding and also with co-activators that have enzymatic activity required for efficient transcriptional activation [265]. This further reinforces SRC-1’s key role in the modulation of gene expression. Of those identified, only PRMT1 and VCP had been previously established as SRC-1 interactors [266]. PRMT1 was first identified to interact with SRC-1 in ovarian cancer cells while, VCP was identified as an SRC-1 interactor in HeLa cells [259]. This suggests a novel SRC-1 interactome in endocrine resistant breast cancer.

Interestingly, the ER was not one of the TFs identified by RIME as interacting with SRC-1, suggesting an ER independent network in this setting. In addition, in a RIME of ER in untreated endocrine sensitive breast cancer cells, SRC-1 was not identified as one of its interactors [231]. Previous studies identified SRC-1-ER binding in an endocrine sensitive breast cancer model was dependent on oestrogen [267]. In light of this, it is tempting to speculate that in endocrine resistant breast cancer, in the presence of tamoxifen, SRC-1 may preferentially modulate other TFs. Moreover, in a clinical study of endocrine resistant breast cancers, SRC-1 protein expression was not correlated with ERα [126]. Together these findings suggest an ER, and therefore oestrogen, independent role for SRC-1 in endocrine resistant breast cancer.

Of the 148 SRC-1 interactors, 27 were TFs. STAT1 was identified as the only TF that had a PWM upstream of all 7 TFs validated from RNA-sequencing. This suggests that STAT1 is the most common regulator of SRC-1-regulated TFs. Immuno-pull down assays confirmed the interaction between SRC-1 and STAT1 observed in the RIME study. While SRC-1 has been reported to interact with STAT3, STAT6 and STAT5 in hepatoma and cervical cancer, respectively, this is the first description of a SRC-1-STAT1 interaction [268-270]. Although initially termed a tumour suppressor, STAT1 has a dual role and evidence for tumour promoting functions have now been described [271]. Elevated STAT1 levels in various cancers correlate with good prognosis [271-273]. Mechanisms of STAT1 tumour suppression include inhibition of cell cycle and promoting apoptosis [274]. In contrast, in breast carcinomas increased activation of STAT1 was detected in compared to normal breast tissue [275]. Elevated levels of STAT1 were reported in aggressive, invasive breast carcinoma compared to a
non-invasive counterpart [276] and high STAT1 expression was linked to poor disease free survival in breast cancer patients [277]. The mechanisms of STAT1 mediated tumour progression are many and varied due to its modulation of diverse cellular processes [271]. STAT1 promotes tumour cell growth and invasion, evasion of the immune system and confers resistance against both local and systemic cancer therapies [271]. The exact mechanisms of how STAT1 switches from a tumour suppressor to an oncogene are not yet established. However, an interesting study of STAT1 in lymphoma identified that, BAL1, modulates different isoforms of STAT1 and regulates altered STAT1 downstream signalling pathways thereby promoting the switch in STAT1’s tumour role [278]. The mechanisms of promoter- and isoform-specific modulation of STAT1 by BAL1 remain to be elucidated. Based on this, STAT1 appears to be a likely candidate for SRC-1’s modulation of disease progression and metastasis.

In summary, the results describe a hub of novel SRC-1-regulated transcription factor targets in endocrine resistant breast cancer (E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1). SRC-1’s regulation of this hub is potentially through complexing with its newly identified transcriptional partner, STAT1.
4. Investigating the role of SRC-1 interactors in the expression of SRC-1-regulated transcription factors.
4.1. Introduction

SRC-1 is primarily characterised as a co-activator of the ER. When oestrogen is bound to the ER, SRC-1 is recruited to its LBD wherein it activates the AF2 domain thereby mediating ligand-dependent ER transcriptional activation \[279\]. SRC-1 simultaneously recruits co-activators such as histone-acetyltransferases (HATs; PRMT1 and CARM1) to enhance gene transcription by decondensing the chromatin \[280\]. While ER’s AF1 domain is primarily involved in ligand-independent signalling, the large co-activator complex formed by SRC-1 facilitates AF1 activation. Synergy between the two AF domains facilitates maximal ER transcriptional activity \[281\]. Upon tamoxifen binding to ER, its AF2 domain is blocked thereby inhibiting ligand-dependent ER transcriptional activation. Interestingly, in the presence of tamoxifen binding, SRC-1 has the ability to directly interact with the AF1 domain and mediate oestrogen-independent signalling \[282\]. As such SRC-1 contributes to the selective agonistic properties of tamoxifen. As described in the previous section, RIME data indicated an absence of ER-SRC-1 binding. To confirm ER independence in this network, the involvement of ER in regulating the TFs identified previously (E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1) will be investigated.

Chapter 3 highlighted STAT1 as a promising candidate for the DNA-binding subunit of the SRC-1 complex. STAT1 was confirmed as interacting with SRC-1 in this model of endocrine resistance. Moreover, network analysis demonstrated the probability of STAT1 binding upstream of each SRC-1-regulated TF. Combined with its recently identified role in promoting tumour progression, investigating the role of STAT1 in this SRC-1-regulated complex is compelling \[271\].

Co-activators like SRC-1 function within highly heterogeneous multimers integrating TFs and chromatin remodellers. These multi-subunit complexes are not static entities \[283\]. They represent a complex machine that is subject to dynamic rearrangements, with many enzymatic activities that together execute the co-activator’s final endeavour; to express a particular set of genes conducive to a phenotypic effect. The nuance of these co-regulator complexes means there are vast possibilities of complex formations depending on the cell type, activation pathway, TF, co-regulator and even post-translational modifications \[283\]. Fluidity in the
composition of transcriptional complexes is an important concept, in which redundant recruitment of TFs to an individual gene is found to confer robustness in its expression [284-287]. In this endocrine resistant setting, the composition of the SRC-1 complex must contribute to the tumours ability to activate transcriptional expression and facilitate survival in oestrogen-independent conditions.

Therefore, this chapter centres on identifying the TF partner of SRC-1 involved in activating the expression of SRC-1-regulated TFs.

**4.2. Aims**

i. To investigate the role of ER, if any, in the activation of SRC-1-regulated TFs

ii. To investigate STAT1 binding at SRC-1-regulated TFs promoters

iii. To examine if STAT1 is necessary for the activation of SRC-1-regulated TFs

iv. To investigate an SRC-1-STAT-1 complex at SRC-1-regulated TF promoters

v. To determine the role of SRC-1 and STAT1 in oestrogen insensitivity.
4.3. Results

4.3.1. Role of 17β-oestradiol in activating transcription of SRC-1-regulated transcription factors

RIME data in the previous chapter indicated a lack of SRC-1-ER binding in this setting. To confirm this finding, the role of ER and oestrogen signalling in activating SRC-1-regulated TFs was investigated. Analysis of ER ChIP-sequencing data in tamoxifen treated LY2 cells reported ER occupancy at the promoter of SRC-1-regulated TFs. The UCSC genome browser was used to visualise the ER peaks present at each SRC-1-regulated TF (Figure 4-1A). To validate the ER ChIP-sequencing observations, ER ChIP-qPCR was performed in LY2 cells after 72 hours steroid depletion followed by 45 minutes tamoxifen treatment. qPCR analysis confirmed significant occupancy of the ER at the promoter region of all seven SRC-1-regulated TFs (Figure 4-1B). Having confirmed ER occupancy at the promoter region of each SRC-1-regulated TF, whether ligand activation of ER promotes their transcription was next queried. LY2 cells were therefore treated with 17β-oestradiol and examined SRC-1-regulated TF mRNA expression by qPCR. No significant changes in mRNA expression of SRC-1-regulated TFs were observed with 17β-oestradiol treatment. Of note, mRNA levels of known ER target gene, TFF1, were queried as a positive control and were found to be driven 3-fold by 17β-oestradiol treatment (Figure 4-1C).
Figure 4-1. ER is recruited to the promoter of SRC-1-regulated transcription factors.  

(A) UCSC genome browser views of ER ChIP-seq profiles in tamoxifen treated LY2 cells. Binding peaks represent ER occupancy at the promoter region of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1. (B) LY2 cells were steroid depleted for 72 hours, treated with tamoxifen 1 x 10\(^{-7}\) M for 45 minutes and ER-associated chromatin was immunoprecipitated. qPCR analysis quantified ER occupancy at the promoter of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 relative to IgG. Recruitment to the TFF1 promoter was included as a positive control. Data represents the average of four independent replicates, normalised to IgG, with error bars demonstrating the S.E.M. *p<0.05.  

(C) LY2 cells were steroid depleted for 72 hours then treated with 17β-oestradiol 1 x 10\(^{-8}\) M for 8 hours. Total RNA was extracted and relative mRNA levels of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 were analysed by qPCR for alterations in expression. TFF1 mRNA expression was investigated as a positive control. Data shown was relative to β-actin expression and represents the average of three independent experiments. Error bars represent the S.E.M. *p<0.05 in comparison to vehicle.
4.3.2. Role of the oestrogen receptor in activating transcription of SRC-1-regulated transcription factors.

Despite ER occupancy at the promoter region of the seven SRC-1-regulated TFs, a lack of transcriptional response to oestrogen signalling was observed. To further investigate this, a knockdown experiment was utilised to examine if ER is involved in activating the transcription of SRC-1-regulated TFs. Despite a significant reduction in ESR1 transcript levels, there was no observable effect on mRNA expression of SRC-1-regulated TFs (Figure 4-2A).

Furthermore, microarray analysis of gene expression in LY2 cells following ER degradation with the SERD ICI182780 (fulvestrant) was conducted previously in our lab. To further validate our findings, this dataset was mined for changes in TFs (E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1) gene expression. No differential expression was observed in SRC-1-regulated TFs gene expression following treatment with ICI182780. (Figure 4-2B). ER and oestrogen signalling are respectively, neither necessary nor sufficient, to drive the expression of SRC-1-regulated TFs.
Figure 4-2. SRC-1-regulated transcription factors are independent of ER transcription. (A) LY2 cells were cultured in steroid depleted media for 48 hours prior to transient transfection with siScramble or siESR1. 24 hours post transfection; cells were treated with tamoxifen $1 \times 10^{-7}$ M for 8 hours. Total RNA extracted and relative mRNA levels of ER, E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 were analysed by qPCR for alterations in expression. Data shown was relative to β-actin expression and represents the average of four independent experiments. Error bars represent the S.E.M. *p<0.05 in comparison to siScramble. (B) The expression of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 was investigated in a gene expression microarray dataset of LY2 cells treated with the SERD ICI 182780 (Fulvestrant). Data shown is from four biological replicates that have been log2-transformed and quantile normalised.
4.3.3. STAT1 occupancy at SRC-1-regulated transcription factor promoters

Following the motif occurrence analysis of SRC-1-regulated TF enhancer/promoter sequences identifying potential binding motifs for STAT1, it was necessary to confirm this association in our model of endocrine resistance. To this end, a ChIP-qPCR experiment was carried out for STAT1 in LY2 cells with the same experimental conditions as that reported for the SRC-1 ChIP-qPCR (i.e. steroid depleted for 72 hours followed by 45 minutes of tamoxifen treatment). The results showed increased STAT1 occupancy at the promoter region of all seven SRC-1-regulated TFs over IgG (Figure 4-3).

![Graph showing relative STAT1 recruitment normalized to IgG](image)

**Figure 4-3. STAT1 occupies SRC-1-regulated transcription factor promoters.** LY2 cells were steroid depleted for 72 hours, treated with tamoxifen $1 \times 10^{-7}$ M for 45 minutes and STAT1-associated chromatin was immunoprecipitated. qPCR analysis quantified STAT1 occupancy at the promoter of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 relative to IgG negative control. Data represents the average of four independent replicates, normalised to IgG, with error bars depicting the S.E.M. *p<0.05.
4.3.4. SRC-1 and STAT1 complex at SRC-1-regulated transcription factor promoters

To confirm the presence of a SRC-1-STAT1 complex at the promoter region of SRC-1-regulated TFs, a sequential ChIP (or ChIP re-ChIP) was performed in cross-linked, tamoxifen-treated LY2 cells. Significant co-occupancy of SRC-1 and STAT1 at the promoter regions of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 was confirmed by qPCR analysis. This finding was consistent regardless of which protein was bait, first SRC-1 bait then re-ChIP for STAT1 (Figure 4-4A), or STAT1 bait and re-ChIP for SRC-1 (Figure 4-4B).

Figure 4-4. SRC-1 and STAT1 co-occupy SRC-1-regulated transcription factor promoters. SRC-1 was immunoprecipitated from cross-linked tamoxifen-treated LY2 cells. The complex was eluted and from this an immunoprecipitation of STAT1 was performed. (B) STAT1 was immunoprecipitated from cross-linked tamoxifen-treated LY2 cells. The complex was eluted and from this an immunoprecipitation of SRC-1 was performed. qPCR analysis confirmed co-occupancy of STAT1 and SRC-1 at SRC-1-regulated TF promoters. Data represents three independent replicates, relative to IgG recruitment. Error bars depict S.E.M. *p<0.05.
4.3.5. Role of STAT1 in activating SRC-1-regulated transcription factors

ChIP-qPCR analysis demonstrated significant STAT1-SRC-1 co-occupancy at the SRC-1-regulated TFs. To investigate if this corresponds to STAT1 being required for SRC-1-regulated TF expression, a STAT1 knockdown experiment was carried out. LY2 cells were transfected with siRNA against STAT1 and its effect on SRC-1-regulated TF mRNA expression was measured by qPCR. The results demonstrated that knockdown of STAT1 does not significantly alter SRC-1-regulated TFs transcript levels (Figure 4-5).

![Graph showing mRNA expression levels](image)

**Figure 4-5. STAT1 is not required for SRC-1-regulated transcription factor expression.** LY2 cells were steroid depleted for 72 hours; during which cells were transfected with a siRNA against STAT1. Total RNA was extracted and qPCR was used to analyse relative STAT1, E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 mRNA expression. β-actin was utilised as a housekeeping gene. Results are expressed as the mean of three independent replicates and error bars represent the S.E.M. *p<0.05 in comparison to siScramble.
4.3.6. Role of SRC-1 in STAT1 occupancy at transcription factor promoters

A two-pronged approach was utilised to investigate the apparent redundancy in the SRC-1-STAT1 complex binding to SRC-1-regulated TF promoters. Firstly, STAT1 was immunoprecipitated from LY2 shSRC-1 cells and compared to that in control cells (shNT). STAT1 was found to significantly occupy the promoters of SRC-1-regulated TFs in the shNT control. Confirmation of SRC-1 protein knockdown was confirmed in Figure 4-6A. In the absence of SRC-1 protein expression, STAT1 occupancy at TF promoters was severely impaired (Figure 4-6B). Next, STAT1 was transiently knocked down in LY2 cells and SRC-1 was immunoprecipitated. Confirmation of STAT1 protein knockdown confirmed in Figure 4-6C. Consistent with our previous finding (Figure 3-4B), SRC-1 was shown to be significantly recruited to SRC-1-regulated TF promoters (Figure 4-6D). The STAT1 knockdown demonstrated a decrease in recruitment to SRC-1-regulated TF promoters however, the results did not reach statistical significance (siScramble vs siSTAT1: E2F7 p=0.07, NFIA p=0.06, DEK p=0.1, SMAD2 p=0.08, SMARCA1 p=0.06, ASCL1 p=0.11, TRPS1 p=0.06)
Figure 4-6. SRC-1 is required for STAT1 occupancy at SRC-1-regulated transcription factor promoters. (A) Confirmation of SRC-1 knockdown as assessed by western blot (n=3), TATA-box binding protein (TBP) used as a loading control. (B) LY2 shNT and shSRC-1 cells were steroid depleted for 72 hours. Cells were then treated with tamoxifen $1 \times 10^{-7}$ M for 45 minutes, cross-linked and STAT1-associated chromatin was immunoprecipitated. qPCR analysis quantified STAT1 occupancy at the promoter of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 in LY2 shNT and shSRC1 cells. Data represents the average of four independent replicates, normalised to IgG, with error bars demonstrating the S.E.M. *p<0.05. (C) Confirmation of STAT1 knockdown as assessed by western blot (n=3). TBP used as a loading control. (D) LY2 cells were transfected with siScramble and siSTAT1 then steroid depleted for 72 hours. Cells were then treated with tamoxifen $1 \times 10^{-7}$ M for 45 minutes, cross-linked and SRC-1-associated chromatin was immunoprecipitated. qPCR analysis quantified SRC-1 recruitment to the promoter of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 in LY2 siScramble and siSTAT1 cells. Data represents the average of four independent replicates, normalised to IgG, with error bars demonstrating the S.E.M. *p<0.05.
4.3.7. Role of SRC-1 and STAT1 in promoting insensitivity to oestrogen signalling

Despite the recruitment of ER to SRC-1-regulated TF promoters, ER and oestrogen were observed to be neither necessary nor sufficient, to drive the transcription of SRC-1-regulated TFs (Section 4.3.1).

We therefore next investigated if SRC-1 and STAT1 mediate oestrogen-independent signalling of these TFs. To test this, LY2 cells were removed from tamoxifen, SRC-1 and STAT1 were individually knocked down and cells were treated with oestrogen to see if the cells would revert to a suspected ‘pre-treatment’ mode of ER-oestrogen driven transcription of the SRC-1-regulated TFs. A significant increase in transcript levels of all seven SRC-1-regulated TFs following oestrogen treatment in the absence of either SRC-1 or STAT1 (Figure 4-7A and B) was observed.
Figure 4-7. SRC-1-regulated transcription factors respond to oestrogen in the absence of SRC-1 or STAT1. LY2 cells were steroid depleted for 48 hours then transiently transfected with siScramble, siSRC-1 (A) or siSTAT1 (B). 24 hours post transfection, all cells were treated with 17β-oestradiol 1 x 10^{-8} M for 8 hours, harvested, total RNA extracted and relative mRNA levels of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 were analysed by qPCR for alterations in expression. Data shown is relative to β-actin expression and represents the average of three independent experiments. Error bars represent the S.E.M. *p<0.05 in comparison to siScramble.
4.4. Discussion

SRC-1 does not contain a known DNA-binding domain. Therefore, in order to regulate gene expression of downstream effector targets, it requires interaction with a DNA-binding factor. The focus of this chapter was to elucidate the SRC-1 transcriptional complex responsible for regulating newly identified target TFs.

RIME data from this study indicated SRC-1 may not bind to ER in the endocrine resistant setting. On further investigation, using in-house ER ChIP-sequencing data of tamoxifen treated LY2 cells, ER binding peaks were observed at the same promoter region as SRC-1. This was an unexpected finding which was validated by ChIP-qPCR. However, in comparison to that of a known ER target gene, TFF1, the recruitment to SRC-1-regulated TFs was low. Despite ER binding, a lack of transcriptional activation of SRC-1-regulated TFs was observed in the presence of oestrogen. This suggests that although ER binds to SRC-1-regulated TF promoters, oestrogen is not required for their expression in an endocrine resistant model. Whether the ER was required for the transcription of these TFs was next investigated. Both siRNA ESR1 knockdown studies and ICI182780 (fulvestrant) data analysis of LY2s revealed ER is not required for the transcription of SRC-1-regulated. Therefore, it was important to explore alternative mechanisms of SRC-1 regulation.

RIME identified the TF STAT1 as an SRC-1 interacting protein and this interaction was validated by Co-IP. While SRC-1 has been reported to interact with STAT3, STAT6 and STAT5 in hepatoma and cervical cancer, respectively, this is the first description of a SRC-1-STAT1 interaction [268-270]. Next, STAT1 was demonstrated to occupy the promoter of each SRC-1-regulated TF. Moreover, the existence of an SRC-1-STAT1 complex at SRC-1-regulated TF promoters was established. Following this, STAT1’s transcriptional regulation of SRC-1-regulated TFs was investigated. Surprisingly, STAT1 was found not to be required for the expression of SRC-1-regulated TFs. This implied that other SRC-1 interactors may be recruited, redundantly, in order to transcribe these specific SRC-1-regulated TFs. Therefore, the recruitment of SRC-1 and STAT1 to SRC-1-regulated TF promoters was examined. Knockdown of SRC-1 protein was observed to impair the occupancy of STAT1 at SRC-1-regulated TF promoters. This suggests that STAT1 occupancy at SRC-1-regulated TFs
is dependent on SRC-1. Conversely, knockdown of STAT1 protein did not significantly prevent SRC-1 recruitment to SRC-1-regulated TF promoter. This result indicates redundancy in SRC-1’s transcriptional partners in regulating these TFs.

The SRC-1-STAT-1 complex was shown to circumvent the need for oestrogen signalling to induce the expression of SRC-1-regulated TFs. Endocrine resistant cells treated with oestrogen showed no further increase in expression of SRC-1-regulated TFs. However, when SRC-1 or STAT1 are knocked down, the expression of these genes increases in response to oestrogen indicating that SRC-1 and STAT1 are important for regulating oestrogen independent transcriptional networks in endocrine resistant breast cancer.

To conclude, in this chapter SRC-1 was found to partner with STAT1 allowing initiation of expression of downstream transcriptional networks. Moreover, the removal of either SRC-1 or STAT1 from this setting introduces TF response to oestrogen signalling.
5. SRC-1-regulated transcription factors promote an endocrine resistant phenotype.
5.1. Introduction

The different mechanisms of endocrine resistance have been discussed in detail. However, irrespective of the type of mechanism, we can define endocrine resistance with one key trait; the ability of a tumour to grow in the presence of endocrine therapy. Endocrine resistant tumours are inextricably linked with having a high metastatic potential. Broadly, metastasis can be categorised into three phases. Firstly, local migration and invasion of the cancer cells to the blood supply. Next, anchorage-independent survival in the blood supply followed by colonisation in distant organs. These cells should have sufficient malignant potential to generate secondary tumours. Therefore, it is suspected that tumour initiating CTCs (undifferentiated breast cancer cells) are responsible for tumour initiation and regrowth of secondary tumours at distant sites.

SRC-1 knockout mouse models have demonstrated a clear role for SRC-1 in breast cancer metastasis [131, 132]. In these models, SRC-1 plays a role in cellular migration and extravasation. SRC-1 expressing mice had increased levels of colony stimulating factor 1 and macrophage recruitment, which in turn stimulate tumour cell migration [131]. Additionally, 83% of mice expressing SRC-1 had detectable CTCs compared with 17% of SRC-1-null mice [131]. Colonization is evidently also augmented by SRC-1 with 85% of SRC-1-expressing mice having metastases compared to 9.5% of SRC-1-null mice [131].

In vitro investigations of SRC-1 have demonstrated a clear role of SRC-1 in breast cancer tumour progression. In an AI-resistant breast cancer cell line, SRC-1 was shown to regulate tumour cell migration and differentiation [213]. In the poorly differentiated MDA-MB-231 breast cancer cell line, SRC-1 inhibition was found to significantly reduce tumoursphere formation [288]. Moreover, reduced SRC-1 expression was found to resensitise tamoxifen-resistant cells to the inhibitory effects of tamoxifen [128]. Unpublished work (carried out by Dr.S.Charmsaz and E.Ward) leading into this research demonstrated that, in vitro, the specific metastatic phenotypes of the endocrine resistance cell line LY2 are in part driven by SRC-1 expression. The proliferative capacity of LY2 cells in the presence of tamoxifen treatment was reduced with decreased SRC-1 expression (Figure 5-1A). The superior
anoikis resistance observed in LY2 cells compared to MCF-7 cells, was reduced with decreased SRC-1 expression and tamoxifen treatment (Figure 5-1B). Reduced SRC-1 expression decreased the number of second generation mammospheres formed by tamoxifen treated LY2 cells (Figure 5-1C). Moreover, the ability to form well-organised mammary acinar structures was repressed by the presence of SRC-1 in tamoxifen treated conditions (Figure 5-1D). Finally, the number of putative stem-like cells (CD24+/CD44+) in tamoxifen treated LY2 cell population was decreased in the absence of SRC-1 expression (Figure 5-1E).

Figure 5-1. SRC-1 regulates an endocrine resistant phenotype. (A) Proliferation of LY2 cells following transfection with siScramble and siSRC-1 treated with tamoxifen 1 x 10^{-7}M. Normalised to siScramble. (B) Anchorage independent growth of tamoxifen 1 x 10^{-7}M treated MCF-7 cells compared with LY2 shNT and LY2 shSRC-1 cells. (C) Primary (P1) and secondary (P2) generation of tamoxifen 1 x 10^{-7}M treated mammospheres formed by LY2 shNT and LY2 shSRC-1. (D) Mammary acini formation of LY2 cells following transfection with siScramble and siSRC-1 treated with tamoxifen 1 x 10^{-7}M. (E) Putative stem cell markers (CD24+/CD44+) of LY2 shNT and shSRC-1 treated with tamoxifen 1 x 10^{-7}M analysed by flow cytometry. All data shown represents the average of three independent experiments. Error bars represent the S.E.M., *p<0.05.
Evident from the aforementioned studies, SRC-1 regulates multiple phenotypes of endocrine resistant breast cancer. Based on the SRC-1 complex and network analysis, it was postulated that the SRC-1-regulated TFs identified in Chapter 3 (E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1) mediate these aggressive endocrine resistant phenotypes.

5.2. Aim
Investigate the role of SRC-1-regulated TFs in tumour progression
5.3. Results

5.3.1. SRC-1 and SRC-1-regulated transcription factor expression in endocrine resistant breast cancer cell lines

The identification of SRC-1-regulated TFs in a cell line model of endocrine resistance prompted the investigation of whether their expression was closely associated with this model (tamoxifen resistant) or whether it was reproducible across multiple models, including AI resistant. Therefore, the expression of SRC-1 and SRC-1-regulated TFs was examined in the endocrine sensitive MCF-7 cell line; the tamoxifen resistant LY2 and TamR cell lines, and furthermore in the AI resistant, LetR cells. All cell lines were cultured in serum free media for 24 hours followed by culture in their own respective media for 24 hours, then harvested. qPCR analysis of mRNA expression found SRC-1 and all SRC-1-regulated TFs to be significantly increased in the tamoxifen resistant LY2 cell line when compared to a sensitive counterpart, MCF-7s (Figure 5-2A). Overall, this followed through to the additional model of tamoxifen resistance where SRC-1, E2F7, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 had elevated transcript levels. Surprisingly, NFIA mRNA was found to be significantly decreased in TamR cells (Figure 5-2B). The role of SRC-1-regulated TFs in the model of AI resistance was substantially less. Only three of the seven SRC-1-regulated TFs (E2F7, NFIA and DEK) had increased mRNA expression in the AI-resistant cell line, LetR, while there was no significant alterations of SMAD2, SMARCA1, ASCL1 or TRPS1 (Figure 5-2C).
Figure 5-2. SRC-1 and SRC-1-regulated transcription factors are highly expressed in endocrine resistant breast cancer cell lines. 

MCF7 (A) LY2, (B) TamR and (C) LetR cells were synced by growing in serum free media for 24 hours followed by 24 hours of culture in their own respective media. Total RNA was extracted and analysed using qPCR for mRNA expression of SRC-1, E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1. Expression was normalised to the expression of β-Actin. All data shown represents the average of four independent experiments. Error bars represent the S.E.M. *p<0.05 in comparison to MCF-7 expression.
5.3.2. SRC-1-regulated transcription factors role in cellular viability in endocrine resistant breast cancer

Having demonstrated the correlation between SRC-1-regulated TF expression and an endocrine resistance model, their role in an endocrine resistant phenotype was subsequently examined. To this end, siRNA against each SRC-1-regulated TF was used to individually knockdown expression of each TF in tamoxifen-treated endocrine resistant LY2 cells. Confirmation of reduced mRNA expression of each SRC-1-regulated TF following siRNA transfection was assessed by qPCR (Figure 5-3A). Post siRNA transfection, cell viability assay demonstrated that reduced expression of E2F7, NFIA, DEK, SMAD2, ASCL1 and TRPS1 decreased the proliferation rate of the LY2 cells. E2F7 and NFIA had the most pronounced effects while, SMARCA1 expression had no significant effect on cellular viability/proliferation (Figure 5-3B).
Figure 5-3. SRC-1-regulated transcription factors promote cellular viability in endocrine resistant breast cancer. (A) LY2 cells were cultured in steroid depleted conditions for 48 hours prior to transient transfection with siScramble, siE2F7, siNFIA, siDEK, siSMAD2, siSMARCA1, siASCL1 or siTRPS1 RNA. 24 hours post transfection, cells were treated with tamoxifen $1 \times 10^{-7}$M for 8 hours, harvested, total RNA was extracted and relative mRNA levels of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 were analysed by qPCR for mRNA knockdown. Expression was normalised to mRNA level of β-Actin. (B) 24 hours post transient transfection, cells were seeded into a 96 well plate and treated with tamoxifen $1 \times 10^{-7}$M for 5 days before assessing cellular viability with MTS reagent. Measurements were normalised to siScramble. All data shown represent the average of three independent experiments. Error bars represent the S.E.M. *p<0.05 in comparison to siScramble.
5.3.3. SRC-1-regulated transcription factors role in cellular motility and anoikis resistance in endocrine resistant breast cancer

The role of SRC-1-regulated TFs in the migratory capacity of LY2 cells was next assessed by SRC-1-regulated TF knockdown in a migration assay utilising a collagen and fluorescent bead coating. The average cell movement for 24 hours was significantly decreased upon reduced E2F7, NFIA, SMAD2, SMARCA1, ASCL1 and TRPS1 expression (Figure 5-4A and B).

Using an agarose assay, the ability of SRC-1-regulated TFs to alter anchorage independent growth was assessed. Following transient transfection, cells were allowed to propagate in suspension in agarose for 14 days. Decreased expression of SMAD2, SMARCA1, ASCL1, TRPS1, and to a lesser extent NFIA, significantly reduced the ability of LY2 cells to grow in anchorage-independent conditions. While reduced E2F7 had no significant effect on colony formation, reduced DEK expression was found to increase the number of colonies growing in agarose (Figure 5-4C).
Figure 5-4. SRC-1-regulated transcription factors promote cellular motility and anoikis resistance in endocrine resistant breast cancer. (A) 24 hours post transient transfection, cells were seeded onto a fluorescent bead/collagen layer and cultured in the presence of tamoxifen for 24 hours. Cells were fixed, stained and their movement through the fluorescent beads was manually tracked on a light microscope using DAPI and TRITC filters. (B) Representative images of the migration patterns formed by LY2 siScramble, siE2F7 siNFIA, siDEK, siSMAD2, siSMARCA1, siASCL1 and siTRPS1. Red scale bar corresponds to 100µm. (C) 24 hours post transient transfection, cells were seeded into 3% agarose and cultured in the presence of tamoxifen $1 \times 10^{-7}$M for 14 days. Colonies greater than 50 µM were counted. Data shown was normalised to siScramble and represents the average of three independent experiments. Error bars represent the S.E.M. *p<0.05 in comparison to siScramble
5.3.4. SRC-1-regulated transcription factors role in mammosphere formation in endocrine resistant breast cancer

Based on the observations that E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 promote the activity of an endocrine resistant phenotype, the ability of SRC-1-regulated TFs in altering the stem cell-like characteristics of LY2 cells was investigated. Stem cells and CSCs alike are principally defined by their ability to self-renew and differentiate, and as such, are likely responsible for the initiation and out-growth of metastatic tumours at a distant site.

Initially the ability of the SRC-1-regulated TFs to affect self-renewal of the LY2 cells was examined. Reducing the expression of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 by siRNA knockdown, decreased the numbers of mammospheres formed in non-adherent conditions (Figure 5-5A) and this was further verified with reduced numbers of second generation mammospheres formed (Figure 5-5B). Overall, reduced expression of E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 resulted in a significant decrease of the self-renewal potential of LY2 cells (Figure 5-5C).
Figure 5-5. SRC-1-regulated transcription factors promote mammosphere formation of endocrine resistant breast cancer cells. (A) 24 hours post transient transfection, cells were seeded into ultra-low adherence plates and cultured in the presence of tamoxifen $1 \times 10^{-7}$ M for 5 days. Mammospheres greater than 50 µM were counted using a light microscope and MFE assessed. (B) Cells from (A) were passaged to produce a single cell suspension and seeded at the same density as the 1st generation in ultra-low adherence plates and cultured in the presence of tamoxifen $1 \times 10^{-7}$ M for 5 days. Mammospheres greater than 50 µM were counted and MFE assessed. Data for both generations was normalised to siScramble. (C) The self-renewing capabilities of LY2 cells following siRNA knockdown was assessed by dividing the number of mammospheres formed in the 2nd generation by the number formed in the first generation. All data shown represents the average of three independent experiments. Error bars represent the S.E.M. *p<0.05 in comparison to siScramble.
5.3.5. SRC-1-regulated transcription factors role in undifferentiated endocrine resistant breast cancer

Loss of expression of SRC-1-regulated TFs reduced the self-renewal capacity of the endocrine resistant LY2 cells as seen from the mammosphere assay. Such loss of self-renewal is often associated with a gain of differentiation. Therefore, the effect of SRC-1-regulated TFs on the differentiation status of these endocrine resistant cells was investigated via protein markers of dedifferentiation and functionally utilising the acini assay.

First, flow cytometry assessment of the differentiation markers CD24 and CD44 was investigated. CD24+/CD44- populations were significantly increased upon knockdown of E2F7, NFIA, SMAD2, SMARCA1 and ASCL1 (Figure 5-6A). Moreover, CD24-/CD44+ showed a significant decrease in this population following knockdown of all TFs, varying from 39%-69% (siDEK and siSMAD2, respectively) (Figure 5-6B).

Next, the ability of these TFs to promote the more undifferentiated phenotype of the endocrine resistant LY2 cells was assessed by an acini forming assay. While knockdown of TFs in LY2 cells did not produce fully mature acini with hollow lumen, their reduced expression promoted increased cellular differentiation. Acini had a superior polarisation (most notably in siSMAD2) with a tendency towards a hollow lumen, compared to that noted in parental LY2 cells (Figure 5-6C).
Figure 5-6. SRC-1-regulated transcription factors promote an undifferentiated phenotype in endocrine resistant breast cancer cells. 24 hours post transient transfection, LY2 cells were treated with tamoxifen $1 \times 10^{-7}$ M for 48 hours. Cells were stained for expression of CD24 and CD44 which was analysed by flow cytometry. (A) CD24$^+$/CD44$^-$ populations and (B) CD24$^-$/CD44$^+$ populations. Cell populations were normalised to that in siScramble. Data shown represents the average of three independent experiments. Error bars represent the S.E.M. *$p<0.05$ in comparison to siScramble. (C) 24 hours post transient transfection, cells were seeded onto a basement membrane matrix and cultured in the presence of tamoxifen $1 \times 10^{-7}$ M for 21 days. Acini were then immunostained for F-actin (red) and the nuclear compartment (blue) and imaged on a confocal microscope. Cross-section images above are representative of the acini formed over three independent experiments. Images taken at 20X. Red scale bar corresponds to 50 µm.
5.4. Discussion

Multiple phenotypes of a cancer cell allow it to evade endocrine therapy and metastasise.

Firstly, the endogenous expression of SRC-1 and SRC-1-regulated TFs in endocrine sensitive and resistant breast cancer cell lines was investigated. SRC-1 was found to be elevated in all three models of endocrine resistance (tamoxifen resistant: LY2 and TamR, and AI resistant: LetR). The overall increased expression of all SRC-1-regulated TFs in tamoxifen resistant cell lines was not replicated in the AI resistant model. This is not wholly surprising as different mechanisms may be at play in AI resistance. Of interest, where other studies have linked several of the SRC-1-regulated TFs to aggressive disease, only E2F7 has been directly associated with tamoxifen resistance [289]. DEK, is highly expressed in breast cancer cell lines while no expression was found in normal breast tissue [290]. TRPS1 is increased in luminal B and basal cell lines compared to luminal A [291]. SMARCA1 expression was moderately increased in aggressive breast cancer compared to non-aggressive counterparts [292]. In contrast, SMAD2 expression was similar across many breast cancer cell lines [293]. This was the first time NFIA and ASCL1 expression has been investigated in breast cancer cell lines.

The role of SRC-1-regulated TFs in breast cancer cellular viability was next examined. Cellular viability is indicative of cellular proliferation and survival. In this study, with the exception of SMARCA1, decreased expression of all SRC-1-regulated TFs reduced the viability of the resistant LY2 cells. This is consistent with others’ findings that E2F7, DEK, SMAD2, SMARCA1 and TRPS1 affect cellular viability in different models of breast cancer [289, 290, 292, 294, 295]. While, this is the first description of a role for NFIA and ASCL1 in breast cancer cellular viability, they have known roles in glioblastoma and lung adenocarcinoma proliferation, respectively [296, 297].

Cellular migration is an essential initial step of the metastatic cascade. It requires a precise balance of signalling cues co-ordinating interactions of adhesion molecules and rearrangement of the cytoskeleton. In this study, the migratory capacity of LY2 cells was observed to be reduced significantly (50% or more) with
decreased expression of all SRC-1-regulated TFs. A role for DEK and SMAD2 in breast cancer cellular migration has been previously described [290, 294]. While NFIA and ASCL1 have defined roles in glioblastoma and lung adenocarcinoma cancer cell migration, respectively, this is the first description of a role in breast cancer cell migration [296, 297]. Moreover, a role for E2F7, SMARCA1 and TRPS1 in cancer cell migration has not been previously described. These data suggest that the SRC-1-regulated TF hub clearly supports the initial steps for metastasis in endocrine resistant breast cancer.

As anchorage-independent growth is a key feature of anoikis resistance, the role of SRC-1-regulated TFs in promoting this metastatic characteristic was investigated. Decreased expression of NFIA, SMAD2, SMARCA1, ASCL1 and TRPS1 was observed to significantly reduce anchorage-independent growth of LY2 cells. E2F7 expression appears to not be necessary, and DEK expression is in fact a hindrance. There is no previous documented role for E2F7, SMARCA1 and TRPS1 in anchorage independent growth of tumour cells. In contrast, NFIA, SMAD2 and ASCL1 were found to promote anchorage independent growth of glioma and lung cancer cells, respectively [298-300]. In contrast to our findings, DEK over-expression in normal human keratinocytes promoted colony formation in soft agar indicating transformation potential [301]. This study provides evidence of a role for each SRC-1-regulated TF in promoting anchorage independent growth in breast cancer. Based on observations from this study, several SRC-1-regulated TFs provide this pre-requisite for the metastatic spread of cancer cells.

As with normal tissues, the cells of a breast tumour are thought to have hierarchical organisation [161]. At the top of this hierarchy are stem cells that play important roles in tumour progression. The presence of CTCs allows the tumour to persist even if the differentiated tumour cells have been eradicated by therapy. Furthermore, once CTCs eventually reach distant organs, these cells must have sufficient malignant potential to generate secondary tumours. Measuring the self-renewal capacity of endocrine resistant cells is one method of assessing their stem cell-like properties.
The role of SRC-1-regulated TFs in endowing self-renewal properties to endocrine resistant breast cancer cells was assessed. A significant decrease in primary and secondary mammosphere formation was observed with reduced SRC-1-regulated TF expression. Each of SRC-1-regulated TFs were demonstrated to regulate both mammosphere formation and self-renewal. While this phenomenon was previously demonstrated by DEK in MCF-7 cells [290] this is the first time a role for each of the other SRC-1-regulated TFs has been described in breast cancer cell self-renewal.

The next assessment of a stem cell-like phenotype was to examine the cell surface markers of endocrine resistant breast cancer cells. The combination CD24 and CD44 is employed in the identification of breast CSCs. This originated from a founding paper that identified the CD24−/CD44+ population of breast cancer cells display stem-cell like characteristics and exclusively retain tumorigenic potential [302]. Upon decreased expression of all SRC-1-regulated TFs, the stem-like population (CD24−/CD44+) of LY2s was significantly decreased with a concurrent increase in the differentiated population (CD24+/CD44−). These data suggest that expression of the SRC-1-regulated TFs in endocrine resistant breast cancer cells promotes the expression of the undifferentiated cell population.

As a final assessment of tumour cell differentiation, the contribution of SRC-1-regulated TFs to the formation of mammary acini was examined. The differentiation status of a tumour denotes its aggressiveness. Clinically, poor differentiation is associated with high tumour grade and a worse prognosis [303]. Well-differentiated epithelial luminal cells should naturally recapitulate the well-ordered mammary architecture as is in vivo [223, 304]. Endocrine resistant LY2 cells form unorganised, undifferentiated 3D structures with no indication of mammary acini structure. Here, gene silencing of SRC-1-regulated TFs, resulted in more differentiated mammary acini. While DEK over-expression in breast cancer cells has previously been documented to enhance this hyperplastic phenotype [305], this is the first documentation of E2F7, NFIA, SMAD2, SMARCA1, ASCL1 and TRPS1 expression promoting undifferentiated acini. Overall, the association of these SRC-1-regulated TFs with poorly differentiated tumours indicates a propensity towards high grade tumours which clinically have a worse prognosis [303].
In summary, six out of seven SRC-1-regulated TFs were shown to contribute significantly to the proliferative ability of endocrine resistant cells. E2F7 and NFIA were clearly the strongest drivers of proliferation. Loss of expression of all SRC-1-regulated TFs had a dramatic effect on the cell’s migratory capacity. Moreover, SMAD2, SMARCA1, ASC1 and TRPS1 were found to be the key drivers of anchorage-independent growth. Lastly, all SRC-1-regulated TFs played an important role in the stem-like features of this endocrine resistant model. (Figure 5-7).

Taken together, in this chapter, the existence of a transcriptional hub under the regulation of SRC-1 driving the phenotypes associated with endocrine resistant breast cancer and malignant tumour progression was identified. However, in a similar fashion to SRC-1, SRC-1-regulated TFs are unlikely to be direct effectors of these phenotypes. Therefore, to understand the SRC-1 regulated molecular network driving endocrine resistance it is necessary to examine the effector genes downstream of the SRC-1-regulated TFs.
Figure 5-7. Key SRC-1-regulated transcription factors in promoting tumour progression. Overview model of the involvement of SRC-1-regulated TFs at each stage of tumour progression. Beginning at the left hand side, the model shows the primary tumour cells migrating through breast stroma. Cells then extravasate and survive in the circulatory system until colonisation at a distant metastatic site represented on the right hand side. Most specific roles for SRC-1-regulated TFs were observed in proliferation and anoikis resistance while other aspects of tumour progression were more sensitive to disruption of any SRC-1-regulated TF e.g. migration.
6. SRC-1-regulated transcription factors and effector target genes show clinical significance in endocrine resistant breast cancer.
6.1. Introduction

The role of SRC-1-regulated TFs in the tumorigenic and malignant progression of endocrine resistant breast cancer cells has been established. We know that tightly regulated transcriptional control is crucial in normal cellular processes, therefore, the impact of alterations in gene expression due to dysregulated TFs in cancer can be vast. Identifying if alterations in the expression of these core SRC-1-regulated TFs exist and elucidating their downstream effector targets is therefore important. A bioinformatics approach and PDX model was employed to investigate this.

Now, more than ever, researchers have generated massive genomic datasets and mining these datasets to identify biologically significant targets is highly useful. iRegulon is a bioinformatics package, powered by huge repositories of publicly available sequencing data, with the capability to identify potential downstream effector targets of SRC-1-regulated TFs. By integrating predicted targets with in-house RNA-sequencing data, genuine downstream effector targets of SRC-1-regulated TFs may be identified. By cross-referencing these effector targets with publically available functional annotation tools (Cytoscape ClueGo; gene ontology visualisation) in addition to our functional data presented here, a comprehensive SRC-1-driven model of an endocrine resistant breast cancer phenotype would be described. This model can then be analysed for clinical significance against in-house and public, patient datasets.

Gene expression profiling has reformed our understanding of breast cancer. Prognostic and predictive tools based on gene expression patterns have been indispensable in the treatment of breast cancer. The seminal study by Perou et al. was the first use of molecular gene expression profiling in breast cancer [306]. Their pam50 gene expression signature was espoused by Prosigna to provide a risk of recurrence for intrinsic tumour subtypes [307]. Moreover, OncotypeDX® uses the expression of 21 genes (16 cancer-related, 5 reference) to predict a patients’ 10-year risk of recurrence and to assess the benefit of adjuvant chemotherapy [308, 309]. Likewise, Mammaprint uses a 70-gene signature to define a patients’ prognosis, and to determine the benefit of adjuvant chemotherapy [310]. While the traditional pathological and clinical features of a breast tumour are a mainstay in
guiding treatment choice, this can occasionally lead to over use of cytotoxic chemotherapy. The use of multigene signatures can more precisely stratify at risk patients. The discovery of additional signalling networks and gene expression patterns associated with a tumour's oncogenic dependency provides the possibility to exploit the tumours' vulnerability via targeted therapy. All the while, overlaps between newly identified signalling networks and current predictive arrays provides further explanation and context for their predictive power.

We have also utilised a patient derived xenograft (PDX) model to examine the impact of alterations in gene expression. This is a superior precursor model compared to cell line derived xenografts; to pre-clinical interventional studies. As CDXs are propagated artificially, the tendency towards genetic or phenotypic drift is higher than PDX, where artificial culture is by-passed [311]. PDX models are an invaluable platform to elucidate tumour progression. They are a clinically relevant model to investigate therapeutic responsiveness and gene expression dynamics in tumour progression. PDXs can provide important evidence to validate in vitro information.

In this chapter, through mining in-house and publicly available data-sets, and investigating PDX models of endocrine resistance, an SRC-1-driven network that contributes to the many stages of malignant progression was observed.

6.2. Aims

i. To investigate the association between SRC-1-regulated TF and their effector targets with disease-free survival in breast cancer patients

ii. To identify the effector target genes of the greater SRC-1 regulated network.
6.3. Results

6.3.1. In Vivo expression of SRC-1-regulated transcription factors

Through in vitro work, the SRC-1-driven network was demonstrated to have functional effects on many aspects of the metastatic cascade. Next, a PDX model of ER positive breast cancer was utilised to examine the clinical relevance of the SRC-1-regulated transcriptional network. Tumour tissue from one endocrine sensitive (primary tumour), and two endocrine resistant (lung and liver metastases) patients, was expanded in NodSCID mice (Details Figure 6-1A). Expression of SRC-1, its co-regulator STAT1, together with SRC-1-regulated TFs, E2F7, NFIA, SMAD2 and ASCL1, was examined in these samples by IHC (Figure 6-1C). (As E2F7, NFIA, SMAD2 and ASCL1 have defined PWMs for subsequent network analysis, this core hub of SRC-1-regulated TFs are the focus of Chapter 6). The PDX tumour displayed maintained biomarker status (ER, PR and Ki67) with that of the patient primary tumour from which they were derived (Figure 6-1B). SRC-1, NFIA and ASCL1 protein expression was observed in both resistant PDXs while no expression was detected in the sensitive PDX tumour. STAT1 and E2F7 protein expression was found across all PDXs. However, this expression was more substantial in the resistant tumours. Finally, SMAD2 protein expression was detected in only one metastatic tumour and not in the sensitive primary.
Table 6.1. Patient derived xenograft (PDX) details

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<td>IDC Stage 3</td>
<td>IDC Stage 3</td>
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</tr>
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</tr>
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<td>Yes</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>Liver and Bone</td>
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<tr>
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<td>Metastatic Lung</td>
<td>Metastatic Liver</td>
</tr>
<tr>
<td>Time to grow to 1cm in NODSCID</td>
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<td>6 months</td>
<td>4 months</td>
</tr>
<tr>
<td>PDX PR status</td>
<td>Positive</td>
<td>Positive</td>
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</tr>
<tr>
<td>PDX HER2 status</td>
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</tr>
</tbody>
</table>

Figure 6-1. SRC-1, STAT1, E2F7, NFIA, SMAD2 and ASCL1 are expressed in endocrine resistant patients in vivo. (A) Table of characteristics describing details of patient history along with tumour details. Darker shade section of the table relates to PDX details. (B) Immunohistochemical analysis of biomarker, ER, PR, and ki76, expression in the sensitive primary tumour and two resistant metastases. (C) Expression of SRC-1, STAT1, E2F7, NFIA, SMAD2 and ASCL1 in the sensitive primary tumour and two resistant metastases assessed by immunohistochemistry. Images were taken at 20X, scale bars in red correspond to 50 μm. Zoomed images at 40X.
6.3.2. SRC-1, STAT1 and SRC-1-regulated transcription factor expression in patient data

This SRC-1-regulated network was identified and interrogated in a cell line model of treatment resistant breast cancer. Discovery work in cell line models is an integral part of molecular cancer research. However, what is found in 2D/3D in vitro cell culture may not always recapitulate what occurs in vivo. Therefore, it was important to transition to patient data to query their expression and relevance. In the previous chapter the expression of SRC-1-regulated TF mRNA transcript was observed to be increased in the endocrine resistant models in contrast to those of endocrine responsiveness. Here, in-house RNA-sequencing data from patient tumours was utilised to examine SRC-1-regulated TF expression in vivo. Three primary tumours which responded well to endocrine therapy and had no record of disease relapse were compared to three primary tumours that relapsed while on treatment, and their resulting matched local recurrences. These patients were ER+/HER+ (Appendix I Table 8.7). The expression of SRC-1, STAT1 and 4 SRC-1-regulated TFs was relatively low in the 3 ‘good’ primaries. Yet, in 2 of the 3 ‘poor’ primaries and their matched local recurrences, a significant increase in SRC-1, STAT1 and SRC-1-regulated TF expression was observed. Of note, no expression of ASCL1 was detected in these breast samples with the exception of one metastasis. The web application Heatmapper was then used to visualise the RNA-sequencing expression counts (Figure 6-2).
Figure 6-2. SRC-1, STAT1 and SRC-1-regulated transcription factors are expressed in poor prognosis patient tumours. The expression of SRC-1, STAT1, E2F7, NFIA, SMAD2 and ASCL1 was examined in 9 patient samples; 3 primary tumours with good response to endocrine therapy (i.e. no relapse to date), 3 samples with poor response to endocrine therapy (i.e. relapsed on therapy) and their 3 matched local recurrences. The web application Heatmapper was used to visualise the RNA-sequencing expression counts.
6.3.3. Expression of SRC-1-regulated transcription factors in breast cancer subtypes

As indicated in the introduction, the pam50 breast cancer subtypes each have their own clinical prognosis. An association of SRC-1-regulated TFs with a particular subtype may therefore indicate a correlation with patient outcome. Therefore, the expression of E2F7, NFIA, SMAD2 and ASCL1 in normal breast tissue and pam50 breast cancer subtypes was queried (by Dr. A. Fagan) using the TCGA dataset (Figure 6-3). E2F7 mRNA was relatively comparable between normal and tumour tissue. In contrast, NFIA mRNA was increased in all breast tumour subtypes (particularly, basal-like) in comparison to normal breast tissue. SMAD2 mRNA was equally expressed across normal and tumour tissue with the exception of increased expression in basal-like subtype. ASCL1 was lowly expressed across all breast samples except luminal B.

Figure 6-3. Expression of E2F7, NFIA, SMAD2 and ASCL1 in pam50 subtypes. mRNA expression of E2F7, NFIA, SMAD2 and ASCL1 was queried in the TCGA dataset of normal breast tissue and breast tumours of PAM50 subtype; luminal A, luminal B, HER2+, basal-like and normal-like. Data is expressed as log2 transformed.
6.3.4. Association of SRC-1-regulated transcription factors with disease survival

Indications from *in vitro* and bioinformatic investigations suggested, E2F7, NFIA, SMAD2 and ASCL1 were associated with tumour progression and disease recurrence. In order to examine the association of E2F7, NFIA, SMAD2 and ASCL1 expression with patient survival, publically available datasets were analysed via the web applications: KM Plotter and BreastMark. mRNA levels in ER positive, tamoxifen treated breast cancer patients were used to determine survival analysis (Figure 6-4). E2F7 expression was found to significantly associate with disease recurrence in both KM Plotter and BreastMark (*p*=0.037, *p*=0.0012416, respectively). NFIA and SMAD2 mRNA expression significantly associated with poor disease-free survival in KM plotter (*p*=0.013, *p*=0.037, respectively) but were found not significant in the BreastMark dataset. ASCL1 mRNA expression correlated with disease recurrence in the BreastMark datasets (*p*=0.0049793).

![Figure 6-4. E2F7, NFIA, SMAD2 and ASCL1 associated with poor prognosis in tamoxifen-treated patients. Kaplan-Meier plots of disease-free survival of ER positive, tamoxifen treated breast cancer patients. Expression of E2F7, NFIA, SMAD2 and ASCL1 investigated in two different databases, KM plotter (high expression in red, low expression in black) and BreastMark (high expression in blue, low expression in red). Log rank *p*<0.05 denoted by *.](image-url)
6.3.5. Identifying effector target genes of SRC-1-regulated transcription factors

Identification of core TFs involved in a perturbed gene network is paramount in establishing the regulatory context of oncogenic programs. An equally instrumental step is to uncover the effector target genes of such TFs, their expression and regulation, which is essential in multifaceted regulatory networks.

Here, iRegulon was used to map the effector target genes of SRC-1-regulated TFs. This work was carried out by N.Cosgrove and Dr.S.Charmsaz. E2F7, NFIA, SMAD2 and ASCL1 were found to have PWMs and were present in the iRegulon dataset. iRegulon analysis identified 201, 232, 211 and 205 effector target genes of E2F7, NFIA, SMAD2 and ASCL1, respectively. On merging these TF-target gene subnetworks, there were 761 genes in total. 79 of the effector target genes had an edge count of two or more (meaning they were regulated by two or more TFs) (Figure 6-5). The cell cycle regulators CDNK1A and CENPF were at the centre of this network, regulated by all 4 SRC-1-regulated TFs. These 79 TF-target genes were taken forward for functional enrichment analysis. Inspection of functional enrichment using ClueGo identified significant enrichment (adj.p<0.001) of 79 effector target genes in 44 pathways (Figure 6-6). These pathways were clustered based on the similarity of biological process terms to each other while maintaining their hierarchy according to the gene ontology. This clusters the specific pathways into a more general parent biological process term. Note, some pathways (e.g. cell cycle) are components of more than one hierarchical biological process and therefore appear more than once. Interestingly, within the top 10 functionally significant terms were positive regulation of cell cycle/proliferation and mitotic process, negative regulation of cell death, response to hormone and abiotic stimulus and cell activation.
Figure 6-5. SRC-1-regulated transcription factor effector target genes. Nodes of effector target genes enriched by E2F7 (201 genes) NFIA (232 genes), SMAD2 (211 genes) and ASCL1 (205 genes) expression as defined by iRegulon. Of the 761 total effector target genes, 79 have an ‘edge’ of 2 or more, are highlighted by the purple lasso.
Figure 6-6. Functional enrichment analysis of effector target genes. Functional enrichment analysis of the 79 effector target genes common to two or more TFs, was performed using ClueGo. Terms have been colour clustered based on their hierarchical gene ontology biological process parent term (highlighted in bold black font). The top axis represents the number of genes from the input (79) that are involved in each pathway. The number at the right-hand end of each row corresponds to the percentage of genes represented in that given term. The top 10 functionally enriched terms have been delineated.
6.3.6. SRC-1 regulation of effector target genes

iRegulon provided identification of the effector target genes of E2F7, NFIA, SMAD2 and ASCL1. However, it did not relay the type of regulation; whether these effector target genes are activated or repressed. Therefore, to add another layer of information to the network, regulation any of the effector target genes by SRC-1 was examined. To this end, the identified iRegulon effector target genes were mapped to the expression values from the LY2 shSRC-1-RNA-sequencing. Of all effector target genes identified, 81 were positively regulated by SRC-1 and 73 were repressed by SRC-1 (log2FC ≥1.2, p-value < 0.01) (Figure 6-7). Functional annotation of these genes lists was carried out using the functional annotation tool, DAVID. Analysis of genes activated by SRC-1 identified pathways in cancer, PI3K signalling and regulation of actin cytoskeleton. While analysis of molecular function revealed transcription factor activity, DNA binding and receptor binding. Biological processes such as cellular motility/migration, cell cycle/cell proliferation and gene transcription were involved in genes activated by SRC-1 (Figure 6-8A). On the contrary, analysis of genes repressed by SRC-1 expression identified pathways in cancer, HTLV-I infection and proteoglycans in cancer. The molecular functions of SRC-1 repressed genes included transferase activity, receptor binding and kinase activity. Finally, genes repressed by SRC-1 activity were found to have biological processes including epithelial cell differentiation, apoptosis and gene expression (Figure 6-8B).
Figure 6-7. SRC-1 regulation of effector target genes. The list of E2F7, NFIA, SMAD2 and ASCL1 effector target genes identified by iRegulon was cross-referenced against those genes identified as being regulated by SRC-1 from RNA-sequencing. 81 genes highlighted in red are activated by SRC-1 in the endocrine resistant LY2 cells. While 73 genes coloured in blue are repressed in the presence of SRC-1 in LY2 cells.
Figure 6-8. Functional annotation of effector target genes, as regulated by SRC-1. The genes identified as being up- or down-regulated based on SRC-1 expression were input into DAVID for functional annotation. The key pathways, molecular functions and biological processes were chosen for representation. (A) KEGG pathway analysis, molecular function and biological process of genes upregulated by SRC-1 in red. (B) KEGG pathway analysis, molecular function and biological process of genes repressed by SRC-1 in blue.
6.3.7. Association of CENPF and CDKN1A (p21) with disease survival

As CENPF and CDKN1A (the two common effector targets of all 4 SRC-1-regulated TFs) were also identified from cell line models of endocrine resistance, the clinical relevance of their expression was next investigated. To this end, the correlation of CENPF and CDKN1A expression with disease recurrence was examined using publically available datasets via the web applications: KM Plotter and BreastMark. mRNA levels in ER positive, tamoxifen treated breast cancer patients were used to determine survival analysis. High expression of CENPF was found to significantly correlate with poor disease-free survival in both datasets (KM plotter p=0.00075 BreastMark p=2.4418e-6) (Figure 6-9A), while high CDKN1A expression was significantly associated with improved disease-free survival in the KM plotter dataset (p=0.046) while approaching significance in the BreastMark cohort (p=0.0556) (Figure 6-9B). However, recalling SRC-1’s regulation of these genes (Figure 6-9C); repression of CDKN1A and activation of CENPF, the expression of the genes was further modelled based on inverse expression. Here, the high expression of activated CENPF and repressed CDKN1A was significantly associated with poor disease free survival in the both datasets (KM plotter p=0.012, BreastMark p=0.0011726) (Figure 6-9D.)
Figure 6-9. SRC-1-regulation of CENPF and CDKN1A promotes poor survival in tamoxifen-treated patients. Kaplan-Meier estimates of disease-free survival in ER positive, tamoxifen treated breast cancer patients according to CENPF (A) and CDKN1A (B) expression. (C) SRC-1’s regulation of CENPF (positive) and CDKN1A (negative) as identified from RNA-sequencing. (D) Kaplan-Meier estimates of disease-free survival in ER positive, tamoxifen treated breast cancer patients according to CENPF expression with inverse CDKN1A expression. Significant log rank value $p<0.05$ denoted by *. 
6.4. Discussion

In this chapter, the clinical relevance of the core hub of SRC-1-regulated TFs was investigated. Moreover, SRC-1’s extended network was identified by defining TF effector target genes. Firstly, the recapitulation of the SRC-1-regulated network in vivo via PDX model was an important finding. SRC-1, NFIA and ASCL1 were expressed exclusively in two models of endocrine resistance. STAT1 and E2F7 were expressed in all models but increased on resistance. SMAD2 was expressed in only one model of resistance which suggests that SMAD2 is not required for all mechanisms of endocrine resistance. In the future, pairing the expression of these SRC-1-regulated TFs with targeted treatment to investigate which therapies can mitigate the malignant progression driven by SRC-1 would be important. The PDX models explored in this chapter would be an optimal starting point for such pre-clinical studies.

Next, the effect of SRC-1-regulated TFs on patient outcome was investigated. Firstly, using in-house patient RNA-sequencing data, SRC-1, STAT1 and SRC-1-regulated TFs exhibited increased expression in primary tumours and metastases that responded poorly to endocrine therapy in comparison to those primaries that are sensitive to treatment, which was confirmed in all patients except one. Thus, as would be expected, this network evidently accounts for some but not all endocrine resistant mechanisms. Of note, these samples were high-risk (ER+HER2+), suggesting an association between SRC-1-regulated TF and poor prognosis.

Using TCGA mRNA data, the association of E2F7, NFIA, SMAD2 and ASCL1 with pam50 breast cancer subtypes was examined. NFIA and SMAD2 had the greatest association with a basal-like subtype, while ASCL1 expression associated with luminal b status. Both basal and luminal b type breast cancers have a poor prognosis [34], suggesting that NFIA, SMAD2 and ASCL1 expression is associated with poor outcome in patients. E2F7 expression was relatively unchanged across the subtypes but notably appeared to be lowest in basal cancers. Finally, survival analysis of breast cancer patients with high SRC-1-regulated TFs expression confirmed their association with poor clinical outcome in ER+ tamoxifen-treated patients.

Next, an endocrine resistant breast cancer gene regulatory network encompassing the SRC-1-regulated TFs and their direct effector target genes
(regulons) was established. This network analysis provides an insight into the regulatory circuits governing breast cancer. Furthermore, it offers insight for future identification of novel interventional targets. Upon merging the TF regulons, 79 genes that were common to two or more SRC-1-regulated TFs were identified. Functional annotation of these genes showed associations with cell cycle, pathways in cancer, apoptosis and differentiation, which are all important in tumour progression. Furthermore, regulation of cell cycle and response to abiotic stimulus were two terms with the greatest numbers of genes involved. The ability of cancer cells to regulate cell cycle and proliferate despite targeted treatment is a hallmark of resistant cancer. Likewise, the capacity to respond to abiotic stimulus suggests these cancer cells have the ability to adapt upon important changes in oxygen including hypoxia.

Continuing from this, pairing the regulon effector targets with the data generated from shSRC-1-RNA-sequencing, it allowed identification of effector genes that may be modulated by SRC-1-regulated TFs in this model of endocrine resistant breast cancer. Moreover, it adds a layer of regulation in terms of function. Functional annotation of these SRC-1 activated and repressed genes lead to some very interesting results. Activation of biological processes such as cellular motility and cell cycle/proliferation complement the functional data in Chapter 5. Indeed, there was an over-representation of cell cycle/proliferation genes regulated by E2F7. This was not surprising as knockdown of E2F7 was found to have a significant effect on cellular viability. Biological processes including epithelial cell differentiation and apoptosis were repressed by SRC-1 and further match the functional data from Chapter 5. The finding that PI3K signalling is activated in the SRC-1 network is of note as PI3K signalling has been suggested as an endocrine resistance mechanism [312-314]. Finally, expression of the two most common SRC-1-regulated TF effector targets, CENPF and CDKN1A, as regulated by SRC-1, demonstrated their expression associates with poor survival for ER+ tamoxifen treated patients.

In summary, through PDX models and bioinformatics analysis, the association of the SRC-1-driven network to treatment resistance and poor patient prognosis was demonstrated. At the core of the extended SRC-1-target gene network were genes found to be important in tumour progression and poor disease-free survival.
7. General Discussion
Breast cancer remains the most predominant cancer diagnosed in women and is the second leading cause of cancer-related mortality. Targeted therapy with tamoxifen has resulted in an estimated survival of over a million women worldwide [315]. Yet, despite this outstanding improvement in patient survival, resistance to endocrine treatment occurs in up to 40% of breast cancer patients [316]. Understanding the molecular mechanisms undermining the success of endocrine therapies would allow for advances in treatment.

To date, important information regarding SRC-1’s role in breast cancer tumour progression has been reported [118, 317]. These do not however, address the full potential of SRC-1 regulatory complexes and downstream effector targets. Full network analysis is required to understand the complexity and power of SRC-1 in promoting breast cancer progression. To this end, global analysis of the SRC-1 transcriptome and interactome in endocrine resistant breast cancer was performed. In line with its title of ‘master regulator’, 3,226 genes were observed to be differentially expressed based on SRC-1 expression (1731 up regulated, 1495 down regulated). As the misregulation of gene expression has an extensive impact on tumour progression, this work prioritised the investigation of TFs and chromatin remodelers. A core hub of 5 TFs (E2F7, NFIA, SMAD2, ASCL1 and TRPS1) and 2 chromatin remodelers (SMARCA1 and DEK) were validated as novel direct TF targets of SRC-1.

SRC-1 TFs and chromatin remodelers were confirmed as mediating key roles in tumour progression. Specifically, E2F7 and NFIA were found to play significant pro-proliferative roles which are important in the early growth of the primary tumour and colonisation of distant metastatic sites. SMAD2, SMARCA1, ASCL1 and TRPS1 were observed as key players in anchorage independence. Meanwhile, all TFs were found to play important roles in cellular migration and an undifferentiated phenotype. These observations reinforce the hypothesis that SRC-1 mediates its pro-metastatic activity via this network of TFs.

An important aim of this work was to identify the mechanism by which SRC-1 regulates downstream TF networks. RIME was therefore performed to identify SRC-1 interacting proteins. The signalling TF, STAT1, was observed to be recruited by, and
complex with, SRC-1 at the promoters of SRC-1-regulated TFs. Although interactions between SRC-1 and STAT3, STAT5 and STAT6 have been previously reported, this is the first time interactions between SRC-1-STAT1 have been described [268-270]. STAT3 and STAT5 are reported to be oncogenic whereas STAT1 is described as both a tumour suppressor and promoter [318]. In terms of endocrine resistance, both STAT3 and STAT5 have a defined role in growth factor mediated tumour progression [319]. Recently, expression and activation of STAT1 has been shown to be enhanced in endocrine resistance, leading to suggestions that STAT1 could represent a viable target in treatment resistant breast cancer [271].

Interestingly, in these studies, STAT1 was not required for the transcription of SRC-1-regulated TFs, which suggests a level of redundancy in SRC-1’s TF partners. Moreover, in this setting, both oestrogen and ER were found to be unnecessary in driving this endocrine resistant TF network. One of the most compelling findings of this work was SRC-1 and STAT1’s circumvention of oestrogen signalling. In the absence of SRC-1 or STAT1 expression, the pro-tumourigenic TFs were driven by oestrogen. Endocrine resistance can be viewed as, the tumours ability to re-express critical steroid targets upon inhibition of oestrogen signalling. This finding is central to SRC-1’s mediation of endocrine resistance; the ability to forgo its association with ER, seek out novel interacting partners and activate TFs that potentiate tumour progression.

The wide-reaching impact of SRC-1’s oncogenic signalling was investigated. Using a bioinformatic approach, the extended regulatory network of SRC-1 that drives the endocrine resistant phenotype was established. At the core of this signalling cascade were genes central to cell cycle, differentiation and pathways in cancer. Moreover, their expression was linked clinically to poor survival on endocrine treatment. Uncovering these downstream networks provides information on how master regulators like SRC-1 execute their function. A greater understanding of acquired resistance affords the opportunity to develop new drugs targeting the source of this dynamic process.

Importantly, this research highlights the significance of aberrant TF activity, from their deregulated expression to the knock-on myriad of mechanisms promoting tumour progression. For many years it was accepted, with the exception of NRs, that
TFs were notoriously difficult to target [320]. This is due to the fact that most TFs do not have cognate ligands. Fortunately, targeting TFs is no longer the task it once was thought to be. Direct and indirect TF inhibitors have recently been described in breast cancer [321]. In terms of the network identified in this study, STAT1 has two known, relatively untested inhibitors [322-324]. While STAT1 knockdown did not affect the transcription of SRC-1-regulated TFs, it did induce their activation via oestrogen signalling. In this setting, perhaps a STAT1 inhibitor combined with endocrine treatment would be successful. However, as STAT1 has both tumour-promoting and tumour-suppressive roles, further research is required as to what induces the switch in STAT1’s tumour role prior to targeting [278]. A more realistic option therefore, might be to target the co-factors that augment dysregulated TFs. Unfortunately, co-factors like SRC-1 are considered even harder to target than TFs. They too lack a LBD and moreover they have large, flexible structures [325]. In more recent years, however, these obstacles are being overcome with the development of SRC-1 inhibitors. In a ‘proof-of-principle’ study by Wang et al., targeting of SRC-1 with the small molecule inhibitor, gossypol, was the first successful inhibition of SRC-1 in breast cancer cells [326]. Despite this encouraging success, the high IC50 values of gossypol render it unsuitable for drug development. Following from this, the glycoside, bufalin, was next identified as successfully reducing SRC-1 expression in breast cancer cells. However, while the IC50 values of bufalin were in range, glycosides are associated with cardiac arrest making them clinically impractical [327]. To overcome this, a recent study describes SI-2, a synthetic compound, originally targeted at AIB1 but displays effective reduction of SRC-1 protein in vitro [328]. Based on its success in vitro, this first in class drug could potentially be expanded to treating breast cancer [288]. By targeting SRC-1, we could negate its dysregulated transcriptional programmes and downstream effector target potentiating breast cancer progression. Nonetheless, regardless of targeting SRC-1, the identification of its dysregulated TFs and downstream effector targets is invaluable for future therapeutic ventures.

Through this research, SRC-1 has been confirmed as a modulator of a large multi-tiered transcriptional response driving endocrine resistant breast cancer. Through functional in vitro assessment, it was shown that it does this through newly
identified SRC-1-regulated TFs, E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1. Along with the identification of the effector target genes from the core-hub, the network identified has the potential to provide more robust prognostic markers in the treatment of endocrine resistant breast cancer.

**Future Work**

Detailed analysis in this study identified a significant role for E2F7, NFIA, DEK, SMAD2, SMARCA1, ASCL1 and TRPS1 in promoting many aggressive phenotypes of an endocrine resistant breast cancer cell. For most of these TFs, this was the first report of having a role in driving breast cancer progression. Moreover, investigation of the core hub of TFs in patient datasets of endocrine resistance and metastasis further revealed their role in driving tumour progression. Therefore, expanding this work to larger patient cohorts (via tissue microarray) followed by mouse xenograft studies would be important to firmly establish this finding.

The SRC-1/STAT1 complex was identified as being important in regulating oestrogen-independent activation of downstream TFs. Furthermore, decreasing the expression of SRC-1 and STAT1 resensitised this transcriptional network to activation by oestrogen signalling. Therefore, future work investigating the inhibition of this complex would provide important information on therapeutic intervention. Dual inhibition, with both STAT1 and ER inhibitors, may mitigate the tumour promoting features of the transcriptional network.

The datasets generated here have paved the way for further modelling of SRC-1’s modulation of breast cancer progression. This current study primarily focused on the TFs downstream of SRC-1. However, the RIME analysis could be expanded to all genes differentially expressed in shSRC-1 RNA-seq dataset to identify ‘tier one’ genes directly regulated the SRC-1 transcriptional complexes followed by ‘tier 2’ genes regulated by the downstream SRC-1-regulated TFs. Moreover, these datasets have laid the foundations for further investigation into the mechanism by which the SRC-1-mediated complexes can transcriptionally regulate downstream targets. TFs, their associated co-regulators, chromatin remodellers and histone chaperones intertwine
to control the position and composition of nucleosomes. RIME identified the histone chaperone, H2AFZ, and the histone linker, H1FX, as complexing with SRC-1. Moreover, the ATPase dependent chromatin remodeller SMARCA1 and the DNA super-coiler, DEK, were transcriptionally regulated by SRC-1 expression. Further investigation of these chromatin remodelling complexes would provide insight into SRC-1’s mechanism of action.
References


141. Ezkurdia, I., et al., *Multiple evidence strands suggest that there may be as few as 19 000 human protein-coding genes*. Human Molecular Genetics, 2014. 23(22): p. 5866-5878.


8. Appendix I

Table 8.1. ChIP and reChIP buffers

<table>
<thead>
<tr>
<th>Lysis Buffer 1</th>
<th>Per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Hepes–KOH, pH 7.5</td>
<td>5 mL (of 1 M)</td>
</tr>
<tr>
<td>140 mM NaCl</td>
<td>2.8 mL (5 M)</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>0.2 mL (0.5 M)</td>
</tr>
<tr>
<td>10% Glycerol</td>
<td>20 mL (50 %)</td>
</tr>
<tr>
<td>0.5% NP-40/Igepal</td>
<td>5 mL (10 %)</td>
</tr>
<tr>
<td>0.25% Triton X-100</td>
<td>2.5 mL (10 %)</td>
</tr>
<tr>
<td>dH2O</td>
<td>64.5 mL</td>
</tr>
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</table>

*(Can store at 4 °C)*

<table>
<thead>
<tr>
<th>Lysis Buffer 2</th>
<th>Per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris–HCl, pH 8.0</td>
<td>1 mL (1 M)</td>
</tr>
<tr>
<td>200 mM NaCl</td>
<td>4 mL (5 M)</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>0.2 mL (0.5 M)</td>
</tr>
<tr>
<td>0.5 mM EGTA</td>
<td>0.1 mL (0.5 M)</td>
</tr>
<tr>
<td>dH2O</td>
<td>94.7 mL</td>
</tr>
</tbody>
</table>

*(Can store at 4 °C)*

<table>
<thead>
<tr>
<th>Elution Buffer</th>
<th>Per 50 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris-HCl pH 8.0</td>
<td>0.5 mL (1M)</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>0.1 mL (0.5 M)</td>
</tr>
<tr>
<td>1 % SDS</td>
<td>5 mL (10 %)</td>
</tr>
<tr>
<td>dH2O</td>
<td>44.35 mL</td>
</tr>
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*(Can store at 4 °C)*

<table>
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<tr>
<th>Re-ChIP Wash Buffer</th>
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</tr>
</thead>
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<tr>
<td>2 mM EDTA</td>
<td>0.4 mL (0.5M)</td>
</tr>
<tr>
<td>500 mM NaCl</td>
<td>10 mL (5 M)</td>
</tr>
<tr>
<td>0.1 % SDS</td>
<td>1 mL (10 %)</td>
</tr>
<tr>
<td>1 % NP40</td>
<td>10 mL (10 %)</td>
</tr>
<tr>
<td>dH2O</td>
<td>78.6 mL</td>
</tr>
</tbody>
</table>

*(Can store at 4 °C)*

<table>
<thead>
<tr>
<th>Re-ChIP Elution Buffer</th>
<th>Per 100 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TE</td>
<td>98 μL</td>
</tr>
<tr>
<td>2% SDS</td>
<td>2 μL (10%)</td>
</tr>
<tr>
<td>15 mM DTT</td>
<td>15 μL (0.1M)</td>
</tr>
<tr>
<td>PI (Roche)</td>
<td><em>(Supplement with PI), (Prepare fresh)</em></td>
</tr>
</tbody>
</table>

*(Can store at 4 °C)*

<table>
<thead>
<tr>
<th>ChIP Dilution Buffer</th>
<th>Per 100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 % SDS</td>
<td>0.1 mL (10 %)</td>
</tr>
<tr>
<td>1.1 % Triton X-100</td>
<td>11 mL (10 %)</td>
</tr>
<tr>
<td>1.2 mM EDTA</td>
<td>0.24mL (0.5M)</td>
</tr>
<tr>
<td>16.7 mM Tris-HCl pH 8.0</td>
<td>1.67 mL (1 M)</td>
</tr>
<tr>
<td>167 mM NaCl</td>
<td>3.34 mL (5 M)</td>
</tr>
<tr>
<td>dH2O</td>
<td>83.65 mL</td>
</tr>
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</table>

*(Supplement with PI + BSA), (Can store at 4 °C)*
Table 9.1. ChIP and reChIP buffers continued

<table>
<thead>
<tr>
<th>Buffer Description</th>
<th>Stock</th>
<th>Final Conc.</th>
<th>Vol. for 50ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM Hepes, pH 8.0</td>
<td>1M</td>
<td>50mM</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>500mM EDTA, pH 8.0</td>
<td>0.5M</td>
<td>1mM</td>
<td>100 µL</td>
</tr>
<tr>
<td>10% NP-40</td>
<td>10%</td>
<td>1.00%</td>
<td>5 mL</td>
</tr>
<tr>
<td>10% DOC</td>
<td>10%</td>
<td>0.70%</td>
<td>3.5 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td></td>
<td>34.8 mL</td>
</tr>
<tr>
<td>8M LiCl</td>
<td>8M</td>
<td>0.5M</td>
<td>3.125 mL</td>
</tr>
<tr>
<td>1 PIC Tablet in 2 mL dH₂O</td>
<td>50X</td>
<td>1X</td>
<td>1 mL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>50 mL</td>
</tr>
</tbody>
</table>

*(Prepare fresh)*

<table>
<thead>
<tr>
<th>Proteinase K Mix</th>
<th>1 Sample</th>
<th>10 Samples</th>
<th>20 Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TE</td>
<td>140 µL</td>
<td>1.4 mL</td>
<td>2.8 mL</td>
</tr>
<tr>
<td>10 mg/ml Glycogen</td>
<td>3 µL</td>
<td>30 µL</td>
<td>60 µL</td>
</tr>
<tr>
<td>Proteinase K 20mg/ml</td>
<td>7 µL</td>
<td>70 µL</td>
<td>140 µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>150 µL</td>
<td>1.5 µl</td>
<td>3 mL</td>
</tr>
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*(Prepare fresh)*

Table 8.2. Western blot buffers

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</thead>
<tbody>
<tr>
<td><strong>RIPA Lysis Buffer</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 % NP-40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% Na-deoxycholate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 % SDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM Tris, pH 8.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tris-buffered Saline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>121.1 g Tris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>175.5 g NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8.3</td>
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<td></td>
</tr>
<tr>
<td><strong>Running Buffer</strong></td>
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</tr>
<tr>
<td>1.92 M Glycine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 mM Trizma Base</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1 % SDS</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Semi-dry transfer buffer</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>390 mM Glycine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>480mM Trizma Base</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.37 % SDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 % methanol (v/v)</td>
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</table>
Table 8.3. 153 Transcription Factors identified from RNA-sequencing

<table>
<thead>
<tr>
<th>AFF1</th>
<th>EPAS1</th>
<th>NMI</th>
<th>TBX10</th>
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</thead>
<tbody>
<tr>
<td>ANKRD10</td>
<td>ERCC6</td>
<td>NR4A1</td>
<td>TEAD1</td>
</tr>
<tr>
<td>ANKRD22</td>
<td>ETV4</td>
<td>ONECUT1</td>
<td>TFAP2A</td>
</tr>
<tr>
<td>ANKRD32</td>
<td>ETV7</td>
<td>OTX1</td>
<td>TRIM29</td>
</tr>
<tr>
<td>ANKRD49</td>
<td>FOSB</td>
<td>PAWR</td>
<td>TRIM33</td>
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<tr>
<td>ANKZF1</td>
<td>FOSL1</td>
<td>PAX8</td>
<td>TRIM5</td>
</tr>
<tr>
<td>APC</td>
<td>GABPB2</td>
<td>PER2</td>
<td>TRIP11</td>
</tr>
<tr>
<td>ARID4A</td>
<td>GLI1</td>
<td>PHF21A</td>
<td>TRIP13</td>
</tr>
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<td>ARID4B</td>
<td>GLI52</td>
<td>PIAS2</td>
<td>TRPS1</td>
</tr>
<tr>
<td>ARID5B</td>
<td>GRHL1</td>
<td>PITX3</td>
<td>TSH2</td>
</tr>
<tr>
<td>ASB9</td>
<td>HDAC4</td>
<td>PML</td>
<td>TULP4</td>
</tr>
<tr>
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<td>HEY2</td>
<td>PMS1</td>
<td>WDHD1</td>
</tr>
<tr>
<td>ATM</td>
<td>HIPK2</td>
<td>PNN</td>
<td>WWP1</td>
</tr>
<tr>
<td>BACH1</td>
<td>HIVEP1</td>
<td>POU2F1</td>
<td>YEATS2</td>
</tr>
<tr>
<td>BARX2</td>
<td>HIVEP2</td>
<td>POU6F2</td>
<td>ZDHHC6</td>
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<tr>
<td>BAZ2B</td>
<td>HSF4</td>
<td>PRPF4B</td>
<td>ZKSCAN1</td>
</tr>
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<td>BDP1</td>
<td>IRF7</td>
<td>RB1</td>
<td>ZNF117</td>
</tr>
<tr>
<td>BIN1</td>
<td>JMJD1C</td>
<td>RBAK</td>
<td>ZNF12</td>
</tr>
<tr>
<td>BRCA1</td>
<td>KLF11</td>
<td>REL</td>
<td>ZNF138</td>
</tr>
<tr>
<td>BRCA2</td>
<td>KLF4</td>
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<td>KLF5</td>
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</tr>
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<td>SHPRH</td>
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<td>LRRFIP1</td>
<td>SIRT1</td>
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<td>SXX1</td>
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Table 8.4. 40 Transcription factors and chromatin remodellers from RNA-sequencing. The 14 TFs/chromatin remodellers molecularly validated are highlighted in blue with the 7 taken forward for gene silencing studies underlined.

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<th>Symbol</th>
<th>Name</th>
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<td>Induce survival in small cell lung cancer cells</td>
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<td>BACH1</td>
<td>BTB domain and CNC homolog 1</td>
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<td>Promotes invasive breast cancer cells</td>
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<td>Involved in HER2 signal attenuation</td>
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<td>BRCA1</td>
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<td>Other</td>
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<td>Upregulation or expression in oligodendrogliomas</td>
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### Table 8.5. 148 SRC-1 interactors identified from RIME

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Table 8.6. 27 transcription factors identified from RIME

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Table 8.7. RNA-sequencing patient data

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9. Appendix II

Publications and presentations throughout this PhD

Publications:

**Browne, AL** Charmsaz, S\(^1\), Vareslija, D\(^1\), Fagan A\(^1\), Cosgrove, N\(^1\), Cocchiglia, S\(^1\), Purcell, S\(^1\), Ward, E\(^1\), Bane, F\(^1\), Hudson, L\(^1\), Hill, ADK\(^1\), Carroll, JS\(^2\), Redmond, AM\(^2\), *Young, LS\(^3\).* *Network analysis of SRC-1 reveals a novel transcription factor hub which regulates endocrine resistant breast cancer.* Oncogene (2018). doi:10.1038/s41388-017-0042-x

Ward E\(^1\), Varešlija D\(^1\), Charmsaz S\(^1\), Fagan A\(^1\), **Browne AL**\(^3\), Cosgrove N\(^1\), Cocchiglia S\(^1\), Purcell S\(^1\), Hudson L\(^1\), Das S\(^2\), O’Connor D\(^2\), O’Halloran P\(^4\), Sims A\(^3\), Hill AD\(^1\), *Young LS\(^3\).* *Epigenome-wide SRC-1 mediated gene silencing represses cellular differentiation in advanced breast cancer.* Clin. Can. Res - *In review*

Communications:

February 2015 - SRC- 1 mediation of cancer cell reprogramming in endocrine resistant breast cancer. Irish Association of Cancer Research *(poster presentation)*

February 2016 - SRC- 1 mediation of cancer cell reprogramming in endocrine resistant breast cancer. Irish Association of Cancer Research *(oral presentation)*


February 2017 – SRC-1 regulation of tumour progression in endocrine resistant breast cancer. Irish Association of Cancer Research. *(poster presentation)*