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IDENTIFICATION OF NOVEL REGULATORS OF ESTROGEN RECEPTOR ALPHA SIGNALLING AND PROLIFERATION IN BREAST CANCER

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RÉSUMÉ

Le cancer du sein est un des cancers les plus fréquents chez les femmes, une canadienne sur neuf étant diagnostiquée au cours de sa vie. Le cancer du sein est une maladie hétérogène et le choix thérapeutique et le pronostic sont guidés par stratification des patientes sur la base de marqueurs moléculaires pour le récepteur des estrogènes α (ERα), le récepteur de la progestérone (PR) et/ou le récepteur membranaire HER2. Plus de 70% des tumeurs mammaires expriment ERα, un facteur de transcription inductible par les ligands. Une stimulation par l'estradiol permet la liaison du récepteur aux éléments de réponse aux estrogènes (ERE) dans les séquences régulatrices de gènes cibles, en association avec des cofacteurs transcriptionnels, et modifie les profils d'expression des gènes, augmente la prolifération cellulaire et accélère la croissance tumorale. Bien qu'il ait été démontré que plusieurs voies de signalisation influencent la fonction de ERα, les gènes capables d'affecter cette fonction restent à caractériser de manière exhaustive.

Dans cette étude, nous décrivons la conception, l'optimisation et l'exécution d'un protocole de criblage de shRNA à l'échelle du génome en vue d'identifier les gènes modulant l'expression de ERα, sa fonction en tant que facteur de transcription et la prolifération dépendante des estrogènes dans une lignée de cancer du sein humaine ERα-positive (luminale) exprimant de manière stable un vecteur rapporteur sous contrôle d'un promoteur répondant aux estrogènes. Nous avons validé notre méthode de criblage en déterminant les effets d'une suppression de l'expression de ERα et de celle d'un de ses cofacteurs connus, NRIP1, comme preuve de principe.

Notre criblage à l'échelle du génome a confirmé plusieurs régulateurs connus de la signalisation par ERα (NCOA1, NCOA2, NRIP1, FOXA1 et GATA3). Nous avons aussi identifié plusieurs régulateurs de l'expression de ERα, incluant l'acétyl-transférase de lysines *KAT6A*. La suppression de l'expression de *KAT6A* diminue l'expression du transcrit de ERα et de sa protéine, and conduit à des profils d'expression des gènes comparables à ceux obtenus suite à la suppression de l'expression de ERα. Nous avons aussi identifié un nombre de candidats régulateurs de la signalisation par ERα, incluant une sous-unité du complexe de remodelage de la chromatine CHRAC. La suppression de l'expression de *CHRAC1* résulte en un effet répressif sur les gènes cibles des estrogènes, y compris ceux jouant un rôle dans le contrôle du cycle cellulaire, la prolifération cellulaire et la réparation de l'ADN, et sur la prolifération de notre modèle cellulaire.

Cette étude a permis de révéler de nouveaux gènes et mécanismes moléculaires de signalisation affectant l'expression et/ou l'activité transcriptionnelle de ERα et la prolifération de cellules ERα-positives, et ouvre de nouvelles avenues pour explorer des approches de prévention et/ou traitement de la résistance aux antiestrogènes.

Mots clés: cancer du sein, récepteur des estrogènes, ERα, criblage haut débit, prolifération

ABSTRACT

Breast cancer remains the most commonly diagnosed cancer among women, with one in nine Canadian women expected to be diagnosed within her lifetime. Breast cancer is a heterogeneous disease, and stratification of patients into cohorts based on the expression of the molecular markers estrogen receptor α (ERα), progesterone receptor (PR) and/or HER2 helps dictate choice of therapy and predict disease prognosis. Over 70% of breast tumours express ERα, a ligand-inducible transcription factor. Stimulation with estradiol (E2) leads to receptor binding to estrogen response elements (ERE) in target gene regulatory sequences in association with transcription cofactors, and results in altered gene expression, increased cell proliferation and accelerated tumour growth. While a number of pathways have been shown to influence ERα signalling, an exhaustive study of genes affecting ERα signalling has not yet been published.

In this study, we describe the design, optimization and execution of an arrayed genome-wide shRNA screening protocol to identify genes modulating ERα expression, signalling and E2-dependent proliferation in a human ERα-positive luminal breast cancer cell line stably expressing a luciferase reporter under the control of an estrogen-responsive promoter. We have validated our screening assays by monitoring the effects of ERα knockdown and knockdown of the known ERα cofactor, NRIP1, as a proof of principle.

Our screen confirmed a number of known regulators of ERα signalling (NCOA1, NCOA2, NRIP1, FOXA1 and GATA3). We also identified several novel regulators of ERα expression, including the lysine acetyl transferase, *KAT6A*. Knockdown of *KAT6A* decreased the expression of ERα transcript and protein, and led to gene expression patterns comparable to those obtained following ERα knockdown. In addition, we identified a number of candidate regulators of ERα signalling, including the chromatin assembly complex CHRAC. Knockdown of *CHRAC1* resulted in a strong repressive effect on estrogen target genes included those involved in cell cycle control, cell proliferation and DNA repair, and decreased proliferation of our model cell line.

This study gives insight into previously unknown genes and molecular signalling pathways affecting expression and/or transcriptional activity of ERα and proliferation of ERα-positive cancer cells, and provides novel avenues to explore to prevent and/or circumvent antiestrogen resistance.

Key words: breast cancer, estrogen receptor, ERα, high-throughput screening, proliferation

TABLE OF CONTENTS

List of Tables

List of Figures

INTRODUCTION

CHAPTER ONE

CHAPTER TWO

DISCUSSION

List of Abbreviations

AF-1 – activation function 1
AF-2 – activation function domain 2
AR – androgen receptor
ARE – antioxidant-response element
CAK – CDK-activating kinase
CBP – page 16
CCND1 – cyclin D1
CDK –cyclin dependent kinase
ChIP – chromatin immunoprecipitation
DBD – DNA-binding domain
E1 – estrone
E2 - 17β-estradiol
E3 – estriol
EGF – epithelial growth factor
EGFR – Epithelial growth factor receptor
EMT – epithelial-mesenchymal transition
eNOS – endothelial nitric oxide synthase
EREs – estrogen response elements
ERα – estrogen receptor α
ESR1 – ERα gene
FBS – fetal bovine serum
FOXA1 – Forkhead box protein 1
FSH – follicle stimulating hormone
GCDFP15 – gross cystic disease fluid protein 15
GR – glucocorticoid receptor
GSK-3 – kinase glycogen synthase-3
HATs – histone acetyl transferases
HDACs – histone deacetylases
LBD – ligand-binding domain
LH – luteinizing hormone
LMTK3 – lemur tyrosine kinase-3
LTED – long-term estrogen-deprived
MDR1 – multidrug resistance gene 1
METABRIC – molecular taxonomy of breast cancer international consortium
NEM – N-Ethylmaleimide
NFYC – nuclear factor gamma
NO – nitric oxide
NRIP1 – nuclear receptor interacting protein 1

PBS –phosphate buffered saline
PIP – prolactin induced protein
PR – progesterone receptor
RAR – retinoic acid receptor
Rb – retinoblastoma protein
REST – RE1 silencing transcription factor
RISC – RNA-induced silencing complex
RNAi – RNA interference
SERDs – selective estrogen receptor downregulators
SERMs – selective estrogen receptor modulators
shRNAs – short hairpin RNAs
siRNAs – short interfering RNAs
SRC – steroid receptor cofactor
TCGA – the cancer genome atlas
TNBC – Triple-Negative Breast Cancer
YY1 – ying-yang one

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This work is the culmination of over six years of sleepless nights, tears of frustration and tension headaches, but equally, moments of pride, lucky mistakes and (short, infrequent) stretches of 'wow, everything is finally working'.

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Best å slutte mens det går vel.

INTRODUCTION

1. BREAST CANCER

1.1 BREAST CANCER EPIDEMIOLOGY IN CANADA

Breast cancer remains the most commonly diagnosed cancer among women. It is estimated that approximately 25,000 women were diagnosed with breast cancer in Canada in 2015, accounting for 26% of all cancer diagnoses. This equates to, on average, one in nine Canadian women diagnosed with breast cancer within her lifetime, with greater risk associated with increasing age (Canadian Cancer Society, 2015). Breast cancer in men is far less frequent, affecting 0.9% of Canadian men, but is also associated with an increased rate of mortality. Diagnosis often occurs at an older age and when the cancer is more advanced (Rizzolo et al., 2013). Breast cancer in men is not well understood and current treatment options are limited to those developed for women. However, many male breast cancers do not harbour the same genetic mutations seen in women and are instead driven by a distinct repertoire of alterations (Piscuoglio et al., 2016), suggesting that existing therapeutics may not be affective in this population.

While one in thirty Canadian women eventually succumbs to her disease, the proportion of women dying of breast cancer has been dropping steadily over time and mortality is at its lowest today since 1950. Consequently, 88% of those diagnosed five years ago are still living today, owing to earlier detection and regular mammography guidelines, as well as ongoing advances in breast cancer research leading to improved diagnostic measures and more effective treatment. However, with the Canadian population expected to grow by 29 percent between the mid-2000s and the year 2030, and with the proportion of Canadians over 65 years of age projected to increase from one in eight to one in four, breast cancer is expected to remain a significant burden on the Canadian health care system, and together with lung, prostate and colorectal, one of the four most frequently diagnosed forms of cancer in Canada (Canadian Cancer Society, 2015).

1.2 BREAST CANCER SUBTYPES

With advancement of molecular methods, particularly high-throughput technologies such as gene expression profiling by microarray and more recently next generation transcriptome sequencing, we have come to understand that breast cancer is not just one disease (Weigelt et al., 2010a). Although breast tumours arise in the same tissue, tumour histology, metastasis to distant sites, therapeutic response to targeted therapy and overall outcome differ greatly between individual patients. Long-observed differences among the histology of individual tumours were corroborated at the molecular level by a pair of seminal studies published in the early 2000s. Using cDNA microarray analysis of breast tumours and normal samples, the authors discerned the existence of multiple molecular subtypes of breast cancer: luminal A and B, normal breast-like, HER2-enriched and basal-like (Perou et al., 2000; Sørlie et al., 2001). Importantly, the distinct molecular subtypes described in these and subsequent studies are correlated with marked differences in clinical outcome, which are summarized in Table 1 (Hu et al., 2006; Sørlie et al., 2001; Sorlie et al., 2003). While molecular subtyping has changed the way we perceive breast cancer, it has been met with some scepticism and has not been widely adopted by clinicians (Ioannidis, 2005; Ioannidis et al., 2009). Rather, individual markers that inform on treatment because they represent targets for specific therapeutic regimen have been used since the identification of these genes and the development of relevant drugs. Thus, patients are currently stratified in the clinic into different cohorts based on the expression of the immunohistochemical markers estrogen receptor α (ERα), progesterone receptor (PR), epithelial growth factor receptor (EGFR) 2 (ErbB2; HER2), or the absence of all three, dictating the choice of therapy (Sims et al., 2007). Of note, molecular subtypes parallel those classes, since Luminal A/B tumours correspond to ER-positive tumours, HER2-enriched tumours generally present a good overlap with HER2-positive tumours identified by immunohistochemistry and FISH, and basal-like tumours are mostly triple-negative tumours. In addition, claudin-low tumours represent a different subtype of triple-negative tumours that can be identified by separate classifiers.

1.2.1 ERα-Positive Breast Cancer

While the exact significance of breast tumour subclassification remains an ongoing debate, the most clear-cut distinction is that between ERα-positive and ERα-negative tumours (Geyer et al., 2009; Gruvberger et al., 2001). ERα-positive tumours represent approximately 70% of all breast tumours and are characterized by an active ERα signalling pathway (Perou et al., 2000; Sørlie et al., 2001) and estrogen-dependent proliferation. As such, ERα-positive breast tumours are treated with anti-hormonal therapies: aromatase inhibitors to block the production of estrogens or antiestrogens that competitively inhibit receptor function. The ERα signalling pathway and currently available hormonal therapies will be discussed in detail in Part II. Estrogen signalling

ERα-positive epithelial cells are found in the normal luminal epithelium of the mammary gland, and thus ER-positive tumours have been labelled luminal tumours even though the cell(s) of origin of these tumours is(are) not clearly identified. There is evidence that luminal breast tumours can be further divided into luminal A and B subtypes; luminal A tumours having higher expression of ERα and ERα-target genes, including PR, lower levels of proliferation markers and a slower rate of proliferation and being thus of lower grade with a better prognosis than luminal B tumours. Luminal B tumours may also express HER2 (see below, Sørlie et al., 2001), and are more likely to express a mutant form of the tumour suppressor p53 (*TP53*; 41% in luminal B vs. 17% in luminal A tumours) (Bertheau et al., 2013). However, based on a recent meta-analysis of published gene expression data, it has been proposed that luminal tumours instead form a continuum and that division of ERα-positive tumours into distinct subgroups based on proliferation is entirely subjective (Geyer et al., 2009; Wirapati et al., 2008).

1.2.2 HER2-Positive Breast Cancer

HER2-positive tumours represent about 20% of all breast tumours (Wolff et al., 2007) and are characterized by the overexpression and increased activity of HER2, a membrane-bound protein and member of the human epidermal growth factor receptor family (Rubin and

Yarden, 2001). HER2, along with other family members, forms homo- or heterodimers, leading to activation of downstream pathways including PI3K/AKT and RAS/MAPK, which in turn regulate cell growth, survival, migration and proliferation (Hynes and Lane, 2005). The HER2 gene (ERBB2) is found in a frequently amplified chromosomal region, amplicon 17q12, along with several genes that may also be required for the growth of this subclass of breast cancer (Sahlberg et al., 2013). In a small number of cases, HER2 is not amplified but is overexpressed, possibly due to transcriptional or post-transcriptional deregulation (Rubin and Yarden, 2001). HER2-positive breast tumours can be positive or negative for ERα, but exhibit high rates of proliferation, increased probability of *TP53* mutation (71%; Sørlie et al., 2001) and are usually of a higher grade and lower probability of survival than ERα-positive breast cancers (Weigelt et al., 2010a).

Trastuzumab (Herceptin®), a monoclonal antibody that targets the extracellular domain of HER2, is the therapy of choice for treatment of HER2-positive breast cancers and is currently approved both in adjuvant therapy and for metastatic disease (Valabrega et al., 2007). Low HER2 expression in normal cells provides a safe therapeutic window and allows for survival of non-cancer cells (Rubin and Yarden, 2001). While trastuzumab is widely used in the clinic, the exact mechanism of action is still poorly understood. Trastuzumab binding may disrupt HER2 interaction with PI3K, leading to downregulation of AKT and inhibition of proliferation (Junttila et al., 2009). It has also been proposed that trastuzumab induces antibody-dependent cytotoxicity (Gennari et al., 2004), and triggers HER2 internalization and degradation via activity of the ubiquitin ligase c-Cbl (Klapper et al., 2000). Since over half of patients with HER2-positive breast cancer are intrinsically resistant to trastuzumab or become resistant over time (Vu and Claret, 2012), the elucidation of how trastuzumab functions in the cell could be a ground-breaking step in aiding the treatment of this subclass of breast cancer.

1.2.3 Triple-Negative Breast Cancer

Triple-negative breast cancers (TNBC) represent the remaining ~15% of breast tumours (Bosch et al., 2010) and are characterized by high tumour grade, younger age of onset and absence of the immunohistochemical markers ERα, PR and HER2. At the transcript level, TNBC is highly heterogeneous and currently lacks specific targeted therapeutics like those that have been developed to treat ERα-positive and HER2-enriched tumours. TNBC is consequently treated with broad-spectrum chemotherapy and is associated with poor prognosis.

1.2.4 Basal-Like Breast Cancer

Approximately 70% of triple-negative breast tumours are designated as basal-like (though not all basal-like tumours are triple-negative) (Bertucci et al., 2008; Geyer et al., 2009; Kreike et al., 2007), and are thought to arise from an aberrant luminal progenitor population (Lim et al., 2009; Visvader and Stingl, 2014). Basal-like tumours express high levels of cytokeratins 5/6 and EGFRs and have an overall poorer outcome than do TNBCs lacking these proteins (Cheang et al., 2008). Additionally, basal-like tumours frequently express mutant *TP53* (88%) with mutations more clearly associated with total loss of function (frameshift, deletion) rather than base substitutions as seen in luminal cases of *TP53* mutation (Bertheau et al., 2013). *TP53* is thought to be a passenger mutation in these tumours, occurring following the loss of PTEN (Martins et al., 2012). In addition, around 20% of basal-like breast tumours harbour mutations in the DNA repair genes BRCA1 or BRCA2 (van der Groep et al., 2004).

1.2.5 Molecular Apocrine Breast Cancer

Characterized by increased expression of androgen receptor (AR), predominantly at the transcript level (Doane et al., 2006), and active AR signalling, as well as a molecular apocrine gene signature, this subclass represents 0.5 to 4% of all breast tumours (Lehmann-Che et al., 2013). Molecular apocrine tumours also frequently express HER2 and/or gross cystic disease fluid protein 15 (GCDFP15), a product of the AR target gene prolactin induced protein (PIP).

Along with AR status, HER2 and GCDFP15 may be useful molecular markers to identify this subclass of breast cancer (Lehmann-Che et al., 2013).

Since molecular apocrine tumours do not express ER but yet have an expression profile resembling that of ER-positive luminal breast tumours, it has been suggested that ER target genes and tumour proliferation are instead under the control of AR in this subgroup. Forkhead box protein A1 (FOXA1) is a pioneer factor, a transcription factor that can directly bind condensed chromatin and recruit additional transcription factors as well as histone and DNA modifying enzymes to initiate transcription. FOXA1 enables recruitment of ERα and AR to their respective response elements (Carroll et al., 2005, 2005; Laganière et al., 2005; Lupien et al., 2008; Sahu et al., 2011). While FOXA1 expression is highly correlated with ERα expression, FOXA1 is also overexpressed in molecular apocrine tumours (Doane et al., 2006); approximately 90% of molecular apocrine cancers are FOXA1 positive (Lehmann-Che et al., 2013). Furthermore, treatment of the molecular apocrine breast cancer cell line MDA-MB-453 with the androgen antagonist bicalutamide results in decreased growth and colony formation (Robinson et al., 2011). Targeting AR therefore, may present a potential therapeutic avenue for treatment of this subclass of breast cancer (Doane et al., 2006), especially in patients who do not benefit from a HER2-directed approach.

1.2.6 Claudin-Low Breast Cancer

The claudin-low subtype comprises tumours with a transcription profile most resembling that of mammary stem cells (Hennessy et al., 2009), with a CD44+/CD24− signature and enrichment in epithelial-mesenchymal transition (EMT) markers and low expression of proteins involved in cell to cell adhesion, characteristically claudins 3, 4 and 7, occludin and E-cadherin (Shang et al., 2012). This subtype of breast cancer is also highly resistant to chemotherapy. Although claudin-low tumours are like the basal-like subtype triple-negative tumours, they express lower levels of genes categorized as part of a proliferation cluster, including the clinical proliferation marker Ki67, indicating that this subtype may be slow-cycling compared to breast tumours with equally poor prognosis (basal-like, HER2-positive,

luminal B) (Prat et al., 2010, 2015) and that targeting proliferative genes in this subtype may be an ineffective approach.

1.2.7 Normal-Like Subtype

Tumour samples classified within the normal-like subtype express genes characteristic of adipose tissue and have a prognosis between that of luminal or basal-like breast tumours (Yersal and Barutca, 2014). The clinical significance and very existence of the normal-like tumour type has been brought into question, as some publications have proposed that the subclass is a technical artefact from contamination of normal mammary cells during biopsy that are then included in subsequent microarray. In fact, studies isolating breast cancer cells through microdissection did not detect cells of the normal-like subtype (Natrajan et al., 2010; Weigelt et al., 2010b).

1.2.8 Neuroendocrine Tumours

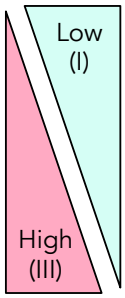
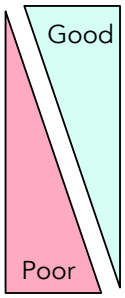
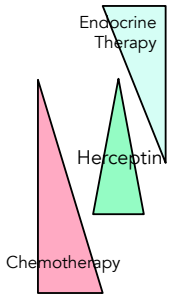
Studies have described a rare (1-4% of all breast cancer cases) and aggressive subtype of breast cancer in which tumour cells express markers specific to neuronal cells including chromogranin, synaptophysin and enolase (Inno et al., 2016), associated with alternative splicing and loss of activity of the RE1 Silencing Transcription Factor, REST. REST binds RE1 sites in regulatory regions of target genes (found in approximately 2000 human genes; Bruce et al., 2004) and recruits histone deacetylases (HDACs) and histone methyltransferases to suppress neuronal gene expression in non-neuronal cells (Huang et al., 1999; Roopra et al., 2000).

1.2.9 Emerging Subtypes

Recent studies using next-generation sequencing technology and epigenetic analysis of primary breast cancer genomes have added to the growing list of molecular subtypes in breast cancer. In particular, the recent Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) study used copy number aberrations and gene expression profiling in 2000 mammary tumours to reveal the existence of as many as ten genetically-unique

breast cancer subtypes (Curtis et al., 2012). Nonetheless, the Cancer Genome Atlas (TCGA) network study published in the same year concluded the existence of only four main phenotypic subtypes of breast cancer (luminal A, luminal B, HER2-positive and triple-negative) using DNA copy number, DNA methylation status, exome, transcriptome and miRNA sequencing and protein arrays (Cancer Genome Atlas Network, 2012). At present, patients are routinely tested for expression of ERα, PR, HER2 and Ki67 by immunohistology and, with limited treatment options available, the clinical implication of subclass stratification of breast tumours beyond ERα, HER2 or triple-negative status remains unclear. The discovery of novel targets and more targeted therapies, especially in triple-negative cancers and in patients who relapse following treatment with hormonal therapy or trastuzumab is therefore a pressing issue in breast cancer research.

Table 1. Characteristics of the main subtypes of breast cancer.

Molecular Subtype	%	Receptor Expression			Histological Grade	Prognosis	Treatment
		ER	PR	HER2			
Luminal A	40	+	+	-			
Luminal B	20	+	+	+/-			
HER2-positive	10-15	-	-	+			
Triple-negative	15-20	-	-	-			

2. ESTROGEN SIGNALLING

Estrogens are generally regarded as female reproductive hormones. While this hormone family does play a significant role in the development and maintenance of female reproductive tissues, estrogens also have a significant influence on multiple other aspects of mammalian physiology in both sexes. In the case of ERα-positive breast cancer, the orchestrated integration of multiple arms of the estrogen-signalling pathway ultimately leads to changes in cell proliferation and tumour expansion.

2.1 ESTROGENS

2.1.1 Estrogen Production

The most potent estrogen produced in humans is 17β-estradiol (E2). Estrone (E1) and estriol (E3), metabolites of E2, are much weaker agonists despite their high affinity to estrogen receptors (Heldring et al., 2007). In premenopausal women, the main site of estrogen production is within the granulosa cells of developing follicles in the ovary. In these cells, estrogen production is stimulated by the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland. Non-ovarian sources of estrogens include the liver, the breasts, the male testes, the adrenal glands, the brain, bone and adipose cells and are especially important in postmenopausal women and those with prior ovariectomy (Blair, 2010).

Estrogens belong to the steroid hormone family. Estrogen production begins with the synthesis of androstenedione from cholesterol. Androstenedione is then either converted directly to estrone, or to E2 through a testosterone intermediate. The final aromatization of androstenedione to estrone, or testosterone to E2, is catalyzed by P450 aromatase monooxygenase (Voet et al., 2006), an enzyme that is expressed by breast cells and is overexpressed in breast cancer (Chen, 1998; Wang et al., 2015). Consequently, small

amounts of estrogen are produced through local aromatization of testosterone in the breast (Nimrod and Ryan, 1975).

While estrogens are produced in both males and females, they are present at significantly higher levels in women of reproductive age, during which time they fluctuate with the menstrual cycle, with concentrations ranging from 20-80 pg/mL in the mid-follicular phase and peaking during the LH surge at 200-500 pg/mL just prior to ovulation. Following menopause, serum E2 declines to prepubertal levels, at approximately 10–20 pg/mL, and can be below 10 pg/mL in women who have undergone oophorectomy. In men, serum E2 concentration is typically below 40 pg/mL but may be increased in association with certain diseases (III and Barbieri, 2013).

Aside from endogenous production of estrogens, humans are also exposed to exogenous estrogens – from hormonal preparations, such as conjugated equine estrogens used for post-menopausal hormone replacement therapy or oral contraceptives, from phytoestrogens and from environmental endocrine disruptors. Phytoestrogens constitute a diverse collection of molecules occurring naturally in plant-derived foods, with estrogenic properties and the ability to initiate and interfere with normal estrogen signalling pathways (Luczak and Leinwand, 2009). A number of these compounds act as selective estrogen receptor modulators (SERMs), contributing to estrogenic action in some tissues while antagonizing it in others. Phytoestrogens may selectively bind one ER over another (see below); the main isoflavones in soy – genistein and daidzein – bind ERβ with a higher affinity (Setchell et al., 2002). Additionally, signalling can be affected by environmental toxins or by-products including Bisphenol A (BPA), a substance found in polycarbonates and epoxy resins with estrogenic properties (Krishnan et al, 1993). Finally, in the context of the laboratory, the pH indicator phenol red found in cell culture media has been shown to possess estrogenic properties (Berthois et al., 1986). Studies on estrogenic signalling, therefore, must be undertaken in phenol red-free media and using fetal bovine serum (FBS) stripped of steroidal hormones.

2.1.2 Estrogens and Mammary Tumourigenesis

While estrogens are necessary for a number of physiological processes, including reproduction and menstrual cycle regulation, maintenance of bone density, cholesterol mobilization and brain function, they are considered causative agents in the development of gynaecological malignancies including endometrial and breast cancers (Shang, 2007). In fact, steroidal estrogens have been listed as carcinogens since 2002 by the US National Toxicology Program following the finding that prolonged exposure is associated with increased risk of endometrial and breast cancers from a number of large-scale hormone-replacement therapy trials and epidemiological studies (Bergkvist et al., 1989; Manson et al., 2003). In fact, estrogen exposure is one of the few well-documented risk factors for breast cancer. Girls who undergo menarche one to two years earlier than the average age (12) have a 2-fold increased risk of breast cancer development (Peeters et al., 1995). Similarly, early menopause or ovariectomy before age 40 can reduce breast cancer risk by nearly 45% as compared to menopause at ages 50-55 (Brinton et al., 1988).

2.2 ESTROGEN RECEPTORS

The field of estrogen biology was dramatically expanded following discovery of an intracellular estrogen binding protein, known today as ERα (Jensen, 1962), and the observation that expression of this protein in breast tumours correlates with endocrine disruption (Jensen et al., 1971). The cloning of the corresponding cDNA subsequently enabled structure function studies and the demonstration of its role as a transcription factor capable of binding DNA specifically and activating expression of target genes (Green et al., 1986, 1986). A second receptor, ERβ, was discovered much later in rat prostate (Kuiper et al., 1996). While knockout of ERα in the mouse is not lethal, absence of the receptor does result in abnormalities in the maintenance of secondary sexual characteristics and functionality of

the reproductive tract in the female (Lubahn et al., 1993). Subsequent ERβ and ERαβ knockouts have shown that neither ER is essential for survival.

The ERα and ERβ proteins are encoded by the genes *ESR1* and *ESR2*, respectively, found at distinct genetic loci (6q25.1-q25.2 and 14q23.2-q23.3). Along with AR and PR, they belong to the nuclear receptor superfamily and have an evolutionarily-conserved modular structure (Figure 1) (Heldring et al., 2007). An A/B region near the N-terminus harbours an activation function 1 (AF-1) domain that is active in isolation and participates in transcriptional activation through coactivator recruitment. The DNA-binding domain (DBD), composed of two zinc fingers, shows 97% sequence similarity between ERα and ERβ and binds to specific genomic estrogen response elements (EREs) to regulate the transcription of estrogen-responsive genes (Mader et al., 1989, 1993a). The hinge region contains the nuclear localization signal. Domain E contains the ligand-binding domain (LBD), to which estradiol binds with similar affinity for both receptors (Escande et al., 2006). Although ERα and ERβ display a great deal of ligand promiscuity, certain features of their respective ligands must be conserved in order for binding to occur. Two residues on either end of the ligand-binding pocket can recognize molecules through hydrogen bonding, while the remainder of the pocket is highly hydrophobic (Zeng et al. 2008). Domain E also contains a second activation function domain (AF-2), whose activity is dependent upon ligand binding. The AF-1 and AF-2 domains of ERα and ERβ are not well conserved, suggesting the two receptors may recruit different cofactors. Finally, the function of the variable C-terminal F region remains to be elucidated (Huang et al., 2010; Le Romancer et al., 2011).

ERα and ERβ have distinct transcriptional activities, cooperating with some common but also some distinct transcriptional cofactors and targeting a distinct subset of genes, often resulting in opposing activities on cellular processes including proliferation and apoptosis (Leitman et al., 2010). Additionally, the receptors show different patterns of tissue distribution. While both receptors are expressed in the uterus, ovaries and testis, ERα is the main receptor in the kidney, pituitary and adrenal gland, whereas elevated levels of ERβ are found in the prostate, lung, bladder and brain (Kuiper et al., 1997). Normal breast tissue

shows diffuse ERα staining in the ductal epithelium but only sporadic nuclear staining for ERβ (Murillo-Ortiz et al., 2008). While ERα is overexpressed in a subset of breast tumours, ERβ is thought to be lost during breast tumourigenesis (Skliris et al., 2003). Unless otherwise specified, the remainder of this work will focus exclusively on ERα.

ERα is one of the few biomarkers currently used in the clinic to stratify breast cancer patients into ERα-positive vs. ERα-negative subgroups for therapy selection. Only 4-15% of normal breast epithelial cells express ERα (Umekita et al., 2007), with low expressors thought to be at lower risk of developing breast cancer (Lawson et al., 2002) and higher expression of the receptor detected even in normal cells of breast cancer patients (Khan et al., 1998).

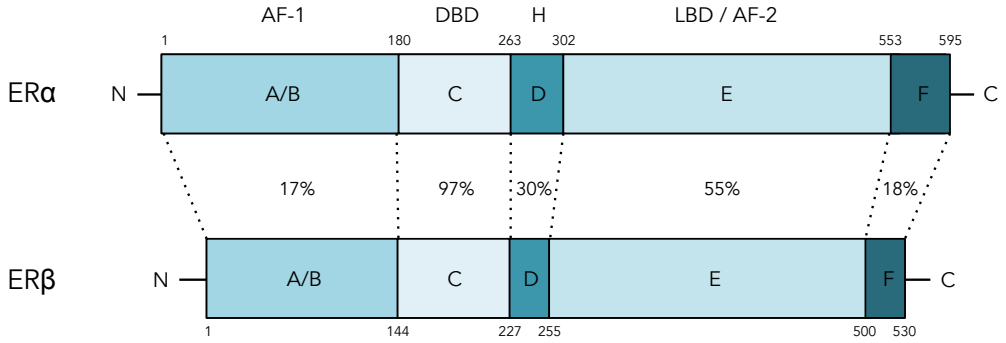


Figure 1. Estrogen receptor α and β protein structures.
ERα and β belong to the nuclear receptor superfamily and have an evolutionarily-conserved modular structure. AF-1: activation function 1, DBD: DNA-binding domain, H: hinge region, LBD: ligand-binding domain, AF-2: activation function 2.

2.3 ESTROGEN RECEPTOR SIGNALLING

The different mechanisms of action of ERα contributing to intracellular signalling upon estrogenic stimulation are summarized in Figure 2.

2.3.1 ERE-Dependent Genomic Estrogen Receptor Signalling

In the absence of ligand, ERα is mostly present in the nucleus of breast cancer cells, with a small fraction detectable in the cytoplasm and near the cellular membrane (Pappas et al., 1995). ERs located within the nucleus mediate genomic signalling pathways when activated by E2 or similar ligands. Monomeric ERs are normally bound to heat shock proteins, Hsp70 and Hsp90, and immunophilins, which maintain the receptors in an inactive state (Pratt and Toft, 1997). E2, being a lipophilic molecule, diffuses across the cellular membrane and binds to ERα or ERβ. The receptor then undergoes conformational changes, releasing it from inactive complexes and resulting in receptor homo- (ERα/ERα or ERβ/ERβ) or heterodimerization (ERα/ERβ), and binding to EREs in the promoters of E2 target genes.

Binding affinity is highest for consensus EREs, which were derived from compilation of response elements found in promoters of various estrogen target genes, and are 15 base pair palindromes consisting of two PuGGTCA motifs separated by three variable base pairs (5'-PuGGTCAnnnTGACCPy-3') (Klein-Hitpass et al., 1988; Mader et al., 1993b). which are found in the promoters of target genes or distal to the site of transcriptional initiation (Bourdeau et al., 2004). This motif is recognized with a high level of specificity by ERs, with EREs present at a frequency of one in every four million base pairs in the genome. Other nuclear receptors recognize similar motifs with different spacing, orientation or different motifs entirely. Experimental symmetric substitution within the recognized PuGGTCA motif abolishes receptor binding in most cases. Studies using chromatin immunoprecipitation (ChIP) have shown that binding of ERα and ERβ to EREs is inducible in cultured cells following treatment with E2 (Sanchez et al., 2002).

Following binding of ligand to ERα, the receptor undergoes conformational changes resulting in the presentation of a co-activator binding groove. Amongst the first cofactors recruited to ERα in a ligand-dependent manner are the SWI/SNF complex (human BRG1/BRM) and the p160 steroid receptor coactivator (SRC) family of coactivators (Heery et al., 1997), comprising three members: SRC1 (nuclear coactivator 1; *NCOA1*), SRC2 (*NCOA2*) and SRC3 (NCOA3). SRC co-activators have acetyltransferase activity and contain multiple functional domains, enabling their interaction with ERα and other nuclear receptors via LXXLL motifs, as well as association with other cofactors, such as the histone acetyl transferases (HATs), E1A binding protein p300 (EP300; p300) and cAMP-response element-binding protein (CBP; Xu et al., 2009). Co-activator recruitment is competitive and mutually exclusive. Binding of one SRC protein prevents others from binding, and a single ERα dimer is thought to bind a single SRC protein (Carraz et al., 2009; Shiau et al., 1998). SRC coactivators also recruit the histone methyltransferases coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase (PRMT; An et al., 2004; Koh et al., 2001). Together, these cofactor complexes modify lysine and arginine residues on histone tails, leading to nucleosome destabilization and an open DNA structure. Finally, recruitment by ERα of the mediator complex, thyroid hormone receptor associated protein/vitamin D receptor-integrating protein (TRAD/DRIP), allows for the interaction between the coactivator complex and the basal transcription machinery (Marino et al., 2006). The overall consequence is a stimulatory effect on ERα target gene expression.

Transcriptional activity for the receptor can be impeded using antiestrogens, which are competitive inhibitors (Oñate et al., 1995) that induce alternative structures of the receptor in which coactivator recruitment is prevented. In breast cancer, response to endocrine treatment is correlated with differential expression of SRC proteins (Hurtado et al., 2008; Osborne and Schiff, 2003). Over-expression of NCOA3, which is frequently amplified in breast and ovarian tumours, correlates with poor tamoxifen response (Anzick et al., 1997). Likewise, expression levels of HATs p300 and CBP are positively correlated with tumour grade (Hudelist et al., 2003).

ERα transcriptional activity can also be initiated through receptor phosphorylation by multiple growth factors resulting in the ligand-independent activation of ERα. Activation of the MAPK signalling pathway by stimulating cells with EGF or IGF results in phosphorylation of serines 118 and 167, leading to receptor activation (Chen et al., 2002; Kato, 2001). Serine 167 can be phosphorylated by AKT, a member of the PI3K cell survival pathway (Campbell et al., 2001). Additionally, the cyclin A/cdk2 complex phosphorylates ERα at serines 104 and 106 (Trowbridge et al., 1997). The aforementioned phosphorylation events take place in the A/B region of the receptor, and allow for recruitment of coactivators interacting with this domain and transcriptional activation without the necessity of ligand binding (Rochette-Egly, 2003).

2.3.2 ERE-Independent Genomic Estrogen Receptor Signalling

ERα has been observed to activate promoters that are devoid of consensus EREs, indicating that other DNA sequences may contribute to transcriptional regulation. Indeed, ERs can tether to transcription factors bound to their own preferred response elements in DNA promoters to activate or repress target genes. This includes interaction with the Fos-Jun complex at AP-1 sites to induce cyclin D1 (*CCND1*) expression (Cicatiello et al., 2004; Gaub et al., 1990), with SP-1 to induce *E2F1* (Kushner et al., 2000; Webb et al., 1999), and with numerous other transcription factors including NFκB, Runx1 and Runx2 (Khalid et al., 2008; Stein and Yang, 1995; Stender et al., 2010).

It has been proposed that ERα opposes the transcriptional activity of the tumour suppressor p53 via this ERE-independent mechanism. On gene targets that are activated by p53 (*CDKN1A*, *PCNA*), ERα interacts directly with p53 and represses p53 transcriptional activation (Liu et al., 2006). However, p53 also represses a subset of target genes, including survivin (*BIRC5*), involved in apoptosis regulation and multidrug resistance gene 1 (P-glycoprotein; *MDR1*), an energy-dependent drug efflux pump. ERα binding at the p53-bound promoters of these two genes reverses p53 repression, leading instead to gene activation (Sayeed et al., 2007)

2.3.3 Nongenomic Estrogen Receptor Signalling

Aside from its well-documented genomic effects, estrogens can activate a number of signal transduction events through rapid signalling events not requiring the production of novel protein. Recent advances in our understanding of rapid steroid hormone signalling were initiated by Claire Szego and colleagues who identified a receptor at the plasma membrane of several cell types, which binds to E2 with high affinity. Brief periods of E2 exposure led to alterations of intracellular Ca2+ and the generation of cAMP (Szego and Davis, 1967). The rapid response in these cells challenged the possibility that the changes observed were due to the traditional genomic pathway through which all steroid hormones were thought to signal.

Unlike most membrane receptors, ERs do not contain a transmembrane domain (Björnström and Sjöberg, 2005). Instead, ERs are associated with the scaffold protein caveolin-1, and are anchored to the membrane in specialized membrane invaginations known as caveolae (Razandi et al., 2003), facilitating rapid signal transduction because these intracellular sites are enriched with a variety of signalling molecules, which are readily available to conduct rapid signalling cascades (Shaul and Anderson, 1998). The association of ERs with caveolin-1 and localization at the membrane requires the S-palmitoylation of the receptor by a palmitoyl acyl transferase enzyme. This posttranslational modification is also crucial for rapid E2 signalling. An alanine substitution at cysteine 447, within the conserved palmitoylation motif, eliminates E2-induced rapid activation of MAPK signalling, even as the receptor is still able to bind its ligand with the same affinity (Acconcia et al., 2005).

Nongenomic ER signalling is thought to play an especially important role in non-reproductive tissues (Simoncini et al., 2006). In the cardiovascular system, rapid estrogen signalling induces vasodilation in a matter of seconds by inducing the synthesis of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) (Murphy, 2011). Additionally, rapid ER signalling induces intracellular calcium mobilization, cyclic AMP production, MAPK and AKT pathway activation and phosphorylation of Src oncoprotein (Sayeed et al., 2007).

Membrane ER have also been identified in breast cancer cells. In MCF-7 cells (an ERα-positive breast cancer cell line), membrane ERs activate MAPK and AKT signalling pathways, both implicated in cell survival and proliferation, and directly interact with HER2 and growth factor receptors IGF-1 and EGF, resulting in their activation (Chung et al., 2002; Kahlert et al., 2000). Although the non-genomic pathway induces rapid responses, enzyme activation and functional changes may persist for longer. For example, in MCF-7 cells (ERα-positive breast cancer cells), E2 induces RAF1 kinase activity, which peaks following five minutes of treatment and is associated with expression of early growth response gene *EGR1*, while expression of a dominant negative RAF1 mutant results in 50% inhibition of E2-induced transcription (Pratt et al., 1998).

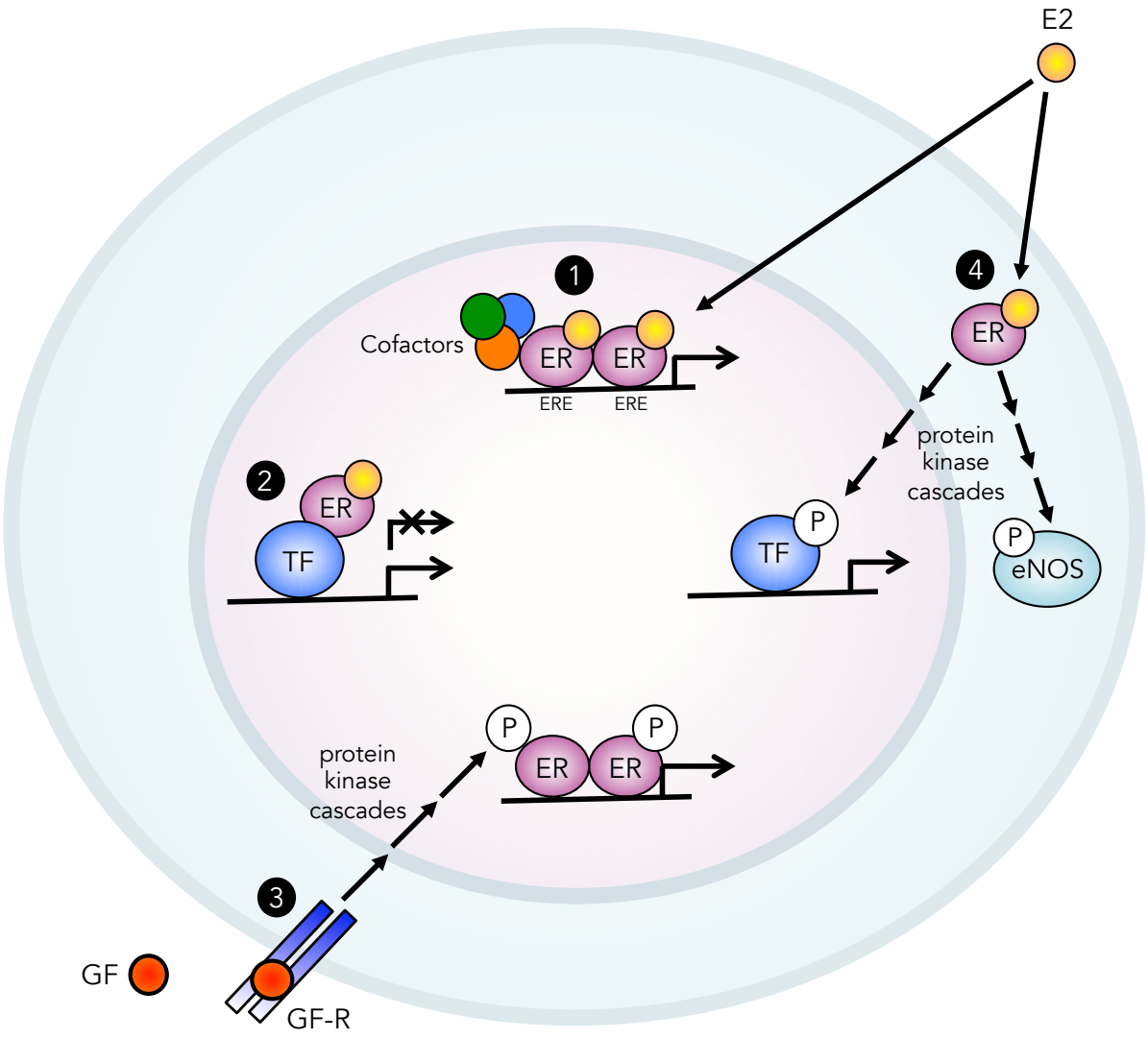


Figure 2. Estrogen receptor signalling.

1) In the classical genomic mechanism of ER action, liganded ERs bind directly to EREs in target gene promoters and recruit additional cofactors to DNA. Coactivators promote chromatin remodelling and stabilization of an active transcription complex. 2) In ERE-independent genomic signalling, liganded ER is tethered to DNA-bound transcription factors (TF) through protein-protein interactions, leading to activation or repression of gene transcription. 3) Growth factors (GF) activate protein-kinase cascades, which can lead to phosphorylation (P) and activation of unliganded ERs at EREs. 4) ERs at or near the cellular membrane can initiate signalling cascades leading to activation of signalling molecules, like eNOS, or to the regulation of gene expression through activation of downstream TFs. Adapted from: Björnström L. et al. (2005) Molecular Endocrinology.

3. REGULATORS OF ERα

3.1 Regulators of ERα Expression

3.1.1 p53

A reciprocal positive feedback loop has been described for ERα and the tumour suppressor protein p53. Exogenous overexpression of p53 (Angeloni et al., 2004) or induction of p53 expression with the DNA damaging agent doxorubicin or ionizing radiation (Shirley et al., 2009) increases ERα mRNA and protein expression in MCF-7 cells. Following doxorubicin treatment, p53 is recruited to the *ESR1* promoter at nt -129 to -40 distal to the transcriptional start site (TSS), while in baseline conditions p53 is found at nt -2094 to -1941 and -350 to 0298 (Shirley et al., 2009). Data from our lab and others has shown that estrogen administration in a second ERα-positive breast cancer cell line, T47D, increases p53 expression (Hurd et al., 1995), which could then also feedback to increase ERα expression.

3.1.2 GATA3

Like p53, GATA3 is also involved in a positive feedback loop with ERα. GATA3 belongs to a family of six transcription factors, GATA1 to GATA6, which bind (A/T)GATA(A/G) consensus motifs through two zinc finger motifs (Ko and Engel, 1993). Conditional knockout of GATA3 in the mouse reveals a role for the transcription factor in pubertal terminal end bud formation, luminal epithelial cell differentiation and mammary gland development (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). In fact, the phenotype seen following GATA3 knockout is reminiscent of that with ERα knockout (Mallepell et al., 2006). *GATA3* expression is highly correlated with that of ERα in breast cancer cell lines and in tumours (Hoch et al., 1999; Lacroix and Leclercq, 2004; Tozlu et al., 2006), while downregulation results in loss of ERα expression and may contribute to tumour dissemination (Dydensborg et al., 2009; Eeckhoute et al., 2007; Kouros-Mehr et al., 2008). Binding of GATA3 to *ESR1* flanking sequences was detected at enhancer sites located upstream of *ESR1* promoters E and F and

was associated with recruitment of the histone acetyltransferase p300 and the demethylase KDM4B and with gain of H3K9/H3K18 acetylation and loss of H3K9me2/3 methylation marks (Eeckhoute et al., 2007; Gaughan et al., 2013; Welboren et al., 2009).

3.1.3 FOXA1

As previously mentioned, FOXA1 is a pioneer transcription factor, which can directly bind and open condensed chromatin (Cirillo et al., 2002) and enable recruitment of ERα and AR to their respective response elements (Carroll et al., 2005, 2005; Laganière et al., 2005; Lupien et al., 2008; Sahu et al., 2011). FOXA1 expression is correlated with that of ERα in breast tumours (van 't Veer et al., 2002), and FOXA1 has been identified as an upstream regulator of ERα expression in mouse and human breast cancer cells (Bernardo et al., 2010). FOXA1 may cooperate with GATA3 to modulate ERα expression, as both transcription factors are associated at several common *ESR1* enhancer regions (Serandour et al., 2013). Three other forkhead box proteins have also been implicated in control of ERα expression in breast cancer cells. Ectopic expression of *FOXM1* in ERα-positive breast cancer cell lines upregulates ERα transcript and protein levels, possibly through formation of a functional transcription factor complex with FOXO3 at the *ESR1* promoter (Guo and Sonenshein, 2004; Madureira et al., 2006). Furthermore, FOXC1 downregulates ERα expression by competing with GATA3 for the same binding regions on the *ESR1* promoter and is associated with histone H3K9 trimethylation, a repressive mark associated with heterochromatin (Yu-Rice et al., 2016).

3.1.4 Additional transcription factors

A number of other proteins have been implicated in regulation of ERα transcriptional expression. In the human heart, inflammatory stimuli activate NFκB, which then binds the F-promoter of the *ESR1* gene, leading to suppression of ERα expression, while E2 treatment or ERα ectopic expression antagonizes NFκB inhibition (Mahmoodzadeh et al., 2009). BCL9-2, a co-activator of Wnt/ß-catenin, has been shown to regulate expression of ERα by interacting

with Sp1 at the proximal promoter of *ESR1* (Zatula et al., 2014). In osteoblasts, ERα complexes with AP-1 factors c-jun, c-fos and ATF-2 following E2 stimulation to modulate activity of the F promoter (nt -117,884 to -117,140) of *ESR1* (Lambertini et al., 2008). Expression of the AP-2 factor, AP-2α correlates with that of ERα in human breast tumours (Turner et al., 1998), and AP-2α and AP-2γ have been shown to activate cloned human *ESR1* promoter in MDA-MB-231 cells (a triple negative breast cancer cell line) (McPherson and Weigel, 1999). Furthermore, AP-2γ binds the *ESR1* promoter in ERα-positive breast cancer cell lines (Woodfield et al., 2009), while knockdown of AP-2γ in these cells reduces ERα transcript and protein expression (Woodfield et al., 2007). Interestingly, AP-2γ binding is dependent on chromatin structure. Overexpression of AP-2γ alongside pre-treatment of ERα-negative cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (AZA) and the histone deacetylase inhibitor trichostatin A (TSA) permits AP-2γ binding and allows for re-expression of ERα (Woodfield et al., 2009).

3.1.5 ERα Autoregulation

Finally, ERα can autoregulate its own expression (Castles et al., 1997). Upon E2 treatment, ERα binds at two sites upstream of *ESR1*: a distal enhancer region, ENH1, and the proximal A promoter, in association with the coactivators p300 and AIB1 at both sites and the histone deacetylase Sin3A at the distal enhancer only. Sin3a is characterized as a repressor of transcription and indeed Sin3a knockdown derepresses expression of ERα in MCF-7 cells treated with E2 (Ellison-Zelski et al., 2009). Depending on coactivator balance therefore, *ESR1* expression can be induced or suppressed by existing ERα protein, and this may contribute to differential ERα expression in human breast tumours.

3.2 Regulators of ERα Transcriptional Activity

3.2.1 ER cofactors

Regulators of ERα transcriptional activity may do so by interfering with any of the signalling pathways in which ERα participates. Altogether, 639 proteins interact directly with nuclear receptors in different cell types (NURSA; www.nursa.org) and many of these have been characterized to act as cofactors of the receptor. As mentioned, ERα transcription cofactors include the histone acetyltransferase SRC/CBP/p300 complex, the histone methyl transferases CARM1 and PRMT1, the Mediator complex and the SWI-SNF chromatin remodelling complex (Burakov et al., 2002; Green and Carroll, 2007; Hall and McDonnell, 2005; Ichinose et al., 1997; Ratajczak, 2001; Smith and O'Malley, 2004). Additionally, components of the ubiquitin ligation and proteasome complexes also act as ERα cofactors (vom Baur et al., 1996; Nawaz et al., 1999; Smith et al., 2002; Verma et al., 2004). On the other hand, corepressors recruited in a ligand-dependent manner such as LCoR and NRIP1 attenuate hormone-induced transactivation and limit ERα transcriptional activity (White et al., 2004). Coactivators and corepressors are not specific for ERα, but are shared with other nuclear receptors or transcription factors.

3.2.2 Transcription Factors

Some of the same transcription factors that regulate ERα expression can also impact ERα transcriptional activity independent of their action at the *ESR1* promoter. Direct protein-protein interaction between ERα and p53 at some ERα target gene promoters (BRCA2, Bcl2, IL-6 and tissue plasminogen activator) (Haldar et al., 1994; Jin et al., 2008; Kunz et al., 1995; Santhanam et al., 1991) interferes with ERα binding to EREs and leads instead to target gene downregulation, while ERα dimerization is unaffected (Liu et al., 1999). Loss of *GATA3* is associated with reorganization of FOXA1 and p300 genome localization and the histone marks H3K4me1 (present at enhancers) and H3K27Ac (indicative of active enhancers; Creyghton et al., 2010), leading to redistribution of ERα binding events and changes in

target gene transcription. It is thought that GATA3 is bound before ERα and modulates enhancer accessibility (Theodorou et al., 2013). Similarly, FOXA1 is a key determinant of ERα activity and FOXA1 binding overlaps with approximately 50% of ERα-binding events (Hurtado et al., 2011).

3.2.3 Nuclear Receptors

ERα transcriptional activity can also be mediated by other proteins in the nuclear receptor superfamily, which compete with ERα for ERE binding and thereby repress ERα target gene activation. Activation of the glucocorticoid receptor (GR) inhibits ERα target gene expression by displacing ERα and NCOA3 from certain EREs in a mechanism also involving FOXA1 and AP-1 binding (Karmakar et al., 2013). PR, currently used as a biomarker of active ERα signalling pathway is not merely a target of ERα but can in turn regulate ERα transcriptional activity. Addition of progesterone in estrogenic conditions results in PR and ERα interaction and redistribution of ERα chromatin binding in a unique gene expression pattern associated with good clinical outcome (Mohammed et al., 2015). There is also evidence that AR plays an inhibitory role in control of ERα signalling by physically interacting with EREs and thereby preventing their binding by ERα (Peters et al., 2009).

3.2.4 Posttranslational Modifiers

Finally, ERα is affected by a number of posttranslational modifications that in turn modulate receptor transcriptional activity. ERα is the target of a number of kinase signalling pathways including mitogen-activated protein kinase (MAPK), protein kinase B (Akt) and the cyclin A/cyclin-dependent kinase 2 (cdk2) complex (Campbell et al., 2001; Chen et al., 2002; Kato, 2001; Trowbridge et al., 1997). Phosphorylation of the receptor is associated with coactivator recruitment and transcriptional activation in the absence of ligand (Rochette-Egly, 2003). Silencing of the serine/threonine protein kinase glycogen synthetase-3 (GSK-3) results in increase proteosomal degradation of ERα, indicating that GSK-3 plays a protective role in receptor stabilization.

ERα protein can also be acetylated by p300/CBP on lysine residues 299, 302 and 303 within the hinge and ligand-binding domain (Wang et al., 2001) and acetylation is thought to decrease ligand sensitivity (Fuqua et al., 2000). Indeed, K303R mutation is thought to be a gain of function mutation in human breast tumours, increasing proliferation in response to low concentrations of E2 (Herynk et al., 2007).

ERα is also a target for SUMOylation by the SUMO-E3 ligases PIAS1 and PIAS3 (Karamouzis et al., 2008) and SUMOylation is known to both enhance and repress transcriptional activity. Work by our group has shown that pure antiestrogens induce SUMOylation of ERα in MCF-7 breast cancer cells, while overexpression of the deSUMOylase SENP1 abrogates the modification and derepresses transcription in the presence of full antiestrogens (Hilmi et al., 2012). Finally, ERα is ubiquitinated for degradation by the ubiquitin E3 ligase CHIP (Fan et al., 2005).

4. HORMONAL THERAPY FOR ER-POSITIVE BREAST CANCER

4.1 Antiestrogens and Aromatase Inhibitors

From as early as the 19th century, scholars noted that regression of metastatic breast tumours could be achieved through surgical oophorectomy (Beatson, 1896), thus early attempts to treat breast cancer were ablative: patients underwent surgery to remove both direct (ovaries) and indirect (pituitary gland) sources of estrogen. Pioneering work in the field by Jensen et al. led to the concept that expression of ERα in mammary tumours is indicative of patient response to endocrine ablation (Jensen et al., 1971). Since then, ERα has been routinely screened for in the clinic and ERα-positive breast tumours are treated with hormonal therapy to either prevent estrogen production from testosterone precursors (aromatase inhibitors) or inhibit estrogen receptor signalling and estrogen-dependent proliferation (antiestrogens).

Aromatase inhibitors (anastrozole, letrozole) inhibit the enzyme aromatase, normally responsible for aromatization of androgens into estrogens. While aromatase inhibitors are used primarily in postmenopausal women, there is evidence that their administration in women at higher risk of breast cancer development may help prevent disease with little to no observed toxicity (Goss et al., 2011).

Antiestrogens are small molecules with a structure similar to that of steroid hormones. Steroid mimics such as tamoxifen were developed in the 1950s and were originally intended for use as contraceptive agents (Herbst et al., 1964; Holtkamp et al., 1960). While their use was limited due to significant toxicity, inhibitory effects on breast cancer were worthy of further exploration. Antiestrogens compete with endogenous estrogens for ERα binding to modify ERα transcriptional activity (Hall et al., 2001) and can be further subdivided into two classes: selective estrogen receptor modulators and selective estrogen receptor downregulators (SERDs), otherwise known as full or pure antiestrogens.

SERMs, including tamoxifen and raloxifene, are so named due to the observation that these molecules have antiestrogenic properties in the breast but behave as agonists in other

tissues. Tamoxifen use results in osteoporosis prevention (Turner et al., 1988; Ward et al., 1993) but increased incidence of endometrial cancer (Assikis et al., 1996). Both effects mimic estrogenic action. To minimize partial agonist activity and combat observed SERM resistance, a second class of antiestrogens was developed (Wakeling, 1993). SERDs, including ICI 182,780 (fulvestrant), are steroidal compounds with long side chains, which accelerate ERα protein turnover through the ubiquitin–proteasome pathway.

As described, E2-bound ERα recruits HATs *NCOA1*, 2 and 3 and CBP/p300 to the complex. Both SERMs and SERDs induce a conformational change to ERα that alters cofactor recruitment (reviewed in Traboulsi et al., 2017). In the presence of tamoxifen, the corepressors NCOR1 and NCOR2 are instead recruited by ERα to repressed genes, and this can be reversed through siRNA-mediated knockdown of NCOR1 and NCOR2 (Lavinsky et al., 1998; Shang & Brown, 2002; Keeton & Brown, 2005). ChIP studies looking at cofactor recruitment over time have linked NCOR1/HDAC3 and NuRD/HDAC1 complex recruitment by ERα in the presence of tamoxifen with loss of histone acetylation marks and decreased RNA Polymerase II at the direct ERα-target gene promoters of *TFF1* and *MYC* (Liu and Bagchi, 2004). The variable action of antiestrogens in different cellular contexts may be due to the balance between ERα coactivators and corepressors of ERα in those cells (Shang and Brown, 2002), whereby the overexpression of coactivators in ERα-positive mammary tumours favours an estrogenic response. In hepatocarcinoma, tamoxifen activity can be redirected through exogenous overexpression of the ERα coactivator SRC1 (*NCOA1*) or the corepressor SMRT (Smith et al., 1997).

Tamoxifen was the first clinically-approved antiestrogen and remains the standard of care for adjuvant treatment of primary breast tumours and for use by high risk women as preventative therapy (Ali et al., 2011; Fisher et al., 1998; Prentice, 1990). However, while antiestrogens are effective in reducing ERα-positive breast cancer progression, a significant proportion of patients relapse on tamoxifen therapy and develop more aggressive metastatic disease even while ERα remains expressed (Musgrove and Sutherland, 2009). In many such cases, tamoxifen withdrawal or subsequent treatment with aromatase inhibitors or

fulvestrant results in disease remission (McDonnell et al., 2015), suggesting that ERα signalling remains active in these tumours to some degree, even in the presence of tamoxifen.

4.2 Mechanisms of Resistance to Endocrine Therapy

Approximately 25% of ER-positive breast cancer patients will develop resistance to hormonal therapy within 10 years of diagnosis (Early Breast Cancer Trialists Collaborative Group, 2005). A number of mechanisms have been identified through which ER-positive breast tumours acquire resistance to antiestrogens, and these include either loss of ERα or alteration of ERα signalling, resulting from either selection of a resistant cell subpopulation present in the original tumour or from development of resistance via genetic or epigenetic changes. However, ERα expression is conserved in the majority of resistant tumours, suggesting ERα signalling has an ongoing role in tumour progression (Johnston, 1997).

Growth factor signalling, including signalling by the HER2 pathway, is often deregulated in antiestrogen-resistant breast cancer cells. For example, the growth of a HER2-overexpressing, ER-positive cell model, MCF-7/HER2-18, is induced in the presence of tamoxifen (20). Furthermore, the levels of coactivators vs. corepressors available in the cell will dictate tamoxifen activity. Amplification of NCOA3 is associated with poorer prognosis of ER-positive tumours and overexpression of NCOA3 correlates with tamoxifen resistance (23). Additionally, insulin-like growth factor receptor 1 (IGFR1) and fibroblast growth factor receptor 1 (FGFR1) activate MAPK and PI3K signalling pathways and this has been linked to lack of response to tamoxifen therapy (24).

An additional mechanism of resistance is the presence of mutations in the ligand-binding domain of ERα (Li et al., 2013; Robinson et al., 2013). Most mutations that have been characterized in resistant tumours (E380Q, L536Q, L536R, D538G, Y537S, Y537C, Y537N) confer a gain-of-function to the receptor allowing it to function in a ligand-independent manner (Jeselsohn et al., 2014; Li et al., 2013; Robinson et al., 2013; Toy et al., 2013). Constitutively-active mutants demonstrate increased S118 phosphorylation levels,

recruitment of NCOA coactivators and ligand-independent tumour growth in xenograft models (Fanning et al., 2016; Merenbakh Lamin et al., 2013; Toy et al., 2013). Several of the above-mentioned mutations (Y537S and D538G) impact ERα conformation, decreasing affinity to ligands (both E2 and tamoxifen) and altering target gene selection (Fanning et al., 2016).

5. RNA INTERFERENCE FOR FUNCTIONAL GENOMICS

5.1 RNAi

Pioneering work by the group of Fire and Mello uncovered the participation of double stranded RNA in suppressing gene expression (Fire et al., 1998) and led to a Nobel prize in 2006. The RNA interference (RNAi) pathway in eukaryotes is initiated by cleavage of long double-stranded RNA molecules into shorter fragments approximately 20 nucleotides in length by Dicer. These are termed short interfering RNAs (siRNAs). While the passenger strand of the double-stranded siRNA structure is degraded, the guide strand becomes incorporated into an RNA-induced silencing complex (RISC). The guide stranded also pairs with a complementary sequence in a target mRNA molecule, inducing cleavage and resulting in gene silencing (Sen and Blau, 2006).

Shortly after the discovery of RNAi, the first systematic analysis of gene repression was undertaken in *Caenorhabditis elegans*, whose genome had been sequenced at the time (Fraser et al., 2000; Gönczy et al., 2000), and this eventually led to genome-wide screening in *C. elegans* (Hamilton et al., 2005; Kim et al., 2005; Simmer et al., 2003) and in *Drosophila melanogaster* (Boutros et al., 2004; DasGupta et al., 2005; Müller et al., 2005). Implementation of RNAi technology in mammalian cells was initially limited, as long double-stranded RNA molecules activate an innate antiviral immune response in the cell (Judge et al., 2005; Marques and Williams, 2005). This can be circumvented by using short (21 nucleotide, double stranded) interfering RNAs (siRNAs) (Elbashir et al., 2001). Today, siRNA-mediated knockdown approaches, both for study of individual genes and on a genome-wide scale, have been widely adopted in functional genomics research, aided through the availability of libraries of chemically synthesized siRNAs and short hairpin RNAs (shRNAs).

shRNAs offer the benefit of stable genomic integration for long-term knockdown in non-dividing cells, through the use of a viral vector carrying an RNAi expression cassette, which produces short double-stranded RNA molecules that are then processed to produce siRNA (Root et al., 2006).

5.2 High-Throughput RNAi Screening Efforts in ERα Biology

With the recent development and wider accessibility of high-throughput technologies, efforts have been made to identify novel regulators of ERα signalling in the context of breast cancer and to delineate genes that may contribute to antiestrogen sensitivity or resistance. These studies are outlined in Table 2.

A number of kinases not previously known to regulate ERα transcriptional activity were reported in a study by Giamas et al. (2011). In this study, the authors performed a kinome siRNA screen (691 kinases and kinase-related genes) in MCF-7 cells with expression of *TFF1* transcript, a direct transcriptional target of ERα, as the primary readout. The authors identified lemur tyrosine kinase-3 (LMTK3) as a potent regulator of ER signalling, which acts by decreasing activity of PKC and AKT, increasing binding of FOXO3 to the *ESR1* promoter, a known regulator of ERα expression described above (Guo and Sonenshein, 2004). LMTK3 silencing leads to abrogated proliferation and reduced tumour volume in ER-positive breast cancer cells only.

A second recent study focusing on a panel of 281 known transcriptional regulators, used high-content microscopy for identification of modulators of expression of a fluorescent protein-tagged ERα following knockdown with siRNA. This group uncovered the E3 ubiquitin ligase UBR5, a known oncogene that was found to regulate ERα protein levels and transcriptional activity, as well as proliferation of ER-positive breast cancer cells (Bolt et al., 2015). Both of these studies were performed in a pooled format with two to three siRNAs per candidate gene.

On a related note, a number of high-throughput studies have focused on identification of genes regulating tamoxifen sensitivity in ER-positive breast cancer, either through gene knockdown in long-term estrogen-deprived (LTED) cells that have adapted to growth in E2-depleted conditions (Bhola et al., 2015; Fox et al., 2011) or in cells grown for long periods of time in the presence of the antiestrogen tamoxifen (Mendes-Pereira et al., 2012).

Table 2. Previous siRNA screening studies in ERα signalling.

Reference	Model	Endpoints	Scope	Protocol	Delivery	Main Finding
Fox EM et al., 2011	MCF-7/LTED (long-term estrogen deprived)	Cell viability (alamar blue)	779 kinases	Transfection of siRNA (pool of 4 siRNA per gene); 4 days	Transfection	LMTK3 decreases PKC activity and AKT phosphorylation, increasing FOXO3 binding to the ESR1 promoter; LMTK3 phosphorylates ERα, protecting it from proteasomal degradation
Giamas G et al., 2011	MCF-7	qPCR for TFF1 vs GAPDH	691 kinases and kinase-related genes	Transfection of siRNA (pool of 2 siRNA per gene); 10nM E2 for 24h	Transfection	The insulin receptor is required for growth of MCF-7/LTED cells
Mendes-Pereira AM et al., 2012	MCF-7	Survival following OHT treatment (500nM) for 21 days	Genome-wide	Pooled shRNA, 72h infection, puromycin selection, OHT treatment (500nM) for 21 days, massive parallel sequencing	Lentiviral infection	Genes whose silencing causes tamoxifen resistance (BAP1, CLPP, GPRC5D, NAE1, NF1, NIPBL, NSD1, RAD21, RARG, SMC3, and UBA3) and genes whose silencing causes sensitivity (C10orf72, C15orf55/NUT, EDF1, ING5, KRAS, NOC3L, PPP1R15B, RRAS2, TMPRSS2, and TPM4)
Bhola et al., 2014	MCF-7/LTED + pGLB-MERE (ERE-regulated Firefly luciferase)	Luciferase and alamarBlue	720 kinases	Kinome-wide siRNA screen using a library targeting 720 kinases	Transfection	PLK1 downregulation results in inhibition of estrogen-independent ER transcriptional activity and growth of LTED cells
Bolt MJ et al., 2015	GFP-ERa: PRL-HeLa	ERα protein levels and nuclear translocation, DNA binding, chromatin remodeling and reporter gene transcriptional output	281 transcriptional modulators and coregulators	Transfection of siRNA (pool of 3 siRNA per gene) for 72h; 10 nM E2 for 30 min	Transfection	UBR5 modulates ERα protein levels and transcriptional output, as well as E2-mediated cell proliferation in breast cancer cells
Marcotte R et al., 2016	77 breast cancer cell lines	shRNA 'dropout' based on DNA sequencing following 6 to 8 population doublings	Genome-wide	Transduction of shRNA (pool of 80k shRNA; MOI 0.3-0.4) for 6 to 8 population doublings, confirmation using alamarBlue following siRNA-mediated knockdown for 7 days	Lentiviral infection	Identification of numerous genes essential in specific subtypes of breast cancer; BRD4 is essential in the luminal subtype

RATIONALE

Breast cancer affects one in eight women in developed countries and is the second cause of death by cancer in this population (Canadian Cancer Society, 2015). While estrogens control normal mammary development, they also contribute to breast tumourigenesis in approximately 70% of breast tumours (Deroo and Korach, 2006; LaMarca and Rosen, 2007) via the expression and activity of ERα (Ascenzi et al., 2006; Hall et al., 2001; Nilsson et al., 2001). While antiestrogens have been developed to prevent the proliferation of ER-positive breast cancer, a significant proportion of patients relapse on tamoxifen therapy and develop more aggressive metastatic disease even while ERα remains expressed (Musgrove and Sutherland, 2009). In many such cases, tamoxifen withdrawal or subsequent treatment with aromatase inhibitors or SERDs results in disease remission (McDonnell et al., 2015), suggesting that ERα signalling remains active in these tumours to some degree, even in the presence of tamoxifen. Furthermore, one of the identified mechanisms for antiestrogen resistance is expression of constitutively active ERα mutants, which can participate in ligand independent activity of the receptor. The wide accessibility of high-throughput screening technologies have allowed for studies that identify regulators of ERα signalling and/or expression. While efforts have been made to identify novel regulators of ERα signalling in the context of breast cancer, none of these has been genome-wide. We hypothesized that a genome-wide shRNA screening approach could identify novel regulators of ERα signalling not previously identified by more targeted studies. Targeting these factors could potentially lead to more effective and personalized treatments for ER-positive breast cancer patients.

Consequently, our aims in the following study were:

1) To design and optimize a genome-wide shRNA screen to identify novel regulators of ERα signalling and expression (Chapter 1); and

2) To execute this screening protocol and explore the possible mechanisms of action of several of our selected candidates (Chapter 2).

CHAPTER ONE:

Development and Optimization of a Multi-Endpoint Genome-Wide shRNA Screen for Genes Affecting ERα Signalling and Estrogen-Induced Proliferation of Breast Cancer Cells

INTRODUCTION

RNA interference represents an efficient approach to perturb gene function in model systems, including cultured cells, and has provided an alternative to genetic loss-of-function experiments aimed at discovering new components of specific cellular functions. Genome-wide shRNA screens can be performed with limited infrastructure requirements in a pooled format using functional assays that are amenable to a selection procedure (Blakely et al., 2011). Typically, this involves comparing the representation of shRNAs in cell populations selected under specific conditions with that in unselected populations. While this approach has been used extensively in survival assays in cultured cell lines, the process of deconvolution of the representation of shRNAs in the presence and absence of selection is not trivial, and biases may occur during the selection process. These may result from population effects; clones carrying different shRNAs may compete against each other for selection. In addition, complex interactions between individual clones may take place.

On the other hand, screens of arrayed libraries bypass these limitations but are associated with their own technical complexities. Typically, robotized facilities need to be used to screen genome-wide shRNA libraries in higher eukaryotes. Each shRNA should be produced with titers high enough to yield an appropriate multiplicity of infection and efficient target gene knockdown. As the screening process takes place over several weeks, assays need to be sensitive and robust, enabling high reproducibility over the screening time frame. As a result, relatively few genome-wide shRNA screens have been successfully performed. Here, we report the design and optimization of such a screen using the Sigma Mission lentiviral library targeting protein-coding genes in the human genome to identify candidates that modulate estradiol signalling in human breast cancer cells.

Estrogen receptor α (ERα) is expressed in luminal breast tumours, where it drives cell proliferation through the regulation of genes controlling the G1/S and G2/M transitions of the cell cycle (Bourdeau et al., 2008; Eeckhoute et al., 2006; Frasor et al., 2003). While therapeutic strategies opposing estrogenic action by blocking estrogen production

(aromatase inhibitors) or signalling (antiestrogens) are the main form of targeted treatment for ERα-expressing breast cancer, one in three breast tumours is intrinsically insensitive to treatment due to lack of the target, ERα, and about 50% of ER-positive breast cancer patients eventually acquire resistance to hormonal therapy and experience local or metastatic relapse (Early Breast Cancer Trialists' Collaborative Group et al., 2011; Palmieri et al., 2014). The identification of genes and/or molecular signalling pathways acting on ERα expression or function is therefore crucial to better understand antiestrogen resistance and to develop novel therapies for luminal breast cancer.

Binding of ligands such as 17-β-estradiol (E2) to ERα results in receptor dissociation from heat-shock proteins and binding to DNA at estrogen response elements (EREs) in association with transcriptional co-factors, leading to changes in gene expression patterns (Figure 1). ERα can also tether to transcription factors to regulate expression of their target genes in an ERE-independent manner, as is the case for ERα interaction with Fos and Jun proteins at AP-1 binding sites (Gaub et al., 1990; Sabbah et al., 1999) or act in a ligand-independent manner following activation by a number of kinase-signalling pathways, including AKT, PKA and MAPK (Al-Dhaheri and Rowan, 2007; Campbell et al., 2001; Thomas et al., 2008). Finally, membrane-associated ERα can initiate rapid signalling events that may ultimately influence gene transcription (reviewed in Björnström and Sjöberg, 2005).

ERα signalling is affected by a number of proteins that modulate its expression and/or activity. The main characterized upstream regulators of ERα gene expression are the transcription factors p53, GATA3 and FOXA1. These proteins modulate transcription by binding to the gene promoter (p53) (Angeloni et al., 2004; Hurd et al., 1995; Shirley et al., 2009) or enhancer regions (FOXA1, GATA3) (Bernardo et al., 2010; Eeckhoute et al., 2006; Serandour et al., 2013). Furthermore, they can bind with ERα to promoters of its target genes to affect its transcriptional activity (Hurtado et al., 2011; Liu et al., 1999). Altogether, 639 proteins interact directly with nuclear receptors in different cell types (NURSA; www.nursa.org). ERα cofactors include the histone acetyltransferase SRC/CBP/p300 complex, the histone methyl transferases CARM1 and PRMT1, the Mediator complex and the

SWI-SNF chromatin remodelling complex (Burakov et al., 2002; Green and Carroll, 2007; Hall and McDonnell, 2005; Ichinose et al., 1997; Ratajczak, 2001; Smith and O'Malley, 2004). Additionally, components of the ubiquitin ligation and proteasome complexes also act as ERα cofactors (vom Baur et al., 1996; Nawaz et al., 1999; Smith et al., 2002; Verma et al., 2004). On the other hand, corepressors recruited in a ligand-dependent manner such as LCoR and NRIP1 attenuate hormone-induced transactivation and limit ERα transcriptional activity (White et al., 2004). Importantly, coactivators and corepressors are not specific for ERα, but are shared with other nuclear receptors or transcription factors.

In this study, we describe the design and optimization of an arrayed genome-wide shRNA screen to identify genes modulating ERα expression, signalling and E2-dependent proliferation. We used available subclones of p53 mt T47D and p53 wt MCF-7 cells carrying a stably integrated estrogen-responsive firefly luciferase gene. Luminescence has been widely used in high-throughput screening applications to identify chemical compounds acting on specific signalling pathways (Fan and Wood, 2007). Here, we also discuss our approach for identifying false-positive hits through the use of an E2-independent luciferase reporter construct. Additionally, since shRNA performance can be affected by the efficiency of lentiviral transduction and epigenetic modifications or activity of the shRNA promoter in a cell-specific context (Hong et al., 2007; Liu et al., 1997), we describe an optimized screening assay in a second, complementary luminal breast cancer reporter cell line, MELN. Finally, we have monitored cell viability using an alamarBlue assay to identify genes essential for survival or mediating the proliferative effects of estrogens in breast cancer cells. We have validated these assays by monitoring the effects of ERα knockdown and the knockdown of the known ERα cofactor, NRIP1 as a proof of principle. Ultimately, our screening approach will help identify novel factors crucial for ERα signalling and may pave the way for the development of new therapeutics for a more effective treatment of breast cancer.

MATERIALS AND METHODS

Cell Culture

The human ERα-positive breast cancer cell lines T47D-KBLuc, T47D and MCF-7 were purchased from ATCC. MELN cells were a kind gift from Dr. Balaguer (Montpellier, France). MCF-7-ARE and T47D-ARE cell lines were generated by infecting MCF-7 and T47D cells, respectively, with lentivirus expressing an inducible antioxidant-response element (ARE) firefly luciferase reporter (Qiagen). All cell lines were maintained in RPMI-1640 medium (Wisent) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate and 1% Penicillin Streptomycin.

Antiestrogen Assays

T47D-KBLuc cells were maintained in phenol red-free RPMI medium (10% dextran-charcoal treated FBS, 2.5 g/L D-glucose, 10 mM HEPES, 1 mM sodium pyruvate, 2% L-glutamine, 1% Penicillin Streptomycin) for 72 hours. Cells were seeded at a density of 10,000 cells per well in 96-well white luminescence plates (BD Falcon). The following day, medium was changed for medium containing the antiestrogens 4-hydroxytamoxifen (OHT) or fulvestrant (ICI 182,780) in combination with E2 (10 nM) or an equal volume of DMSO. Antiestrogens were administered with a starting concentration of 5 uM, and 1:3 dilutions thereafter across 11 concentrations. Cells were incubated for 24 hours at 37°C. Plates were washed once with PBS. Cells were lysed and D-luciferin substrate (Nanolight Technologies) was added. Plates were read on a luminescence counter following eight minutes of incubation in the dark at room temperature.

Automated High-Throughput Screening

For all assays, cells were seeded manually. Media changes and virus addition was performed using a Biomek FX pipettor with 96-well head (Beckman). Plates were maintained at 37°C for the duration of each assay (four or eight days). Plate washes for primary screening were

performed using the Biotek ELx405 plate washer, or with the Biomek FX pipettor for subsequent assays. Fluorescence (alamarBlue assays) and luminescence readings were performed using the Envision plate reader (enhanced luminescence, Perkin-Elmer; primary screening) and the POLARstar plate reader (BMG; secondary screening).

shRNA Libraries and Lentivirus Production

We utilized the genome-wide shRNA library from Sigma, which targets 16,083 protein-coding genes in the human genome. shRNA in this library is cloned into the pLKO.1 backbone, which also contains bacterial ampicillin and mammalian puromycin resistance markers. For our primary, genome-wide shRNA screen, three different shRNA clones per gene for each of 16,083 genes was produced as lentivirus to ease shRNA delivery to T47D-KBLuc cells. Lentivirus was produced using the Mirus transfection reagent, as per the manufacturer's recommended protocol. In brief, human embryonic kidney cells (HEK 293) were seeded at an initial seeding density of 22,000 cells per well in 96-well plates. Cells were transfected with a mixture of lentiviral packaging plasmids (SHP001, Sigma) and shRNA (200 ng) in combination with the transfection reagent (Mirus). Lentivirus was collected 48 and 72 hours following transfection and stored at -80°C before use. Viral titers were determined for randomly selected wells (~4% of wells in each 96-well plate) by infecting HeLa cells with a 1:10,000 dilution of lentiviral supernatant, and selecting with puromycin (1 ug/mL) for five days. Colonies were visualized by crystal violet staining and colony counts in each well were used to estimate the number of viral particles per milliliter of viral supernatant. An average titer for all lentiviruses produced was established by taking the mean of viral titers for all wells in the titration. For our screen, viral titers were estimated at ten million viral particles per milliliter. Taking into consideration the drop in viral titers by ~50% at the first freeze-thaw, we used a final titer of five million viral particles per milliliter to calculate the volume of viral supernatant to add to each well.

Short-term Luciferase and Cell Viability Assays

T47D-KBLuc and MELN cells were seeded manually at a density of 5,000 or 2,000 cells per well, respectively, in opaque white 96-well plates (BD Falcon, primary screening or Greiner CELLSTAR, subsequent assays), in phenol red-free RPMI (Wisent) supplemented with 10% heat-inactivated, charcoal dextran-stripped FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2% L-glutamine and 1% Penicillin Streptomycin. The following day, media was replaced with fresh media supplemented with 2 ug/mL polybrene and 25 nM E2. Cells were transduced in an arrayed format at an average multiplicity of infection (MOI) of 10. Each plate also contained control wells with no virus, non-targeting shRNA, shRNA against the estrogen receptor and wells treated with vehicle (0.1% DMSO). Following four days of infection, media was replaced with media containing a 1:50 dilution of alamarBlue® (Invitrogen). Plates were incubated for three hours at 37°C, after which absorbance was read. Cells were washed with PBS, and incubated with a solution of N-Ethylmaleimide (NEM)-containing lysis buffer and luciferin substrate (Nanolight Technology) and incubated at room temperature in the dark for eight minutes. Luminescence was read on the Envision plate reader (Perkin Elmer). The primary screening protocol and plate layout is summarized in Figure 2.

Counter-Screening Assays

T47D-ARE and MCF-7-ARE cells were seeded at a density of 10,000 and 5,000 cells per well, respectively, in Greiner CELLSTAR opaque white 96-well plates (Sigma) in phenol red-free RPMI (Wisent) as previously described. The following day, media was changed for hormone-free media containing 2 ug/mL polybrene. Cells were infected at an MOI of approximately 10 lentiviral particles per cell. 15 hours prior to alamarBlue® addition, media was changed for media containing 10 μM DL-Sulforaphane to stimulate the Nrf2 pathway governing luciferase reporter expression in these cells. alamarBlue® and luciferase assays were performed as described above for primary screening.

Long-Term Luciferase and Proliferation Assays

T47D-KBLuc and MELN cells were seeded at a density of 2,000 and 1,000 cells per well, respectively. The following day, media was replaced with fresh media supplemented with 2 ug/mL polybrene and 25 nM E2. Cells were infected as above. Following four days of infection/E2 treatment, media was changed to fresh media containing 25 nM E2. AlamarBlue® and luciferase assays were performed following eight total days of infection/E2 treatment, as described above.

Western Blotting

T47D-KBLuc were seeded manually at a density of 5,000 cells per well in transparent 96-well plates (Costar 3595) and treated and infected as described for primary screening. Following four days of infection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and stored at -20°C. Whole cell extracts were prepared by addition of 90 uL per well of lysis buffer containing 50 mM Tris-Cl pH7.5, 150 mM NaCl, 2% SDS, 0.5% Triton X-100, 1% NP40, 20 mM N-ethylmaleimide and a cocktail of protease and phosphatase inhibitors. Plates were incubated on ice for 10 minutes. Lysates were heated at 95°C for five minutes and 30 uL of lysate was loaded onto 8% SDS-PAGE gels, separated and transferred to a PVDF membrane. Membranes were incubated with a monoclonal rabbit ERα antibody (60C; Millipore). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and the Western Lightning ECL Pro (Perkin Elmer) kit. Membranes were stripped and reblotted using a mouse anti-β-actin mouse antibody (AC-15; Sigma) to normalize for total protein. ERα and β-actin protein expression was quantified using Image J software (Schneider et al., 2012).

Z-factor Calculation

To determine if each assay was sufficiently robust for high-throughput screening in 96-well plates, a Z-factor was calculated using the following formula:

$$\text{Z-factor} = 1 - \frac{3(\sigma_P + \sigma_N)}{|\mu_P - \mu_N|}$$

where σ_P is the standard deviation of the positive control and σ_N is the standard deviation of the negative control (shNT), μ_P is the mean of the positive control and μ_N is the mean of the negative control in each assay. For this purpose, alternating columns of a 96-well plate were infected with non-targeting shRNA (shNT) or shRNA targeting the positive control for that assay (shER or shNRF2) (Figure 2).

Data Analysis

Primary high-throughput screening data is representative of a single assay. Percent inhibition of the E2-induced luciferase reporter was calculated using the following formula:

$$\% \text{ inhibition} = 100 - \left(\frac{x - \text{Background}}{\text{shNT} - \text{Background}} \text{ x } 100 \right)$$

where x is the luciferase read for each individual hit and shNT is the mean of four wells treated with non-targeting shRNA on each plate.

qRT-PCR and Transcriptome Sequencing

T47D-KBLuc cells were seeded in 24-well plates at a density of 60,000 cells per well in phenol-red free RPMI. The following day, media was replaced with fresh media supplemented with 2 ug/mL polybrene and 25 nM E2 or an equal volume of DMSO. Cells were transduced with shRNA at an MOI of 10. Following four days of infection, cells were washed twice with ice-cold PBS and RNA was extracted using the RNeasy mini kit (Qiagen).

500 ng of RNA was reverse-transcribed using an oligo dT primer to confirm target knockdown by qRT-PCR.

Transcriptome sequencing was performed at the genomics facility at our institute. RNA integrity was verified on the Agilent Bioanalyzer 2100. 500 ng of total RNA was used to prepare RNA-Seq libraries using the KAPA RNA Stranded Library kit with PolyA capture following the manufacturer's protocol (Roche). Sequencing was performed on an Illumina HiSeq2000. Samples were demultiplexed using Casava 1.8.2 (Illumina, San Diego, CA). Gene expression in the presence of each of the two shRNAs against each hit was compared to that in controls, corresponding to two non-targeting controls (shNT+E2, shGFP+E2) and to non-infected cells (Ni+E2), while gene induction by E2 was determined by comparing non-infected, E2-treated cells (Ni+E2) with DMSO-treated cells (Ni-E2). Mapping of reads to transcripts in the human genome (Ensembl annotation GRCh38.85) was performed with Kallisto (Bray et al., 2016), and differential expression was assessed both at the transcript and gene level using Sleuth (Pimental et al., 2016). For this study, differential gene expression values were filtered using a beta-value (b-value) cut-off of ± 0.4. The b-value is a bias estimator generated by Sleuth that is analogous to fold change in gene expression.

RESULTS

Global screening strategy

The overall screening pipeline is summarized in Figure 3. For primary screening, we designed a genome-wide arrayed assay using three unique clones of shRNA targeting each of 16,083 protein-coding genes in the human genome, to identify those hits with two or more shRNAs affecting reporter gene expression. The top ~600 candidates of interest are selected based on maximal effect on luciferase (ER transcriptional activity) and minimal effect on fluorescence (cell viability) and are then tested in subsequent secondary screens with five shRNAs per gene both to reproduce primary screening results and to select hits that are most likely to affect ERα signalling. Finally, the most promising ~30 genes (effectors of ER signalling and long-term proliferation assays with minimal effect in counterscreening assays) are interrogated further, through focused assays to confirm down-regulation of shRNA target gene expression and to examine the impact of each hit on the transcriptome.

Genome-wide screen for modulators of ERα signalling

Estrogens directly regulate gene expression by binding to EREs in the promoters of target genes. The T47D-KBLuc cell line is an ER-positive human breast cancer cell line that stably expresses a luciferase reporter downstream of three consensus EREs. The cell line was originally developed for screening of chemicals for estrogenic and antiestrogenic properties (Wilson et al., 2004) and has been used in a number of studies for monitoring water quality and screening environmental toxins for estrogenic activity (Kunz et al., 2016; Leusch et al., 2010; Pop et al., 2015). We have also used T47D-KBLuc cells to monitor the antiestrogenic potential of novel hybrid molecules that combine antiestrogenic and histone deacetylase inhibitor activity (Mendoza-Sanchez et al., 2015).

In this study, we have explored T47D-KBLuc cells as a model for the identification of genes contributing to ERα signalling using an shRNA knockdown approach. Concentrations

of E2 in the nanomolar range potently and reproducibly induced expression of the luciferase reporter in this cell line (Figure 4). E2-stimulated luciferase activity can be efficiently repressed through the addition of the antiestrogens 4-hydroxytamoxifen (OHT) (Figure 4A) or fulvestrant (ICI 182,780) (Figure 4B). Additionally, knocking down ERα expression using shRNA (TRCN0000003300) at an MOI of 10 for four days resulted in decreased expression of ERα protein (Figure 5A) and suppression of E2-induced reporter vector activity (Figure 5B). T47D cells express an altered version of the tumour suppressor p53, carrying a missense mutation at residue 194, within the zinc-binding domain (O'Connor et al., 1997; Schafer et al., 2000). p53 has been previously shown to regulate ERα expression in MCF-7 cells (Angeloni et al., 2004; Shirley et al., 2009). Of interest, knockdown of p53 also affected E2-induced reporter vector activity in T47D-KBLuc cells (Figure 5B), suggesting an impact on ERα expression or activity in spite of the presence of mutant p53. NRIP1 is an ERα corepressor that is recruited in the presence of E2 via LXXLL motifs (Heery et al., 1997, 2001). As predicted, knockdown of *NRIP1* using shRNA resulted in increased E2-stimulated transcription from the reporter gene (Figure 5B). Therefore, we conclude that the T47D-KBLuc cell line represents a sensitive model to study the contribution of regulators of ERα expression to its transcriptional activity.

We tested the robustness of this assay in 96-well plates seeded with T47D-KBLuc cells (5,000 cells per well) treated with E2 and transduced with shNT or shER (TRCN0000003300; MOI 10; four days) in alternating columns, as shown in Figure 7A. This cell seeding density was found to be optimal, both to maximize the luminescence signal, and to ensure cells were actively proliferating following four days of incubation with E2 without reaching confluency in the well. Simultaneous addition of E2 and lentiviruses 24 hours after manual seeding was chosen to minimize robotic time and associated costs. Moreover, a single E2 treatment (25 nM) four days prior to luminescence reading was sufficient to maintain signal for the duration of the assay in T47D-KBLuc cells (Figure 6A). Z-factor calculations determined this assay to be robust and suitable for high-throughput screening (Z-factor of 0.51; Figure 7B). While a similar Z-factor was achieved by infecting cells with

lentiviruses at MOI 15 (data not shown), we opted to use the lowest viral concentration that still produced a robust assay to minimize off-target effects.

Knocking down essential genes is expected to decrease T47D-KBLuc cell survival and thus luciferase expression in the presence of E2. To flag such genes, we implemented a cell viability assay using the reagent alamarBlue®, which exploits the mitochondrial reductive processes of living cells to convert the dye resazurin to fluorescent resorufin (O'Brien et al., 2000; Rampersad, 2012). While suppression of E2-signalling through down-regulation of ERα eventually leads to decreased proliferation in ERα-positive breast cancer cells, our four-day knockdown was not long enough to produce a discernable effect on T47D-KBLuc proliferation in the presence of E2 (Figure 7C). Therefore, the alamarBlue assay could effectively be used to monitor E2-independent variations in cell viability and to correct for any inconsistencies in cell seeding without being affected by suppression of E2 signalling.

Secondary Screens

MELN cells, a second cell model of ER-positive breast cancer

The T47D-KBLuc cell line, which expresses ERα and its target gene, the progesterone receptor (PR), is representative of luminal breast cancer. However, ERα-positive tumours can exhibit a great deal of variability in their genetic background, manifested in differences in expression level or mutational status of genes found upstream of the ERα signalling pathway and targeted by our screen. For example, T47D cells express lower levels of ERα protein than do MCF-7 cells, another ERα/PR-positive model of luminal breast cancer (Andruska et al., 2012) (Figure 8A). Furthermore, the tumour suppressor gene *TP53* is mutant in 26% of luminal breast tumours (Bertheau et al., 2013), as it is in the T47D-KBLuc cell line. We therefore sought to determine whether identified shRNA hits are specific to the T47D-KBLuc cell line or if the effect of each shRNA can be extended to other models of ERα-positive breast cancer. To address this question, a reporter assay was developed in MELN cells,

which are MCF-7 breast cancer cells expressing a luciferase reporter downstream of a single ERE and the β-globin promoter (Balaguer et al., 2001). ERα, expressed at higher levels than in T47D-KBLuc cells, drives expression of the luciferase gene in the presence of E2 (0.1 or 25 nM) with a lower fold induction than in T47D-KBLuc cells (3.3-fold in MELN vs. 11.2-fold in T47D-KBLuc), likely due to the presence of only one ERE in the MELN reporter promoter versus three in the luciferase promoter of T47D-KBLuc cells. It is also possible that the β-globin promoter yields higher levels of basal activity than the TATA box found in the T47D-KBLuc reporter vector. Similar to T47D-KBLuc cells, E2-induced expression can be efficiently knocked down by addition of antiestrogens (OHT, 10nM or ICI, 100 nM) (Figure 8B). Furthermore, the ERE-luciferase reporter activity in MELN cells is also induced with a single E2 treatment four days prior to luminescence reading (Figure 6B) and is repressed with shER (TRCN0000003300) administered for four days at an MOI of 10, yielding a robust assay with a Z-factor of 0.62 (Figure 8C).

Counterscreen development in T47D and MCF-7 cells to eliminate false positives

While regulators of ERα signalling are expected to regulate ERE-driven luciferase gene expression, the final luminescence readout can also be influenced by ERα-independent confounding factors. For instance, knockdown of genes affecting overall transcription or translation processes will reduce luciferase gene expression independently of ERα. Furthermore the luminescence readout requires oxygenation of firefly luciferin using ATP, magnesium ions and oxygen, yielding oxyluciferin, an unstable, light-emitting molecule (Leitão and Esteves da Silva, 2010). Any shRNA interfering with these processes will also present as a false positive in our screening approach.

In order to eliminate false-positive hits from our primary screen results, we produced reporter cell lines in MCF-7 and T47D cells (MCF-7-ARE and T47D-ARE cell lines, respectively) in which the luciferase gene is under the control of an antioxidant response

element (ARE; Figure 9). When treated with the antioxidant DL-Sulforaphane (DLS), which modifies Keap1 cysteine residues to disrupt the Keap1-Nrf2 complex (Hu et al., 2011), MCF-7-ARE and T47D-ARE cells respond with a significant increase in luminescence, which is impaired in cells where Nrf2 is knocked down with shRNA. Importantly, this response is not affected by administration of E2 (25 nM) or by knockdown of ERα (Figure 10). The assay was robust in T47D-ARE cells (Z-factor = 0.54; Figure 11A), but not in MCF-7-ARE cells (Z-factor = 0.25; Figure 11B), indicating that T47D-ARE cells can be used as a suitable counter-screen to eliminate false-positive 'hits' from our primary screening data.

shRNA effects on E2-induced proliferation

Regulation of target gene expression by ERα in ERα-positive breast cancer cells ultimately leads to cell proliferation and tumour expansion. Indeed, increased expression of ERα or administration of E2 in MCF-7 cells that have been growth-arrested by estrogen deprivation promotes S–phase entry and cellular proliferation (Liao et al., 2014). E2 treatment is followed by a marked increase in cyclin D1 expression, and activation of Cdk4 through association with cyclin D1, an essential step in G1 phase progression (Altucci et al., 1996; Doisneau-Sixou et al., 2003; Foster and Wimalasena, 1996). E2 also affects expression of Myc, E2Fs, *FOXM1* and B-Myb, all of which are transcription factors that control expression of cell cycle genes. It follows that any gene affecting ERα-signalling in our screening approach may also have an impact on ERα-positive breast cancer cell proliferation. To determine at which time point this impact can be observed, we monitored the impact of ERα knockdown on T47D-KBLuc cell and MELN cell proliferation both in a short-term (four day) (Figure 7C; Figure 12B) and longer term (eight day) assays (Figure 12A; Figure 12C). While little effect could be detected after four days, extension of the assay to eight days with a complete media change and E2 replenishment at day four revealed a perceptible difference between shNT and shER samples. This assay was robust (Z-factor = 0.57) in MELN cells (Figure 12C), but not in T47D-KBLuc cells, the Z-factor remaining below 0.5 in this cell line (Figure 12A). As mentioned,

T47D cells express lower levels of ERα protein than MCF-7 (Andruska et al., 2012) (Figure 8A), possibly resulting in lower sensitivity to estrogens.

Target Confirmation and Mechanistic Assays

Testing for effects on expression of the estrogen receptor

Hits in our screen could affect ERα-signalling at any point in the signalling cascade (Figure 1). Target knockdown may affect ERα activity as a transcription factor due to absence or altered activity of essential cofactors. For example, siRNA-mediated depletion of the SRC coactivator *NCOA1* leads to decreased activation of the ERα target gene *TFF1* without modulation of ERα protein expression (Karmakar et al., 2009). Alternatively, some hits may influence the expression of the receptor itself, leading to alterations in ERα signalling due to variations in ERα protein levels.

In order to directly identify hits affecting ERα protein expression, we explored the feasibility of using a high-throughput western blot assay in 96 well plates using T47D-KBLuc cells. Limiting amounts of protein extract prevented standardization of protein levels. Instead, cells were lysed directly in the plate and loaded onto a polyacrylamide gel without protein quantification. Since different shRNAs may affect cell proliferation to variable extents, and to correct for seeding variations, normalization at the protein detection step is desirable. We therefore monitored the ratio between ERα protein (60C antibody from Millipore) and β-actin protein (Figure 13A). Ratios between ERα and β-actin were calculated using Image J software (NIH) (Figure 13B). This approach confirmed a significant decrease ($p<0.05$) in relative ERα protein expression (86%) following transduction of shER (Figure 13C). While not feasible on a genome-wide scale due to the limited number of samples that can be processed at one time (resulting in over 4,000 polyacrylamide gels from a genome-wide screen), this assay is useful to discern changes in ERα protein expression in a small subset of samples where it is hypothesized that knockdown of a particular gene alters levels of ERα.

Confirmation of Target Knockdown

The Sigma Mission shRNA library used in this study contains shRNA designed by the Broad Institute, using an algorithm to target genes specifically and minimize off-target effects (http://portals.broadinstitute.org). However, not all shRNAs have been validated to actively repress their target gene. Furthermore, performance of shRNA can be affected by the efficiency of lentiviral transduction or the activity of the shRNA promoter. Additionally, epigenetic modifications within the host cell may lead to silencing of shRNA expression (Hong et al., 2007; Liu et al., 1997). Target knockdown confirmation is not feasible on a genome-wide scale due to the high cost associated with RNA extraction and qRT-PCR and the necessity to acquire and optimize efficient primers for each of >16,000 genes tested. However, it is a necessary step once a short list of gene hits has been selected, to ensure target knockdown is successful and that observed effects are not simply the consequence of off-target events.

In order to confirm knockdown of ERα, we isolated total RNA four days following transduction of two distinct shRNAs in T47D-KBLuc cells, using the same culture conditions as for primary screening, and performed qRT-PCR to measure expression of target gene mRNA. Transduction of each of two shRNAs targeting ERα (TRCN0000003300 and TRCN0000003301), resulted in a 74% and 60% repression of *ESR1* transcript expression, respectively, relative to shNT (Figure 14A). Similarly, knockdown of the ERα corepressor *NRIP1* (shRNAs: TRCN0000019782 and TRCN0000019779) resulted in a 60% and 47% repression of *NRIP1* transcript expression, respectively (Figure 14B).

Transcriptome Characterization

Finally, to confirm the influence on ERα-signalling predicted by our ERE-luciferase reporter assays and to further understand the overall consequences of each shRNA-mediated knockdown, we optimized transcriptome sequencing from ~500 ng of RNA (25,000 cells), to be performed on a small number of samples (~30 genes, two shRNA per gene) where >45% target gene knockdown was confirmed. Gene expression in the presence of the two shRNAs

against *ESR1* or *NRIP1* was compared to that in three controls, corresponding to two non-targeting controls (shNT-E2, shGFP-E2) and to three samples of non-infected cells (Ni-E2). Mapping of reads to transcripts in the human genome (Ensembl annotation GRCh38.85) was performed with Kallisto (Bray et al., 2016), and differential expression was assessed both at the transcript and gene level using Sleuth (Pimental et al., 2016). As expected, knockdown of ERα resulted in an opposite regulation of direct E2 target gene expression (genes regulated by E2 treatment and containing an ERE upstream of the gene promoter) as compared with E2 treatment alone (Figure 15). Furthermore, comparison of overall gene expression changes following ERα knockdown or E2 treatment revealed a negative correlation (R=-0.7) between the two conditions (Figure 16A), indicating that, as expected, genes upregulated by E2 treatment are downregulated by the knockdown of ERα. This expression pattern is expected following the knockdown of any gene directly regulating expression of ERα.

NRIP1 has been identified as a corepressor of ERα and other nuclear receptors (Cavaillès et al., 1995; Lee et al., 1998), that acts both through direct interaction with ERα and by competing with ERα for coactivator binding (Treuter et al., 1998). More recently, NRIP1 has been characterized as a regulator of ERα-signalling during mammary gland development where it promotes the generation of mitogenic signals necessary for the maintenance of mammary epithelium and stroma during puberty (Nautiyal et al., 2013). Our transcriptome profiling of T47D-KBLuc cells following *NRIP1* knockdown reveals that *NRIP1* acts as a repressor of expression of a select subset of direct ERα-target genes (Figure 15). However, unlike shER, comparison of overall gene regulation by shNRIP1 and E2 treatment does not reveal a clear correlation (Figure 16B). Moreover, our transcriptome data reveals that knockdown of *NRIP1* has no discernable effect on ERα expression (Figure 15). This expression pattern is expected following the knockdown of any gene that acts as a cofactor with ERα on a preferred subset of ERα target genes without influencing ERα expression.

DISCUSSION

Unlike previous studies that addressed only subsets of genes such as kinases (Giamas et al., 2011) or select transcriptional regulators (Bolt et al., 2015), our screening strategy encompasses over 16,000 protein-coding human genes in an unbiased manner. We therefore expect to identify hits from pathways not previously known to regulate ERα signalling. While a comprehensive approach such as ours is more informative than a targeted screen, it comes with its own set of practical considerations. First, the cost of screening, which includes the purchase of assay plates and tips, lentivirus production and robotic time, can be considerable. To offset additional costs, we utilized an inexpensive reporter gene assay rather than opting for more costly assays such as qRT-PCR of select ER target genes. Secondly, extensive time and effort is necessary to design and optimize assay conditions prior to screening initiation. Primary genome-wide screening itself is a lengthy process. With three shRNAs per gene and over 16,000 genes to assay, our primary screen will require over 600 96-well plates. Our robotic protocol can accommodate 40-60 plates per day, and with each assay taking six days (Figure 3), primary screening is expected to proceed for roughly 10-15 weeks. Secondary screening assays with ~600 gene hits and five shRNAs per gene (40 viral plates) can all be accommodated simultaneously, with six viral plates per run. The entire secondary screening process is expected to take six to seven weeks. To reduce costs and improve assay reproducibility we opted to perform certain steps manually (cell seeding, luciferase substrate addition, treatment of counterscreen cell lines with DL-Sulforaphane, addition of positive and negative controls on each assay plate) while others were robotized (virus addition and media changes for proliferation assays, addition of alamarBlue® substrate).

Many screening approaches have used siRNA constructs to achieve target gene knockdown, which produce only transient effects and limit screens to those for rapidly visible phenotypes. On the other hand, shRNAs, as used in this study, undergo stable incorporation in the genome of the host cell (Campeau and Gobeil, 2011), allowing for longer-term

proliferation assays that would not be possible using siRNA. Furthermore, use of siRNAs requires cell lines that are amenable to transfection, while shRNA delivery through lentiviral transduction assures high delivery efficiency across all cell types, whether dividing or not. However, use of lentiviruses necessitates elevated biosafety containment ($BSL2^{+}$) and may not be possible at every facility (Campeau and Gobeil, 2011).

Our screen is designed in an arrayed format in 96-well plates, in which luminescence measurements are derived from a cell population transduced with a unique shRNA in each well. This approach is expected to maximize relevant hits as compared to a pooled format, as arrayed screening does not require competitive selection of cells for a desired phenotype and/or deconvolution of differential representation of shRNAs after vs. before selection. The results of shRNA screening are however not unequivocally quantitative. The relative percent inhibition of luminescence attributed to each shRNA may not be directly related to the abundance of the given target protein (Barrows et al., 2010). Because viral titers are estimated by titration of a small selection of wells, it is expected that variations in infection efficiency and individual viral titer may confound some results and contribute to false negative reads. Furthermore, as most shRNA constructs have not been validated on their target, and performance of shRNA is affected by shRNA promoter activity and epigenetic silencing in a cell context specific manner (Hong et al., 2007; Liu et al., 1997), and by mRNA abundance and protein half-life of the target, it is plausible that none of the shRNA for a given gene efficiently represses its target, resulting in false negatives. Finally, off-target effects are frequent with shRNAs, potentially resulting in false positive candidates. Identification of hits with at least two different shRNAs is therefore necessary for good confidence in the significance of the observed effects. In short, completely comprehensive screening on such a large scale is not possible.

Our counterscreening assay was designed to eliminate false positives by identifying those genes that affect both the E2-dependent ERE-luciferase and E2-independent ARE-luciferase reporters. However, it is possible that genes affecting ER-signalling will also affect antioxidant signalling in a specific manner and are not false-positives, but rather genes with

roles in multiple signalling pathways. For example, AIB1 (amplified in breast cancer-1; SRC-3) associates with ERα in ER-positive breast cancer cells (Azorsa et al., 2001) and acts as a selective coactivator of ERα transcriptional activity (Wagner et al., 2013). However, AIB1 also interacts with Nrf2 transactivation domains (Kim et al., 2013) and activates Nrf2 to induce proliferation (Chen et al., 2012). While a counterscreen can provide useful supplementary information, hit selection must be undertaken on a gene-by-gene basis and counterscreening data should not categorically dictate whether a given gene warrants inclusion in subsequent assays.

The screening strategy described here is expected to identify both upstream regulators of ERα expression and modulators of ERα function. Expected hits that could regulate ERα expression may include transcription factors that regulate transcription of the *ESR1* gene and proteins that regulate ERα mRNA or protein stability and degradation (including ubiquitin ligases). Hits that regulate ERα function may include proteins that act as cofactors of ERα on DNA to regulate expression of its target genes, including epigenetic enzymes and transcription factors acting as ERα cofactors or cooperating with it for transcriptional activation, as well as enzymes that post-translationally modify ERα or any of its cofactors. Alternatively, identified hits could indirectly regulate ERα expression by modulating expression of an intermediary target. Finally, it is plausible that our screen will identify proteins that are regulators of both expression and function of ERα. Indeed, FOXA1 has been reported to both regulate transcriptional expression of the *ESR1* gene and overlap with approximately 50% of ERα-binding events (Carroll et al., 2005; Hurtado et al., 2011; Lupien et al., 2008).

While the mechanism of action of each hit will not be clear from reporter screening data alone (regulators of expression and of transcriptional output are expected to yield similar luciferase readings), these will be clarified following analysis of ERα protein expression and transcriptome analysis, with regulators of ERα expression expected to have a transcriptome pattern largely overlapping that of the receptor, especially when considering primary estrogen target genes. Impact on genes associated with cell cycle control will further

provide information on the capacity of genes to inhibit proliferation of ER-positive breast cancer cells. Estrogen receptor cofactors from transcriptome analysis are expected to be more difficult to identify from transcriptome analysis, as their impact may be gene-specific even on direct estrogen target genes. This can be observed for NRIP1, which has a repressive effect on up-regulated direct E2 target genes, but has mixed effects on those genes repressed by E2 stimulation. Finally, it is likely that the observed impacts of shRNA-mediated suppression of candidate genes on transcriptomes is dependent on effective suppression of the target by two different shRNAs, and that addition of a third shRNA is likely to result in greater confidence in differentially regulated genes.

We expect that this screening project will identify previously unknown genes and/or molecular signalling pathways upstream of ERα expression and/or transcriptional activity, elucidate their mechanism of action through transcriptome analysis following shRNA-mediated knockdown and provide novel avenues to explore to prevent and/or circumvent antiestrogen resistance. Additionally, our screening approach could potentially be adapted to screen for regulators of other nuclear receptors that drive different types of cancers, including the androgen receptor in prostate cancer or in breast tumours in the molecular apocrine subclassification.

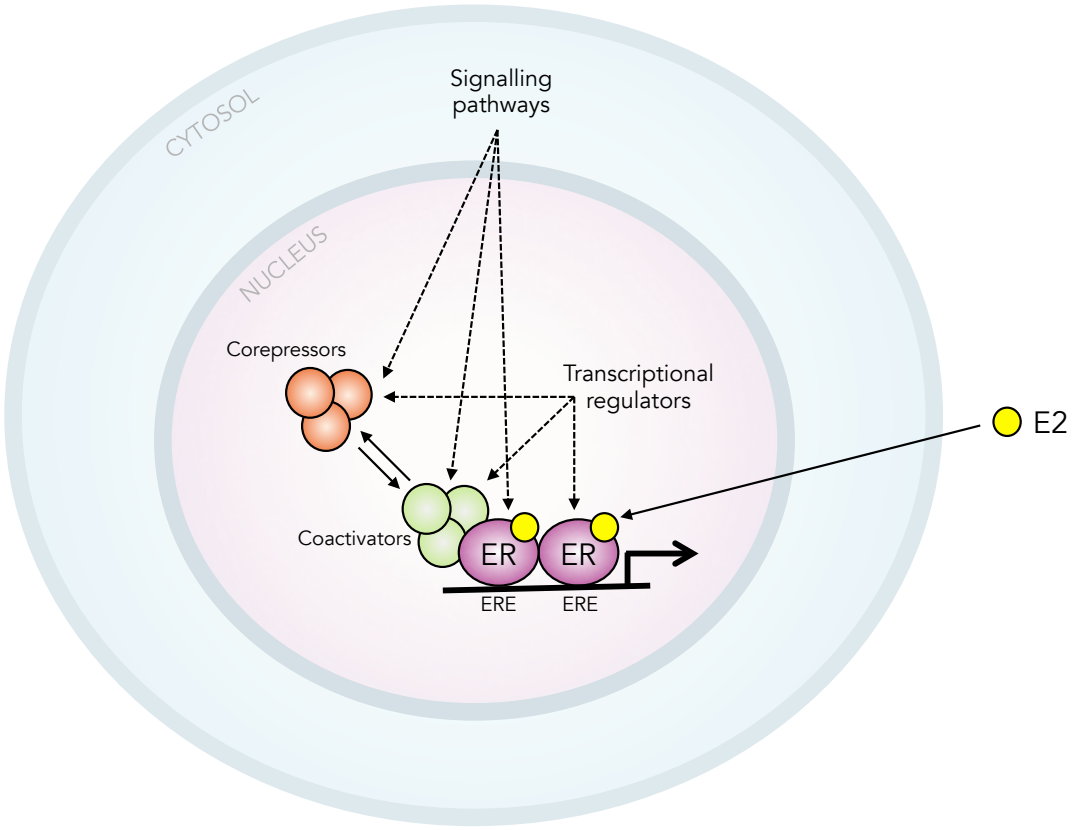


Figure 1. Genomic ERE-dependent signalling and factors that may influence the pathway.

In the classical genomic mechanism of ER action, liganded ERs form dimers and bind directly to EREs in target gene promoters. Additional cofactors are recruited to DNA: coactivators promote chromatin remodelling and stabilization of an active transcription complex leading to expression of ERα target genes, while corepressors inhibit ERα transcriptional activity. ER genomic signalling can be influenced by upstream transcriptional regulators that modulate the level of expression of ERα and/or the balance of coactivators and corepressors available to bind the complex. Additionally, upstream signalling pathways can influence ERα expression levels or ligand-independent ERα activation through phosphorylation events and may equally regulate cofactor expression or activity.

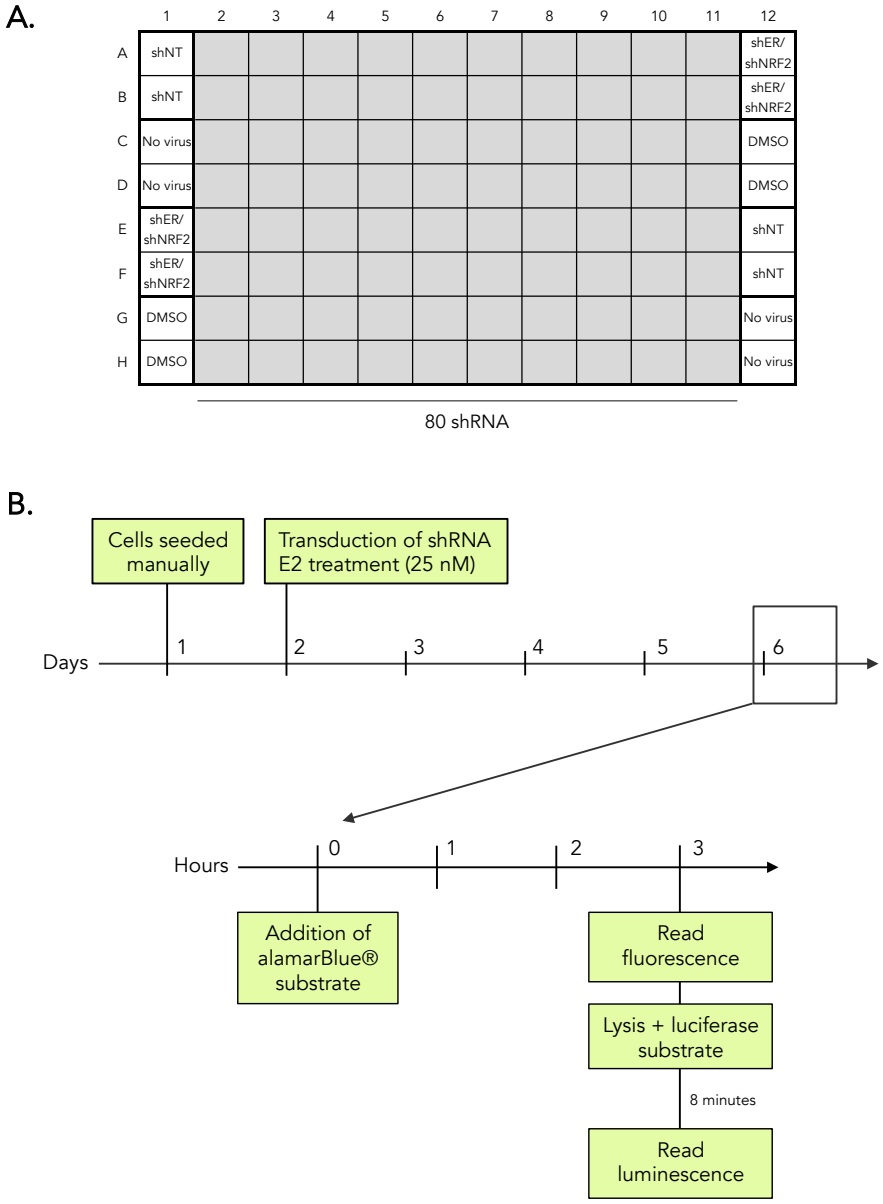


Figure 2. Plate design and primary screening workflow.

Each plate in the screen contained control wells with no virus, non-targeting shRNA (shNT), shRNA against the positive control for each assay (shER or shNRF2) and wells treated with vehicle only (0.1% DMSO), along with 80 wells containing individual shRNAs. B) For the primary screen, cells were seeded manually in hormone-depleted media. The following day cells were treated with E2 (25 nM) and transduced with shRNA-containing lentivirus. Following four days of infection, cell viability (alamarBlue assay) and ER-signalling (luciferase assay) were assessed.

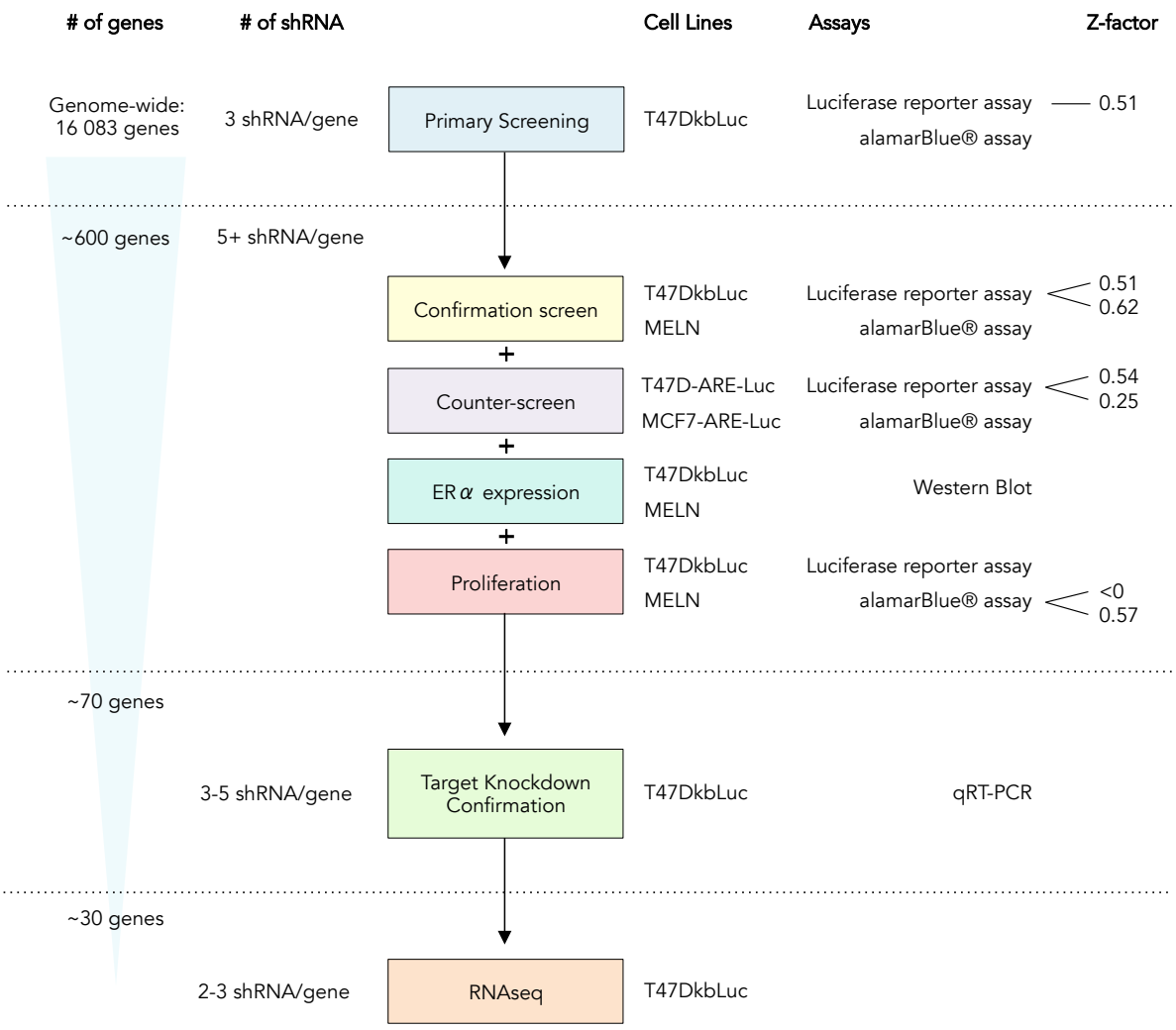


Figure 3. Summary of Screening Pipeline.

The genome-wide primary screen will comprise three unique clones of shRNA in an arrayed format, targeting each of 16,083 protein-coding genes in the human genome. The top ~600 candidates of interest are then tested in subsequent secondary screens with five shRNAs per gene. Down-regulation of shRNA target gene expression is confirmed for the most promising ~70 hits and ~30 genes with efficient target knockdown (>45%) are interrogated through transcriptome sequencing.

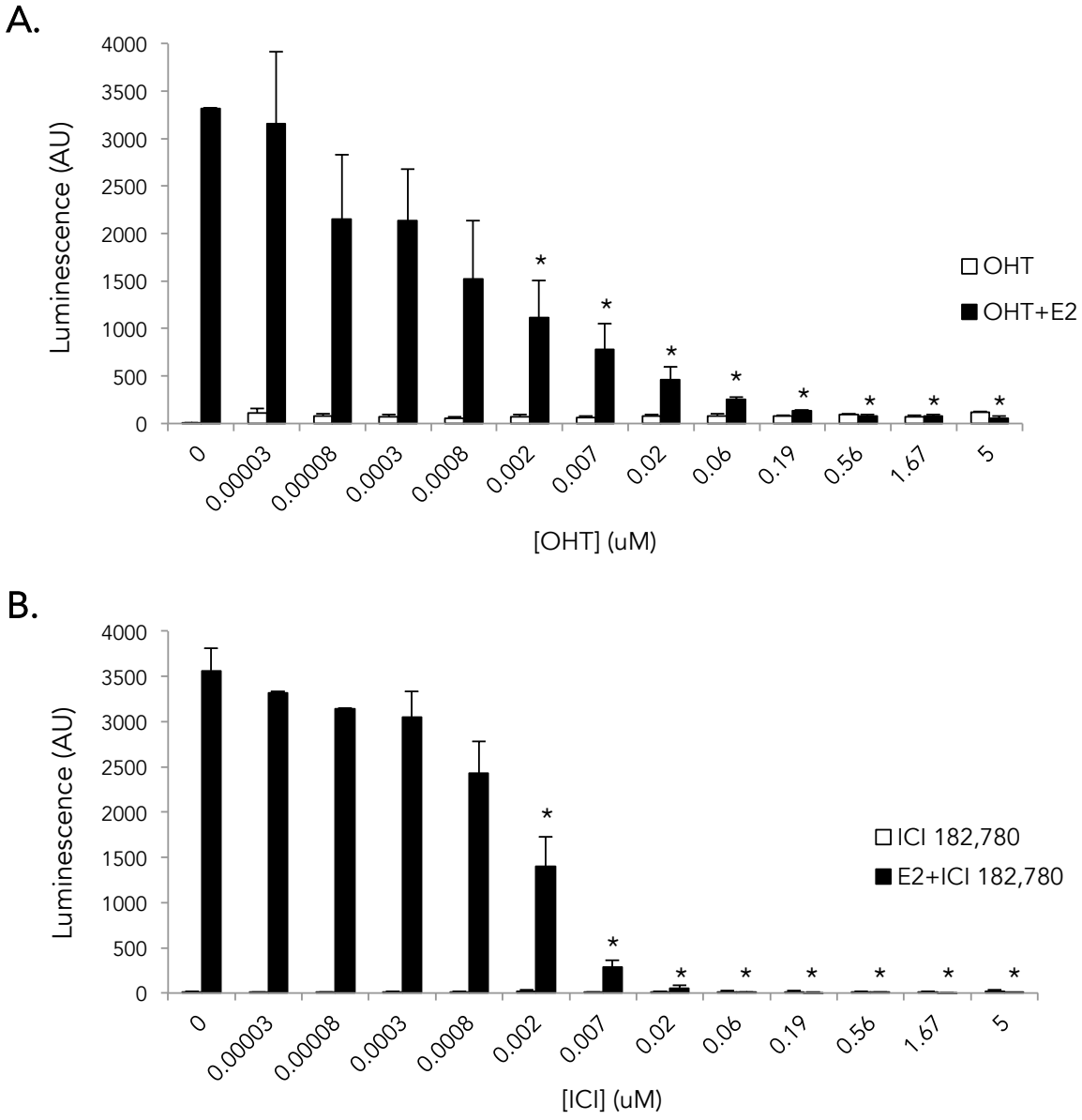


Figure 4. T47D-KBLuc cells are responsive to estrogen and antiestrogens.
T47D-KBLuc cells express an ERE-luciferase reporter that is induced upon E2 treatment (10 nM, 24 hours) and can be inhibited by co-administration of increasing doses of the antiestrogens A) 4-hydroxytamoxifen (OHT) or B) fulvestrant (ICI 182,780). Data represents means ± SEM from three independent experiments. *Denotes $p<0.05$ as compared to E2 treatment alone.

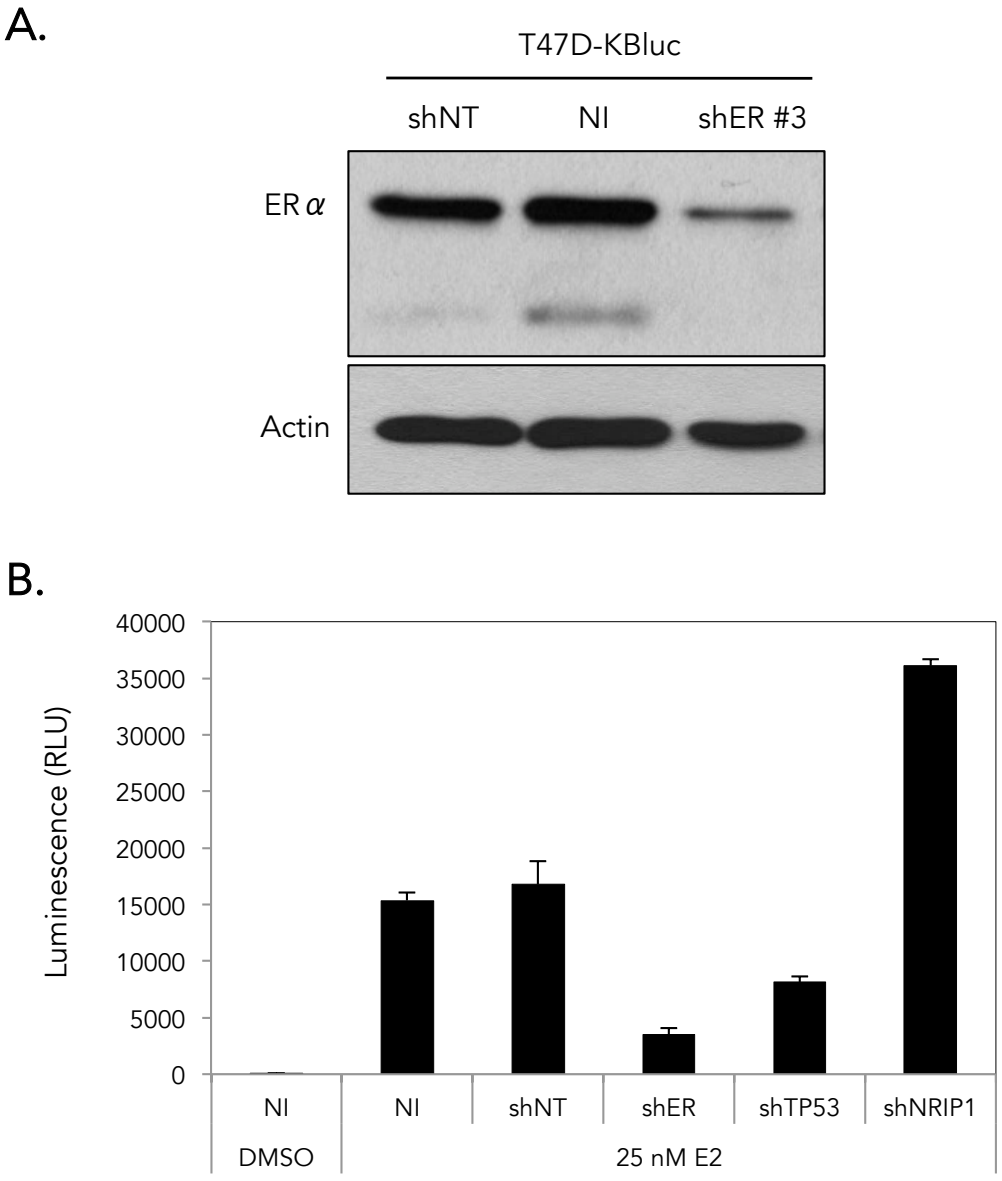


Figure 5. ERE-luciferase reporter expression in T47D-KBLuc cells is altered by shRNA targeting ERα or known effectors of ERα signalling.

A) T47D-KBLuc cells express endogenous ERα protein, which is decreased by transducing cells with shER for four days. B) The ERE-luciferase reporter is inducible with 25 nM E2, and is unaffected by transduction of non-targeting shRNA. Knockdown of ERα reduces the luminescence signal, while knockdown of *TP53* or *NRIP1* decreases or increases luminescence, respectively, in a manner consistent with the role of these transcription factors in ERα signalling.

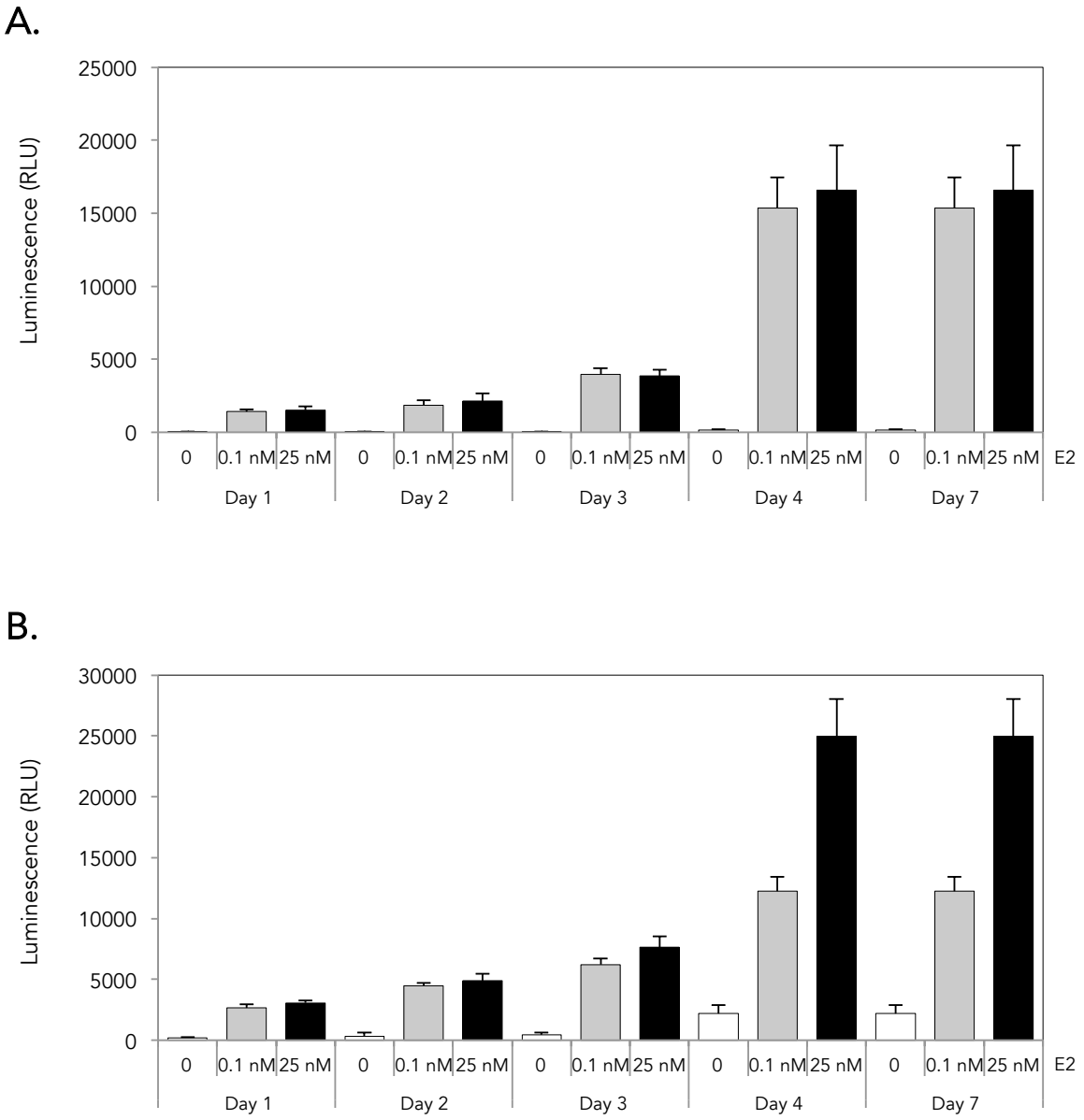


Figure 6. A single treatment of T47D-KBLuc and MELN cells is sufficient to maintain luciferase activity for the duration of the four-day ERE-luciferase assay.

A) T47D-KBLuc cells and B) MELN cells were seeded in white media at a density of 5,000 or 2,000 cells per well, respectively. The following day, cells were treated with 0.1 nM or 25 nM E2 or vehicle (DMSO). Luminescence was read following one, two, three, four or seven days of treatment.

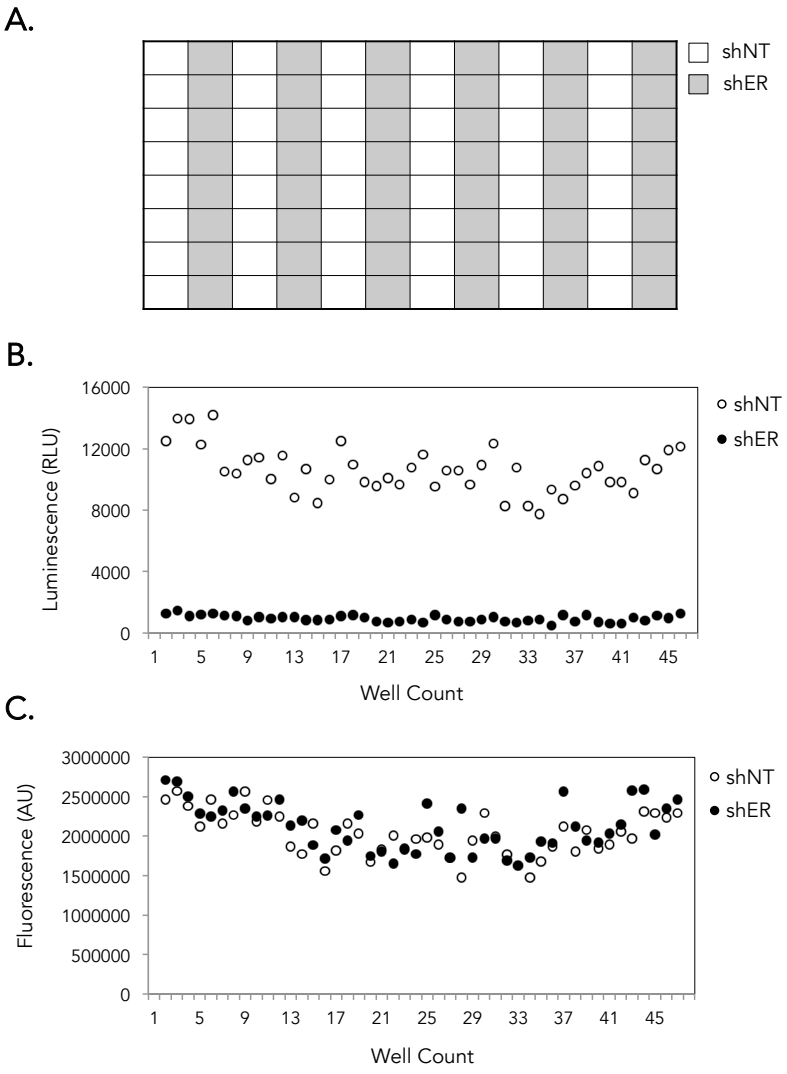


Figure 7. The ERE-luciferase reporter assay in T47D-KBLuc cells is robust and amenable to high-throughput screening.

A) To calculate the Z-factor for all high-throughput assays, cells were seeded in 96-well plates, treated with 25 nM E2 or 2 uM DLS, as described, and alternating columns were infected with non-targeting shRNA (shNT, negative control) or shRNA targeting the positive control in each assay (shER or shNRF2). The mean and standard deviation of 48 wells in each treatment group were used to calculate the Z-factor (see Materials and Methods). B) The ERE-luciferase reporter assay in T47D-KBLuc cells is robust with a Z-factor of 0.51. C) Cell viability, as measured using the alamarBlue® assay, is unaffected in T47D-KBLuc cells following four days of ERα knockdown and fluorescence readout can be used to monitor E2-independent variations in cell viability and to correct for inconsistencies in cell seeding.

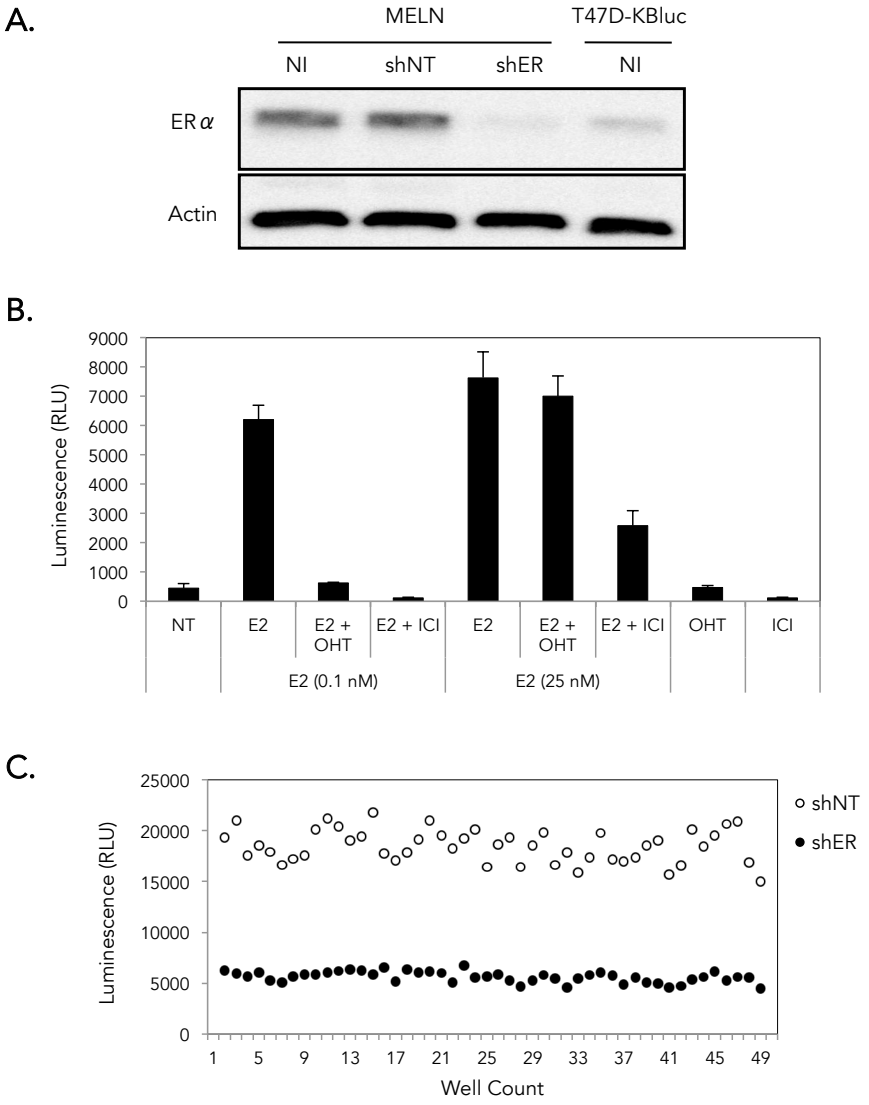


Figure 8. The ERE-luciferase assay in MELN cells is robust and can be used to confirm hits from the primary screen.

A) MELN cells express higher levels of ERα protein than T47D-KBLuc. ERα is efficiently knocked down using shRNA. B) The ERE-luciferase reporter in MELN cells is inducible with 0.1 nM or 25 nM E2 (24h treatment) and can be inhibited by co-administration of the antiestrogens OHT (10 nM) or ICI 182,780 (100 nM). C) The ERE-luciferase reporter assay in MELN cells is robust with a Z-factor of 0.62.

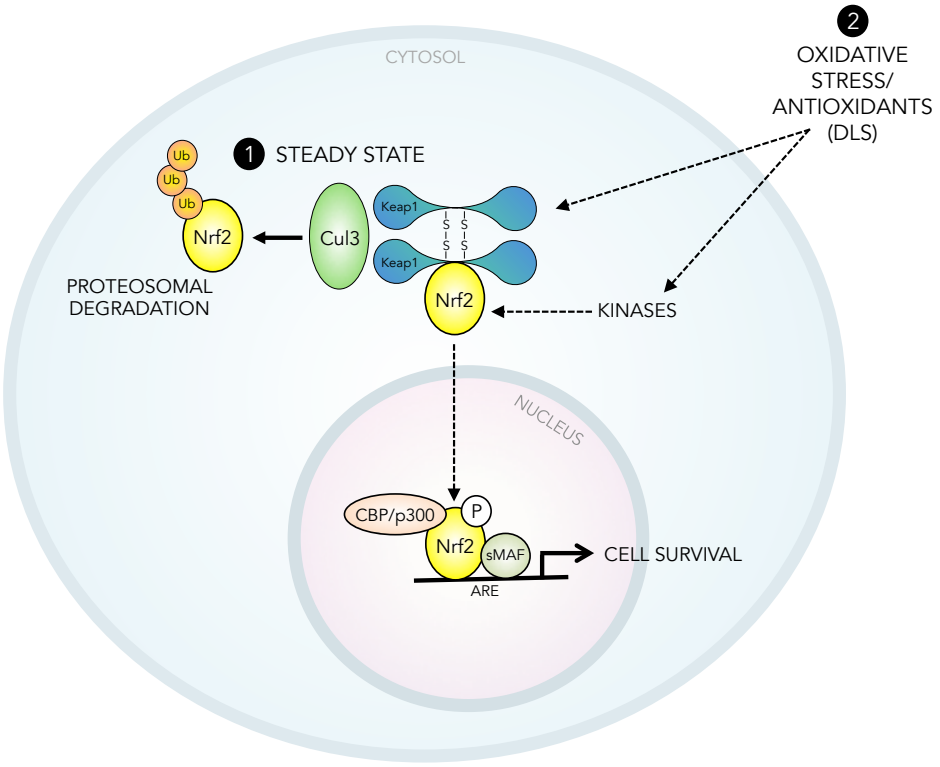


Figure 9. The antioxidant signalling pathway.

A) Under steady state conditions, Nrf2 is sequestered in the cytosol and actively ubiquitinated by Cul3 for proteasomal degradation. B) In response to oxidative stress, which activates kinase pathways that phosphorylate Nrf2, or following treatment with antioxidants like DL-Sulforaphane (DLS), which disrupts the Nrf2-Keap1 interaction, Nrf2 detaches from its inhibitory complex and translocates to the nucleus. Nuclear Nrf2 forms heterodimers with small MAF protein (sMAF) and binds to antioxidant response elements (ARE) in the promoters of target genes, leading to transcription of detoxifying enzyme genes and cell survival. (Adapted from Pamplona and Costantini, 2011).

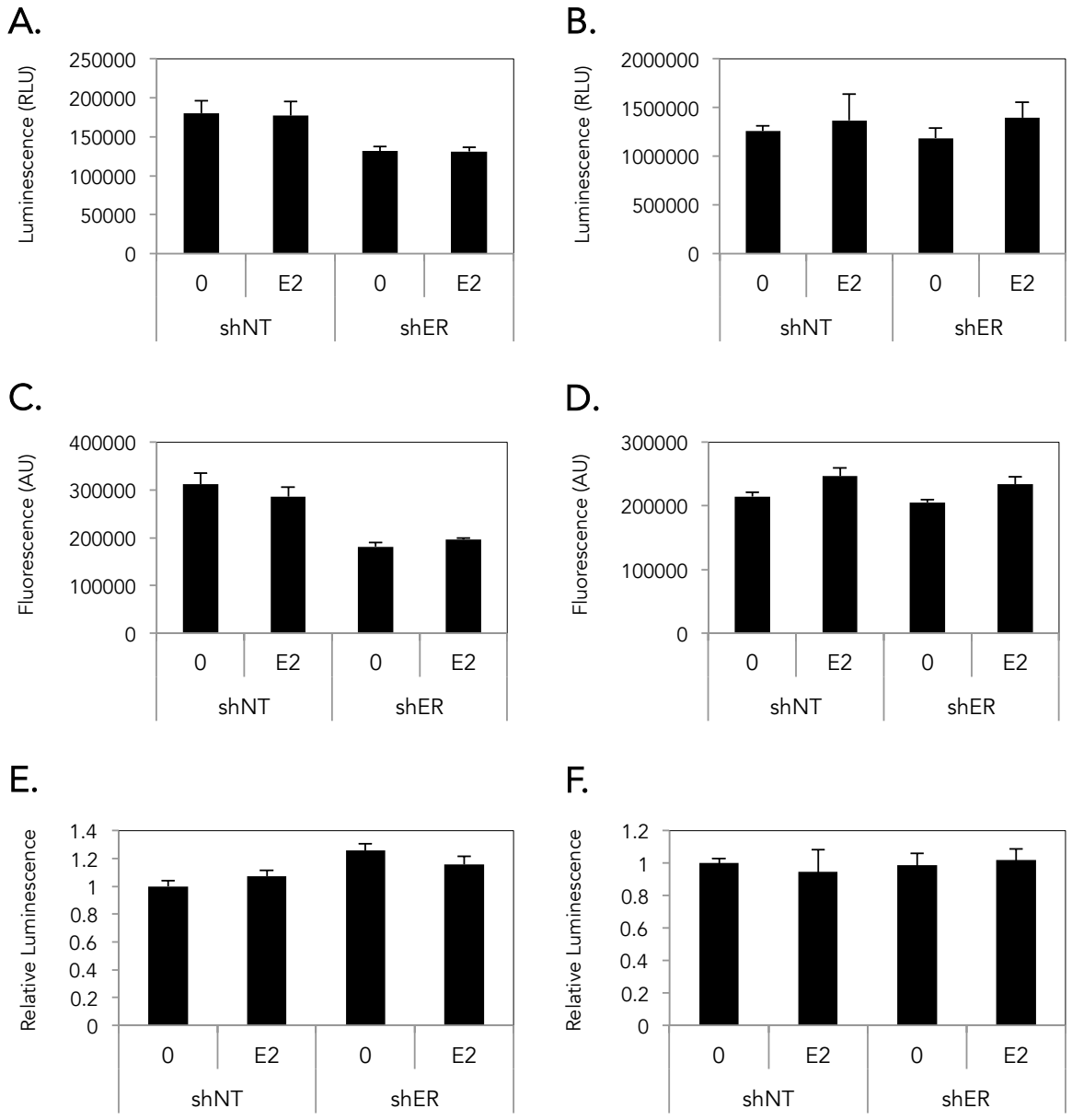


Figure 10. T47D-ARE and MCF-7-ARE reporter expression is not influenced by E2 treatment or knockdown of ERα.

T47D-ARE and MCF-7-ARE were treated with E2 (25 nM) and transduced with non-targeting shRNA (shNT) or shRNA targeting ERα (shER) for four days. A) T47D-ARE and B) MCF-7-ARE luciferase reporter activity is shown. C) and D) Small differences in luminescence between treatments are reflected in the alamarBlue® cell viability assay. E) and F) Normalizing luminescence to alamarBlue® fluorescence corrects for differences due to cell proliferation.

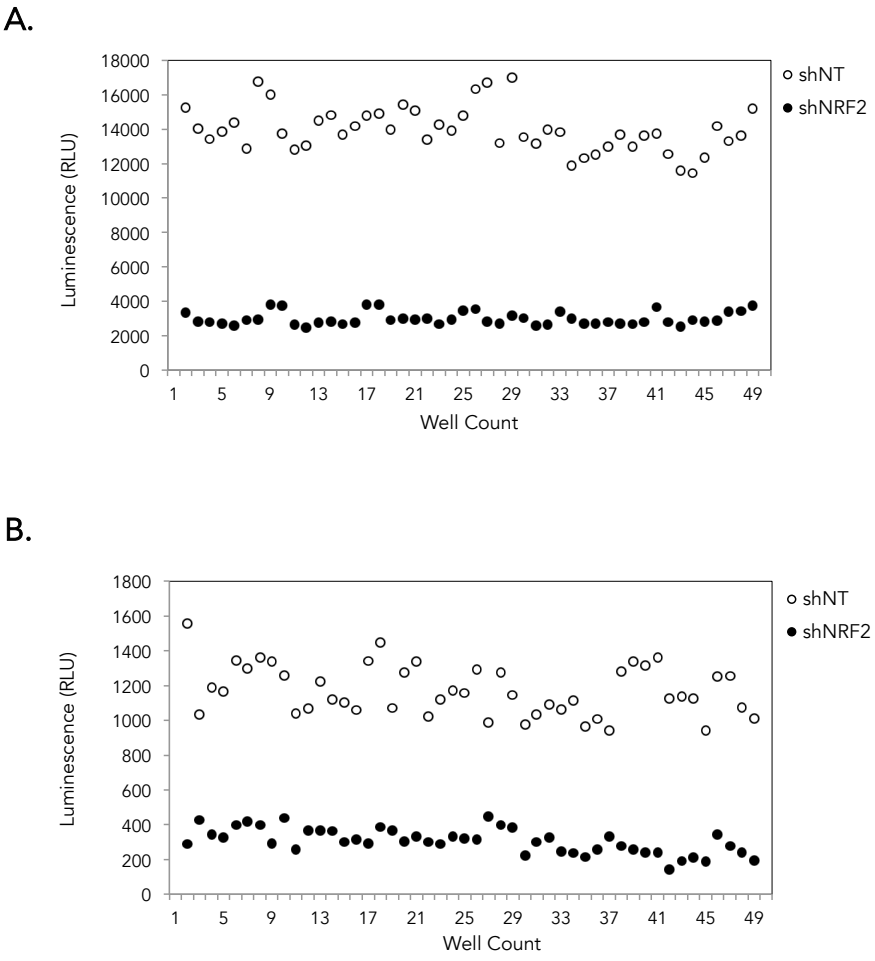


Figure 11. Optimization of luciferase assays in ARE-reporter cell lines, MCF-7-ARE and T47D-ARE, to eliminate false-positive hits.

MCF-7-ARE and T47D-ARE cell lines were generated by transducing MCF-7 and T47D cells, respectively, with an inducible antioxidant-response element (ARE) luciferase reporter. Cells were seeded in 96-well plates and transduced in alternating columns with non-targeting shRNA or shRNA against Nfr2 (shNRF2), for a total infection time of four days. Sixteen hours prior to assay completion, cells were treated with the antioxidant DL-Sulforaphane to induce the luciferase reporter. Luminescence counts (RLU) in each well were used to calculate a Z-factor for each assay. A) The assay is robust in T47D-ARE cells (Z-factor = 0.54) and amenable to high-throughput screening. B) The assay in MCF-7 cells is not optimal for high-throughput screening (Z-factor = 0.25).

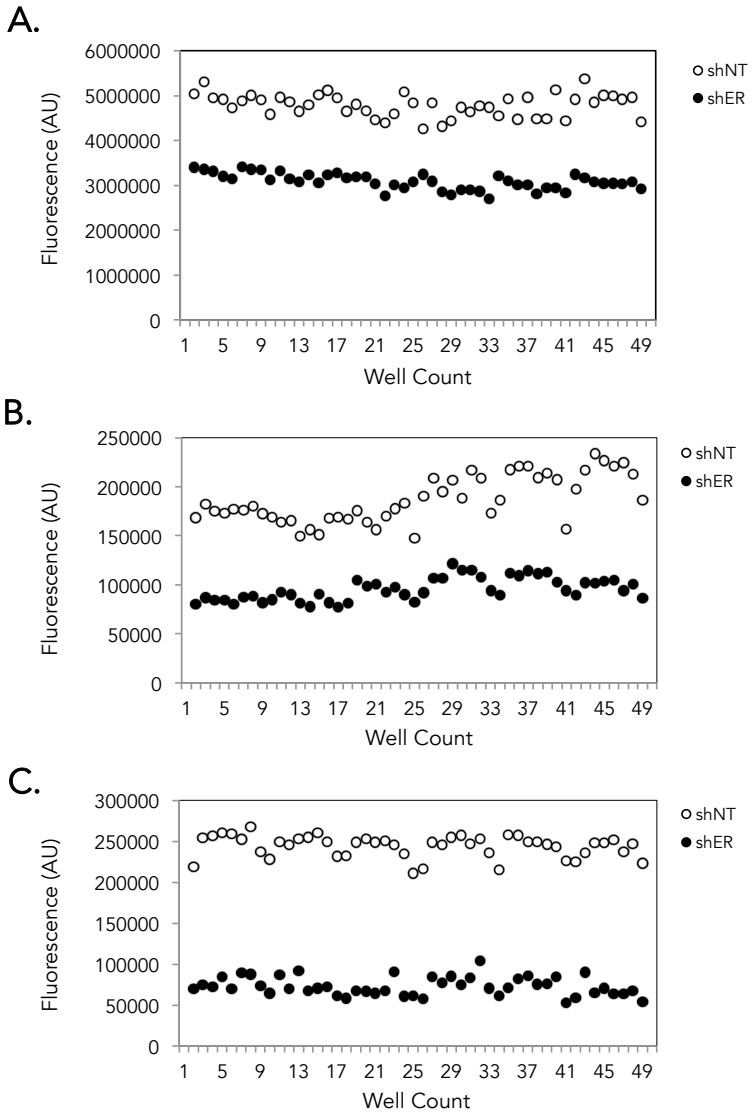


Figure 12. Cell viability assays to identify regulators of E2-dependant proliferation.
A) MELN cells were seeded at 2,000 cells per well in hormone-depleted median in 96-well plates. The following day, alternating columns were transduced with non-targeting shRNA (shNT) or shRNA targeting ERα (shER) at an MOI of 10 in the presence of E2 (25 nM). Cell viability (alamarBlue® assay) in each well was determined following four total days of shRNA administration/E2 treatment. B) T47D-KBLuc cells (2,000 cells per well) or C) MELN cells (1,000 cells per well) were seeded in hormone-depleted media in 96-well plates. The following day, alternating columns were transduced with non-targeting shRNA (shNT) or shRNA targeting ERα (shER) at an MOI of 10 in the presence of E2 (25 nM). Following four days of infection, media was replaced and again supplemented with E2 (25 nM). Cell viability (alamarBlue® assay) in each well was determined following 8 total days of shRNA administration/E2 treatment. A) The long-term cell viability assay is robust in MELN cells (Z-factor = 0.57) and amenable to high-throughput screening. The assay is not optimal in T47D-KBLuc cells (Z-factor < 0).

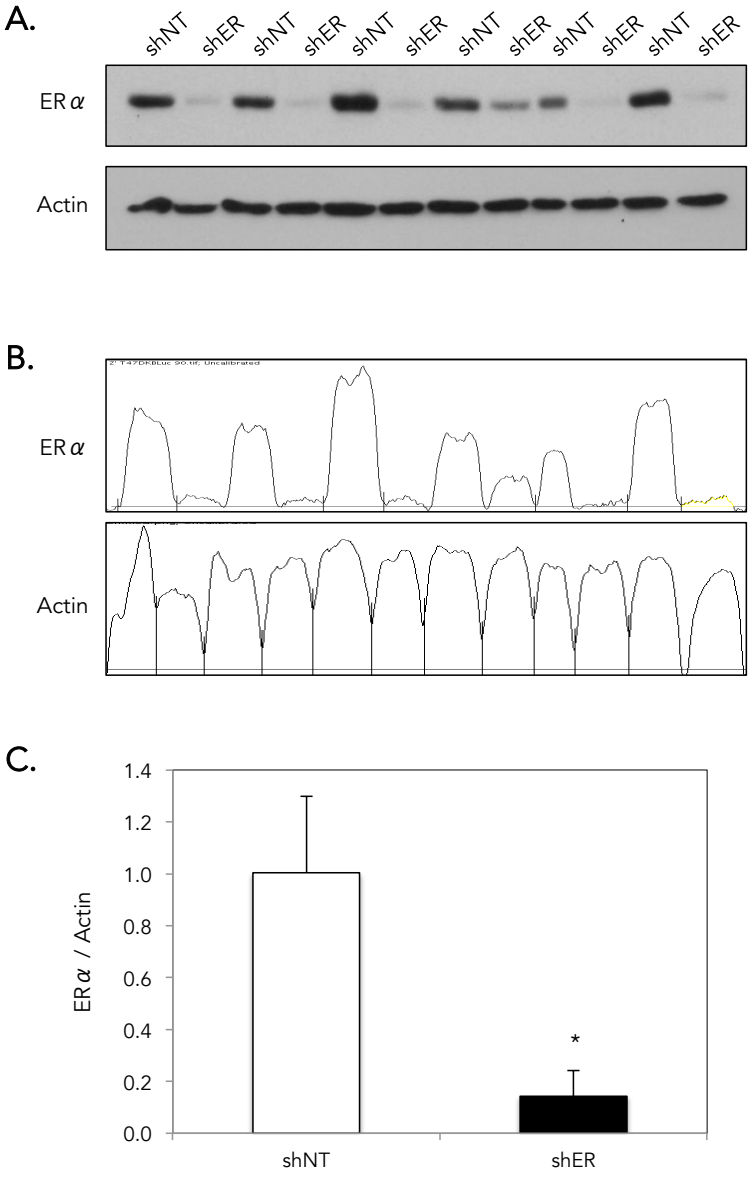


Figure 13. High-throughput Western blot analysis to identify genes regulating ERα protein expression.

A) T47D-KBLuc cells were seeded in 96-well plates and transduced in alternating columns with non-targeting shRNA (shNT) or shRNA against ERα. Six wells were selected at random from each condition for Western blot analysis of ERα and actin protein expression in whole cell lysates. B) ERα and actin protein expression was quantified using Image J software (NIH). C) Normalizing ERα to actin protein levels indicates a significant decrease (86%) in relative ERα protein expression following transduction of shER. *Denotes $p<0.05$ as compared to shNT (Student's T-test).

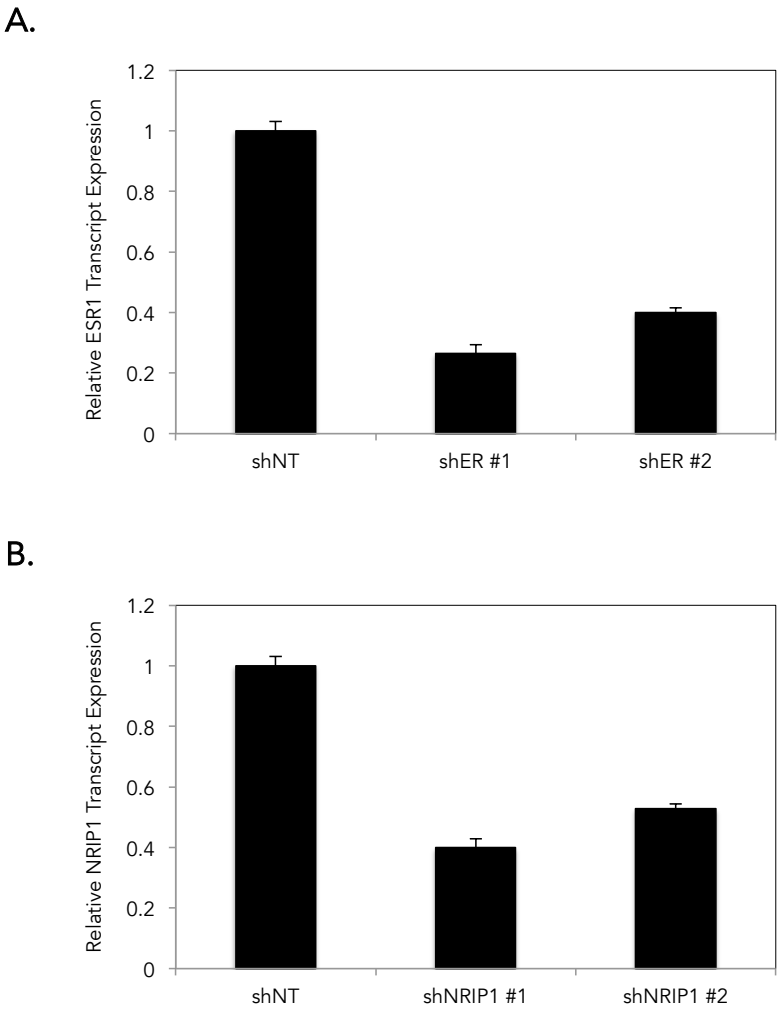


Figure 14. Confirmation of target gene knockdown by quantitative RT-PCR.
T47D-KBLuc cells were seeded in hormone depleted media at 50,000 cells per well in 24-well plates. The following day, cells were transduced with non-targeting shRNA (shNT) or shRNAs targeting A) ERα (TRCN0000003300: shER #1 or TRCN0000003301: shER #2) or B) *NRIP1* (TRCN0000019782: shNRIP1 #1 or TRCN0000019779: shNRIP1 #2) at an MOI of 10 in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted, reverse transcribed and quantified for A) *ESR1* or B) *NRIP1* transcript expression. Transcript expression was normalized to three housekeeping genes (RPLP0, TBP, YWHAZ) and is presented relative to mRNA expression of each transcript following transduction of non-targeting shRNA (shNT).

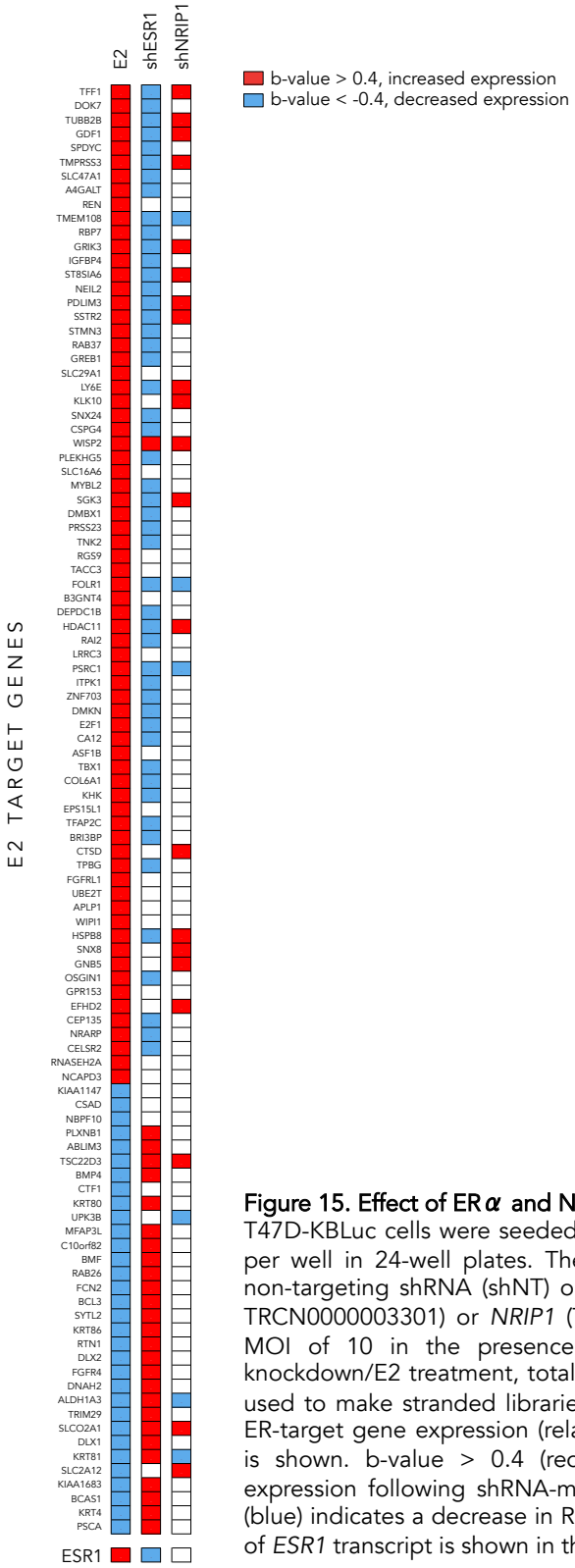


Figure 15. Effect of ERα and NRIP1 knockdown on direct E2 target genes. T47D-KBLuc cells were seeded in hormone depleted media at 50,000 cells per well in 24-well plates. The following day, cells were transduced with non-targeting shRNA (shNT) or shRNA targeting *ESR1* (TRCN0000003300, TRCN0000003301) or *NRIP1* (TRCN0000019782, TRCN0000019779) at an MOI of 10 in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted and 500 ng of RNA was used to make stranded libraries using a PolyA capture kit (Illumina). Direct ER-target gene expression (relative to non-infected cells, shNT and shGFP) is shown. b-value > 0.4 (red) indicates an increase in RNA transcript expression following shRNA-mediated knockdown, while a b-value < -0.4 (blue) indicates a decrease in RNA transcript expression. Relative expression of *ESR1* transcript is shown in the bottom panel.

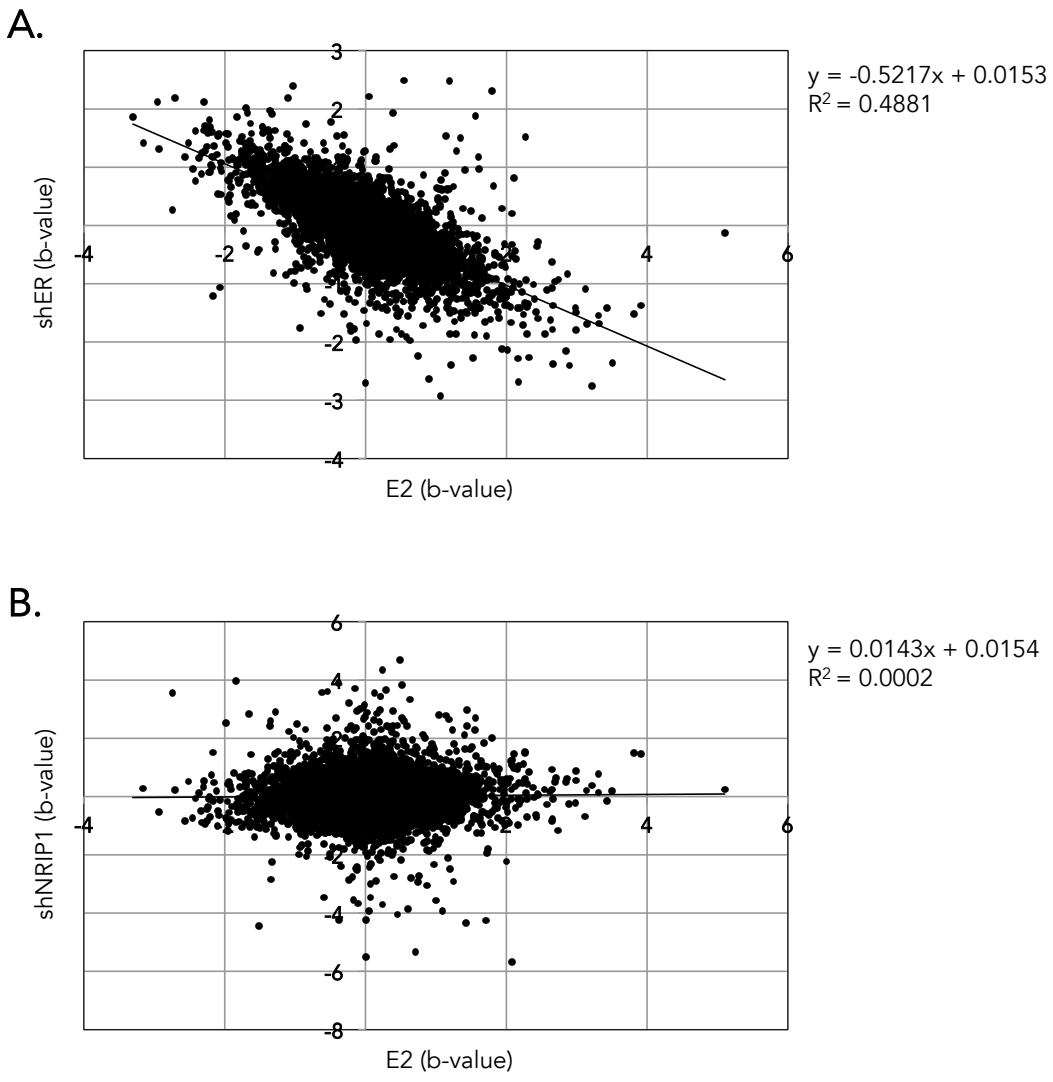


Figure 16. Effect of ERα and *NRIP1* knockdown on total transcript expression.
T47D-KBLuc cells were seeded in hormone depleted media at 50,000 cells per well in 24-well plates. The following day, cells were transduced with non-targeting shRNA (shNT) or shRNA targeting A) ERα (TRCN0000003300, TRCN0000003301) or B) *NRIP1* (TRCN0000019782, TRCN0000019779) at an MOI of 10 in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted and 500 ng of RNA was used to make stranded libraries using a PolyA capture kit (Kappa). Relative transcript expression (b-value, relative to non-infected cells, shNT and shGFP) is plotted as compared to non-infected samples treated for four days with E2 (25 nM). b-value > 0 indicates an increase while a b-value < -0 indicates a decrease in RNA transcript expression.

CHAPTER TWO:

Identification of regulators of estrogenic signalling in ER-positive breast cancer cells by whole genome shRNA screening

INTRODUCTION

Breast cancer affects one in eight women in developed countries and is the second cause of death by cancer in this population (Canadian Cancer Society, 2015). Estrogens control both normal mammary development and breast tumourigenesis (Deroo and Korach, 2006; LaMarca and Rosen, 2007) via estrogen receptor α (ERα) (Ascenzi et al., 2006; Hall et al., 2001; Nilsson et al., 2001). ERα acts as a ligand-dependent transcription factor that controls expression of a network of target genes via direct binding to DNA at specific estrogen response elements (Bourdeau et al., 2004; Mader et al., 1989, 1993; Sanchez et al., 2002) and recruitment of a vast array of coactivators and corepressors through its transcriptional regulators regions AF1 and AF2 (Green and Carroll, 2007; Hall and McDonnell, 2005; Ratajczak, 2001; Smith and O'Malley, 2004). Estrogenic stimulation of ER-positive breast cancer cells leads to the up-regulation of an array of proliferative genes controlling the G1/S and G2/M transitions (Bourdeau et al., 2008; Eeckhoute et al., 2007; Frasor et al., 2003). Therapeutic strategies opposing estrogenic action either by blocking estrogen production (aromatase inhibitors) or estrogenic signalling (antiestrogens) (Ali et al., 2011; Brodie, 2002; Traboulsi et al., 2017) represent the main form of targeted treatment in breast cancer. However, one third of breast tumours are not sensitive to these treatments (ER-negative tumours), and about 50% of ER-positive breast cancer patients eventually relapse. Sensitivity to endocrine therapy of breast cancer is determined both by ERα expression levels and by its activity as a regulator of cell proliferation. However, much remains to be clarified about mechanisms controlling estrogenic signalling in normal breast epithelial cells and their deregulation during breast tumourigenesis.

Gain in ERα expression levels in ER-positive tumour cells compared with normal epithelial cells is thought to underlie the role of ERα as a tumourigenesis driver. Contrary to early reports (Albertson, 2008; Holst et al., 2007), *ESR1* (the gene coding for ERα) is amplified in only 2.6% of tumours in the Cancer Genome Atlas (TCGA) breast cancer dataset and this amplification does not correlate with an overall increase in gene expression (Ma and

Ellis, 2013). *ESR1* is deleted or mutated in fewer than 1% of cases in primary tumours, although mutations are found in about 20% of tumours progressing to metastasis after treatment with endocrine therapy (reviewed in Traboulsi et al., 2017). However, methylation of *ESR1* is inversely correlated with its level of expression. Accordingly, combined use of DNA methyltransferase inhibitors and histone deacetylase inhibitors has been found to lead to de novo expression of ERα in ER-negative cell lines, pointing to epigenetic remodelling as the main mechanism controlling ERα expression (Keen et al., 2003; Ottaviano et al., 1994; Sabnis et al., 2011; Yan et al., 2003; Yang et al., 2001).

ESR1 is transcribed from multiple promoters controlling tissue-specific expression. Resulting transcripts differ in their 5'UTR but not the coding region (Grandien et al., 1997).*ESR1*promoters are relatively weak, being devoid of consensus TATA, CCAAT or GC boxes (Kos et al., 2001). Promoters A-C, located within 3 kb of upstream flanking sequences, and promoters E and F, at -151 and -117 kb, respectively, from the transcriptional start site downstream of promoter A, are used in breast cancer cells (Eeckhoute et al., 2007; Gaughan et al., 2013; Grandien et al., 1997; Kos et al., 2001). Enhancers affecting *ESR1* expression have been characterized upstream of promoters E and F (Eeckhoute et al., 2007; Gaughan et al., 2013). In addition, a recent study found that both inherited and sporadic single nucleotide variants found in enhancer or promoter sequences affect a broader network of regulatory elements in the *ESR1* gene and contribute to upregulation of *ESR1* expression in breast cancer patients (Bailey et al., 2016).

The main characterized upstream regulators of *ESR1* expression are transcription factors p53, GATA3 and FOXA1, whose expression/activity as transcription factors is associated with the ER-positive (luminal) breast cancer phenotype. p53 is a tumour suppressor that acts as a transcription factor binding to chromatin at specific elements but also via protein-protein interactions with other transcription factors (Verfaillie et al., 2016; Zhu et al., 2015). A reciprocal positive feedback loop has been reported between p53 and ERα in the ER-positive MCF-7 breast cancer cell line. Ectopic expression of p53 or its induction by doxorubicin or ionizing radiation in MCF-7 cells increases ERα levels, and

conversely knockdown of p53 decreases *ESR1* expression; this regulation is mediated via association of p53 with *ESR1* promoter A (Angeloni et al., 2004; Hurd et al., 1995; Shirley et al., 2009). Conversely, estrogens increase p53 expression in MCF-7 cells. In addition, ligand-independent protection of p53 from hdm2 by ERα was reported (Liu et al., 2000). Accordingly, loss of p53 expression is associated with a triple-negative phenotype (Cancer Genome Atlas Network, 2012). However, many p53 missense mutations are found in ER-positive tumours (45% in ER-positive tumours vs. 75% in HER2-positive and 84% in triple negative tumours, particularly in Luminal B tumours, a subset of ER-positive tumours with worse prognosis (Sørlie et al., 2001; Sorlie et al., 2003). Of note, mutations in p53 can have variable outcomes in terms of transcriptional regulation and protein-protein interactions (Freed-Pastor and Prives, 2012; Polotskaia et al., 2015; Zhu et al., 2015), suggesting the possibility of residual or gain of function activity in ER-positive tumours.

GATA3 belongs to the GATA1-6 family of zinc finger transcription factor binding (A/T)GATA(A/G) consensus motifs. GATA3 plays roles in mammary gland development and luminal epithelial cell differentiation (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006, 2008). *GATA3* expression is highly correlated with that of *ESR1* in breast cancer cell lines and tumours (Hoch et al., 1999; Lacroix and Leclercq, 2004; Tozlu et al., 2006), and its downregulation results in loss of *ESR1* expression and may play roles in tumour dissemination (Dydensborg et al., 2009; Eeckhoute et al., 2007; Kouros-Mehr et al., 2008; Lee et al., 2014). Binding of GATA3 to *ESR1* flanking sequences was detected at enhancer sites located upstream of *ESR1* promoters E and F and was associated with recruitment of p300 and KDM4B recruitment and with H3K18/H4K12 acetylation marks as well as loss of H3K9me2/3 methylation marks (Eeckhoute et al., 2007; Gaughan et al., 2013; Kong et al., 2011; Serandour et al., 2013; Welboren et al., 2009). Of interest, FOXA1 is co-associated with GATA3 at several enhancers in the *ESR1* gene (Serandour et al., 2013) and may cooperate with it for regulation of ERα expression in mouse and in human breast cancer cells (Bernardo et al., 2010).

Known ERα cofactors include the histone acetyl-transferase SRC/CBP/p300 complex, the histone methyl transferases CARM1 and PRMT1, the Mediator complex and the SWI-SNF chromatin remodelling complex (Burakov et al., 2002; Green and Carroll, 2007; Hall and McDonnell, 2005; Ichinose et al., 1997; Ratajczak, 2001; Smith and O'Malley, 2004). In addition, components of the ubiquitin ligation and proteasome complexes also act as ERα cofactors (vom Baur et al., 1996; Gong et al., 2010; Nawaz et al., 1999; Smith et al., 2002; Verma et al., 2004). On the other hand, corepressors recruited in a ligand-dependent manner such as LCoR and NRIP1 limit transcriptional activation by ERα (White et al., 2004). Altogether, 639 factors have been shown to interact with nuclear receptors in different cell types (Atlas, N.R.S.), but it still remains unclear which cofactors are important for ER signalling in breast cancer cells. Cofactors NCOA3 (20q13.12) and NCOA6 (20q11.22) are amplified in about 9% and 4%, resp., of breast tumours (with partial overlap), which may drive estrogen signalling in ER-positive tumours (Anzick et al., 1997; Lee et al., 1999). On the other hand, ERα cofactors ARID1A (1p36.11) and SPEN (1p36.21-1p36.13) were shown to be lost in T47D breast cancer cells via loss of heterozygocity and non-sense mediated mRNA decay and act as tumour suppressor genes (Légaré et al., 2015; Mamo et al., 2012).

In this work, we have developed a loss-of-function approach using shRNA-mediated gene knockdown to discover new modulators of ERα expression and function in ER-positive breast cancer cells. We have used the p53mut, ER-positive T47D breast cancer cell line carrying an ERE-containing reporter vector (T47D-KBLuc, Wilson et al., 2004) as a model of luminal B breast cancer cell line and have performed a genome-wide screen of the Sigma lentiviral shRNA library in the presence of estradiol. Secondary assays were used to eliminate some non-specific hits and select candidates for further characterization by transcriptome analysis. We report for the first time the role of several proteins including the lysine histone acetyltransferase *KAT6A* as upstream modulators of *ESR1* expression.

MATERIALS AND METHODS

Cell Culture

The human ERα -positive breast cancer cell lines T47D-KBLuc, T47D and MCF-7 were purchased from ATCC. MELN cells were a kind gift from Dr. Balaguer (Montpellier, France). MCF-7-ARE and T47D-ARE cell lines were generated by infecting MCF-7 and T47D cells, respectively, with lentivirus expressing an inducible antioxidant-response element (ARE) firefly luciferase reporter (Qiagen). All cell lines were maintained in RPMI-1640 medium (Wisent) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate and 1% Penicillin Streptomycin.

Automated High-Throughput Screening

shRNA Libraries and Lentivirus Production

For our primary genome-wide shRNA screen, lentiviruses were produced for delivery of shRNAs targeting 16,083 protein-coding genes (Mission shRNA library, Sigma, three different shRNA clones per gene). Lentivirus production was performed in 96-well plates using the Mirus transfection reagent, as per the manufacturer's recommended protocol (Sigma). Viral titers were determined for randomly selected wells (~4% of wells in each 96-well plate) by infecting HeLa cells with a 1:10,000 dilution of lentiviral supernatant, and selecting with puromycin (1 ug/mL) for five days. Colonies were visualized by crystal violet staining and colony counts in each well were used to estimate the number of viral particles per milliliter of viral supernatant. An average titer for all lentiviruses produced was established by taking the mean of viral titers for all wells in the titration. For our screen, viral titers were estimated at ten million viral particles per milliliter. Taking into consideration an estimated drop in viral titers by ~50% at the first freeze-thaw, we used a final titer of five million viral particles per milliliter to calculate the volume of viral supernatant to add to each well.

Short-Term Luciferase and Viability assays

A step-wise screening protocol has been designed (Kulpa et al. in preparation) and is summarized in Figure 1. In brief, we used the Mission shRNA library from Sigma, which targets 16,083 protein-coding genes in the human genome. For all assays, cells were seeded manually in opaque white 96-well plates in hormone-depleted phenol red-free RPMI media. The following day, media was replaced with fresh media supplemented with 2 ug/mL polybrene (Sigma), supplemented with 25 nM E2 (for estrogen-inducible reporter cell lines only). Cells were transduced in an arrayed format at an average MOI of 10 with three (primary screen) or five (secondary screens) unique shRNAs targeting each gene of interest. Each plate also contained control wells with no virus, non-targeting shRNA (shNT), shRNA against ERα or NRF2 and wells treated with vehicle (0.1% DMSO). Media changes and virus addition were performed using a Biomek FX pipettor with 96-well head (Beckman). Plates were incubated at 37°C for four days. ARE assay plates were treated with 10 μM DL-Sulforaphane 16 hours prior to luciferase reading. Media was replaced with media containing a 1:50 dilution of alamarBlue® (Invitrogen). Plates were incubated for three hours at 37°C, after which absorbance was read (590 nm). Cells were washed with PBS, and incubated with a solution of N-Ethylmaleimide (NEM)-containing lysis buffer and luciferin substrate (Nanolight Technology) and incubated at room temperature in the dark for eight minutes. Luminescence was read on the Envision plate reader (Perkin Elmer; primary screening) or POLARstar plate reader (BMG; secondary screening).

Long-Term Luciferase and Proliferation Assays

Cells were seeded and transduced as described above. Following four days of infection/E2 treatment, media was changed to fresh media containing 25 nM E2. AlamarBlue® and luciferase assays were performed following eight total days of infection/E2 treatment.

Data Analysis

Percent inhibition of the E2-induced luciferase reporter was calculated using the following formula:

$$\% \text{ inhibition} = 100 - \left(\frac{x - \text{Background}}{\text{shNT} - \text{Background}} \times 100 \right)$$

where x is the luciferase read for each individual hit and shNT is the mean of four wells treated with non-targeting shRNA on each plate. shRNA clones inhibiting luminescence by (+/-) 60% were designated as "hits" in the primary screen. shRNA producing a (+/-) 25% inhibition of fluorescence in the alamarBlue assay were considered effectors of T47D-KBLuc cell viability. The luciferase cut-off was adjusted to (+/-) 30% for the secondary and counter screens due to lower titers in subsequent rounds of lentivirus production.

qRT-PCR and Transcriptome Sequencing

T47D-KBLuc cells were seeded in 24-well plates at a density of 60,000 cells per well in phenol-red free RPMI. The following day, media was replaced with fresh media supplemented with 2 ug/mL polybrene and 25 nM E2 or an equal volume of DMSO. Cells were transduced with shRNA at an MOI of 10. Following four days of infection, cells were washed twice with ice-cold PBS and total RNA was extracted using the RNeasy mini kit (Qiagen). 400-500 ng of RNA was reverse-transcribed using an oligo dT primer and target knockdown was confirmed by qRT-PCR on the Viia7 (Thermo Fisher Scientific), and normalized to expression of housekeeping genes RPLP0, TBP and YWHAZ.

Transcriptome sequencing was performed at the genomics facility at our institute. RNA integrity was verified on the Agilent BioAnalyzer 2100. 400-500 ng of total RNA was used to prepare RNA-Seq libraries using the KAPA RNA Stranded Library kit with PolyA capture following the manufacturer's protocol (Roche). Sequencing was performed on an Illumina HiSeq2000. Samples were demultiplexed using Casava 1.8.2 (Illumina, San Diego,

CA). Gene expression in the presence of each of the two shRNAs against each hit was compared to that in three controls, corresponding to two non-targeting controls (shNT+E2, shGFP+E2) and to non-infected cells (Ni+E2), while gene induction by E2 was determined by comparing non-infected, E2-treated cells (Ni+E2) with DMSO-treated cells (Ni-E2). Mapping of reads to transcripts in the human genome (Ensembl annotation GRCh38.85) was performed with Kallisto (Bray et al., 2016), and differential expression was assessed both at the transcript and gene level using Sleuth (Pimental et al., 2016). For this study, differential gene expression values were filtered using a beta-value (b-value) cut-off of ± 0.4. The b-value is a bias estimator generated by Sleuth that is analogous to fold change in gene expression.

Western Blotting

T47D-KBLuc were seeded at a density of 50,000 cells per well in 24-well plates (Costar) RPMI media. The following day, media was supplemented with 2 ug/mL polybrene and cells were transduced with lentiviruses containing shRNAs at an average MOI of 10. Following four days of infection, cells were washed twice with ice-cold phosphate-buffered saline (PBS). Whole cell extracts were prepared using lysing buffer containing 20 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 2 mM EDTA and a cocktail of protease and phosphatase inhibitors. Samples were incubated on ice for 10 minutes and sonicated for 15 minutes. 60 ug of lysate was loaded onto 7 or 15 SDS-PAGE gels, separated and transferred to a PVDF membrane. Membranes were incubated with the following antibodies overnight: *KAT6A* (mouse 4D8, Santacruz), ERα (rabbit 60C; Millipore), p53 (mouse DO-1, Santacruz), acetyl-H3K9 (mouse ab12179, abcam), total H3 (rabbit, abcam), β-actin (mouse, Sigma). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and the Western Lightning Clarity (BioRad) kit.

RESULTS

Identification of modulators of ERα signalling via arrayed genome-wide shRNA screening in T47D-KBLuc cells

In order to identify modulators of estrogenic response in ER-positive breast cancer cells, we used a clonal reporter cell line carrying a stably-integrated estrogen reporter vector, the T47D-KBLuc cell line (Wilson et al., 2004). These cells express the luciferase gene under the control of three estrogen response elements and a TATA box and initiator element. Luciferase activity is induced more than 40-fold by addition of E2 (25 nM) for four days and this activation is inhibited by antiestrogens or by infection with an shRNA against , the gene coding for ERα. The robustness of this assay was excellent in a 96-well plate format (Z-factor = 0.51), enabling high-throughput screening of an arrayed shRNA library (Mission lentiviral library, Sigma) including shRNAs against 16,083 genes (average of five shRNAs per gene). For the primary screen (Figure 1), three shRNAs per gene were chosen for a total of 48,249 shRNAs arrayed on 604 plates. Because the primary screen needed to be carried out over a period of about eight weeks, we used positive (shRNA against *ESR1*) and negative (non-targeting shRNA) controls in each plate (see Fig. 2A for plate design). Lentiviruses produced in 96 well plates were used to infect T47D-KBLuc cells plated in phenol red-free DMEM medium containing 5% charcoal-stripped serum and supplemented with E2 (25 nM) at an MOI of 10. Four days later, a luciferase assay was performed. The frequency distribution of hits in this assay is shown in Figure 2A. A cut-off of +/- 60% was selected to identify hits that either induced or inhibited luciferase activity. While 10,568 genes were not identified by any shRNA in the screen as being regulators of estrogen signalling, possibly including a significant number of false negatives due to lack of efficient inhibition by shRNAs in the library, 5,515 genes were identified as hits with at least one shRNA modulating estrogenic response by more than 60%. This included 107 genes identified by three shRNAs and 926 genes identified by two shRNAs (Figure 2B). In some cases, shRNAs had opposite impacts on reporter vector activity, suggesting random effects. Encouragingly, enrichment analysis

performed using Ingenuity pathway analysis software (IPA) with the list of hits identified with two or three shRNAs indicated enrichment in estrogen, glucocorticoid and androgen signalling, as well as molecular mechanisms of cancer and ERK and PI3K signalling (Table S1).

In addition, an alamarBlue assay was performed to identify shRNAs with marked impact on cell survival, as this is expected to affect the luciferase read-out. The frequency distribution indicated that most shRNAs had little impact of cell survival under these conditions (Figure 2C). Using a 25% cut-off, 13 genes had three of three shRNAs suppressing survival and 49 genes had two of three shRNAs with significant impact (Figure 2D; Table S2). These hits, which did not include , were flagged as potentially essential genes but were not excluded from the selection process for secondary assays. Some of these hits had a drastic effect on cell proliferation suggesting induction of cell death. For instance, *USP19* (three shRNAs with over 50% inhibition of cell proliferation) has been previously found to drive cell proliferation by deubiquitinating the p27Kip1 ubiquitin ligase KPC1 in fibroblasts and in MCF10A immortalized breast epithelial cells, but not in transformed MCF-7 or MDA-MB-231 cells (Lu et al., 2009, 2011). In addition, it has been shown to stabilize CORO2A, a component of the NCoR nuclear receptor corepressor complex, and to down-regulate retinoic acid receptor (RAR) activity in adipose cells (Lim et al., 2016). RARs have antiproliferative and pro-apoptotic activity in breast cancer cells (Appleyard et al., 2004; Raffo et al., 2000; Seewaldt et al., 1997; Wetherall et al., 1984). USP19 was also identified as a regulator of DNA repair, and its loss could contribute to DNA instability (Wu et al., 2016). Other hits had more modest effects on cell proliferation. For instance, the transcription factor ying-yang one (YY1, two shRNAs each with impact around 25 to 35%) plays roles in the control of cell proliferation and apoptosis, with different impacts on prognosis depending on cancer type (Nicholson et al., 2011), and has been proposed to extend the Myc transcription network in embryonic stem cells (Vella et al., 2012).

Based on results of the primary screen, we selected 615 genes (40 96-well plates with 80 test shRNAs per plate) for further analysis in secondary assays based on numbers of

shRNAs with impact on the luciferase reporter vector (two or three for novel candidates with effects >60% in the same direction, one for known ERα coregulators to be included as additional positive controls with NRIP1 in the secondary screens, or for additional components of the same pathways as hits identified with two or more shRNAs), absence of drastic impact (defined as >30% with two shRNAs) on cell survival, and functional information.

Selection of hits in secondary assays and confirmation of impact on target gene expression.

In the secondary screens (Figure 1), five shRNAs against each of the 615 selected hits were produced and used in a confirmation screen in T47D-KBLuc cells, but also in MELN cells, which are derived from MCF-7 cells and carry a stably integrated reporter vector where luciferase expression is driven by one ERE and the β-globin promoter (Balaguer et al., 2001). MELN cells carry a different complement of genetic aberrations than T47D cells (e.g. they are p53 positive, but *GATA3* mutant; (Adomas et al., 2014; Usary et al., 2004)), therefore we do not necessarily expect hits identified in T47D cells to be replicated in MELN cells. In addition, the efficiency of gene knockdown by shRNAs may be different in the two cell lines. Nevertheless, observation of an effect in both ER-positive cell lines was used to prioritize hits in the selection of hits for mechanistic analyses. The impact of shRNAs against positive controls *ESR1*, *NCOA2*, and *NRIP1* on inhibition of luciferase activity is shown in Figure 3, illustrating inhibition by most shRNAs against *ESR1* and *NCOA2*, but on the other hand activation of the ERE-Luc reporter vector by shRNAs against *NRIP1* (see Figure S1 for shRNA distribution of selected hits).

As many genes may affect the luciferase reporter assays non-specifically, for instance via modulation of global transcription, mRNA stability or translation efficiency, or via modulation of ATP levels, which could affect luciferase activity, we incorporated a counter-screen in T47D cells. We used a luciferase reporter vector containing an antioxidant response element, which is bound and activated by the transcription factor nuclear factor

erythroid-derived 2-like 2 (Nrf2) in response to oxidative or electrophilic stress or in cells treated with antioxidants. Importantly, this assay was robust in T47D cells (Z-factor = 0.54) and the activity of the reporter vector was not affected by administration of E2 (25 nM) or by knockdown of ERα (not shown). Therefore, this assay should help eliminate false positives due to non-specific impact on gene transcription or luciferase activity (selected examples in Figure S2).

Genes affecting ERα -mediated signalling are expected to also have an impact on ER-positive breast cancer cell proliferation in the presence of estrogens. We monitored the impact of ERα knockdown on T47D-KBLuc cell and MELN cell proliferation both in a short-term (four days) and longer-term (eight days) assay. While little effect of *ESR1* suppression could be detected after four days, extension of the assay to eight days with a complete media change and E2 replenishment at day four revealed three shRNAs inhibiting growth by more than 25% in MELN cells (Figure S3).

Hits were prioritized for the next step, validation of target knockout, based on number of shRNAs having an impact (30% or more) on ERE-luciferase activity, observation of effects in MELN cells (30% or more with at least one shRNA) but minimal effects on ARE-Luc reporter activity in T47D cells (no more than one shRNA with impact >30%) and minimal effects on growth of ER-positive cells at four days (no more than one shRNA with impact >25%). Suppression of target genes by shRNAs was investigated for prioritized hits using the shRNAs that were effective in modulating the estrogenic response of the reporter cell lines in the validation screen (Figure 4). Suppression of at least 50% of the endogenous gene level by two different shRNAs, as assessed by RT-qPCR, was selected as a criterion for further analysis of the impact of gene suppression on the transcriptome of T47D-KBLuc cells.

Assessment of the impact of hits on ERα signalling by transcriptome analysis

For those targets effectively suppressed by at least 2 shRNAs (27 candidate genes), transcriptome analysis was performed in the same culture conditions as for the screens (charcoal stripped medium in the presence of 25 nM E2, four days). Gene expression in the

presence of the two best targeting shRNAs was compared to that in three controls, corresponding to two non-targeting controls (shNT-E2, shGFP-E2) and to non-infected cells (Ni-E2). Mapping of reads to transcripts in the human genome was performed with Kallisto (Bray et al., 2016), and differential expression was assessed both at the transcript and gene level using Sleuth (Pimental et al., 2016).

We first assessed the impact of shRNA-mediated suppression of *ESR1* expression on transcriptomes compared to E2 treatment for four days by comparing beta values generated by Sleuth under each treatment condition. The coefficient of correlation (R) obtained was -0.7, indicating a very good negative correlation between the impact of E2 and that of shRNAs against *ESR1* (not shown). This value was even higher (-0.89) when focusing on a set of direct ERα target genes, selected by the presence of an ERα-associated region in ChIP-Seq experiments within 25 kb of the transcriptional start site overlapping with the presence of a predicted estrogen response element and regulation by E2 in T47D cells (Figure 5). On the other hand, the R value for the comparison between b values obtained for the regulation by two different shRNAs against the transcription factor GATA3, an upstream regulator of ERα in T47D breast cancer cells, and for E2 regulation of the same set of direct ERα target genes was much lower (-0.46), likely reflecting both partial suppression of ERα levels and regulation of other target genes (Figure 5). Suppression of the nuclear receptor cofactor *NCOA2* correlated even more weakly (-0.11) with mostly an impact on loss of expression of ERα target genes, consistent with a role of *NCOA2* as a co-activator of ERα.

We then compared treatment with two shRNAs against each selected hit gene with E2 regulation of direct ERα target genes (Figure 5 and Figure S3). R values between those observed for *ESR1* and *NCOA2* were obtained for several hits, including the histone acetyltransferase *KAT6A* (R=-0.81), the chromatin remodelling protein *CHRAC1* (R=-0.48), the transcription factors *EGR1* (R=-0.75), *YY1* (R=-0.55) and *NFYC* (R=-0.53), the ubiquitin ligase *WWP2* (R=-0.65), the peptidyl-prolyl isomerase *PPID* (R=-0.59) and the heat shock protein *HYOU1* (R=-0.37). Individual b values for each direct *ESR1* target gene are shown in a heat map (Figure 5B). Other hits had R values around -0.3 (*DIXDC1*) or below (Figure S4).

NRIP1 was the only factor whose suppression showed a positive (albeit relatively weak; R= 0.26, not shown) correlation with E2 signalling, in keeping with a function as a corepressor of ERα.

As estrogenic signalling has a proliferative effect in ER-positive breast cancer cells, we next evaluated the impact of suppression of hit genes compared to that of *ESR1* on expression of estrogen target genes with roles in cell proliferation in T47D cells. We selected a cluster of genes highly correlated in the TCGA breast cancer dataset and associated with cell cycle control, cell proliferation and DNA repair terms in gene set enrichment analysis (Lemieux, S et al. in preparation). E2 positively regulated most genes expressed in T47D cells in this cluster (b>0.4, Figure 5C), while shRNA-mediated suppression of *ESR1* expression resulted in down-regulation of all significantly regulated genes (b<-0.4, Fig 5D). For all selected hits with an R value greater than -0.5, shRNA-mediated suppression of expression resulted in more than 94% of regulated proliferative genes being repressed (b<-0.4, Figure 5C). Other hits with lower R values had more mixed positive/negative impacts on proliferative genes; for instance, suppression of *NFYC* and *PPIL1* negatively affected only 67% and 63%, respectively, of significantly regulated genes (b<-0.4, Figure 5C and Figure S5).

We next assessed whether suppressing expression of hit genes had an impact on *ESR1* expression at the transcript level by plotting b values obtained in each case. Interestingly, suppression of several hits with the highest R values compared to E2 regulation of direct *ESR1* target genes had a negative impact on regulation of *ESR1* expression (Figure 6A-B), similar or higher than that of *GATA3*, a known positive regulator of *ESR1* (Figure 6A). On the other hand, suppression of the cofactor *NCOA2* did not negatively affect *ESR1* expression in T47D cells (Figure 6A). Together, these results suggest that several hits identified have a significant positive impact on estrogenic signalling mediated by ERα in T47D cells at least in part via up-regulation of *ESR1* expression.

KAT6A, in association with mutant p53 in T47D cells, induces *ESR1* expression and high expression is associated with good prognosis in breast tumours.

The hit whose suppression was most correlated with the impact of *ESR1* shRNA treatment on direct ERα target genes (Figure 5) was the Lys acetyl-transferase KAT6A, initially identified as a translocation partner with other Lys acetyl transferases (CBP, p300, SRC2/TIF2) in acute myeloid leukemia as Monocytic leukemia zinc finger protein (MOZ, Champagne et al., 2001; Troke et al., 2006). This 2004 amino acid protein contains a MYST domain responsible for its catalytic activity, an N-terminal repression domain and a C-terminal activation domain and acts as a cofactor for several transcription factors with roles in leukemia, such as AML and the ETS family transcription factor, PU.1 (*SPI1*) (Rokudai et al., 2013).

KAT6A RNA is expressed in most breast tumours with highest overall levels in ER-positive tumours (Luminal A and Luminal B classes), and lowest levels in HER2-positive tumours (Figure S6A). *KAT6A* is amplified in 11% of breast tumours, and amplification correlates with higher levels of expression (Figure S6B-C). In addition, several mutant forms of p53 induce *KAT6A* expression via interaction with Ets (Zhu et al., 2015). Highest expression of *KAT6A* in breast tumours (TCGA dataset) correlates with a positive ERα status and enrichment in luminal B tumours (Figure S6D). In cell lines, it was expressed at high levels in T47D cells (p53 mutant) and in ZR75 cells (amplified) compared to MCF-7 cells (5-fold lower than T47D cells, not shown).

Transcriptional regulation of direct ERα target genes upon suppression of *KAT6A* with two different shRNAs in T47D cells was remarkably correlated with that observed for the same genes after shRNA-mediated suppression of *ESR1* expression (Figure 5). Remarkably, suppression of *KAT6A* led to gene regulation that was more markedly correlated with the impact of shESR1 treatment on the whole transcriptome (R=0.53, Figure S7L) than suppression of ERα cofactors *NCOA1*-3 (Figure S7N-P), suggesting a key role of *KAT6A* in the control of *ESR1* expression. Down-regulation of E2-induced target genes (*GREB1, PGR*) and up-regulation of E2-repressed genes (*BCAS1, KRT4*) upon either *ESR1* or *KAT6A* down-regulation was first verified in RT-qPCR experiments and mirrored the impact of shESR1

(Figure 7C). Further, down-regulation of ERα protein levels was observed when *KAT6A* expression was suppressed with two of three shRNAs (Figure 8A); similar downregulation of ERα protein (Figure 8B) or *ESR1* mRNA (Figure 8C) were observed using guide RNAs rather than shRNAs. In addition, shRNA-mediated suppression of *KAT6A* in MCF-7 cells also lowered ERα expression in these cells (not shown). Although detection of the *KAT6A* protein was very weak, depletion of global H3K9Ac levels was observed with two out of three shRNAs against *KAT6A*, correlating with loss of ERα expression (Figure 8A).

Expression of *KAT6A* has been reported to be induced by mutant p53 in triple negative cells (Zhu et al., 2015). Interestingly, suppression of the mutated version of p53 expressed in T47D cells also led to apparent loss of *KAT6A* expression and of acetylated H3K9, suggesting that this regulation is preserved with the mutant version of p53 present in T47D cells. *KAT6A* has been shown to interact with p53 through multiple domains in MCF-7 cells, increasing p53 acetylation at K120 and 382 and transcriptional activity on target genes such as p21 (Rokudai et al., 2009, 2013). p53 acts as an upstream modulator of *ESR1* in MCF-7 cells (Angeloni et al., 2004; Shirley et al., 2009). Of interest, shRNA-mediated suppression of p53 expression by shRNAs also led to suppression of *ESR1* (Figure 8A), suggesting that p53 maintains the capacity to regulate *ESR1* expression in T47D cells in spite of the presence of a mutant form of the protein.

Altogether, our data suggest that *KAT6A* is induced by p53 in T47D cells, and in turn modulates p53 activity as an upstream regulator of *ESR1* expression. A prediction of this conclusion is that *KAT6A* should have an impact on prognosis in breast tumour cells that mirrors that of *ESR1*. Indeed, using the KMplotter tool (Győrffy et al. 2009), we observed in a meta-dataset of breast tumour samples with associated transcriptomes that both *ESR1* and *KAT6A* were good prognostic factors (Figure 9AB). Of note, however, *KAT6A* was a bad prognostic factor in ER-positive tumours, suggesting that it could drive proliferation of these tumours and lead to resistance to hormonal therapy (Figure 9C).

DISCUSSION

While ERα-expressing luminal breast tumours can be targeted by blocking estrogen production (aromatase inhibitors) or signalling (antiestrogens), the prevalence of acquired resistance even while ERα continues to be expressed supports the further study of factors influencing estrogen signalling and the mechanisms controlling expression of ERα in breast cancer cells.

Our genome-wide shRNA screening effort identified a number of known ERα cofactors as modulators of estrogen signalling in our model system, the T47D-KBLuc cell line, including members of the steroid receptor cofactor (SRC) family of coactivators (*NCOA1*, *NCOA2* and NCOA3) as activators of ERα signalling, and nuclear receptor interacting protein 1 (*NRIP1*) as an overall corepressor on direct estrogen target genes in our reporter assays. We also confirmed the activating roles of GATA3 and FOXA1, two pioneer transcription factors that have been implicated both in ERα signalling and expression (Eeckhoute et al., 2007; Lupien et al., 2008; Theodorou et al., 2013).

As anticipated, our comprehensive genome-wide approach also identified a number of novel regulators of ERα with different functions and likely mechanisms of action that may have been overlooked by a more selective, targeted screening approach. These include transcription factors such as nuclear factor gamma (NFYC), one subunit of a trimeric complex that binds CCAAT motifs in the promoters of a variety of genes. NFYC did not affect *ESR1* expression, but behaved as an activator in our reporter assays, although it has been identified as a corepressor of the mineralocorticoid receptor, another steroid receptor (Murai-Takeda et al., 2010). NFYA and NFYB, the remaining two members of the trimeric complex were not selected beyond primary screening as each had only a single shRNA hit, which however also supported an activating role. The three NFY subunits may indeed participate together in ERα signalling. Interestingly, knockdown of NFYC did not correlate significantly with *ESR1* down-regulation when considering the entire transcriptome (R=0.17), suggesting that NFYC has a unique set of target genes distinct from those regulated by the

ERα signalling. This is unsurprising considering that the CCAAT motif recognized by NFY factors is over-represented in human promoters and enhancers (Dolfini and Mantovani, 2013). However, knockdown of NFYC results in decreased expression of direct E2 target genes (R=-0.53, Figure 5A); this was mostly due to decreased expression of all upregulated direct ERα targets, while the majority of down-regulated targets were also decreased (Figure 5B), suggesting NFYC is an overall positive regulator of gene expression (Figure 5A, Figure S7R) in T47D cells. It is possible that NFYC may act in estrogen signalling as a cooperating transcription factor bound to regulatory sequences of a significant fraction of estrogen target genes either to its own target sites or via direct or indirect (through common cofactors) interaction with ERα bound to its cognate regulatory sites.

Another interesting coregulatory candidate is CHRAC1, a chromatin remodelling protein not previously known to affect ERα signalling. The *CHRAC1* gene is frequently amplified in breast cancer as part of the 8q24.3 amplicon (Figure S8) and is thought to be a driver gene regulating proliferation of these tumours. Accordingly, knockdown of *CHRAC1* results in decreased cell viability and inhibited anchorage-independent growth (Mahmood et al., 2014). Similarly, *CHRAC1* was flagged early on in the screening protocol as its knockdown results in decreased viability of T47D-KBLuc cells at short time points. Transcriptome analysis of T47D-KBLuc cells following *CHRAC1* knockdown reveals no significant effect on *ESR1* expression, but a correlation with *ESR1* knock-down on direct *ESR1* target gene expression (R=0.48) that was higher than that observed with the known transcriptional cofactor *NCOA2* (R=0.33). In addition, *CHRAC1* knockdown resulted in a strong repressive effect on a cluster of genes involved in cell cycle control, cell proliferation and DNA repair (Figure 5C), including a significant downregulation (b<-0.4) of transcription factors *FOXM1*, *MYBL2*, *E2F1* and *E2F2*, which play roles in cell cycle control. Although this effect may possibly take place through E2-independent as well as dependent mechanisms, we note that *CHRAC1* represents a good prognosis marker in a meta-dataset of breast tumours, and that it retains good prognosis power in the ER-positive tumour subgroup (Figures S9-10). This suggests that *CHRAC1* amplification does not drive estrogen-

independent growth of breast tumours, which would result in resistance to antiestrogen therapies.

In addition, knockdown of several candidate genes had an impact on *ESR1* gene expression, suggesting that their role in affecting E2 signalling is mediated at least in part via the mechanism. The most significant effect on *ESR1* gene expression was obtained by knock-down of *KAT6A*, a member of the MYST family of histone acetyltransferases (HATs) that plays a central role in erythroid and myeloid cell differentiation and is required for hematopoietic stem cell maintenance (reviewed in Perez-Campo et al., 2013). *KAT6A* participates in frequent chromosomal translocations in acute myeloid leukemia, whereby the MYST domain (responsible for HAT activity) is fused with other transcriptional coactivators including *CBP, p300, TIF2* and *NCOA3* (Borrow et al., 1996; Carapeti et al., 1998, 1999, Chaffanet et al., 1999, 2000; Esteyries et al., 2008; Liang et al., 1998). KAT6A can self-acetylate and has been shown to acetylate lysine residues on H2B, H3K14 and H4K5/8/12/16 in vitro (Champagne et al., 2001; Holbert et al., 2007; Kitabayashi et al., 2001) and H3K9 in vivo (Voss et al., 2009). Furthermore, KAT6A interacts with and acetylates non-histone substrates including the transcription factors p53, RUNX1, RUNX2 and NFκB to modulate regulation of their target genes (Bristow and Shore, 2003; Chan et al., 2007; Pelletier et al., 2002; Rokudai et al., 2009, 2013). Of interest, several of these transcription factors are upstream regulators of *ESR1* gene expression (Angeloni et al., 2004; Bragt et al., 2014; Lambertini et al., 2007; Shirley et al., 2009).

In this study, knockdown of *KAT6A* potently repressed ERα signalling in the T47D-KBLuc cell line with little effect on the ARE reporter (Figure S2M), a pattern similar to that following knockdown of *ESR1* (Figure S2G). Notably, the effect of knock-down was milder in MELN cells, possibly indicating differential *KAT6A* involvement in ERα signalling in this cell line, in keeping with its lower expression in MCF-7 cells (not shown). Furthermore, *KAT6A* repression led to an overall gene expression pattern similar to that following *ESR1* depletion, and, like *ESR1* knockdown, negatively correlated with direct ERα target gene expression following E2 treatment. Accordingly, *KAT6A* was found to be a regulator of *ESR1* transcript

and protein expression (Figure 7-8. While this work was in progress, a separate study reported *KAT6A* as an epigenetic activator of *ESR1* expression, a positive regulator of T47D cell proliferation in mouse models and a bad prognosis marker in ER-positive breast tumours (Yu et al., 2016), confirming the conclusions of our study and therefore the validity of our screening approach.

It remains to be elucidated by what mechanism *KAT6A* affects ERα expression. In our study, transcriptome analysis revealed a potent suppression of *GATA3*, *TP53*, *RUNX1* and *RUNX2* transcript expression following *KAT6A* knockdown, and these transcription factors have been implicated in control of *ESR1* expression and/or the ERα signalling pathway (Angeloni et al., 2004; Bragt et al., 2014; Chimge et al., 2016; Dydensborg et al., 2009; Eeckhoute et al., 2007; Khalid et al., 2008; Lambertini et al., 2007; Shirley et al., 2009; Stender et al., 2010). Of interest, p53 has been shown to interact with the *ESR1* proximal promoter and *KAT6A* binding was detected on that promoter (Yu et al., 2016), although it remains unclear whether the two regulators are recruited as a complex to the same site. *KAT6A* may modify the acetylation of histones on the *ESR1* promoter. Alternatively, ERα expression may equally be affected by *KAT6A*-mediated acetylation of non-histone substrates, including the transcription factors mentioned above or ERα itself. Because ERα autoregulates its transcription (Castles et al., 1997; Ellison-Zelski et al., 2009) and acetylation of ERα is known to increase ligand-independent activity (Fuqua et al., 2000; Herynk et al., 2007), it is indeed possible that *KAT6A*-mediated acetylation of ERα protein contributes to *ESR1* expression in T47D cells.

Our screen also identified several other novel regulators of *ESR1* transcript expression, namely EGR1, GNG7, *WWP2* and YY1, whose knockdown correlates to varying degrees with knockdown of ERα on direct E2 target gene expression and proliferative genes. EGR1 is an immediate early target gene activated following E2 stimulation in breast and uterus (Kim et al., 2014; Pratt et al., 1998) and transcript expression was significantly upregulated following E2 stimulation in our study (Figure S11), suggesting it may also play a role in a positive feed-back on *ESR1* expression. While EGR1 has not previously been

identified as a regulator of *ESR1* expression, it is deleted in ER-negative breast tumours (Ronski et al., 2005) suggesting it may also play a role upstream of the receptor in breast carcinoma. GNG7, *WWP2* and YY1 transcript expression were unaffected by E2 stimulation or knockdown of *ESR1*. The transcription factor YY1 has not been previously implicated in *ESR1* transcriptional control, although YY1 has been described as a potential tumour suppressor in breast cancer (Zhou et al., 2016). *WWP1*, an E3-ubiquitin ligase related to *WWP2*, is associated with ERα status in breast cancer cell lines and suppression has been shown to decrease ERα in the literature (Chen et al., 2009). While a positive role of *WWP1* on *ESR1* expression was not supported by our studies, this may be due to inefficient suppression of *WWP1* or to a mechanism of action occurring at the level of ERα protein rather than at the transcription level. Finally, GNG7 is a small G protein gamma subunit that is frequently repressed in head and neck cancer (Demokan et al., 2013; Hartmann et al., 2012). Its connection with *ESR1* expression remains unclear at the moment, but could stem from downstream signalling events such as MAPK or PI3K signalling.

It is likely that some of our hits affect ERα signalling in an indirect manner, by modulating expression or activity of other genes that then affect ERα. Our transcriptome analysis reveals that a number of hits cross-regulate other hits, including those chosen as positive controls whose role in ERα signalling is well documented. For example, *NCOA2* expression is repressed by knockdown of *CHRAC1*, DIXDC1, MLLT1, NCOA3, NCOA6, *WWP2* and YY1, but not by *ESR1* (Figure S11), indicating that *NCOA2* repression in this case is not just a consequence of inhibited E2 signalling. Due to the duration of our assay, any of these hits could regulate *NCOA2*, which would then go on to modulate ERα signalling. A more comprehensive time course study with or without addition of a protein synthesis inhibitor would be necessary to clarify whether effects on ER target genes are direct and/or indirect consequences of hit knockdown.

ERβ is expressed in T47D cells, albeit likely at low levels considering the difference in transcript abundance between ERα and ERβ. Knockdown of *ESR2* significantly increased ERE-luciferase reporter activity with only a single shRNA in our primary screen, and so was

not assayed in subsequent protocols. Nonetheless, ERβ may participate in gene expression changes seen following knockdown of our selected hits. Notably, *ESR2* transcript expression is significantly upregulated following E2 stimulation, and is affected by knockdown of a number of genes, both positively (*FOXA1, GATA3, MLLT1, NRIP1* and *YY1* shRNAs upregulate) and negatively (*GNG7, KAT6A, NCOA1, NFYC, PPIL1, RAD21* and *WWP2* shRNAs downregulate) (Figure S12). ERβ may participate in regulating those genes that were differentially affected by E2 stimulation and *ESR1* knockdown. Alternatively, the discrepancy between genes differentially regulated by E2 or *ESR1* may be due to incomplete knockdown of ERα protein, leading to residual transcriptional activity and therefore significant regulation of only a fraction of E2 targets. Certain genes (*TFF1, GREB1*) may be more sensitive to small fluctuations in ERα protein levels.

While we have identified a number of promising candidate regulator genes, we expect many other regulators of ERα signalling were dismissed as false negatives due to lack of effective suppression by available shRNAs. The field of RNA interference has recently been complemented by the availability of CRISPR/Cas9 technology, which allows for gene knockout rather than knockdown at the clonal level, and results in stronger average knockdown effects at the population level. However, most screens published to date using this technique (Korkmaz et al., 2016; Park et al., 2016; Phelps et al., 2016; Shalem et al., 2015; Wang et al., 2014; Zhang et al., 2016), have used a pooled format, necessitating assays that can be amenable to selection procedures.

It is equally possible that our assay protocol introduced biases and resulted in lack of detection of some ERα regulators. For example, our chosen reporter cell line (T47D) or our luciferase promoter sequence or structure excluded those cofactors that require a specific repertoire of expressed coactivators or cooperating transcription factors to function. Regulators unable to activate luciferase expression from the integrated ERE3-TATA-Luc reporter construct would therefore have been excluded even if they do regulate a certain subset of ERα target genes. Most obviously, this would include those transcription factors to which ERα tethers at alternate response elements that do not contain consensus ERE.

Future experiments will be needed to address the mechanisms of action of candidate modulators, in particular by characterizing their interactome in T47D cells, and to study the general impact of these modulators in both ER-positive and ER-negative breast cancer cells. Nevertheless, our genome-wide screen identified potential targets for modulation of ERα expression, such as *KAT6A*, and for amplification of ERα signalling, such as *CHRAC1*, which are deregulated in breast cancer. These studies may therefore lead to the development of novel therapeutic approaches targeting tumours with overactive ERα signalling.

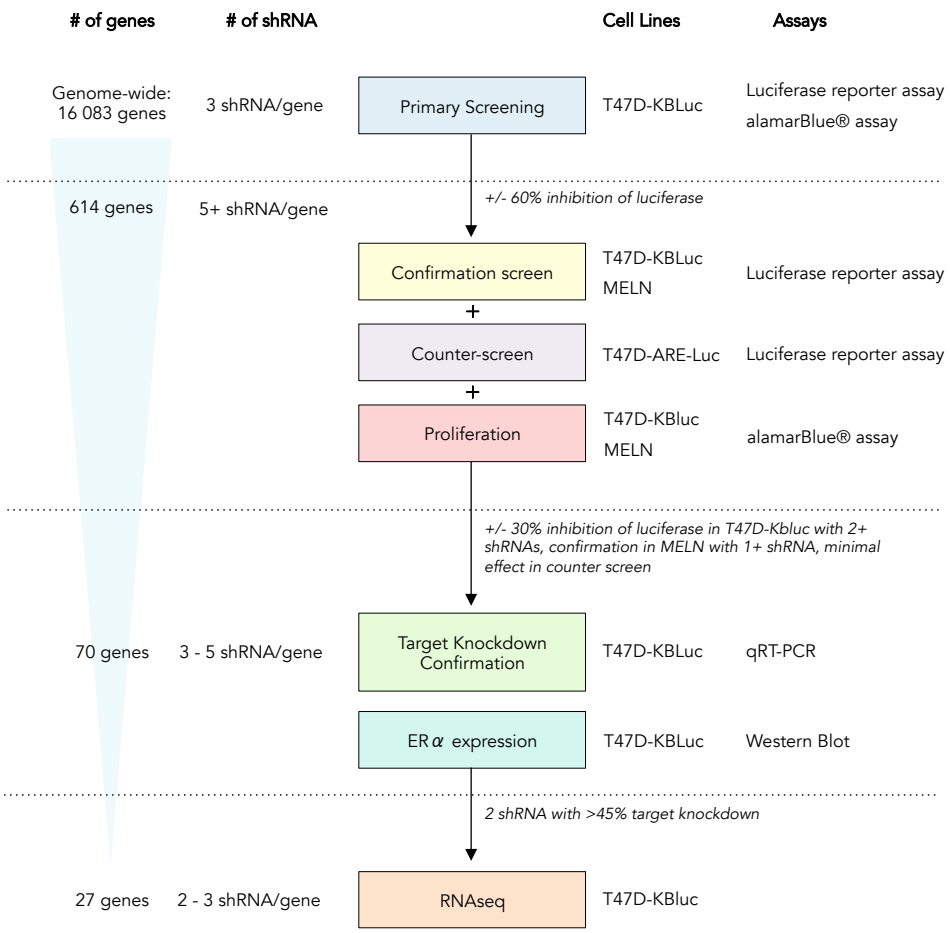


Figure 1. Summary of Screening Pipeline.
Genome-wide primary screening was based on ERE-luciferase reporter activity in T47D cells following shRNA-mediated knockdown of each of 16,083 protein coding genes in an arrayed format with three unique clones of shRNA for each gene. The top 614 candidates of interest were tested in subsequent secondary screens with five shRNAs per gene, to confirm primary screening observations in two ER-positive breast cancer cell lines, eliminate non-specific hits and characterize the effect of gene knockdown on cell proliferation in the presence of E2 (25 nM). Down-regulation of shRNA target gene expression in T47D-KBLuc cells was confirmed for the most promising 70 hits and samples with efficient target knockdown (>45%) by two individual shRNAs for each gene were characterized through transcriptome sequencing.

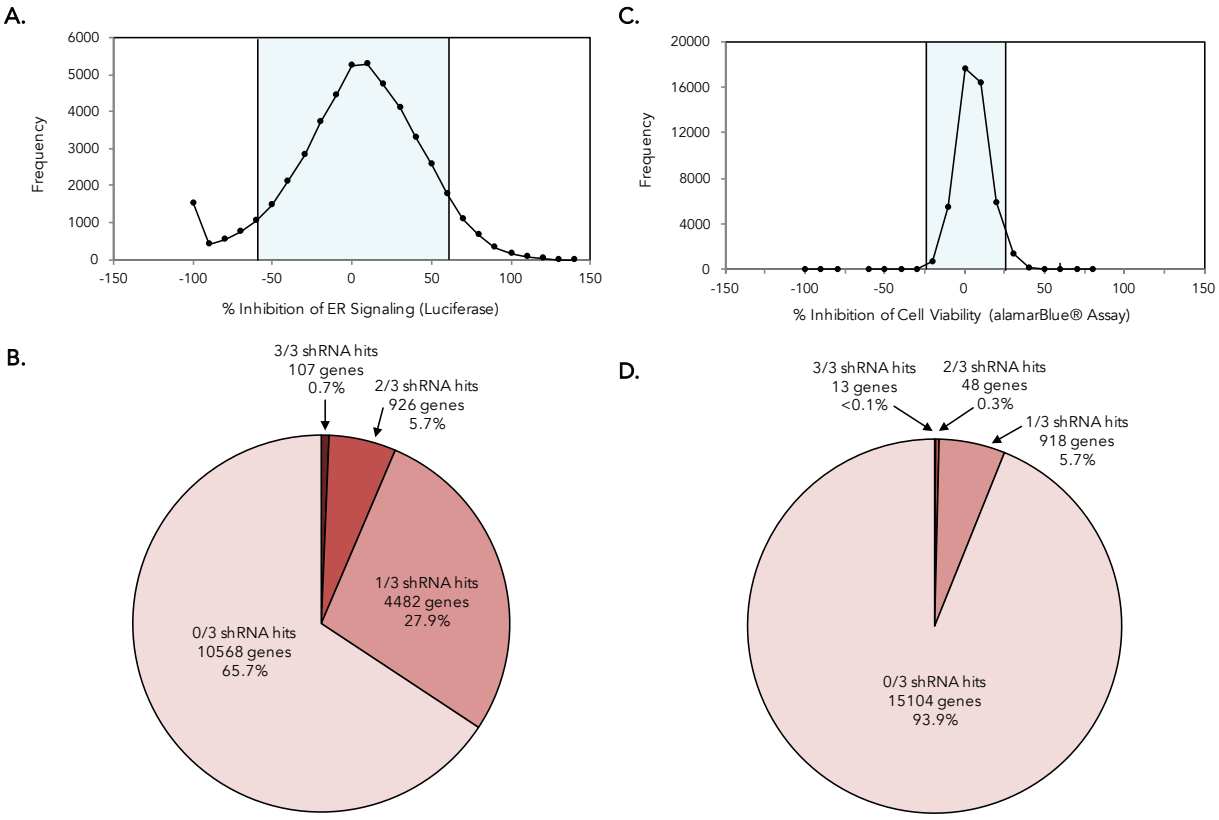


Figure 2. Distribution of hits in the primary screen.
For the primary genome-wide screen (16,083 protein-coding genes, three shRNAs/gene), T47D-KBLuc cells were transduced with shRNAs (MOI 10) in the presence of E2 (25 nM). Cell viability (alamarBlue fluorescence assay) and ER-signalling (ERE-luciferase reporter activity) were assessed following four days of knockdown/E2 stimulation. A) Percent inhibition of ER signalling based on luciferase reporter expression is plotted against the number of shRNAs falling within that % inhibition. All shRNA falling outside the cut-off (+/-60% inhibition; the non-significant area is indicated in blue) are considered hits. B) Distribution of assayed genes based on number of hits for each gene. While most genes (94%) have at most one of three shRNAs outside the cut-off, a number of genes (1033) have 2/3 or 3/3 shRNAs significantly affecting ER-signalling in the T47D-KBLuc cell line. C) Percent inhibition of cell viability based on an alamarBlue® assay is plotted against the number of shRNAs falling within that % inhibition. D) Distribution of assayed genes based on number of shRNAs for each gene with an impact on cell proliferation of >25 or <-25% . While nearly all genes (99.6%) have at most one of three shRNAs outside the cut-off, a small number of genes (62) have 2/3 or 3/3 shRNAs significantly affecting cell viability in the T47D-KBLuc cell line (see Table S2).

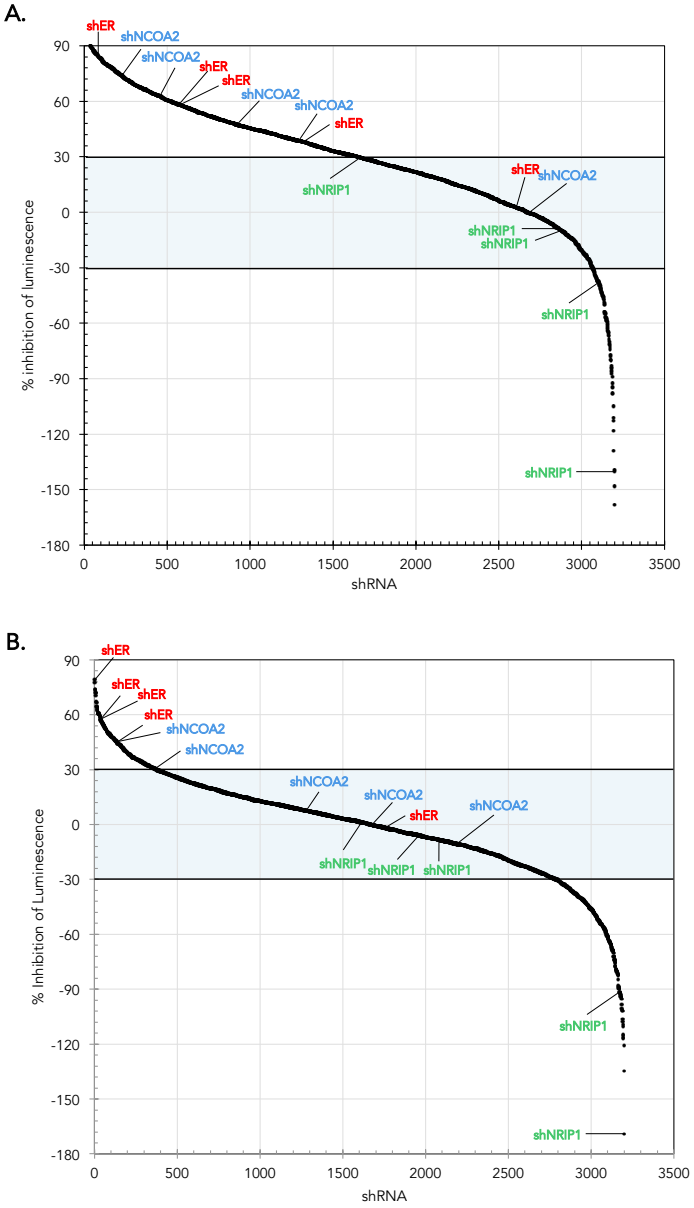


Figure 3. Hit impact on luciferase activity in T47D-KBLuc and MELN cells in secondary screens.
A) T47D-KBLuc and B) MELN cells were transduced with shRNA (MOI 10) in the presence of E2 (25 nM). ER-signalling (ERE-luciferase reporter activity) was assessed following four days of knockdown/E2 stimulation. Percent inhibition of luminescence for each of 3,200 shRNAs used in the secondary screen (614 genes, 5 or more shRNA per gene) is shown. Labelled data points represent shRNAs targeted against known effectors of the ER signalling pathway, namely ERα, NRIP1 and NCOA2. Values represent TRCN ID numbers for each individual shRNA from the Sigma Mission shRNA library. The cut-off for hit selection in the secondary screen was +/-30% (see Figure S1 for supplementary data on selected hits).

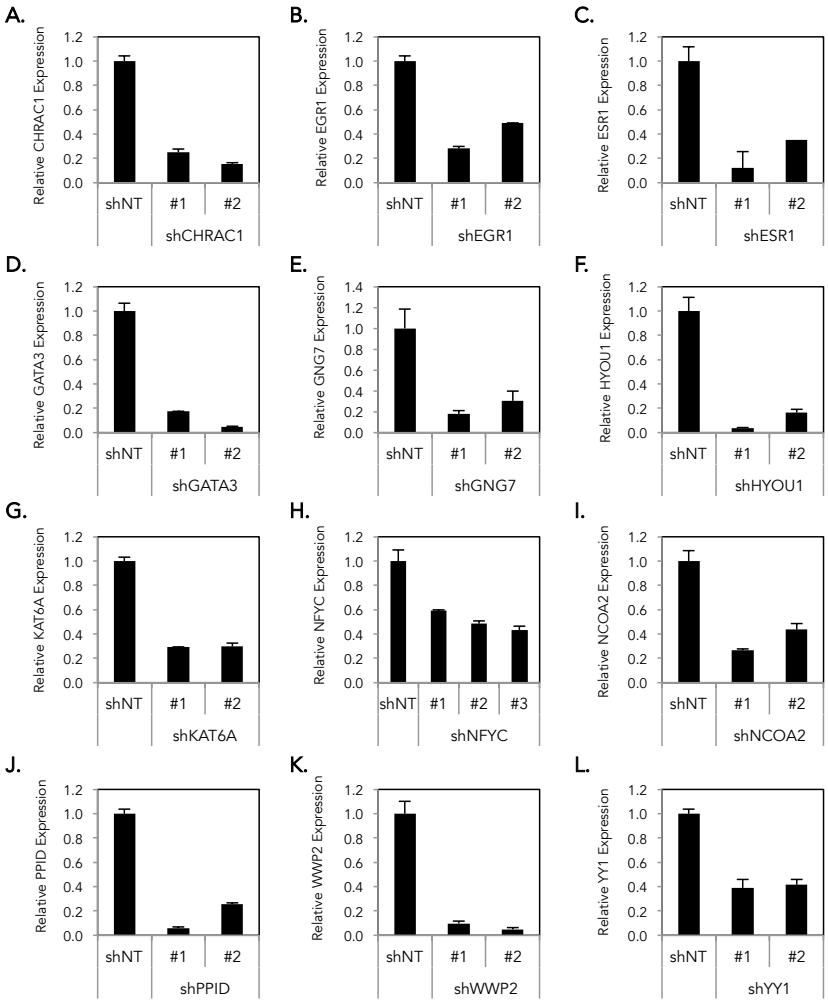


Figure 4. Confirmation of target gene knockdown by quantitative RT-PCR.
T47D-KBLuc cells were transduced with non-targeting shRNA (shNT) or shRNA targeting A) CHRAC1, B) EGR1, C) ESR1, D) GATA3, E) GNG7, F) HYOU1, G) KAT6A, H) NCOA2, I) NFYC, J) PPID, K) WWP2 or L) YY1 (MOI 10), with two to three unique shRNAs per gene in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted and transcript levels were assessed by qRT-PCR and normalized to housekeeping genes RPLP0, YWHAZ and TBP.

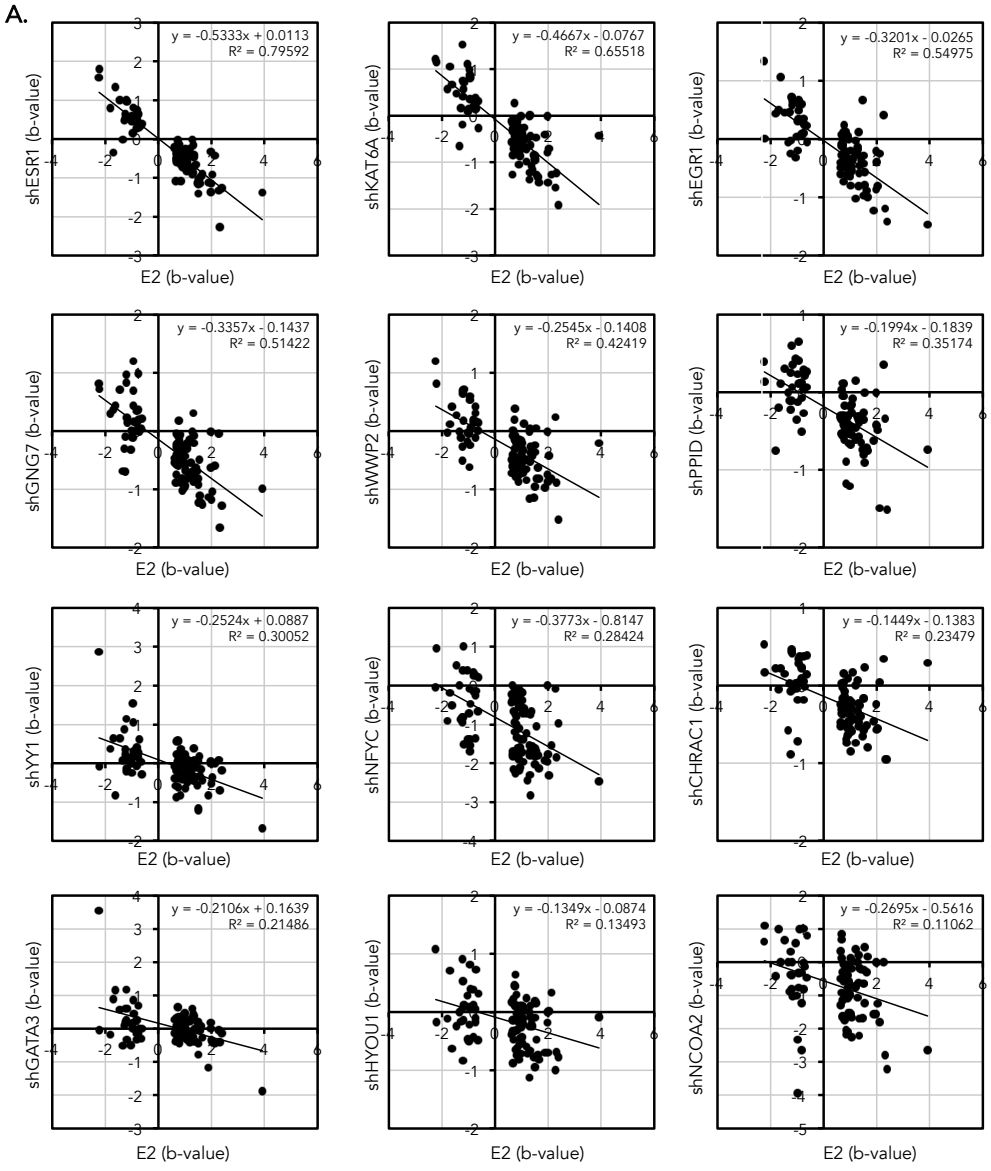


Figure 5. Direct E2 target gene expression following shRNA-mediated gene knockdown.
A) T47D-KBLuc cells were transduced with non-targeting shRNA (shNT), shGFP or shRNA targeting ESR1, KAT6A, EGR1, GNG7, WWP2, PPID, YY1, NFYC, CHRAC1, GATA3, HYOU1 or NCOA2 (MOI 10), with two unique shRNAs per gene in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted and transcriptomes were sequenced for each sample. Direct ERα target gene expression (relative to non-infected cells, shNT and shGFP) is shown on the y-axis of each panel, plotted against gene expression following treatment with E2 (25nM) for four days. The trendline for each plot is calculated by a simple linear regression and is displayed with the R^2 for each dataset. Plots are presented ordered by decreasing R^2 value. b-values > 0.4 indicate increased gene expression, while b-values < -0.4 indicate decreased expression.

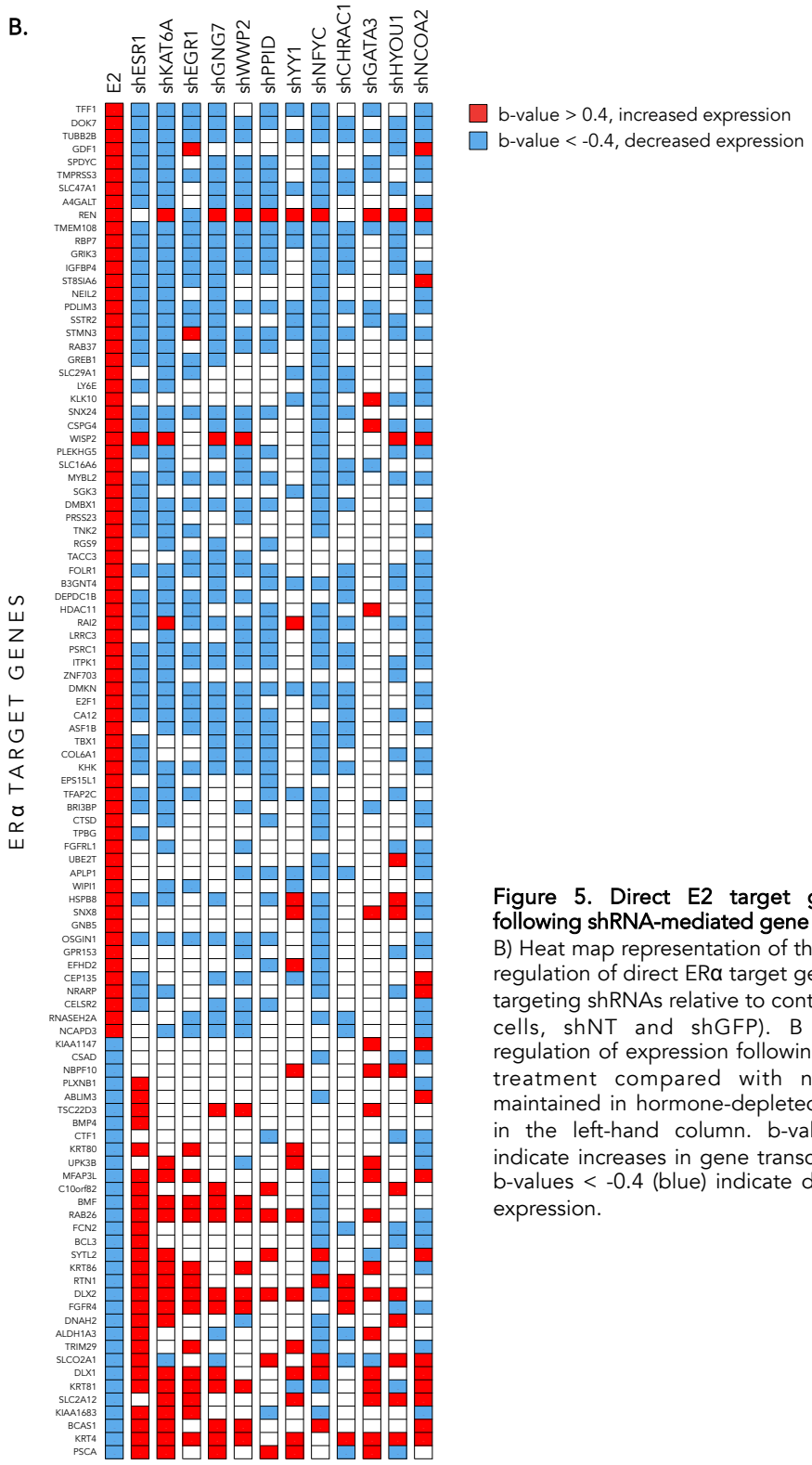


Figure 5. Direct E2 target gene expression following shRNA-mediated gene knockdown.
B) Heat map representation of the b values for the regulation of direct ERα target gene expression by targeting shRNAs relative to controls (non-infected cells, shNT and shGFP). B values for the regulation of expression following four days of E2 treatment compared with nontreated cells maintained in hormone-depleted media is shown in the left-hand column. b-values > 0.4 (red) indicate increases in gene transcript levels , while b-values < -0.4 (blue) indicate decreases in gene expression.

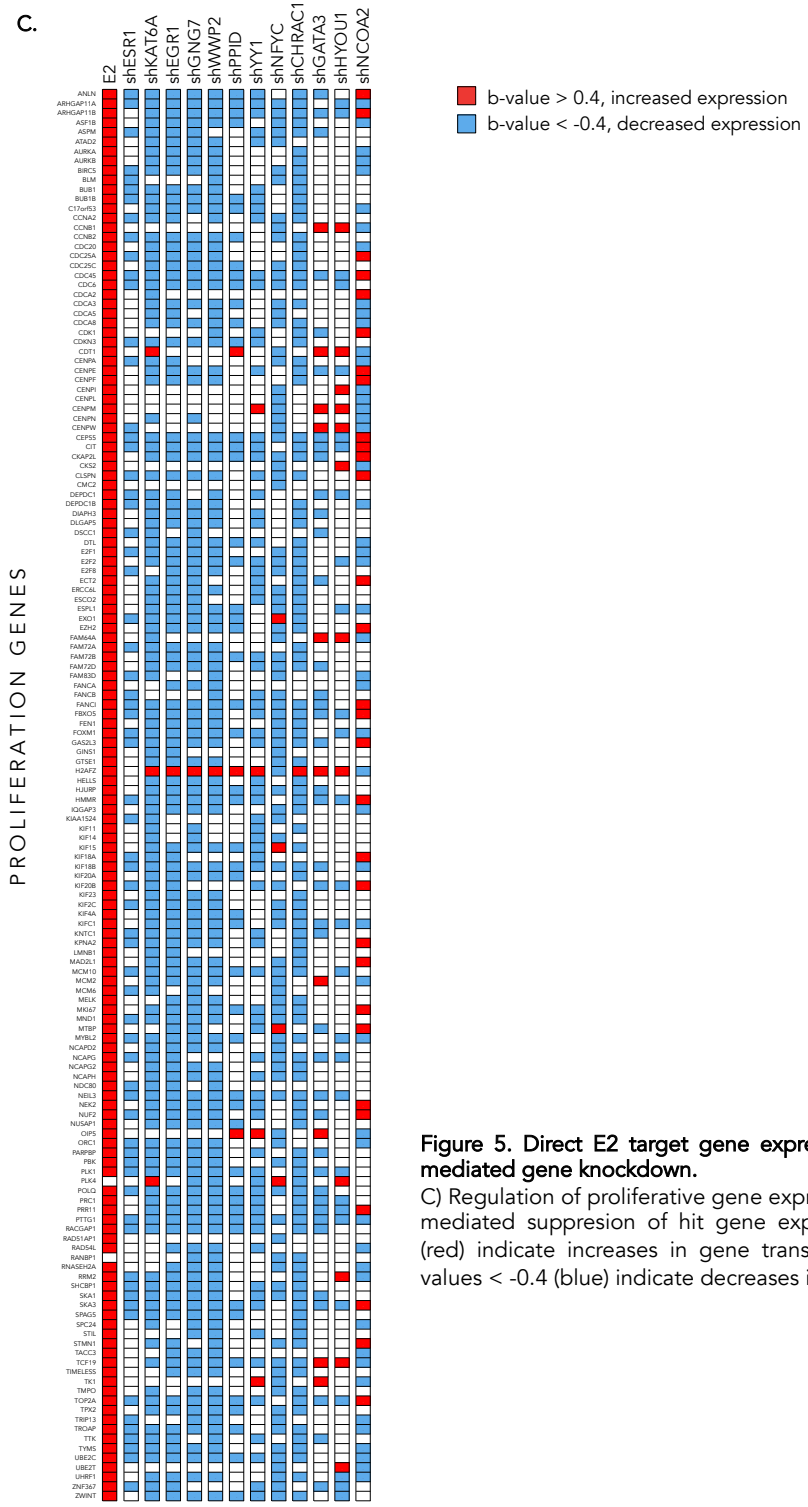


Figure 5. Direct E2 target gene expression following shRNA-mediated gene knockdown.

C) Regulation of proliferative gene expression following shRNA-mediated suppresion of hit gene expression. b-values > 0.4 (red) indicate increases in gene transcript levels , while b-values < -0.4 (blue) indicate decreases in gene expression.

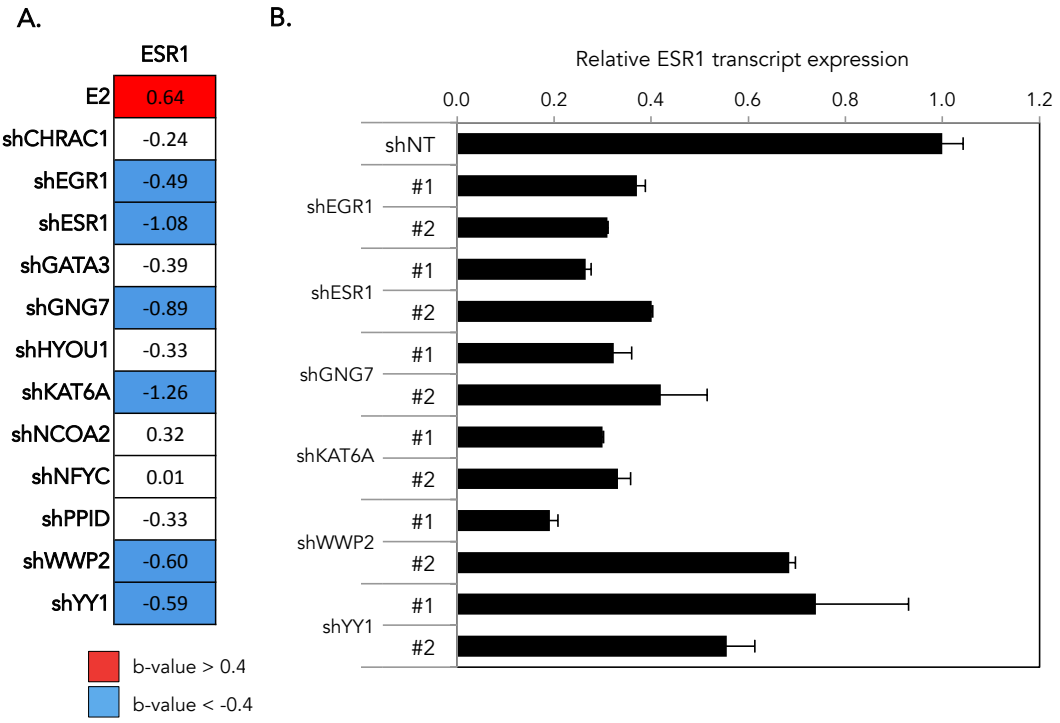


Figure 6. Impact of shRNA-mediated knockdown of hit genes on ERα expression

A) Regulation of ESR1 transcript expression in transcriptome analysis after transduction of T47D-KBLuc cells with non-targeting shRNA (shNT), shGFP or 2 shRNAs targeting CHRAC1, EGR1, ESR1, GATA3, GNG7, HYOU1, KAT6A, NCOA2, NFYC, PPID, WWP2 or YY1 (MOI 10), in the presence of E2 (25 nM). E2 treatment or knockdown of EGR1, ESR1, GNG7, KAT6A, WWP2 and YY1 markedly affects ESR1 transcript levels. b-values > 0.4 (red) indicate an increase in RNA transcript expression following shRNA-mediated knockdown, while b-values < -0.4 (blue) indicate a decrease in RNA transcript expression. B) Repression of ESR1 transcript expression by knockdown of EGR1, ESR1, GNG7, KAT6A, WWP2 and YY1 (four days in the presence of 25 nM E2) was confirmed by RT-qPCR. ESR1 transcript levels were normalized to housekeeping genes RPLP0, TBP and YWHAZ.

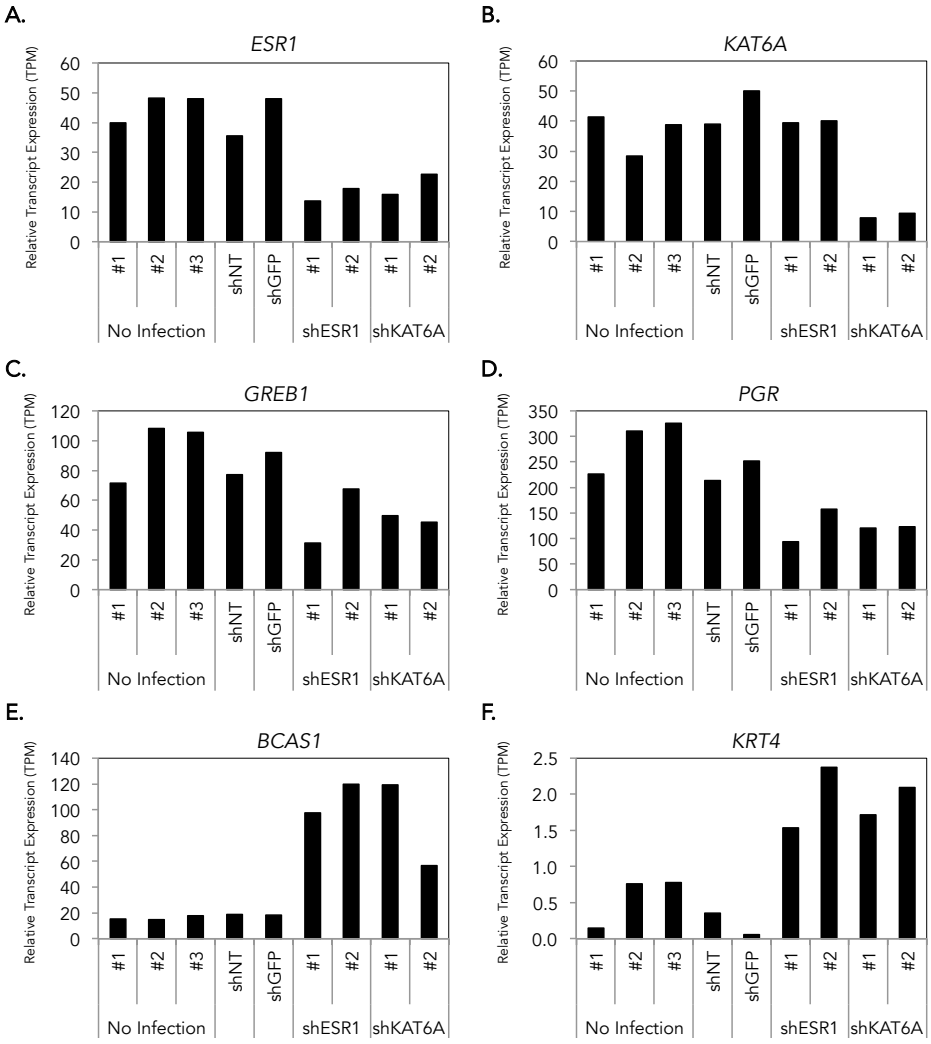


Figure 7. Impact of shRNA-mediated knockdown of KAT6A on expression of E2 target genes.
Regulation of E2 target genes in T47D-KBLuc cells transduced with non-targeting shRNA (shNT), shGFP or with either of two shRNAs targeting ESR1 or KAT6A (MOI 10) in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted and transcriptomes were sequenced for each sample. Knockdown of both A) *ESR1* or B) *KAT6A* resulted in repression of E2-induced target genes C) *GREB1* and D) *PGR* but induction of E) *BCAS1* and F) *KRT4*, genes normally repressed by E2.

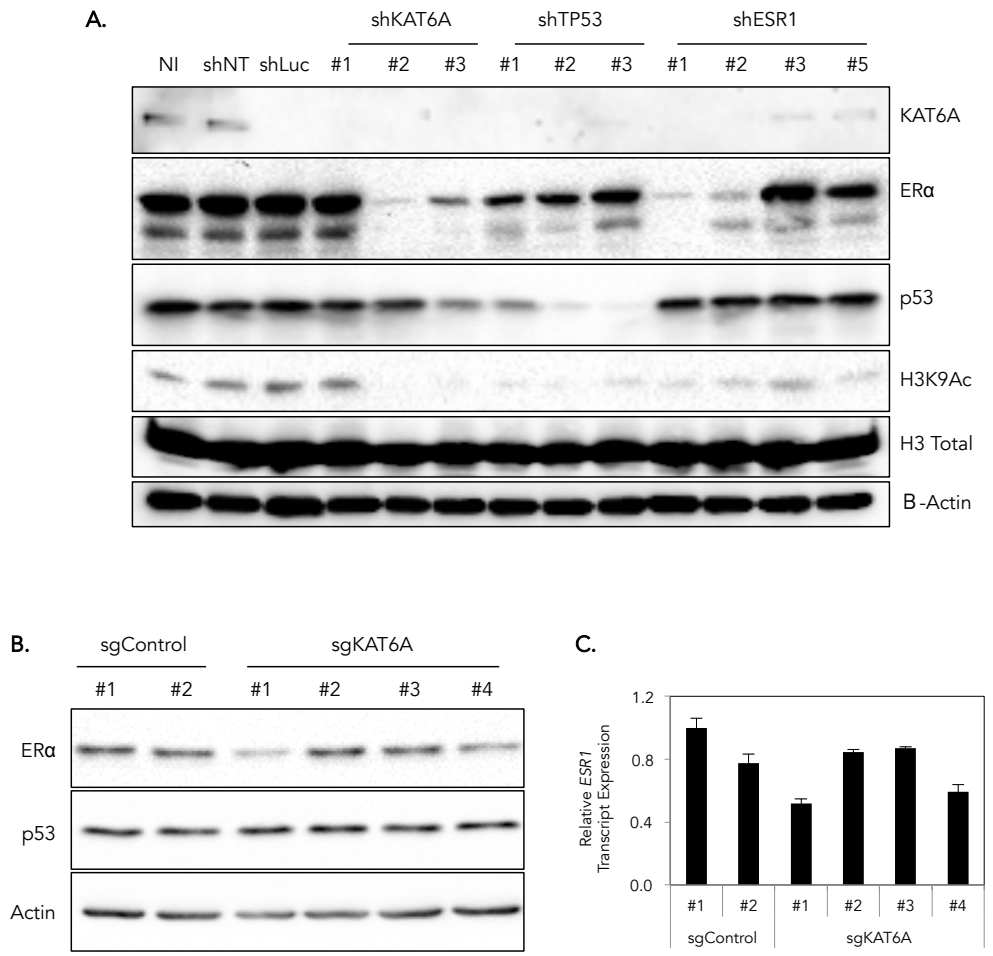


Figure 8. KAT6A suppression by shRNA or sgRNA results in decreased ERα levels.
A) T47D-KBLuc cells were transduced with non-targeting shRNA (shNT), shLuc or with shRNAs targeting *ESR1*, *KAT6A* or *TP53* (MOI 10). Following four days of knockdown, total protein was extracted. KAT6A, ERα, TP53, H3K9Ac, total H3 and β-actin protein expression was assessed by Western analysis B) T47D-KBLuc cells were transduced with control sgRNA, or one of four unique sgRNAs targeting *KAT6A*. Cells were selected with puromycin to retain the transduced population. Total protein was extracted and protein expression of ERα, TP53 and β–actin was assessed by Western analysis C) Total RNA was extracted from the selected sgRNA-transduced populations. *ESR1* transcript levels were assessed by qRT-PCR and normalized to housekeeping genes *RPLP0*, *YWHAZ* and *TBP*.

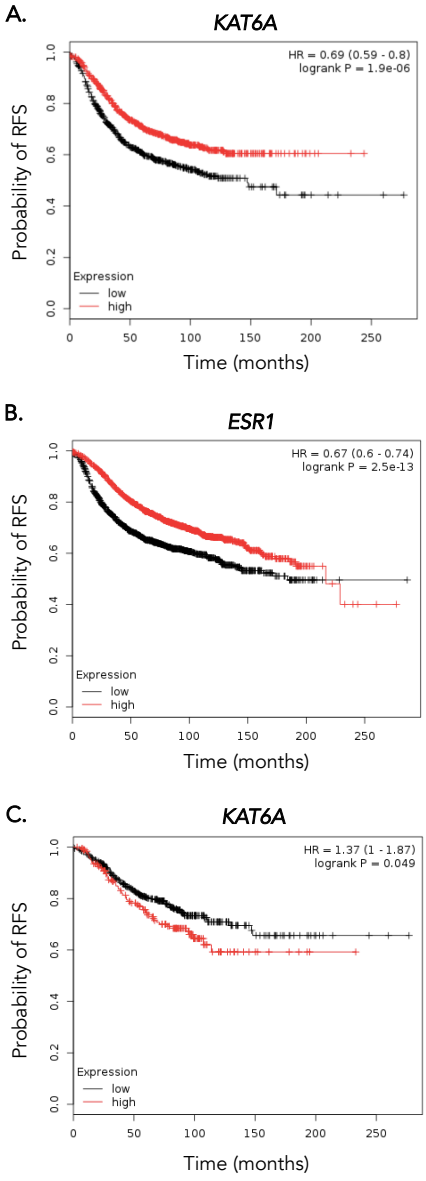


Figure 9. Increased expression of *KAT6A* or *ESR1* in human breast tumours predicts recurrence free survival. Kaplan Meier plots were generated using the KM plotter software <kmplot.com> (Győrffy et al. 2009). Breast cancer patient data was stratified based on A) *KAT6A* (1764 patients) or B) *ESR1* (3951 patients) expression as compared to the median. Patients with high expression of *KAT6A* or *ESR1* had a significantly better probability of survival ($p=1.9^{-6}$ and 2.5^{-13}, respectively) than those with low expression of either gene. C) On the other hand, in an ER-positive tumour sub-cohort, patients with high expression of *KAT6A* had a significantly worse probability of recurrence-free survival (p=0.049) than those with lower expression.

Table S1. Top 100 canonical signalling pathways significantly enriched in the gene set identified by 2-3 shRNAs with impact on luciferase reporter activity in T47D-KBluc cells

Canonical Pathways	p-value
G-Protein Coupled Receptor Signaling	1.58E-19
Inositol Phosphate Metabolism	3.98E-11
Estrogen Receptor Signaling	1.62E-09
CREB Signaling in Neurons	1.82E-08
Cardiac Hypertrophy Signaling	2.63E-08
cAMP-mediated signaling	3.31E-08
Glucocorticoid Receptor Signaling	3.80E-08
CCR3 Signaling in Eosinophils	6.17E-08
IL-8 Signaling	1.23E-07
CXCR4 Signaling	2.40E-07
Huntington's Disease Signaling	3.09E-07
Role of NFAT in Cardiac Hypertrophy	3.24E-07
IL-12 Signaling and Production in Macrophages	3.24E-07
Protein Kinase A Signaling	3.39E-07
Endothelin-1 Signaling	4.17E-07
Thrombin Signaling	4.47E-07
Axonal Guidance Signaling	6.46E-07
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	9.12E-07
Molecular Mechanisms of Cancer	9.55E-07
EIF2 Signaling	1.66E-06
Androgen Signaling	2.45E-06
Atherosclerosis Signaling	2.69E-06
LPS-stimulated MAPK Signaling	2.75E-06
Ephrin Receptor Signaling	2.82E-06
Regulation of eIF4 and p70S6K Signaling	3.47E-06
Synaptic Long Term Depression	4.17E-06
Clathrin-mediated Endocytosis Signaling	5.13E-06
mTOR Signaling	5.75E-06
Phospholipase C Signaling	6.76E-06
GNRH Signaling	1.02E-05
Cholecystokinin/Gastrin-mediated Signaling	1.02E-05
HIF1 α Signaling	1.17E-05
Nicotinate and Nicotinamide Metabolism	1.29E-05
RAR Activation	1.35E-05
Synaptic Long Term Potentiation	1.58E-05
NF κ B Signaling	2.04E-05
Neuropathic Pain Signaling In Dorsal Horn Neurons	2.14E-05
Fc Epsilon RI Signaling	3.02E-05
P2Y Purigenic Receptor Signaling Pathway	3.02E-05
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	3.09E-05
NRF2-mediated Oxidative Stress Response	3.39E-05
Dendritic Cell Maturation	3.39E-05
Bladder Cancer Signaling	3.63E-05
B Cell Receptor Signaling	3.63E-05
IL-17 Signaling	4.17E-05
fMLP Signaling in Neutrophils	4.37E-05
Corticotropin Releasing Hormone Signaling	4.57E-05
Assembly of RNA Polymerase II Complex	5.37E-05
Relaxin Signaling	5.50E-05
Natural Killer Cell Signaling	7.76E-05

Canonical Pathways	p-value
Signaling by Rho Family GTPases	8.13E-05
SAPK/JNK Signaling	8.13E-05
ERK/MAPK Signaling	8.51E-05
FGF Signaling	8.71E-05
Colorectal Cancer Metastasis Signaling	8.91E-05
Integrin Signaling	1.00E-04
FLT3 Signaling in Hematopoietic Progenitor Cells	1.07E-04
HMGB1 Signaling	1.32E-04
Germ Cell-Sertoli Cell Junction Signaling	1.32E-04
Breast Cancer Regulation by Stathmin1	1.41E-04
G α 12/13 Signaling	1.48E-04
Protein Ubiquitination Pathway	1.51E-04
Glioblastoma Multiforme Signaling	1.74E-04
NGF Signaling	1.95E-04
p38 MAPK Signaling	1.95E-04
RANK Signaling in Osteoclasts	2.00E-04
Nucleotide Excision Repair Pathway	2.09E-04
Renin-Angiotensin Signaling	2.29E-04
Role of NFAT in Regulation of the Immune Response	2.40E-04
Role of MAPK Signaling in the Pathogenesis of Influenza	2.45E-04
PI3K Signaling in B Lymphocytes	2.51E-04
Sphingosine-1-phosphate Signaling	2.63E-04
Xenobiotic Metabolism Signaling	2.82E-04
Role of IL-17A in Arthritis	3.24E-04
Hereditary Breast Cancer Signaling	3.47E-04
HGF Signaling	3.47E-04
Melatonin Signaling	4.07E-04
Leptin Signaling in Obesity	4.27E-04
Calcium Signaling	4.37E-04
Chronic Myeloid Leukemia Signaling	4.57E-04
Pancreatic Adenocarcinoma Signaling	4.57E-04
CD28 Signaling in T Helper Cells	5.13E-04
Dopamine-DARPP32 Feedback in cAMP Signaling	5.25E-04
Role of Tissue Factor in Cancer	5.25E-04
NF κ B Activation by Viruses	5.50E-04
Growth Hormone Signaling	6.31E-04
Gap Junction Signaling	6.76E-04
eNOS Signaling	7.24E-04
Leukocyte Extravasation Signaling	7.94E-04
Aldosterone Signaling in Epithelial Cells	8.13E-04
Glioma Signaling	8.13E-04
TGF- β Signaling	9.33E-04
LXR/RXR Activation	9.33E-04
p53 Signaling	9.55E-04
PTEN Signaling	1.15E-03
Cardiac β -adrenergic Signaling	1.20E-03
JAK/Stat Signaling	1.38E-03
Acute Myeloid Leukemia Signaling	1.45E-03
Melanocyte Development and Pigmentation Signaling	1.45E-03
Prostate Cancer Signaling	1.70E-03

Data was generated using Ingenuity Pathway Analysis (IPA) from Ingenuity Biosystems (Qiagen)

Table S2. Genes significantly affecting T47D-KBLuc cell viability.

Predicted Regulation	NM_ID	GENE	Name	% Inhibition of Cell Viability		
				Clone A	Clone B	Clone C
Positive Regulators	NM_178827	*FLJ35834*	hypothetical protein FLJ35834	-37.94	-28.64	-25.91
	NM_024557	*RIC3*	resistance to inhibitors of cholinesterase 3 homolog (C. elegans)	-45.08	-33.36	-13.15
	NM_021958	*HLX1*	H2.0-like homeobox 1 (Drosophila)	-47.46	-26.21	-17.81
	NM_001064	*TKT*	transketolase (Wernicke-Korsakoff syndrome)	-26.6	-38.6	-18.89
	XM_499454			-20.75	-29.83	-26.79
	NM_000041	*APOE*	apolipoprotein E	-25.42	-29.55	-18.63
	XM_059399	*CIB4*	calcium and integrin binding family member 4	-14.51	-32.23	-25.96
	NM_139174	*LOC161931*	testis nuclear RNA-binding protein-like	-27.36	-14.6	-27
	NM_030876	*OR5V1*	olfactory receptor, family 5, subfamily V, member 1	-30.65	-28.1	-8.85
	NM_152272	*CHMP7*	CHMP family, member 7	-26.3	-26.71	-6.03
	NM_001795	*CDH5*	cadherin 5, type 2, VE-cadherin (vascular epithelium)	-25.05	-39.61	23.13
	NM_021235	*EPS15L1*	epidermal growth factor receptor pathway substrate 15-like 1	-27.16	-25.67	14.02
	NM_198452	*PNCK*	pregnancy upregulated non-ubiquitously expressed CaM kinase	-27.67	18.62	-25.16
Negative Regulators	NM_005622	*ACSM3*	acyl-CoA synthetase medium-chain family member 3	36.75	-18.43	25.74
	NM_198285	*LOC349136*	hypothetical protein LOC349136	27.63	29.24	-5.44
	NM_003403	*YY1*	YY1 transcription factor	25.99	-5.76	31.24
	NM_002377	*MAS1*	MAS1 oncogene	-4.95	28.8	28.56
	NM_207420	*SLC2A7*	solute carrier family 2 (facilitated glucose transporter), member 7	-3.78	32.37	25.37
	NM_014801	*PCNXL2*	pecanex-like 2 (Drosophila)	26.79	28.85	2.46
	XM_380021			-2.28	36.4	26.46
	NM_002880	*RAF1*	v-raf-1 murine leukemia viral oncogene homolog 1	26.65	26.83	8.82
	NM_001826	*CKS1B*	CDC28 protein kinase regulatory subunit 1B	30.61	4.98	28.83
	NM_001907	*CTRL*	chymotrypsin-like	25.88	30.49	8.43
	NM_005348	*HSP90AA1*	heat shock protein 90kDa alpha (cytosolic), class A member 1	35.65	25.43	4.32
	NM_006328	*RBM14*	RNA binding motif protein 14	25.65	33.87	8.97
	NM_203494	*USP50*	ubiquitin specific peptidase 50	6.99	35.65	25.99
	NM_006494	*ERF*	Ets2 repressor factor	7.91	32.43	28.61
	NM_001011	*RPS7*	ribosomal protein S7	11.21	33.38	25.78
	NM_002067	*GNA11*	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	31.53	12.97	27.67
	NM_017444	*CHRAC1*	chromatin accessibility complex 1	29.83	11.01	32.03
	NM_152227	*SNX5*	sorting nexin 5	27.1	38.07	8.67
	NM_178174	*TREML1*	triggering receptor expressed on myeloid cells-like 1	12.47	31.94	29.89
	NM_015202	*KIAA0556*	KIAA0556 protein	40.92	26.96	6.82
	NM_012189	*CABYR*	calcium binding tyrosine-(Y)-phosphorylation regulated (fibrousheathi	37.33	25.47	12.55
	NM_016632	*ARL17P1*	ADP-ribosylation factor-like 17 pseudogene 1	17.42	26.01	32.71
	NM_023921	*TAS2R10*	taste receptor, type 2, member 10	10.53	26.65	39.5
	NM_002445	*MSR1*	macrophage scavenger receptor 1	21.21	29.59	25.92
	NM_144640	*IL17RE*	interleukin 17 receptor E	37.53	5.01	34.5
	NM_005663	*WHSC2*	Wolf-Hirschhorn syndrome candidate 2	34.64	1.59	43.01
	NM_004890	*SPAG7*	sperm associated antigen 7	22	27.94	29.47
	NM_003126	*SPTA1*	spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	37.95	6.53	37.46
	NM_002630	*PGC*	progastricsin (pepsinogen C)	28.18	32.18	23.53
	NM_007176	*C14orf1*	chromosome 14 open reading frame 1	44.32	25.67	14.86
	XM_377949	*LOC402282*	similar to RAB guanine nucleotide exchange factor (GEF) 1	56.49	4.16	25.48
	NM_014244	*ADAMTS2*	ADAM metallopeptidase with thrombospondin type 1 motif, 2	42.52	29.27	14.74
	NM_018918	*PCDHGA5*	protocadherin gamma subfamily A, 5	38.17	15.67	36.85
	NM_006082	*K-ALPHA-1*	alpha tubulin	13.59	43.79	36.52
	NM_006384	*CIB1*	calcium and integrin binding 1 (calmyrin)	36.5	27.04	30.55
	NM_130465	*TSPAN17*	tetraspanin 17	25.11	56.57	14.48
	NM_000661	*RPL9*	ribosomal protein L9	30.21	34.92	31.14
	NM_199000	*LHFPL3*	lipoma HMGIC fusion partner-like 3	30.78	44.2	31.43
	NM_015371	*HS322B1A*	glutathione S-transferase theta pseudogene	36.48	34.43	40.77
	NM_013248	*NXT1*	NTF2-like export factor 1	57.14	51.33	10.21
	NM_022047	*DEF6*	differentially expressed in FDCP 6 homolog (mouse)	49.64	23.66	46.63
	NM_207308	*NUP210L*	nucleoporin 210kDa-like	61.25	40.38	28.23
	NM_002429	*MMP19*	matrix metallopeptidase 19	49.91	45.42	44.2
	NM_182485	*CPEB2*	cytoplasmic polyadenylation element binding protein 2	43.12	44.89	60.06
	NM_153757	*NAP1L5*	nucleosome assembly protein 1-like 5	67.81	46.57	37.75
	NM_020717	*RP11-119E20.1*	KIAA1202 protein	58.73	49.08	44.84
	NM_138493	*C6orf129*	chromosome 6 open reading frame 129	64.53	58.84	31.46
	NM_015715	*PLA2G3*	phospholipase A2, group III	61.65	47.86	51.85
	XM_496642	*USP19*	ubiquitin specific peptidase 19	59.05	54.64	56.97

Genes listed show a significant regulation of T47D-KBLuc cell viability (>25 or <-25% inhibition of alamarBlue fluorescence) following knockdown with two or three independent shRNAs and are sorted by increasing average effect.
Predicted positive regulators: Genes whose knockdown represses T47D-KBLuc cell viability
Predicted negative regulators: Genes whose knockdown increases T47D-KBLuc cell proliferation

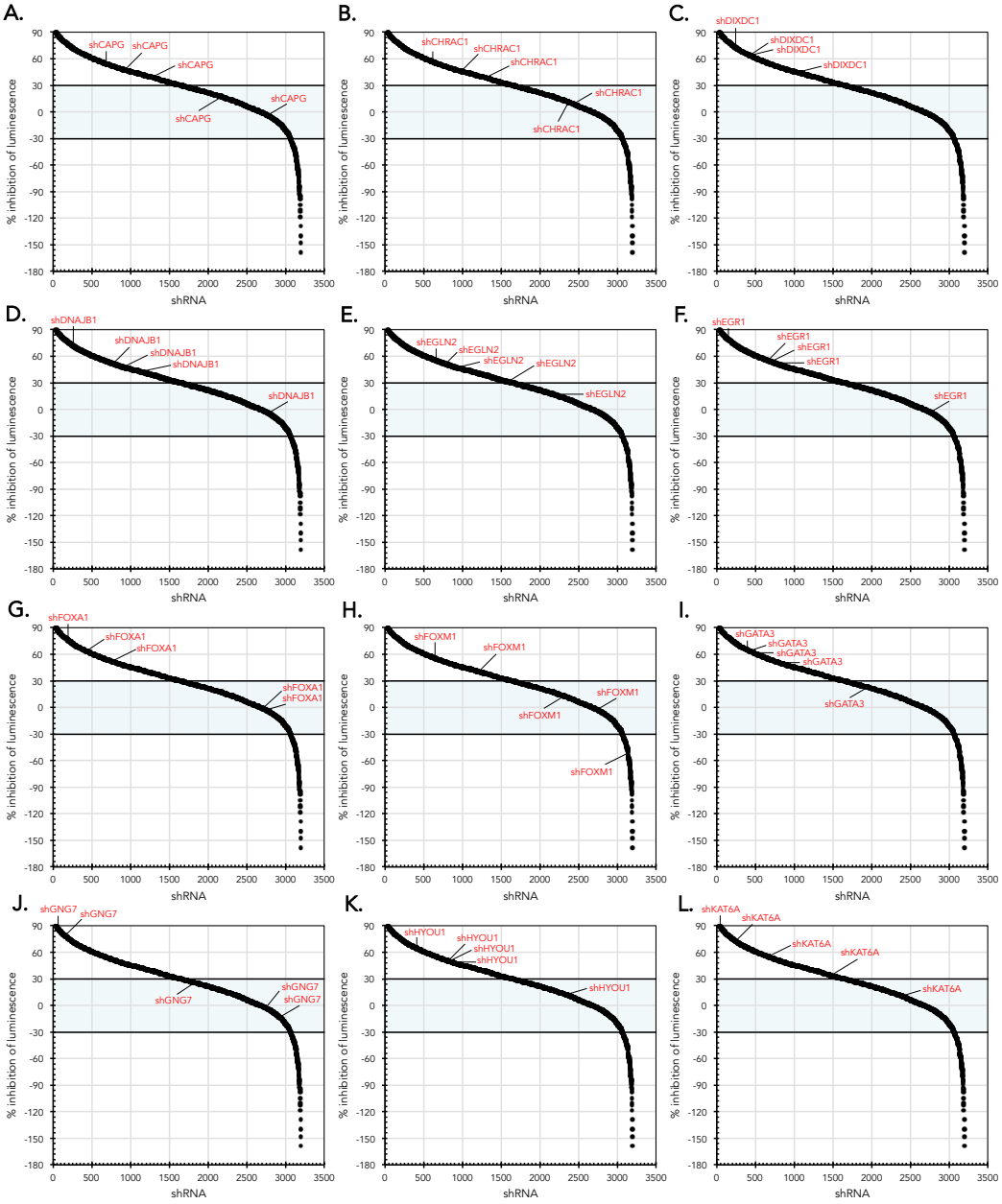


Figure S1. Distribution of luciferase data for select hits from secondary screening.

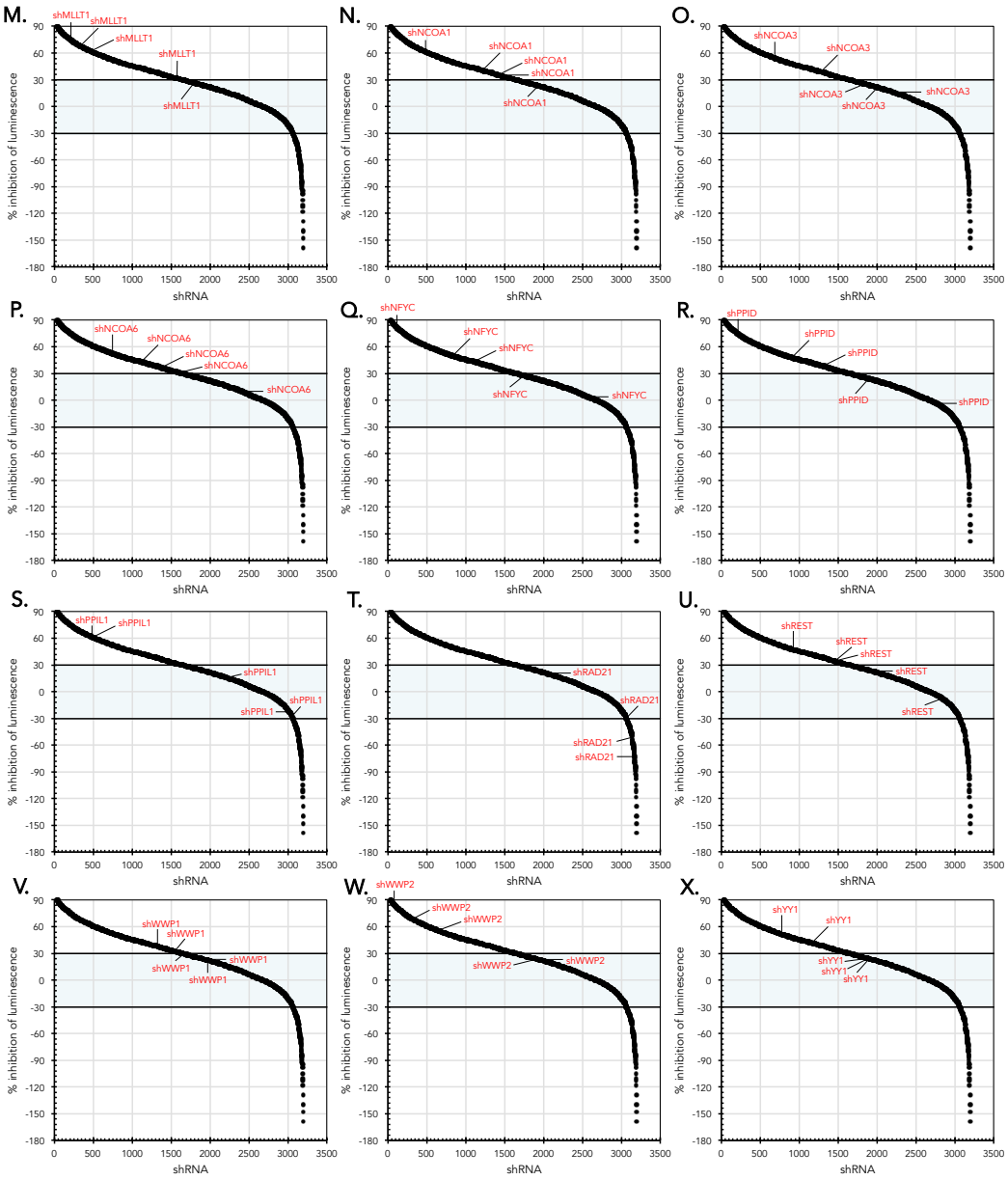


Figure S1. Distribution of luciferase data for select hits from secondary screening.
T47D-KBLuc cells were transduced with shRNA (MOI 10) in the presence of E2. ER-signalling (ERE-luciferase reporter activity) was assessed following four days of knockdown/E2 stimulation. Percent inhibition of luminescence for each of 3,200 shRNAs used in the secondary screen (614 genes, 5+ shRNA per gene) is shown. Labelled data points represent shRNAs targeted against A) *CAPG*, B) *CHRAC1*, C) *DIXDC1*, D) *DNAJB1*, E) *EGLN2*, F) *EGR1*, G) *FOXA1*, H) *FOXM1*, I) *GATA3*, J) *GNG7*, K) *HYOU1*, L) *KAT6A*, M) *MLLT1*, N) *NCOA1*, O) *NCOA3*, P) *NCOA6*, Q) *NFYC*, R) *PPID*, S) *PPIL1*, T) *RAD21*, U) *REST*, V) *WWP1*, W) *WWP2* and X) *YY1*. The cut-off for hit designation (>30%, <-30%) is demarcated in blue.

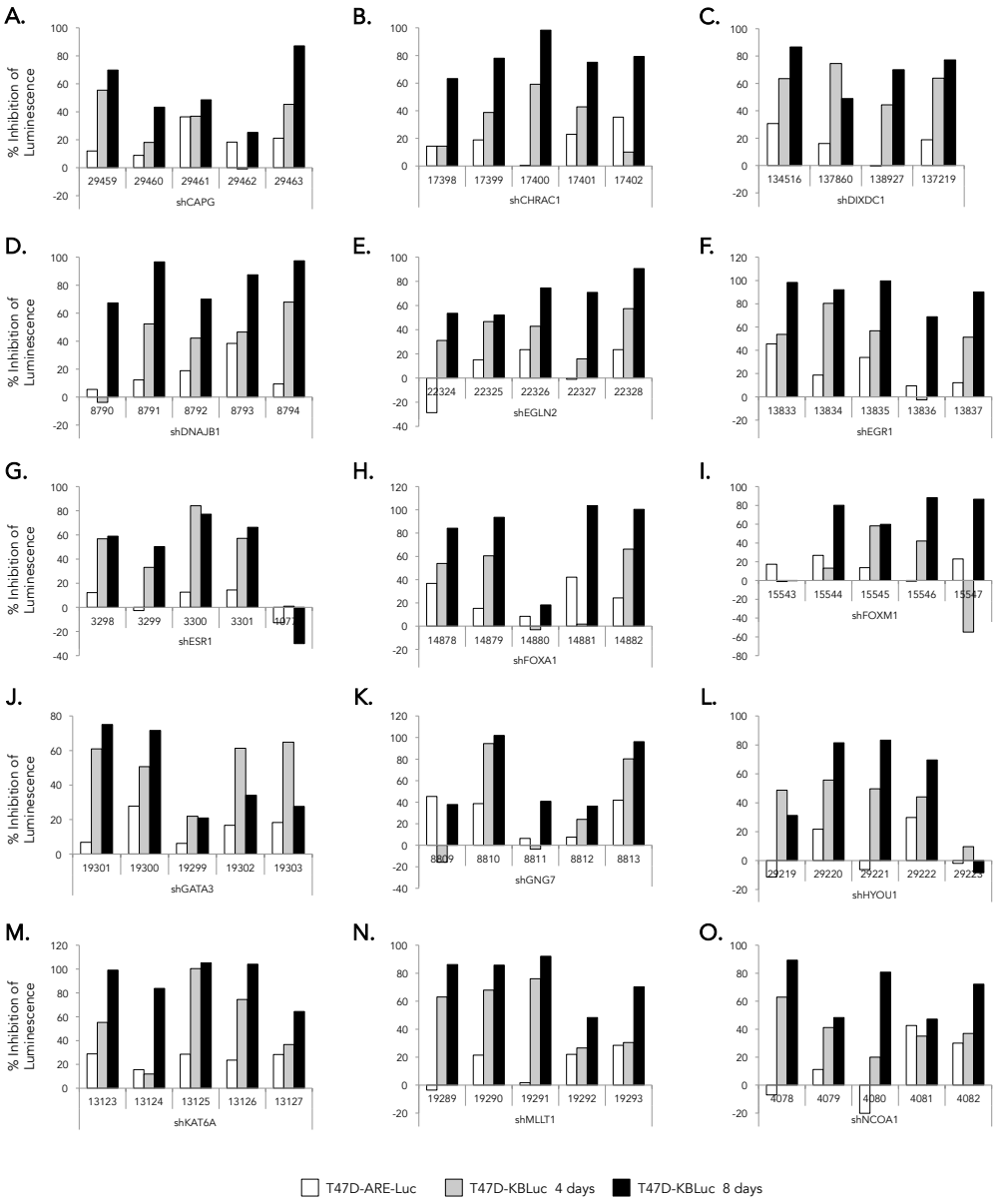


Figure S2. Differential impact of 'hit' shRNA on ARE and ERE luciferase reporter activity

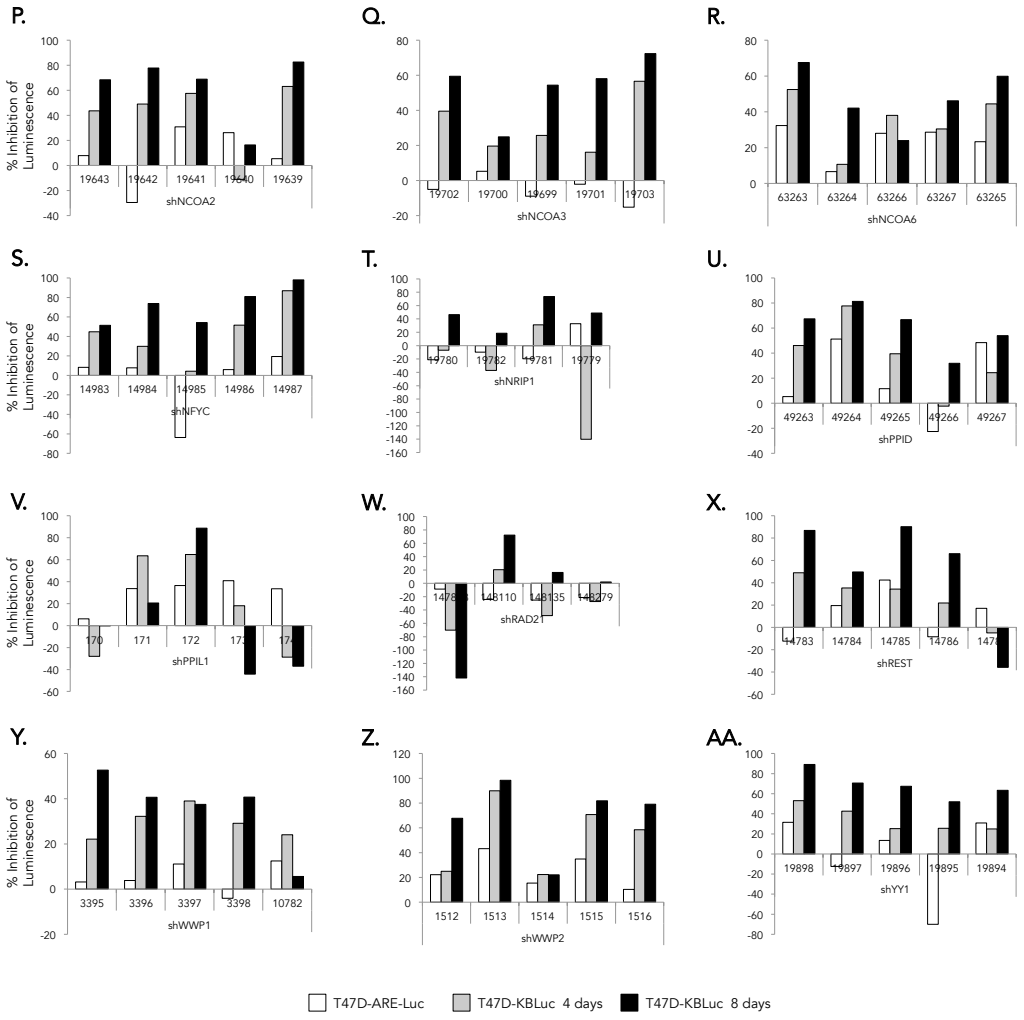


Figure S2. Differential impact of 'hit' shRNA on ARE and ERE luciferase reporter activity
T47D-ARE-Luc and T47D-KBLuc cells were transduced in 96 well plates with lentiviruses (MOI 10) targeting A) *CAPG*, B) *CHRAC1*, C) *DIXDC1*, D) *DNAJB1*, E) *EGLN2*, F) *EGR1*, G) *ESR1*, H) *FOXA1*, I) *FOXM1*, J) *GATA3*, K) *GNG7*, L) *HYOU1*, M) *KAT6A*, N) *MLLT1*, O) *NCOA1*, P) *NCOA2*, Q) *NCOA3*, R) *NCOA6*, S) *NFYC*, T) *NRIP1*, U) *PPID*, V) *PPIL1*, W) *RAD21*, X) *REST*, Y) *WWP1*, Z) *WWP2* and AA) *YY1*, in the presence of NRF2 agonist DL-Sulforaphane (T47D-ARE-Luc; 10 nM, 16 hours) or E2 (T47D-KBLuc; 25 nM, 4 days). Luminescence was read following four days (white: T47D-ARE-Luc and grey: T47D-KBLuc 4 days) or eight days (black: T47D-KBLuc 8 days) of knockdown and is plotted as percent inhibition of luminescence as compared to non-treated control wells.

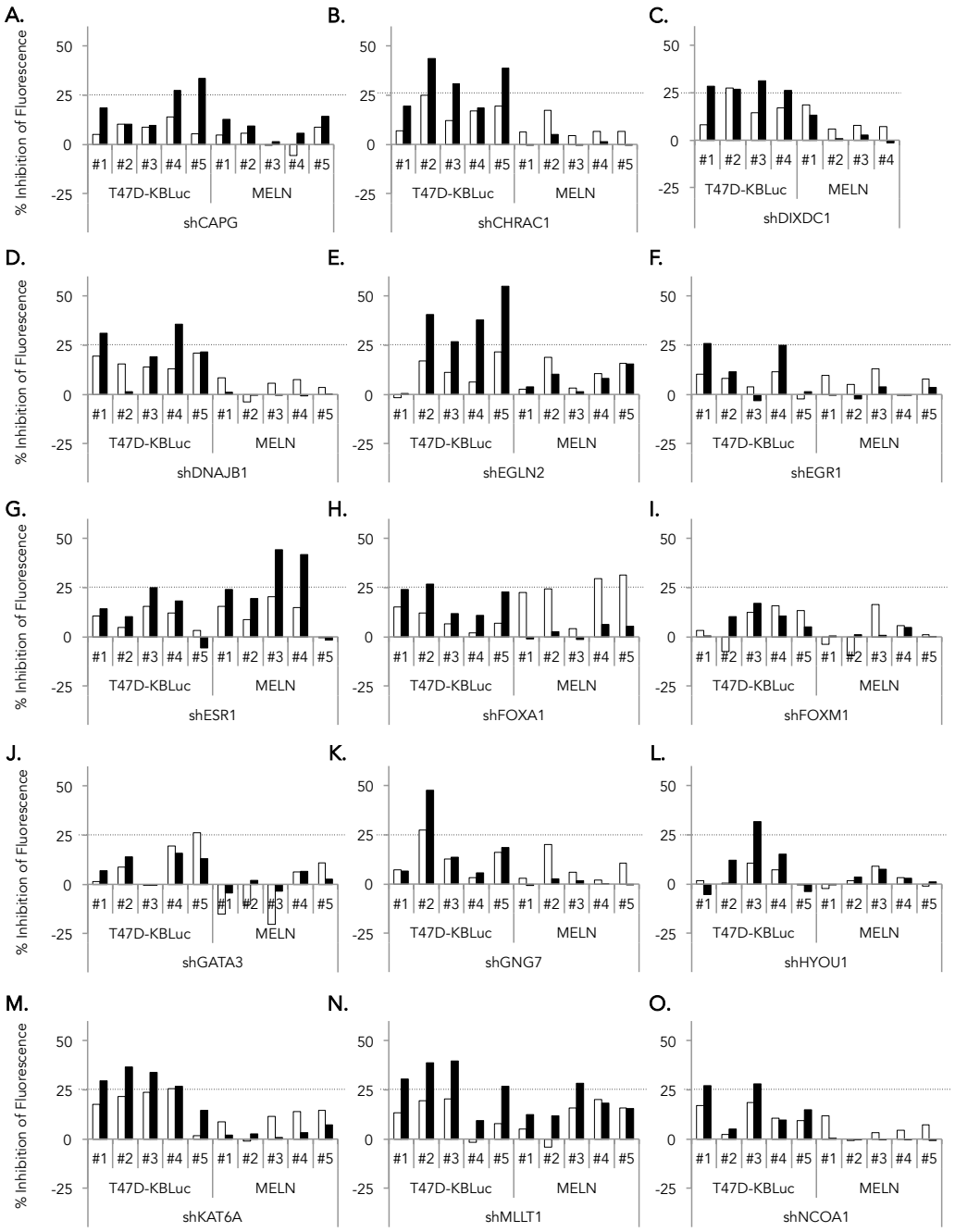


Figure S3. shRNA impact on T47D-KBLuc and MELN cell viability following four or eight days of knockdown.

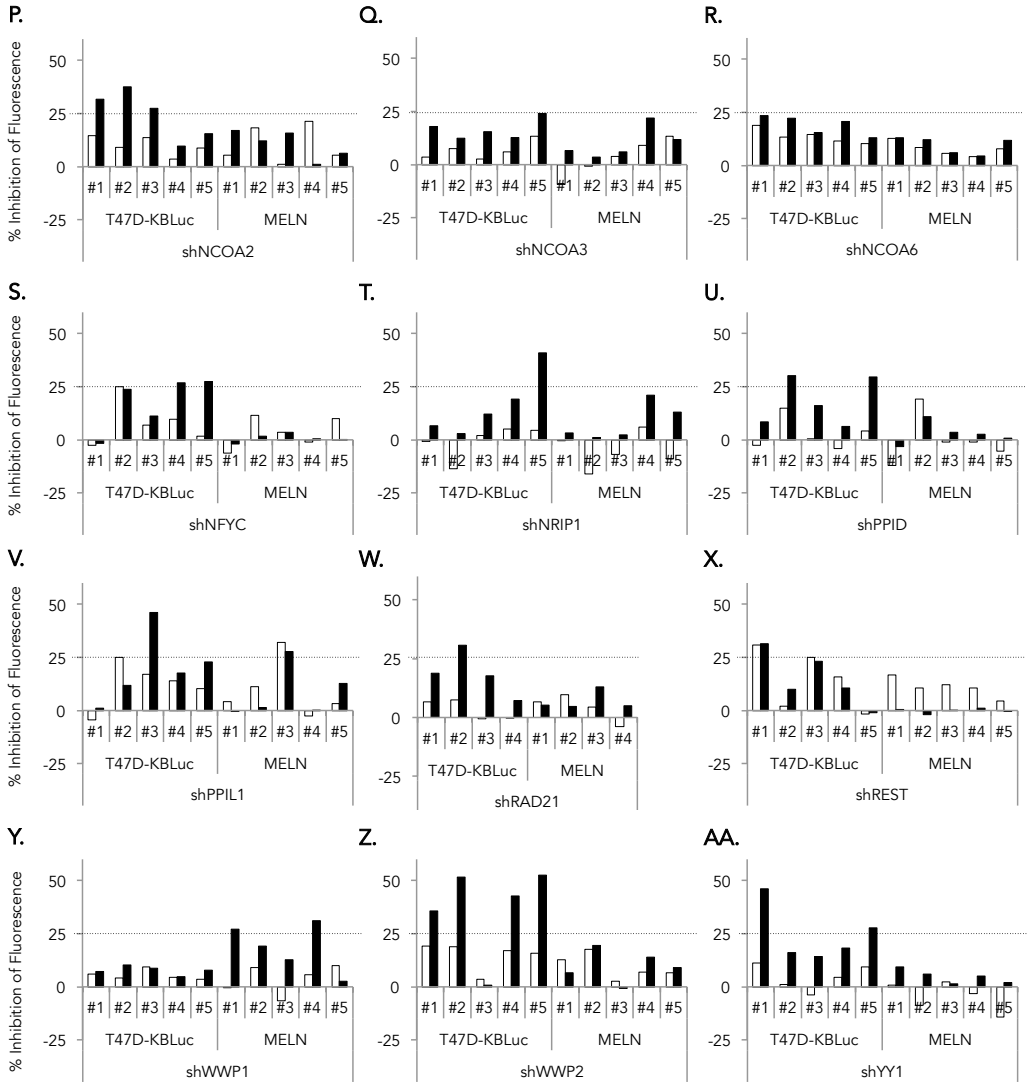


Figure S3. shRNA impact on T47D-KBLuc and MELN cell viability following four or eight days of knockdown.
T47D-KBLuc and MELN cells were transduced in 96 well plates with lentiviruses (MOI 10) targeting A) *CAPG*, B) *CHRAC1*, C) *DIXDC1*, D) *DNAJB1*, E) *EGLN2*, F) *EGR1*, G) *ESR1*, H) *FOXA1*, I) *FOXM1*, J) *GATA3*, K) *GNG7*, L) *HYOU1*, M) *KAT6A*, N) *MLLT1*, O) *NCOA1*, P) *NCOA2*, Q) *NCOA3*, R) *NCOA6*, S) *NFYC*, T) *NRIP1*, U) *PPID*, V) *PPIL1*, W) *RAD21*, X) *REST*, Y) *WWP1*, Z) *WWP2* and AA) *YY1*, in the presence of E2 (25 nM, 4 days). Cell viability was measured by alamarBlue assay following four days (white) or eight days (black) of knockdown and is plotted as percent inhibition of fluorescence as compared to non-treated control wells.

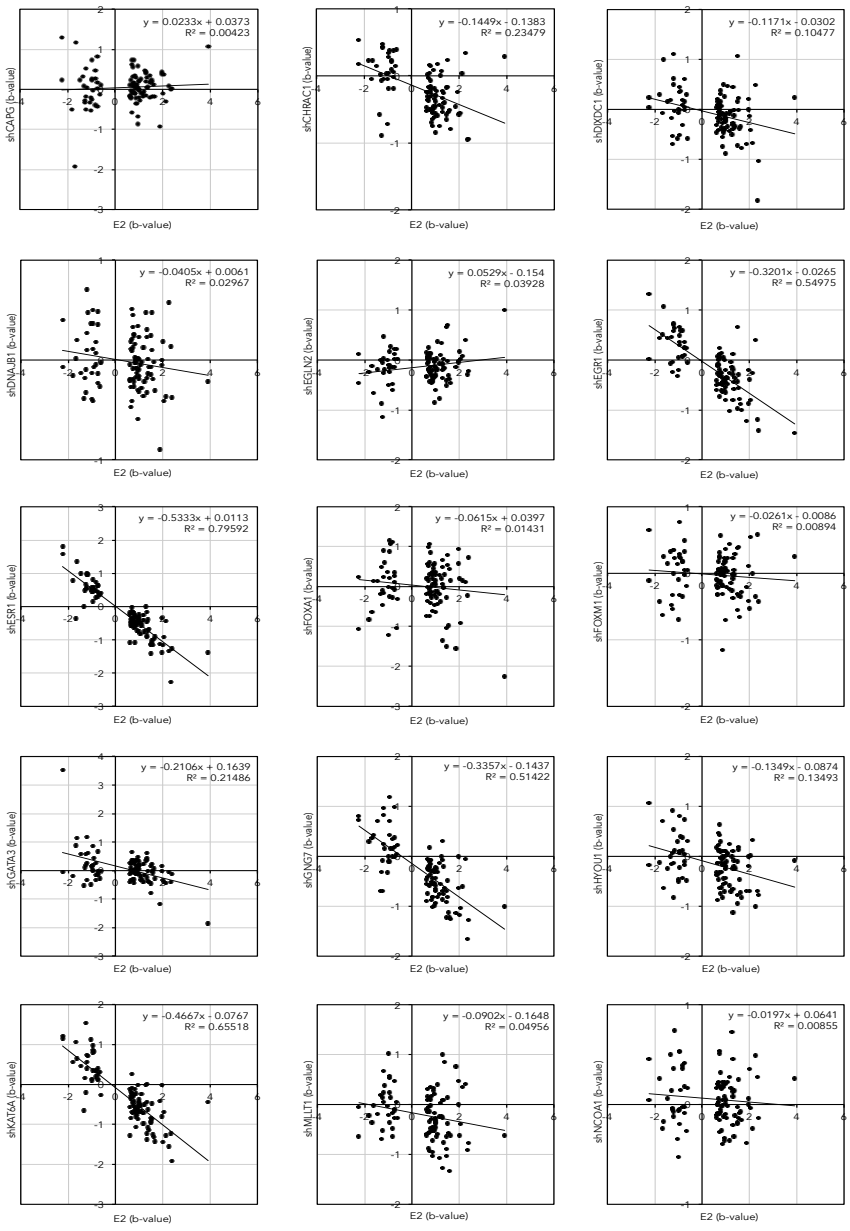


Figure S4. Direct E2 target gene expression following shRNA-mediated gene knockdown.

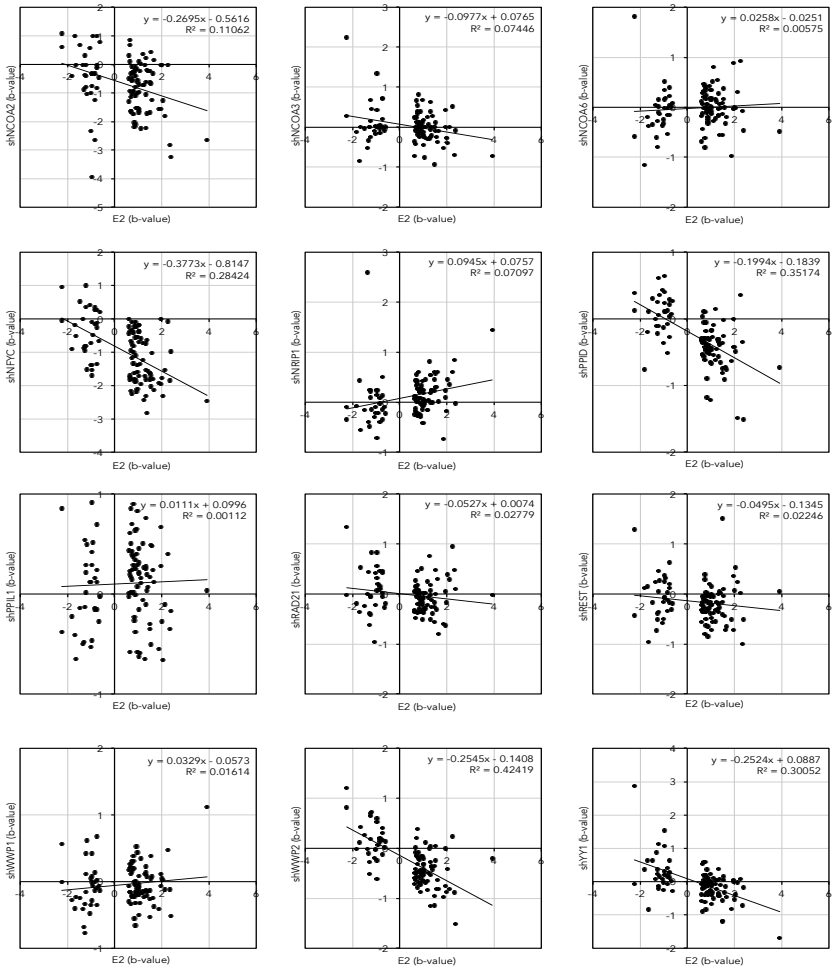


Figure S4. Direct E2 target gene expression following shRNA-mediated gene knockdown.
T47D-KBLuc cells were transduced with non-targeting shRNA (shNT), shGFP or shRNA targeting *CAPG*, *CHRAC1*, *DIXDC1*, *DNAJB1*, *EGLN2*, *EGR1*, *ESR1*, *FOXA1*, *FOXM1*, *GATA3*, *GNG7*, *HYOU1*, *KAT6A*, *MLLT1*, *NCOA1*, *NCOA2*, *NCOA3*, *NCOA6*, *NFYC*, *NRIP1*, *PPID*, *PPIL1*, *RAD21*, *REST*, *WWP1*, *WWP2 or YY1* (MOI 10), with two unique shRNAs per gene in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted and transcriptomes were sequenced for each sample. Direct ERα target gene expression (relative to non-infected cells, shNT and shGFP) is shown on the y-axis of each panel, plotted against gene expression following treatment with E2 (25 nM) for four days. The trendline for each plot is calculated by a simple linear regression and is displayed with the R^2 for each dataset.

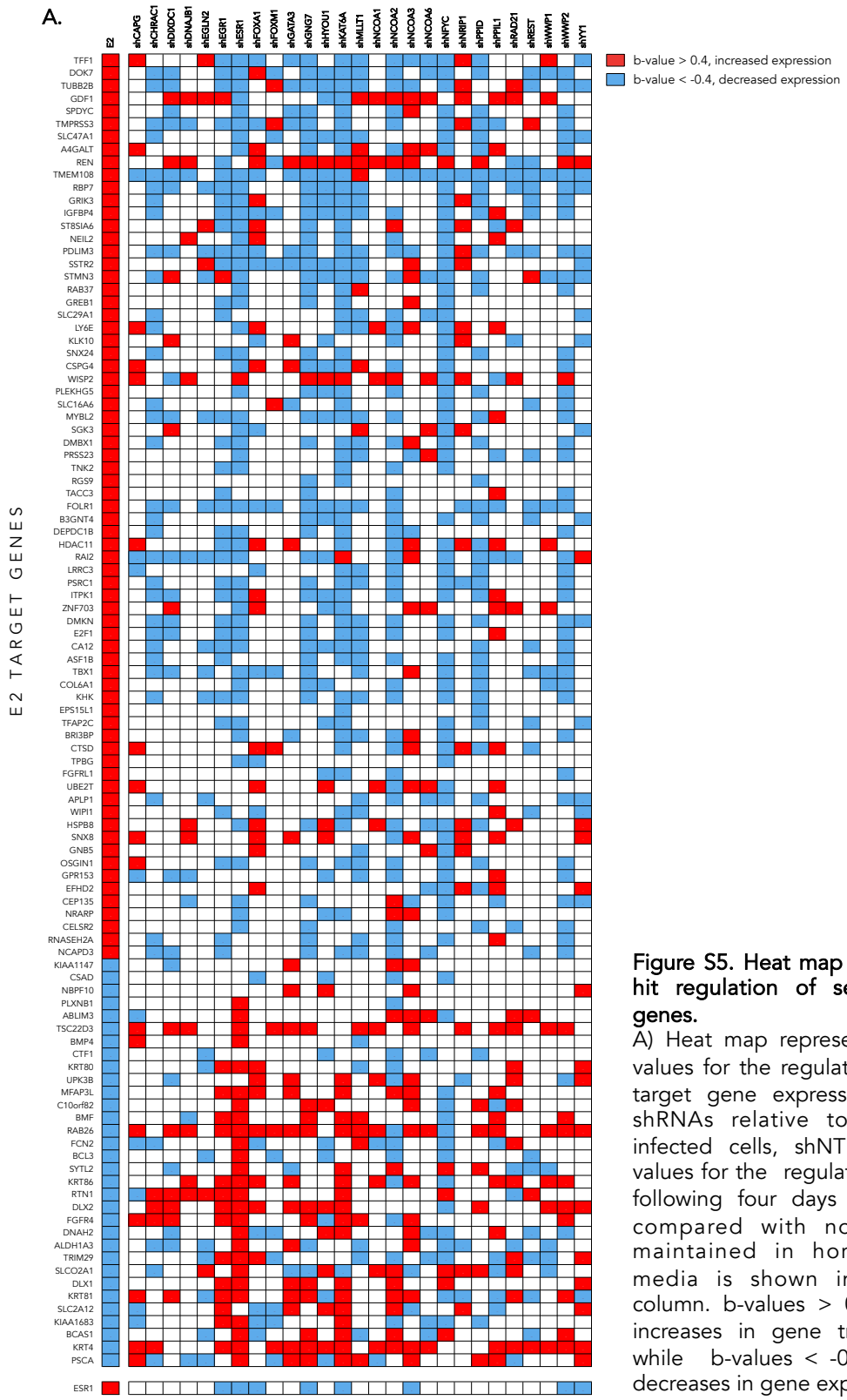


Figure S5. Heat map representation of hit regulation of select subsets of genes.

A) Heat map representation of the b values for the regulation of direct ERα target gene expression by targeting shRNAs relative to controls (non-infected cells, shNT and shGFP). B values for the regulation of expression following four days of E2 treatment compared with non-treated cells maintained in hormone-depleted media is shown in the left-hand column. b-values > 0.4 (red) indicate increases in gene transcript levels , while b-values < -0.4 (blue) indicate decreases in gene expression.

B.

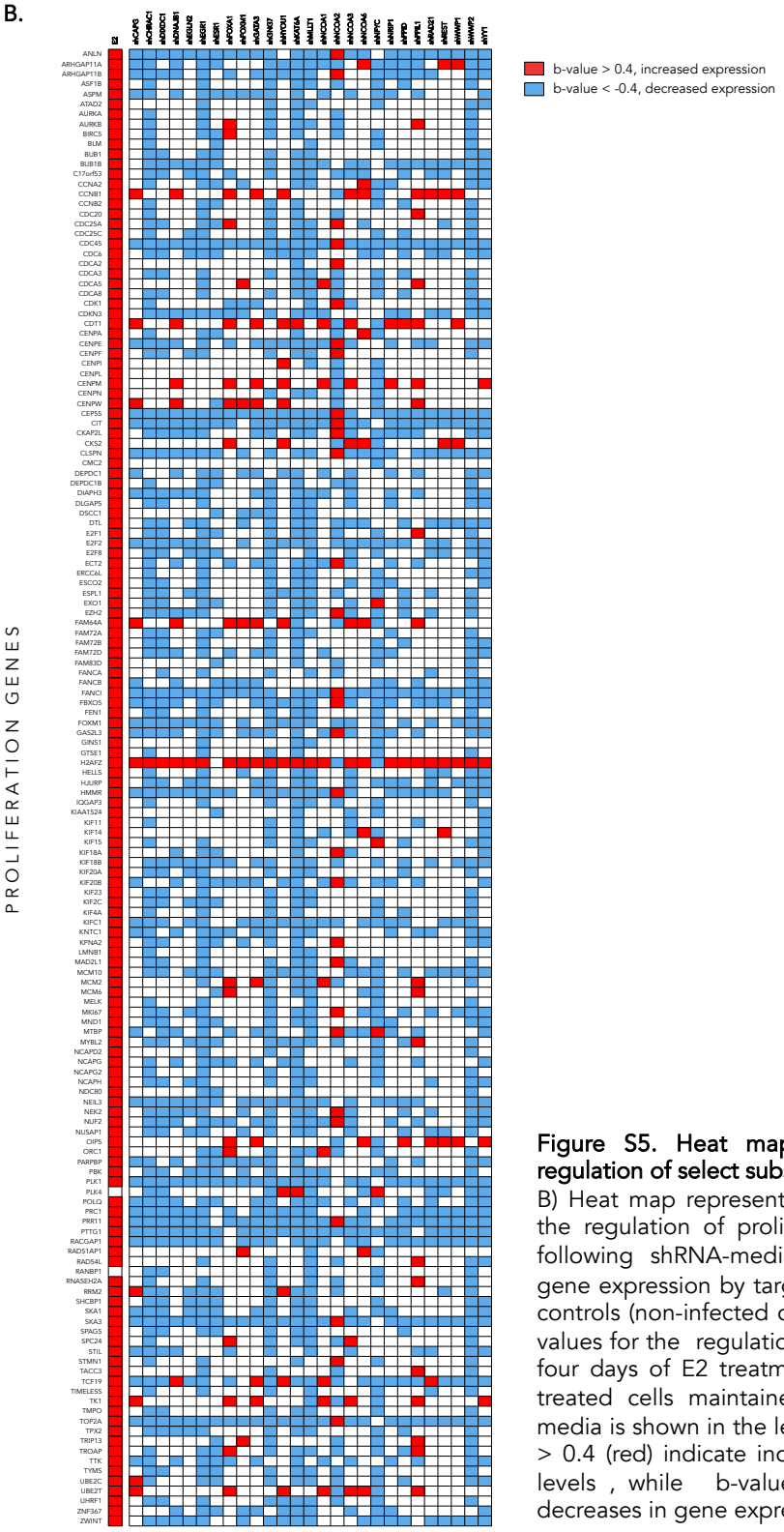


Figure S5. Heat map representation of hit regulation of select subsets of genes.

B) Heat map representation of the b values for the regulation of proliferative gene expression following shRNA-mediated suppression of hit gene expression by targeting shRNAs relative to controls (non-infected cells, shNT and shGFP). B values for the regulation of expression following four days of E2 treatment compared with non-treated cells maintained in hormone-depleted media is shown in the left-hand column. b-values > 0.4 (red) indicate increases in gene transcript levels , while b-values < -0.4 (blue) indicate decreases in gene expression.

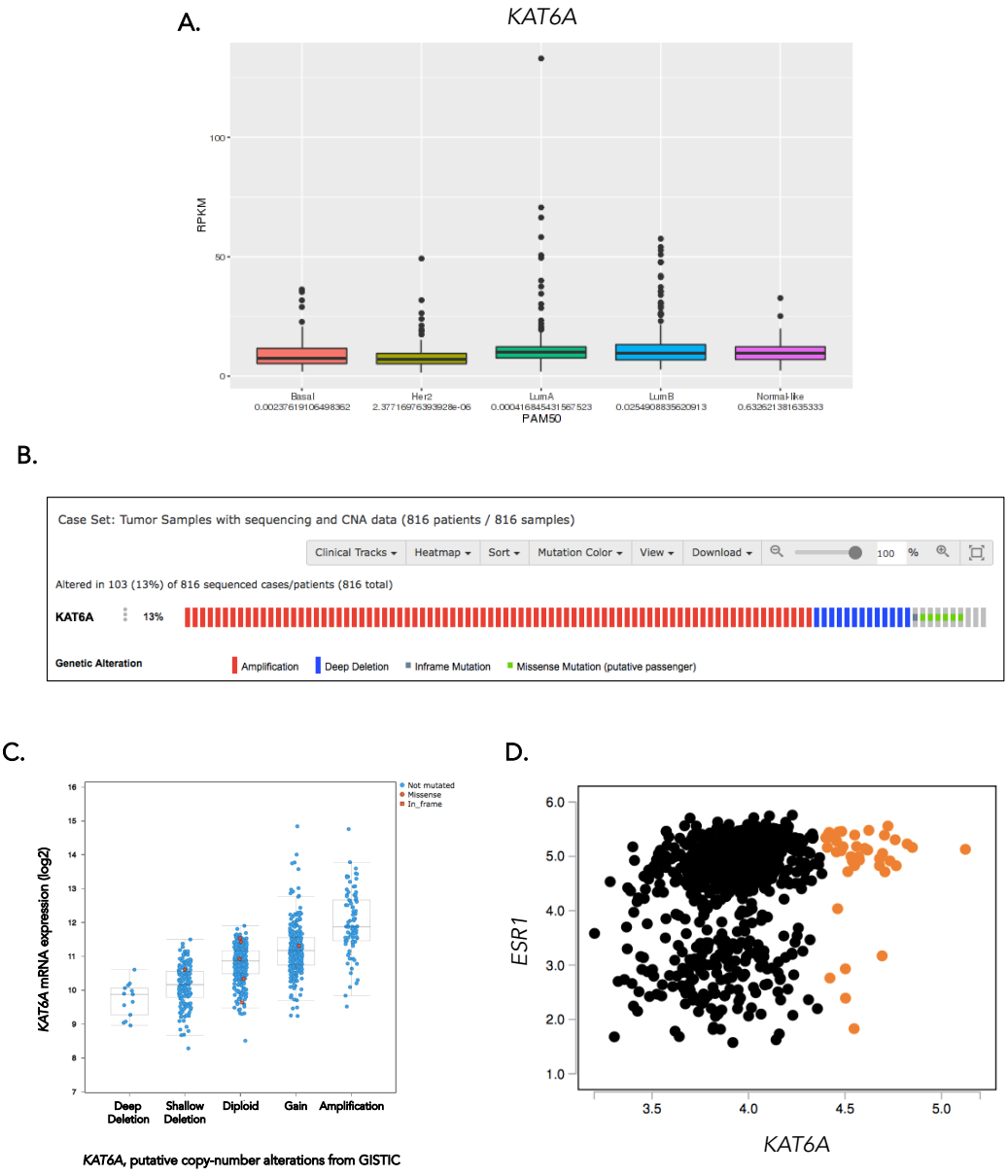


Figure S6. *KAT6A* is amplified and overexpressed in breast tumours

A) Box plot representation of *KAT6A* mRNA distribution in breast tumours (TCGA dataset). B) Genetic aberrations affecting *KAT6A* (TCGA dataset, cBioPortal). C) Correlation between predicted copy number and mRNA levels of *KAT6A* (TCGA dataset, cBioPortal). D) Tumours with highest levels of *KAT6A* (~5%, highlighted in orange) are mainly ER-positive and are enriched in luminal B tumours (odds ratio 4.8, $p=2.6^{-6}$).

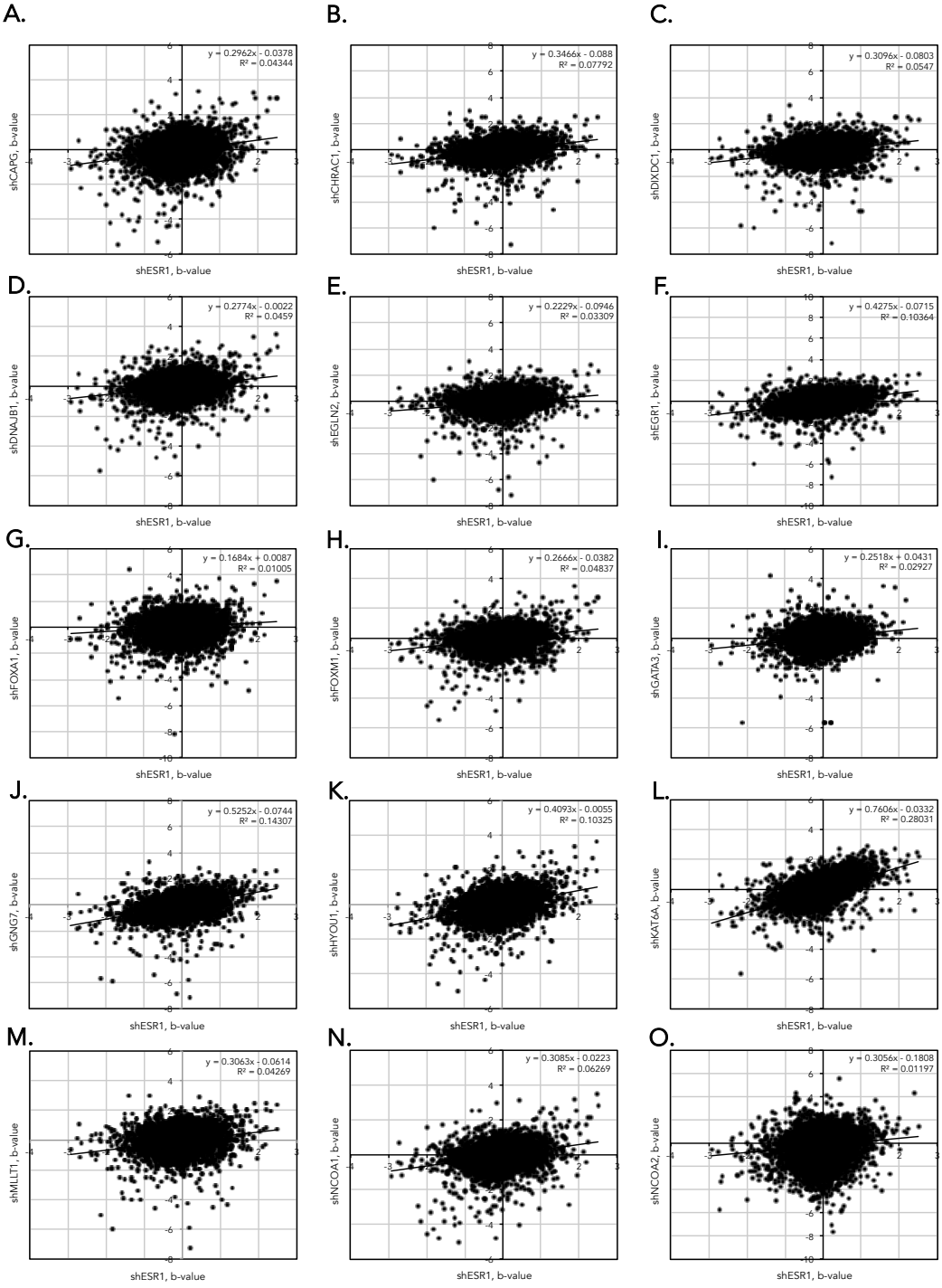


Figure S7. Impact of selected hit knockdown on the entire T47D-KBLuc cell transcriptome.

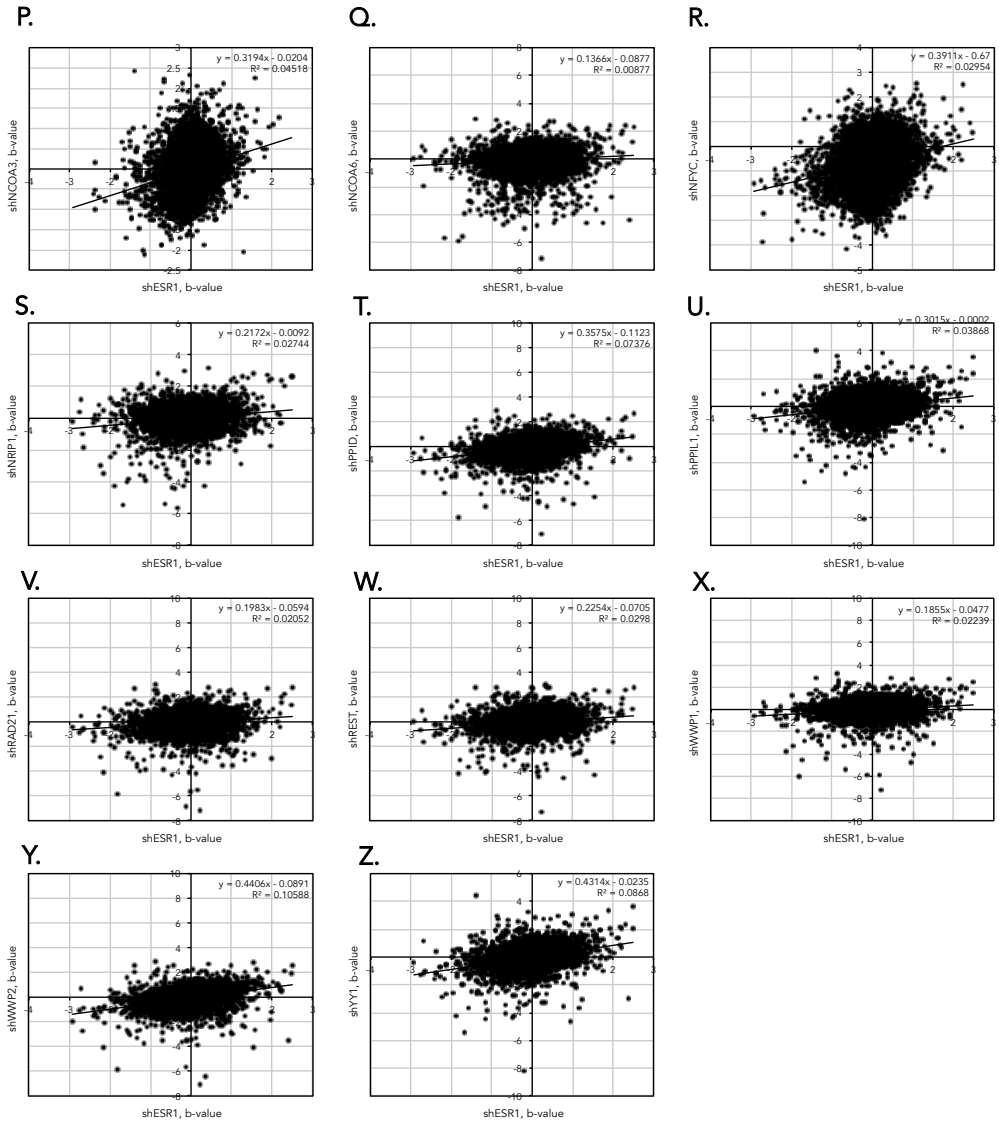


Figure S7. Impact of selected hit knockdown on the entire T47D-KBLuc cell transcriptome.
T47D-KBLuc cells were transduced with non-targeting shRNA (shNT), shGFP or shRNA targeting A) *CAPG*, B) *CHRAC1*, C) *DIXDC1*, D) *DNAJB1*, E) *EGLN2*, F) *EGR1*, G) *FOXA1*, H) *FOXM1*, I) *GATA3*, J) *GNG7*, K) *HYOU1*, L) *KAT6A*, M) *MLLT1*, N) *NCOA1*, O) *NCOA2*, P) *NCOA3*, Q) *NCOA6*, R) *NFYC*, S) *NRIP1*, T) *PPID*, U) *PPIL1*, V) *RAD21*, W) *REST*, X) *WWP1*, Y) *WWP2* or Z) *YY1* with two to three unique shRNAs per gene at MOI 10) in the presence of E2 (25 nM). Following four days of knockdown/E2 treatment, total RNA was extracted and transcriptomes were sequenced for each sample. Fold regulation by two shRNAs against each target (relative to non-infected cells, shNT and shGFP) is shown on the y-axis of each panel, plotted against gene expression following transduction of two unique shRNAs against ESR1. The trendline for each plot is calculated by a simple linear regression and is displayed with the R^2 for each dataset.

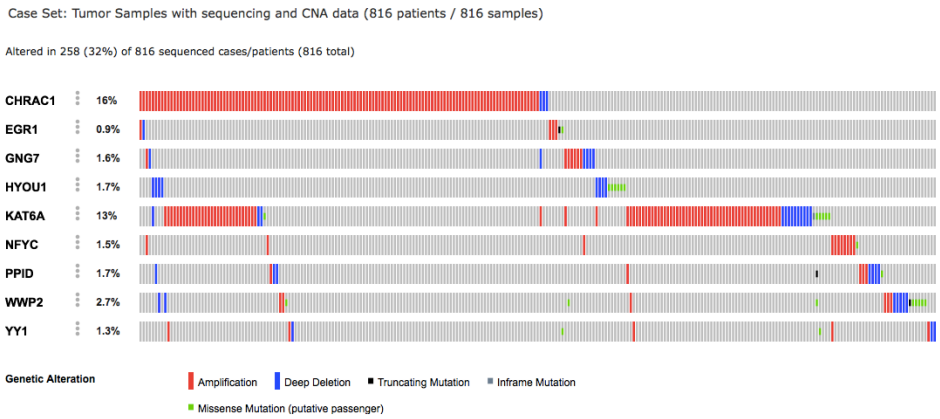


Figure S8. Genetic aberrations affecting candidate ERα regulators in breast tumours.
Genetic aberrations affecting candidate genes in the Cancer Genome Atlas breast cancer dataset are illustrated using the cBioportal web site (www.cbioportal.org). Tumours with amplifications are shown in red, those with deletions in blue and tumours with mutations in black, grey or green according to the nature of the mutation (truncating, in frame or missense).

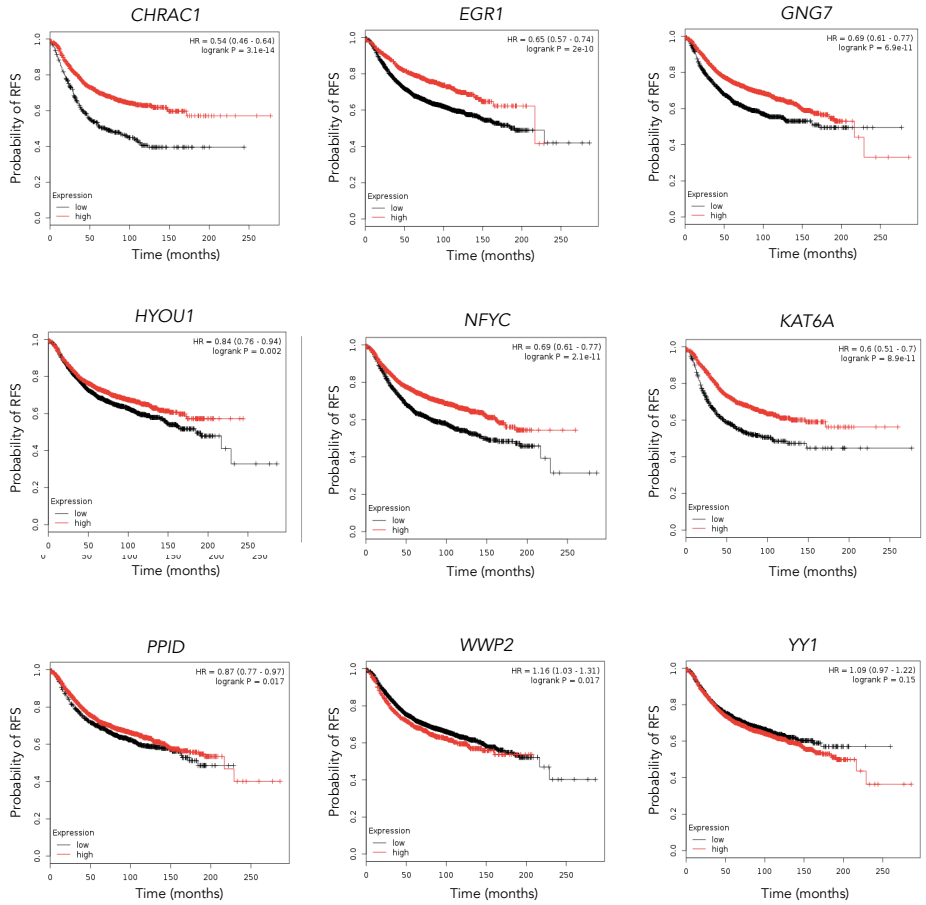


Figure S9. Role of candidate ERα modulators as prognostic biomarkers in breast tumours.
Kaplan Meier plots were generated using the KM plotter software <kmplot.com> (Győrffy et al. 2009) using patient information from the NCBI's Gene Expression Omnibus (GEO; Affymetrix HGU133A and HGU133+2 microarrays) for probability of recurrence-free survival using an auto-selected cut-off for expression of candidate genes in all breast tumours. P-values (P) and hazard ratios (HR) are indicated.

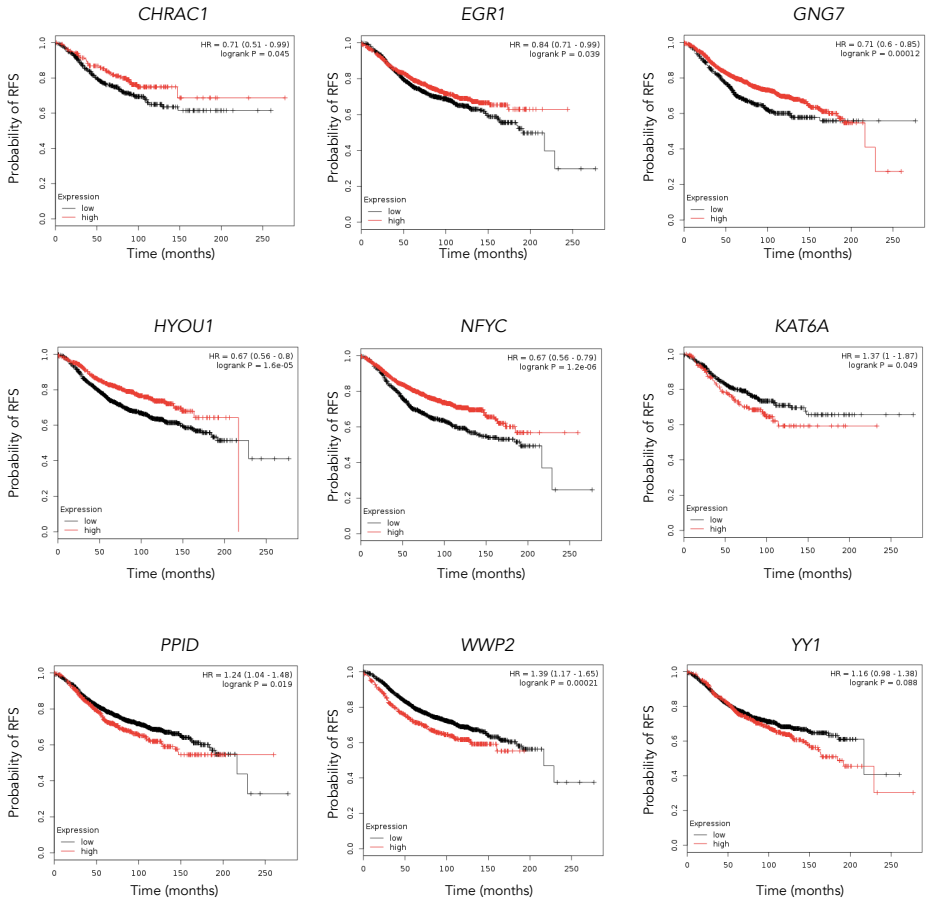


Figure S10. Candidate ERα modulators as prognostic biomarkers in ER-positive breast tumours.
Kaplan Meier plots were generated using the KM plotter software <kmplot.com> (Győrffy et al. 2009) using patient information from the NCBI's Gene Expression Omnibus (GEO; Affymetrix HGU133A and HGU133+2 microarrays) for probability of recurrence-free survival using an auto-selected cut-off for expression of candidate genes. Only ER-positive tumours in the metadataset were considered. P-values (P) and hazard ratios (HR) are indicated.

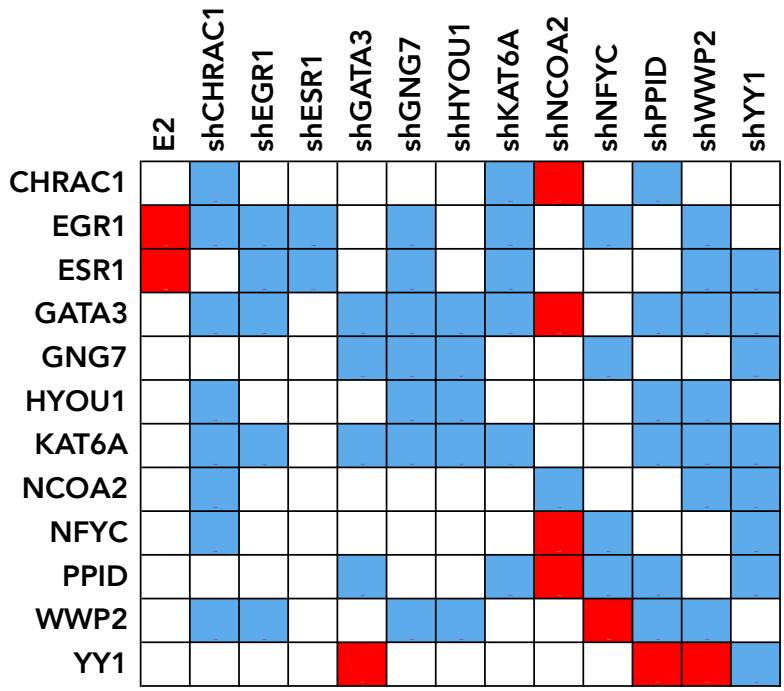


Figure S11. Cross-regulatory relationships amongst hits in our screen.
Heat map showing cross-regulation of CHRAC1, EGR1, ESR1, GATA3, GNG7, HYOU1, KAT6A, NCOA2, NFYC, PPID, WWP2 and YY2 knockdown on transcript expression of all other hits. b-values > 0.4 (red) indicate an increase in RNA transcript expression following shRNA-mediated knockdown, while b-values < -0.4 (blue) indicate a decrease in RNA transcript expression.

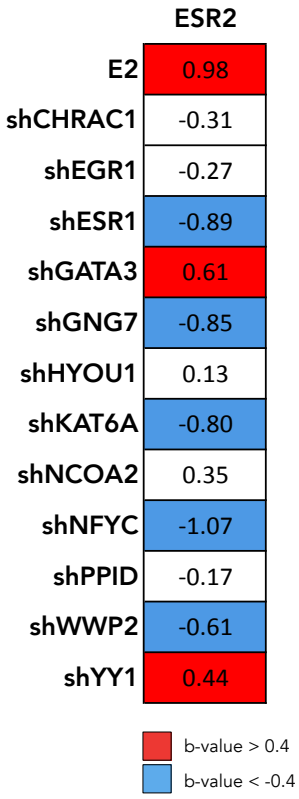


Figure S12. Impact of shRNA-mediated knockdown of hit genes on ERβ expression
Regulation of ESR2 transcript expression in transcriptome analysis after transduction of T47D-KBLuc cells with non-targeting shRNA (shNT), shGFP or two unique shRNAs targeting CHRAC1, EGR1, ESR1, GATA3, GNG7, HYOU1, KAT6A, NCOA2, NFYC, PPID, WWP2 or YY1 (MOI 10), in the presence of E2 (25 nM). E2 treatment or knockdown of ESR1, GATA3, GNG7, KAT6A, NFYC, WWP2 and YY1 markedly affects ESR2 transcript levels. b-value > 0.4 (red) indicates an increase in RNA transcript expression following shRNA-mediated knockdown; b-value < -0.4 (blue) indicates a decrease.

DISCUSSION

1. A PROTOCOL TO IDENTIFY REGULATORS OF LIGANDED, ERE-DEPENDENT ERα SIGNALLING

While ERα-expressing luminal breast tumours can be targeted by blocking estrogen production (aromatase inhibitors) or signalling (antiestrogens), the prevalence of acquired resistance even while ERα continues to be expressed supports the further study of factors influencing estrogen signalling and the mechanisms controlling expression of ERα in breast cancer cells.

The screening strategy described here was designed to identify those factors that impact ERα transcriptional activity. This will include direct coactivators or corepressors that bind with ERα at ERE consensus sequences in target gene promoters, but also transcriptional regulators upstream of ERα or any of its cofactors. Furthermore, genes involved in signalling pathways that eventually impact ERα transcriptional activation or the expression or activity of its cofactors could potentially impact our reporter assay only if their knockdown affects ERα activity at ERE consensus sequences in target gene promoters (for schematic representation, see Chapter 1, Figure 1).

ERα is also known to contribute to ERE-independent transcription, through tethering to other transcription factors including with the Fos-Jun complex, SP-1, NFκB, Runx1, Runx2 and p53 at their preferred DNA binding sites (Cicatiello et al., 2004; Gaub et al., 1990; Khalid et al., 2008; Kushner et al., 2000; Liu et al., 2006; Sayeed et al., 2007; Stein and Yang, 1995; Stender et al., 2010; Webb et al., 1999). Our screening protocol is not designed to identify those factors that selectively impact ERE-independent ERα signalling. However, tethering occurs via protein-protein interactions between transcription factors (direct or indirect via shared cofactors), and is often reciprocal. Therefore, the influence of other transcription factors on ERα signalling on EREs may suggest the possibility of tethering of ERα to sites recognized by these factors if they are interacting with ERα. This could be further investigated by co-immunoprecipitation and ChIP/ChIP-Seq experiments. Furthermore, ERα can be activated by a number of growth factor signalling pathways and activated in a ligand dependent or independent manner through posttranslational

modifications, but this often requires stimulation of a growth factor receptor with the appropriate ligand. For example, treatment with EGF or IGF will stimulate membrane receptors EGFR or IGFR, respectively, which will activate downstream signalling pathways such as the MAPK signalling pathway, leading to ERα phosphorylation (S118 and S167), and resulting in unliganded receptor activation (Chen et al., 2002; Kato, 2001). The composition of our assay media may therefore influence the factors we identify through this mechanism of ERα activation.

Finally, some factors may require a specific chromatin context or repertoire of coactivators to function. Transcription factors unable to activate or repress luciferase expression from the T47D-KBLuc promoter would therefore have been excluded even if they do regulate a certain subset of ERα target genes or regulate ERα target genes in other cell lines. T47D cells express a mutant version of p53, and while p53 mutants often preserve some functional capacity, any factors cooperating only with wild-type p53 would have been missed by our primary screening design.

In short, while our genome-wide screening approach is comprehensive in scope, there remain many avenues to explore before an exhaustive list of factors regulating ERα signalling or expression can be generated.

2. CONFIRMATION OF KNOWN REGULATORS

Our genome-wide shRNA screening effort successfully confirmed a number of known ERα cofactors in our model system, the T47D-KBLuc cell line, including members of the steroid receptor cofactor (SRC) family of coactivators (*NCOA1*, *NCOA2* and NCOA3) as activators of ER signalling, and nuclear receptor interacting protein 1 (*NRIP1*) as a repressor on ER target genes in our reporter assays. Note however that the correlation between the suppression of these factors and that of *ESR1* was not as high as in the case of upstream regulators of ER expression, due to gene-specific effects of these coactivators and to their ER-independent action, likely via interaction with other transcription factors. We also confirmed via

transcriptome sequencing the activating roles of GATA3 and FOXA1, two pioneer factors of ERα that have been implicated both in ERα signalling and expression (Bernardo et al., 2010; Carroll et al., 2005; Eeckhoute et al., 2007; Kouros-Mehr et al., 2006; Serandour et al., 2013; van 't Veer et al., 2002)

Additionally, Ingenuity Pathway Analysis (IPA; a software package from Qiagen for interpreting trends in large-scale datasets) of our genome-wide ER-reporter screening data revealed a number of canonical pathways significantly enriched in our dataset (genes identified with two or three of three shRNAs) that are known to be involved in ERα signalling, including glucocorticoid and androgen receptor signalling as well as PI3K, mTor and ERK signalling. Encouragingly, ER signalling emerged as one of our most significantly represented canonical signalling pathways.

In particular, we identified a number of members of the phosphatidylinositide 3'-OH kinase/Akt/mTOR-signalling pathway, including AKT3, PDK1, RPS6KB1 and RICTOR as activators, and PTEN as a repressor of ER signalling, with regulatory effects consistent with their roles as activators or repressors of the Akt signalling cascade. The PI3K signalling pathway has previously been implicated in ER signalling (Ciruelos Gil, 2014) and activation is known to result in ER phosphorylation by AKT and S6 kinase 1 (RPS6KB1) and transcriptional activation in a ligand-independent manner (Campbell et al., 2001). Hyperactivation of this pathway is thought to be a key mechanism underlying endocrine resistance (Barone et al., 2009; Cavazzoni et al., 2012; Miller et al., 2011) that could allow ER-positive cells to adapt to growth in conditions of estrogen deprivation. Furthermore, we also identified several members of the MAPK/ERK pathway, also consistent with an activating role of this pathway in ERα signalling. Indeed, MAPK activation has been shown to phosphorylate serine 104, 106 and 118 on ERα, leading to ligand-independent transactivation (Chen et al., 2002; Thomas et al., 2008) (Figure 1).

3. OTHER SCREENING EFFORTS FOR REGULATORS OF ERα SIGNALLING

This study is not the first screening approach to aim to identify regulators of ERα signalling. However, unlike previous studies that addressed only subsets of genes such as kinases (Giamas et al., 2011) or select transcriptional regulators (Bolt et al., 2015), our screening strategy encompassed over 16,000 protein-coding human genes in an unbiased manner. Furthermore, previous screening approaches have used siRNA constructs to achieve target gene knockdown, which produce only transient effects and limit screens to those with rapidly visible phenotypes. shRNAs, as used in this study, undergo stable incorporation in the genome of the host cell (Campeau and Gobeil, 2011), allowing for longer-term proliferation assays that would not be possible using siRNA.

Despite technical differences between the study described here and published screening studies in this field, we have identified some of the same regulators of ERα signalling and/or expression. Using a microscopy-based pooled siRNA approach in HeLa cells ectopically expressing a GFP-tagged ERα, Bolt et al. identified the E3 ubiquitin ligase EDD1 (UBR5) as a regulator of ERα protein levels and transcriptional output (Bolt et al., 2015). EDD1 was also identified as a hit in our primary and secondary screens, but was not promoted to the transcriptome sequencing step due to insufficient target knockdown. Interestingly, from our screening data, EDD1 appears to be a positive regulator of ERα signalling, whereas in the Bolt et al. study, EDD1 knockdown leads to increased ERα transcriptional output due to increased ERα protein levels, indicating EDD1 is a repressor in this study. Discrepancies between our finding and that of Bolt et al. may be due to their use of siRNA, the model cell line (HeLa vs T47D-KBLuc), or the duration (24h vs. four days in our study) or concentration of E2 treatment (10 nM vs. 25 nM in our study). Bolt et al. also identified several members of the Mediator complex, and MED4, MED8, MED9, MED18 and MED21 were all identified as single hits in our primary screen.

Genome-wide screening efforts have been undertaken to identify essential genes in breast cancer. Marcotte et al. (2016) screened 77 breast cancer cell lines to identify driver genes contributing to the growth and survival of specific subtypes of breast cancer.

Interestingly, one of the luminal subtype-specific essential genes identified by the group was *KAT6A*, which in our study controls expression of *ESR1*. This study identified several other luminal-specific drivers that were found in our study to be either positive (*ACVR1B*, *GRM4*, *TLE3*) or negative (*BLNK*, *CLK1*, *ZNF652*) regulators of ER signalling. How these genes contribute to the ERα pathway and to maintenance of the luminal subtype remains to be elucidated.

In another genome-wide knockdown study, Mendes-Pereira et al. (2012) aimed to identify genes whose silencing contributes to tamoxifen resistance or sensitivity. This study identified RAD21, a component of the cohesin complex involved in chromosome organization during cell cycle, as a gene whose silencing causes tamoxifen resistance, and here we detected stimulation of ERE-luciferase reporter activity in both T47D-KBLuc and MELN cells following *RAD21* knockdown, suggesting that RAD21 has a repressive role in E2 signalling. However, this was not supported by our transcriptome results following *RAD21* knockdown. Gene expression correlation between *RAD21* knockdown and E2 treatment was low even when focusing on direct ERα target genes. Impact of shRAD21 on upregulated estrogen target genes was indeed mixed, while repressed target genes were mostly (but not always) up-regulated. The impact of shRAD21 on proliferative target genes was however globally positive, including on expression of *E2F1*, *E2F2* and *FOXM1*, suggesting RAD21 may drive proliferation of ER-positive cells in an ER-independent manner. *RAD21* is amplified in breast cancer, in amplicon 8q23, and amplification is thought to drive proliferation in breast tumours (Mahmood et al., 2014). While we found no significant effect of *RAD21* knockdown on viability of T47D-KBLuc or MCF-7 cells or on *ESR1* expression, there was a weak but overall repressive effect on expression of proliferative genes. Further growth assays and transcriptome studies performed in the presence of tamoxifen are required to fully understand the role of RAD21 in antiestrogen sensitivity. The Mendes-Pereira screening study also identified a number of other genes that contribute to antiestrogen sensitivity and were found to be negative regulators of ER signalling in our study, namely *BAP1*, *CLPP*, *NF1*, *NIPBL*, *NSD1*, *RARG*, *EDF1*, *ING5* and *KRAS*.

In a kinome-wide siRNA screen for regulators of tamoxifen resistance in long-term estrogen deprived MCF-7 cells, Bhola et al. identified Polo-like kinase 1 (PLK1) as a gene whose downregulation resulted in inhibition of ligand-independent ER transcriptional activity and growth of antiestrogen resistant cells (Bhola et al., 2015). Interestingly, PLK1 was identified in our primary screen (albeit with only one hit) as a gene whose knockdown increases ERE-luciferase reporter activity. It remains to be seen if knockdown of PLK1 also regulates ER activity following tamoxifen treatment of the T47D-KBLuc cell line.

4. DISCOVERY OF NOVEL REGULATORS OF ERα TRANSCRIPTIONAL ACTIVITY

As anticipated, we also identified a number of novel regulators of ERα that may have been overlooked by a more selective, targeted screening approach. These include the nuclear transcription factor NFYC, the chromatin assembly complex *CHRAC1*, and the histone acetyltransferase *KAT6A*. These have all been discussed in detail in Chapter 2.

There were, however, several other interesting hits that emerged from our screen. RE1 Silencing Transcription Factor (REST) is a transcriptional suppressor which, by binding to RE1 sites in regulatory regions of target genes (found in approximately 2000 human genes (Bruce et al., 2004)), recruits histone deacetylases (HDACs) and histone methyltransferases to suppress neuronal gene expression in non-neuronal cells (Huang et al., 1999; Roopra et al., 2000). In small cell lung cancer, alternative splicing of REST mRNA results in a truncated and inactive version of the repressor and overexpression of REST target genes, conveying a neuroendocrine phenotype. REST activity is also lost in a subset of aggressive breast tumours through alternative splicing (Wagoner et al., 2010). Neuroendocrine tumours are an aggressive subclass of breast tumours that express some markers specific to neuronal cells including chromogranin, synaptophysin and enolase, have also been described (Inno et al., 2016). In our screen, knockdown of REST led to repression of ERE-luciferase activity but correlation on direct ER target gene regulation as compared to E2 stimulation was weak, in keeping with an absence of influence on *ESR1* expression. REST suppression down-regulated most affected E2-induced direct targets, but had mixed effects on suppressed

direct target genes, suggesting that REST is an activator of ER signalling. As expected however, REST knockdown resulted in increased expression of neuronal-specific genes, including synaptophysin, in T47D-KBLuc cells, confirming successful knockdown. It also resulted in induction of a subset of proliferative genes, including *E2F2*/8, suggesting a proliferative effect of REST loss-of-function in breast cancer.

Our screen also identified a number of heat-shock proteins whose knockdown downregulates ER reporter activity. One that we explored further is hypoxia upregulated 1 (HYOU1), which is overexpressed in a small number of invasive breast cancers. Overexpression of HYOU was reported as being associated with indicators of poor prognosis, including markers of metastasis, and loss of ERα (Stojadinovic et al., 2007). In our study, HYOU1 was seen as a positive regulator of ER reporter activity, and its modulation of gene expression following repression inversely correlated with E2 stimulation, indicating that HYOU1 is an activator of the ER-signalling pathway. Interestingly, HYOU1 is deleted in a subset of breast tumours (Figure S10).

Other examples from the heat-shock protein family include HSP70 family members HSPA1B, HSPA4, and HSPA9B, which were confirmed in secondary screens in T47D cells but not in MELN cells. In addition, another hit, FKBP52 (FKBP4; 52-kDa FK506-binding protein), an immunophilin whose loss is associated with endometriosis (Hirota et al., 2008) and whose expression positively correlates with that of ERα in breast cancer cell lines (Ward et al., 1999). FKBP52-ERα binding has been reported *in vitro* (Byrne et al., 2016), indicating FKBP52 may be an ERα co-chaperone as has been reported for progesterone, androgen and glucocorticoid receptors (Cheung-Flynn et al., 2005; Riggs et al., 2003). We noted induction of *FKBP52* transcript expression following treatment of T47D-KBLuc cells with E2, while expression was repressed by the knockdown of ERα in our study. Induction of *FKBP52* expression has been previously reported in MCF-7 cells, and this induction can be inhibited by treating cells with the antiestrogen ICI 182, 780 or with a protein synthesis inhibitor, indicating that *FKBP52* is a direct transcriptional target of ERα signalling that may feed back to positively regulate receptor activity (Kumar et al., 2001). Kaplan-Meier analysis of *FKBP52*

expression in human breast tumours indicates no significant difference in probability of recurrence-free survival between high and low expressors when all patients are considered (Figure 2A). However, when looking at only ER-positive tumours, high expression of *FKBP52* is associated with a significantly lower probability of recurrence-free survival than low expressors (p=0.0017; Figure 2B), marking *FKBP52* as a marker of bad prognosis in the ER-positive subgroup.

Many other interesting hits identified in our secondary screens were not included in transcriptome studies, either due to inconsistent effects between primary and secondary screens, between T47D and MCF-7 cells, or due to inefficient suppression of their targets as measured by qRT-PCR. This study has provided a wealth of knowledge that will surely be interrogated time and again in future single-gene-centric projects.

The mechanism of action by which most of the hits identified in this study affect ER signalling remains to be elucidated. The factors identified here may be regulators of ER expression, as is likely the case for *EGR1*, *GNG7*, *WWP1* and *YY1*, or may be cofactors with gene specific activity. We noted an overall stronger impact on E2 target genes by those hits that affect *ESR1* expression. In comparison, the correlation between E2 regulation and hit knockdown for most identified hits that do not significantly impact levels was relatively weak, possibly due to a role as cofactors for multiple transcription factors or to gene-specific effects on subsets of ER target genes. As mentioned, NFYC has been identified as a corepressor of the mineralocorticoid receptor (MLR), and *NR3C2*, the gene encoding for MLR, is expressed in T47D-KBLuc cells and is induced following knockdown of *NFYC*. Moreover, *NR3C2* knockdown results in a weak but overall positive regulation of ERE-luciferase reporter activity (albeit below our set cut-off). It is therefore plausible that discrepancies between *ESR1* and *NFYC* knockdown are due to MLR signalling, or signalling by another transcription factor not yet identified as an NFYC target.

NFYC knockdown, as does knockdown of all other genes interrogated by transcriptome sequencing in this project, results in regulation of the target transcript and a subset of E2 target genes, but also in the cross-regulation of other upstream regulators

identified in this study (Figure 3). It is likely that some of our hits affect ERα signalling in an indirect manner, by modulating expression or activity of other genes that then affect ERα. As mentioned in Chapter 2, a number of hits regulate *NCOA2* expression, which may contribute to their role in ERα signalling. A striking number of gene knockdowns also significantly downregulate GATA3 and/or NCOA6 transcript expression and activate expression of YY1, and it remains to be confirmed if GATA3, NCOA6 or YY1 protein levels are also altered by these shRNAs. A more comprehensive time course study with or without addition of a protein synthesis inhibitor would be necessary to clarify whether effects on ER target genes are direct and/or indirect consequences of hit knockdown.

Future experiments will be needed to address the mechanisms of action of candidate modulators, in particular by characterizing their interactome in T47D cells. It remains to be seen if hits in our screening protocol affect ERα signalling in other ER-positive cell lines or in T47D cells only. While we included MCF-7 cells in our secondary screening protocol, inactivity on a luciferase reporter vector in high-throughput assays does not necessarily translate to lack of effect on endogenous ER target gene expression. Furthermore, for those hits that affect ERα transcript and/or protein expression (*KAT6A*, EGR1, *WWP2*, YY1), it would be interesting to explore if modulation of *KAT6A*, EGR1, *WWP2* or YY1 protein levels in ER-negative cell lines can help to re-express ERα protein and thereby re-sensitive those cells to antiestrogen treatment.

5. CONFOUNDING FACTORS

While our screening effort was genome-wide, a fully exhaustive approach at such a large scale is not possible. Furthermore, there were a number of variables in our screening strategy that likely led to elimination of potential true regulators along the way.

As described in Chapter 1, we opted for an arrayed screening format to maximize relevant hits and because presence or absence of ERα signalling is not necessarily a phenotype that can be selected for from amongst a population of cells. Furthermore, it allowed us to bypass the sequencing and deconvolution step, which can introduce a number

of biases to the data. However, results of shRNA screening are not unequivocally quantitative. The relative percent inhibition of luminescence attributed to each shRNA may not be directly related to the abundance of the given target protein (Barrows et al., 2010). Viral titers are estimated by titration of a small selection of wells and so it is expected that variations in infection efficiency and individual viral titer may confound some results and contribute to false negative reads. Furthermore, as most shRNA constructs have not been validated on their target, and performance of shRNA is affected by shRNA promoter activity and epigenetic silencing in a cell context specific manner (Hong et al., 2007; Liu et al., 1997), and by mRNA abundance and protein half-life of the target, it is plausible that none of the shRNA for a given gene efficiently represses its target, resulting in false negatives.

The field of RNA interference has recently been complemented by the availability of CRISPR/Cas9 technology, which allows for complete gene knockout at the level of DNA. While a handful of screens have been published using this technique (Korkmaz et al., 2016; Park et al., 2016; Shalem et al., 2015; Wang et al., 2014), these have mostly been pooled screens, necessitating assays (like cell viability) that are amenable to selection procedures. Due to the random nature of mutations introduced in each cell, CRISPR/Cas9 produces a heterogeneous population in a single well. In this study, we have seen that CRISPR/Cas9-mediated knockdown of *KAT6A* in a population of T47D-KBLuc cells is no better than that achieved using shRNA, and in fact while cells in that experiment were selected for successful transduction (puromycin), only two of the four sgRNAs tested produced a discernable reduction in ERα protein or RNA levels following 'knockout' of *KAT6A*. For the extension of our study, CRISPR/Cas9 could be used to further explore factors contributing to ERα expression, by stably expressing a fluorescent protein-tagger ERα and sorting cells after sgRNA transduction for those that have either lost or increased fluorescence, and therefore ERα expression. FACS-sorting cells after sgRNA transduction for those that have either lost or increased fluorescence, and therefore ERα expression. Alternatively, insertion of GFP directly in the genomic sequence of *ESR1* would provide a reporter vector for *ESR1* expression levels.

6. GENES SUPPRESSING PROLIFERATION OF BREAST CANCER CELLS

Knockdown of nearly all hits significantly decreased expression of *FOXM1*, a transcription factor that regulates expression of genes involved in G2/M transition (*CCNB1*) and maintenance of chromosome segregation (*CENPF*) (Wonsey and Follettie, 2005), and a target of ERα (Millour et al., 2010), suggesting that most hits can potentially modulate proliferation of T47D cells. While most hits did not show a profound impact on proliferation in the alamarBlue assay following four or eight days of knockdown (including *FOXM1* itself), it is possible that extension of the assay timeframe or selection for cells successfully transduced with shRNA would show a greater impact on T47D cell growth. This being said, we noticed an overall trend whereby genes that affect multiple proliferation-associated transcription factors (*FOXM1, E2F1, E2F2, MYBL2*) had a more pronounced effect on proliferation in the alamarBlue assay than those which affected just one of these. Knockdown of *DIXDC1, KAT6A, MLLT1, NCOA2* and *WWP2* significantly represses expression of all four proliferation-associated transcription factors and has a far more pronounced effect in the alamarBlue assay than does knockdown of *NCOA6* or *NRIP1*, for example, each of which significantly represses *E2F2* expression only. From a clinical perspective, those hits affecting T47D cell proliferative pathways may be the most interesting to explore in future studies. In addition, a full review of genetic alterations affecting hits identified in the primary and/or secondary screens may identify additional interesting new candidates for future validation studies similar to the ones conducted to date.

7. ONE PROTOCOL, MANY APPLICATIONS?

The screening protocol described herein is particular to the exploration of ERα biology in ER-positive breast cancer however, it has provided our lab with the tools and expertise necessary to potentially extend this type of study to other biological problems. For example, the molecular apocrine subgroup of breast tumours expresses androgen receptor (AR) and it is thought to drive the proliferation of this subgroup much like ERα drives proliferation in

luminal breast cancers (Doane et al., 2006). Treatment of the molecular apocrine breast cancer cell line MDA-MB-453 with the androgen antagonist bicalutamide results in decreased growth and colony formation (Robinson et al., 2011) but to date, no targeted therapy exists in the clinic to specifically address molecular apocrine tumours. A similar screening strategy as the one described here could be undertaken in a molecular apocrine breast cancer cell line expressing a luciferase reporter vector under the control of an androgen response element promoter, and could provide a wealth of knowledge on a more aggressive subtype of breast cancer that we currently know very little about.

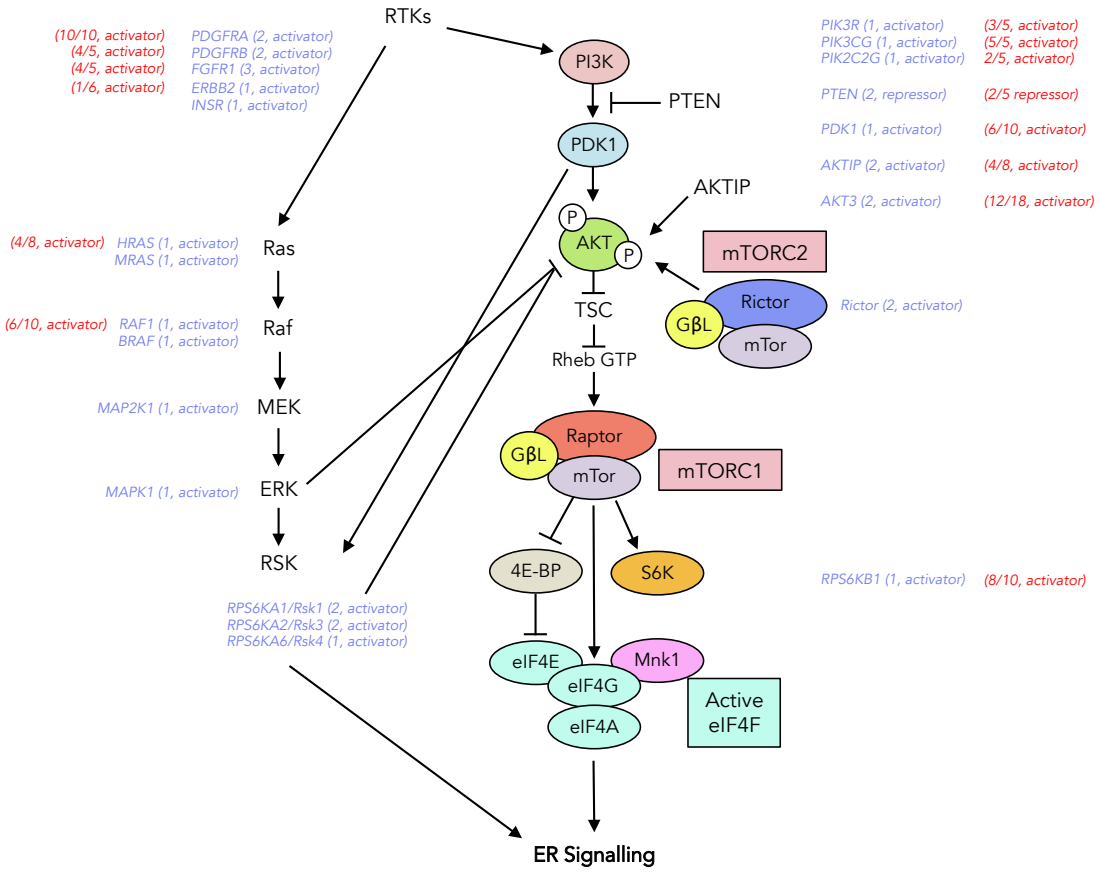


Figure 1. The PI3K/Akt/mTor and MAPK/ERK pathways affect ERα signalling.
Multiple members of the PI3K/Akt/mTor and MAPK/ERK pathways were hits in our primary (blue) and confirmatory (red) luciferase reporter screening assays in T47D-KBLuc cells. The number of effective shRNAs at each stage of the screening protocol is shown, along with the prediction of whether a gene is an activator or repressor of ERα signalling based on luciferase data.

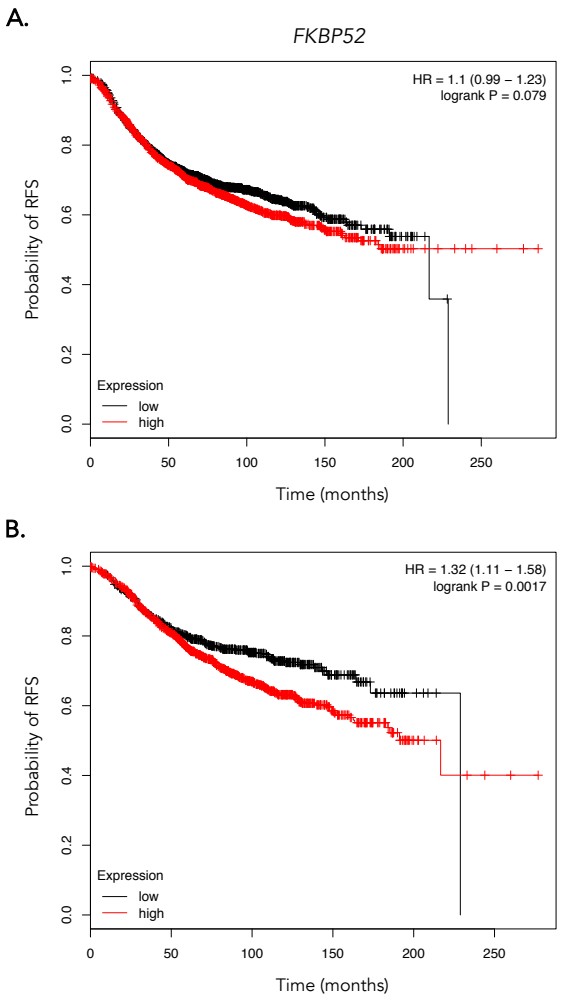


Figure 2. Increased expression of *FKBP52* in ER-positive human breast tumours predicts poor outcome.

Kaplan Meier plots were generated using the KM plotter software <kmplot.com> (Győrffy et al. 2009) using patient information from the NCBI's Gene Expression Omnibus (GEO; Affymetrix HGU133A and HGU133+2 microarrays), EGA and TCGA datasets. Breast cancer patient data was stratified based on *FKBP52* expression in A) all breast tumours (1764 patients) or B) ER-positive tumours (3951 patients). While *FKBP52* expression was not significantly correlated with recurrence-free survival in all tumours (p=0.079), high expression of *FKBP52* in ER-positive breast tumours was associated with a significantly lower probability of survival (p=0.0017).

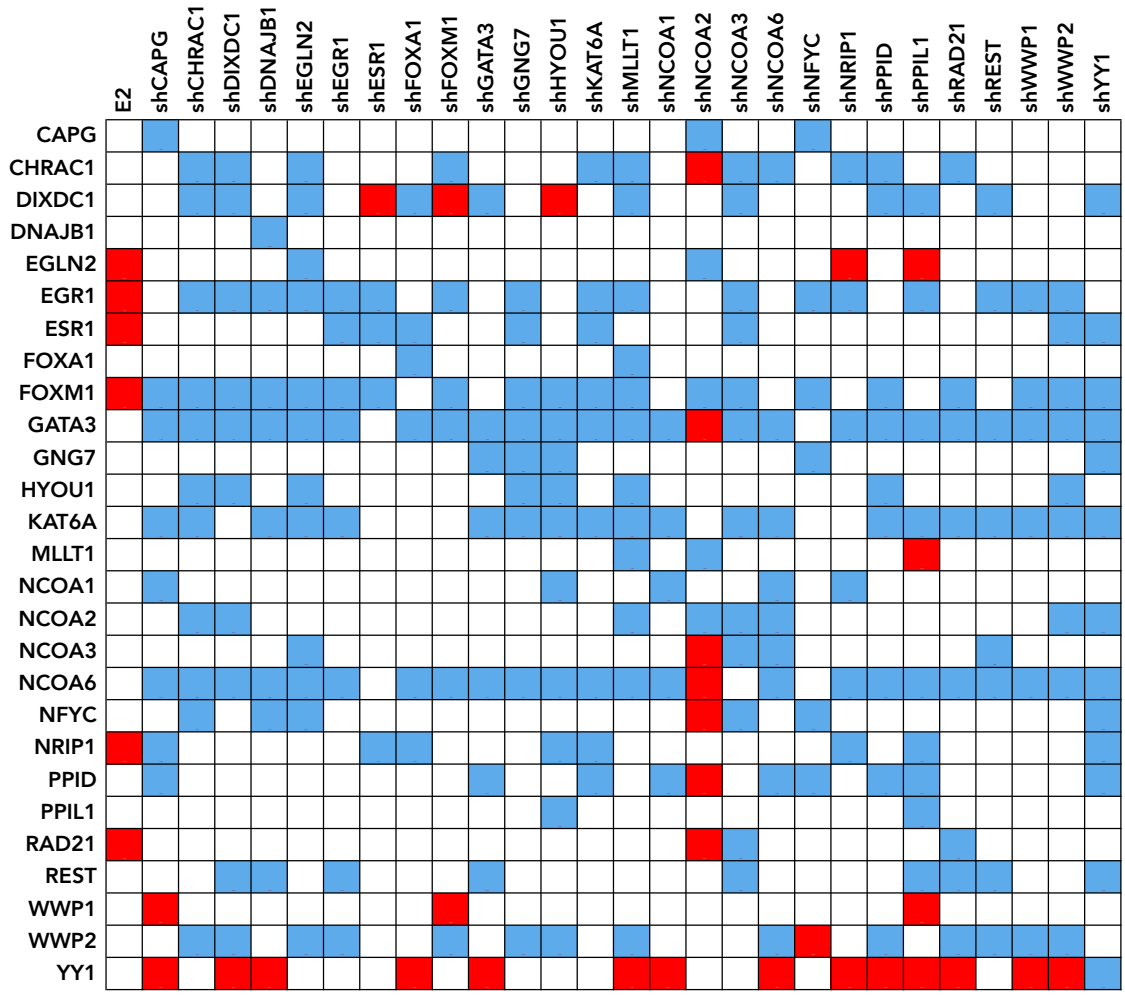


Figure 3. Cross-regulatory relationships amongst hits in our screen.

Heat map showing regulation of all 27 selected hits on the transcript expression of the remaining genes of interest following transcriptome sequencing. b-values > 0.4 (red) indicate an increase in RNA transcript expression following shRNA-mediated knockdown, while b-values < -0.4 (blue) indicate a decrease in RNA transcript expression.

CONCLUSION

This study represents the first genome-wide arrayed shRNA screen for modifiers of ER signalling and/or expression in breast cancer. Due to the modest impact of shRNAs on gene expression, potential side effects and need for high reproducibility of assays for high-throughput screens, we optimized the assays used for this study and developed a step-wise validation process to increase chances of identifying bona-fide regulators of ERα signalling. Our work therefore presents a valuable contribution in terms of method development that could also be applied to other loss or gain of function screens in arrayed formats.

Our results confirmed a number of known regulators of ERα-mediated transcriptional activity and provide a more profound characterization of their mechanisms of action through transcriptome sequencing, revealing gene-specific effects of several ERα coregulators and suggesting their action as coregulators of other transcription factors. Follow-up studies will build on the analysis of these datasets to better understand the role of each coregulator in estrogen signalling and in breast cancer cell response to antiestrogens.

Additionally, our studies identified novel genes whose products modulate ERα expression in T47D luminal breast cancer cells, such as *KAT6A, EGR1, GNG7, WWP2* and *YY1*, and those that impact ERα signalling, such as *CHRAC1, HYOU1, NFYC and PPID*. All factors had global stimulatory effects on cell proliferation, suggesting pro-tumourigenic roles that will be investigated in further studies using *in vitro* and *in vivo* models.

Notably, both *KAT6A* and *CHRAC1* are amplified in breast cancer and may drive estrogenic signalling. Our findings may therefore lead to the development of novel therapeutic approaches targeting tumours with associated genetic defects, or alternatively targeting overexpression of these factors to impose more effective and personalized treatments for ER-positive breast cancer patients.

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