

Cytidine Deaminases are Regulators of Estrogen Receptor Activity in Breast Cancer Cells

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Abstract

Breast cancer is the most common cancer worldwide, with 1.38 million women diagnosed with the disease each year. Estrogens play a critical role in the development and progression of breast cancer, their action being mediated by the estrogen receptors (ER), ER α and ER β , which are the members of the nuclear receptor superfamily of transcription factors. This understanding has led to the development of endocrine therapies aimed at inhibiting ER action by competitive binding to the ER (anti-estrogens), or using inhibitors of estrogen biosynthesis (aromatase inhibitors). Determining the mechanisms by which ER regulate gene expression will aid our understanding of the role of ER in breast cancer progression, response and resistance to endocrine therapies. Upon binding estrogen, ER drives the expression of estrogen responsive genes through the orderly recruitment of co-regulators that act by remodelling and modifying chromatin, ultimately promoting RNA polymerase II recruitment and transcription initiation. Previous work from our laboratory has shown that the APOBEC3B cytosine deaminase acts as an ER α transcriptional coactivator in reporter gene assays. Here, I have developed these initial observations and demonstrate that APOBEC3B is important for the regulation of estrogen responsive genes and breast cancer cell growth. I show that APOBEC3B is recruited to the promoters of estrogen-responsive genes and interacts with ER α . Studies carried out to identify the molecular mechanisms by which APOBEC3B regulates the expression of estrogen-responsive genes included its potential role in DNA demethylation and identified a role for APOBEC3B in DNA strand break formation at the promoter of the estrogen regulated pS2 gene. Together, these studies identify APOBEC3B as an important new coregulator of ER α that is required for the regulation of gene expression by estrogen in breast cancer cells.

Statement of originality

I hereby declare that this submission is my own work, or if not, it is clearly stated and fully acknowledged. To the best of my knowledge and belief, this report contains no material previously published or written by any other person, nor material which, to a substantial extent has been accepted for the award of any degree or diploma of the Imperial College London, or other institution of higher learning, except where due acknowledgement has been made in the text.

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Abbreviations

AF-1	Activation function-1
AF-2	Activation function-2
ACF	APOBEC complementation factor
APS	Ammonium persulphate
AP-1	Activator Protein-1
AR	Androgen receptor
APOBEC	Apolipoprotein B mRNA editing, enzyme catalytic
AID	Activation induced cytidine deaminase
ATM	Ataxia-telangiectasia mutated
APE1	Apurinic/apyrimidinic endonuclease
BER	Base excision repair
Biotin-11-dUTP	Biotin-11-deoxyuridine triphosphate
CARM-1	Coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
CREB	cAmp response element –binding
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
ChIP-Seq	Chromatin immunoprecipitation sequencing
ChIP-chip	Chromatin immunoprecipitation with microarray
CHIA-PET	Chromatin Interaction Analysis by Paired-End Tag Sequencing
CSR	Class switch recombination
DNA	Deoxyribonucleic acid
dsDNA	Double strand DNA
DBD	DNA binding domain
DSS	Double charcoal stripped serum
DTT	Dithiothreitol
ddH ₂ O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DCIS	Ductal carcinoma <i>in situ</i>
DES	Diethylstilbestrol

DNA-PK	DNA dependent protein kinase
DNMT	DNA methyltransferase
DRIP	D3 receptor interacting protein
DND1	Dead end protein
DSB	Double strand DNA break
ERE	Estrogen response element
EGFR	Epidermal growth factor receptor
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
E2	17 β estradiol
FCS	Fetal calf serum
FEN1	Flap structure-specific endonuclease 1
FXR	Farnesoid X receptor
GSK3	Glycogen synthase kinase 3
GR	Glucocorticoid receptor
GST	Glutathione
GFP	Green fluorescence protein
HA	Human influenza hemagglutinin
GADD45 α	Growth arrest and DNA-damage-inducible protein
HRP	Horseradish peroxidase
H ₂ O ₂	Hydrogen peroxide
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HR	Homologous recombination
γ H2AX	Phosphorylation of histone protein H2AX on serine 139
HSP	Heat-shock protein
ICI	ICI 182 780
IP	Immunoprecipitation
LBD	Ligand binding domain
LXR	Liver X receptor
LB	Luria broth
LRH-1	Liver receptor homolog 1
MAPK	Mitogen activated protein kinase

mg	Milligram
ml	Milliliter
μg	Microgram
MR	Mineralocorticoid receptor
meCpG	Methylated CpG
MBD	Methyl-CpG binding domain
5meC	5-methyl cytosine
5hmC	5-hydroxymethyl cytosine
5hmU	5-hydroxymethyl uridine
miRNA	MicroRNA
NR	Nuclear receptor
NLS	Nuclear localization signal
NONO	non-POU domain-containing octamer-binding protein
NCOR	Nuclear receptor corepressor
NHEJ	Non-homologous end joining
NuRD	Nucleosome remodeling and deacetylation
OHT	4-hydroxytamoxifen
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pCAF	p300/CBP associated factor
PKC	Protein kinase C
PKA	Protein kinase A
PCR	Polymerase chain reaction
PR	Progesterone receptor
Pol II	RNA polymerase II
PSF	Polypyrimidine tract binding (PTB) protein associated splicing factor
PPAR	Peroxisome proliferators activated receptor
RT	Reverse transcription
RT-PCR	Real-time PCR
RNA	Ribonucleic acid
RRM	RNA recognition motif
RAR	Retinoic acid receptor
RNAi	RNA interference

SRC	Steroid receptor coactivator
SHM	Somatic hypermutation
SRA	Steroid receptor RNA activator
SWI/SNF	Switch/Sucrose non-fermentable
SDS	Sodium dodecyl sulphate
SMRT	Silencing mediator of retinoic and thyroid hormone receptor
SERM	Selective estrogen receptor modulator
SP-1	Specificity Protein 1
ssDNA	Single strand DNA
siRNA	Small interference RNA
TCA	Trichloroacetic acid
TR	Thyroid receptor
TOP2B (Topo II β)	Topoisomerase II β
TEMED	Tetramethylethylenediamine
TRAP	Thyroid hormone receptor associated protein
TDG	Thymine DNA glycosylase
TdT	Terminal deoxynucleotide transferase
XRCC1	X-ray repair cross-complementing protein 1

1. Introduction

1.1 Breast cancer

With 1.38 million women diagnosed each year, Breast cancer (BC) is one of the most common cancers seen worldwide, comprising 23% of cancers in women and 10.9% of all cancers. BC is most prevalent in Western countries, with over 45,000 women being diagnosed annually in the UK and, more than 200,000 women in the USA, thereby constituting 31% and 28% of all cancers seen amongst women in these populations. BC accounts for more than 12,000 and 40,000 death annually in the UK and USA (Jemal et al., 2010). Indeed, the incidence of BC has increased over the past three decades, partly due to more public awareness of the disease and the implementation of screening programmes. Nonetheless, in spite of increases in incidence, BC mortality rates have declined in the developed countries over the same period. This decline is likely to be due to earlier detection and the use of multidisciplinary treatment approaches, involving surgery, radiation, chemotherapy and, most importantly, adjuvant endocrine therapies, such as antiestrogens (Ali et al., 2011; Jones and Buzdar, 2004; Lin et al., 2010). However, despite these advances in treatment, BC remains a major cause of cancer mortality in women, the mortality being due, at least in part, to *de novo* and acquired resistance to therapies, the mechanisms for which remain unclear.

1.1.1 Estrogens and Breast Cancer

Estrogens are steroid hormones that have widespread biological functions. The primary roles of estrogens are in the development and maintenance of female characteristics including the reproductive organs and the mammary gland (Gruber et al., 2002). Estrogens are also important in maintaining normal physiological functions in other tissues including skeletal, cardiovascular and central nervous systems (Gruber et al., 2002). The primary source of estrogen production in premenopausal women is the ovary. Theca cells in the ovary synthesis the steroid hormones androstenedione and testosterone, which are subsequently converted by the P450 enzyme aromatase, in granulosa cells to 17 β -estradiol (E2). Ovarian estrogens, released into the bloodstream act by binding to estrogen receptor in target tissue (Gruber et al., 2002). Cyclical

ovarian estrogen is required to maintain normal mammary ductal formation and stimulate the proliferation of normal breast epithelial cells (Anderson et al., 1998).

A connection between BC and estrogens has long been recognized. In 1896, George Beatson demonstrated that bilateral oophorectomy resulted in remission in premenopausal women with metastatic BC (Love and Philips, 2002). More recent epidemiological studies have demonstrated that the lifetime exposure of estrogens is a key risk factor in BC development. Thus, early age of onset of puberty and late menopause are important risk factors (Clemons and Goss, 2001). Following the onset of menopause and decline in ovarian estrogen production, extragonadal sites, such as adipose tissue, brain, bone and vascular tissues, become the chief sources of estrogen, this occurring through the aromatisation of adrenal androgens by aromatase (Clemons and Goss, 2001). Indeed, there is considerable evidence to show that aromatase is highly expressed in breast tumours and surrounding adipose tissue and the local production of estrogen synthesis may be important for BC progression (Miki et al., 2007; O'Neill et al., 1988). Local concentrations of estrogens have been found to be significantly elevated in tumour-containing breast tissues compared to normal breast tissues, reflecting aromatase overexpression and elevated aromatase activity (Bulun et al., 2009). However, a recent study examining the expression of estrogen-regulated genes in breast tumours with plasma estradiol levels showed that there is a significant association between circulating estrogen levels and expression of estrogen-regulated genes, indicating an important role for circulating estrogen in BC (Dunbier et al., 2010), in addition to the potential importance of local estrogen biosynthesis in BC in post-menopausal women.

1.2 Outline of the mechanisms of estrogen action

Estrogens play key roles in proliferation and differentiation of estrogen target cells through the action of the transcription factors, estrogen receptor α (ER α) and estrogen receptor β (ER β). 70-80% of all breast tumours express ER α and ER α is a positive prognostic marker, as well as being a predictor of response to endocrine therapies (Fisher et al., 2001). In patients with ER α positive BC, endocrine therapy is well tolerated and has milder side effects compared to cytotoxic therapies (Buzdar, 2001). The majority of ER α positive BC patients respond to tamoxifen treatment. However,

despite continued expression of ER α , many of the patients who initially present with localised disease and all of the patients with metastatic disease who respond to tamoxifen treatment, develop resistance to tamoxifen over time (Ali and Coombes, 2002). Interestingly, many of these resistant tumours still respond to other anti-estrogens (e.g. Fulvestrant) or to aromatase inhibitors (e.g. Anastrozole), indicating that ER α continues to be a key regulator of tumour growth in tamoxifen-resistant BC (Ali and Coombes, 2002). Resistance to other anti-estrogens, as well as to aromatase inhibitors also arises. Hence, resistance to hormonal therapies is an important clinical problem in the treatment of BC patients.

The estrogen receptors, ER α and ER β , are members of the nuclear receptor (NR) superfamily of transcription factors. It has been estimated that 15-25% of epithelial cells in normal breast express ER α , whereas 80-85% of epithelial cells express ER β ; only ER α positive cell numbers fluctuate during the menstrual cycle (Ali and Coombes, 2002; Fox et al., 2008). Interestingly, non-proliferating cells co-express ER α and ER β and it is believed that ER-positive cells promote the proliferation of neighbouring ER-negative epithelial cells (Clarke et al., 1997; Roger et al., 2001). Compared to the normal breast epithelial cells, in breast tumours ER α expression increases several fold, with 75% of the cells expressing high levels of ER α in low grade ductal carcinoma *in situ* (DCIS) and 30% of cells expressing low levels of ER α in high grade DCIS (Herynk and Fuqua, 2004; Leygue et al., 1998). In early BC, ER α expression increases and ER β expression decreases, while expression of ER α and ER β declines in invasive BC (Bardin et al., 2004; Foley et al., 2000; Leygue et al., 1998; Roger et al., 2001). The relative levels of ER α and ER β are essential determinants of the estrogen response in a given cell. A rise in levels of ER β expression relative to ER α has been shown to decrease ER α response to estrogen and reduce the partial agonistic action of tamoxifen, whilst augmenting its antagonistic activity (Pettersson et al., 2000). In a mouse model it was observed that in the absence of ER β , the expression of estrogen induced genes was approximately 85% higher in bone cells, suggesting that ER β is a negative regulator of ER α and the estrogen response (Lindberg et al., 2003).

1.3 Estrogen in the development of the normal breast and breast cancer

The mammary gland is a highly specialized organ required for the production of milk for the newborn (Sternlicht, 2006). Human breast development is a progressive process initiated during embryonic life. The main spurt of growth occurs at puberty and is dependent on the high levels of estrogens produced by ovary, as well as progesterone. After puberty, the menstrual cycle, pregnancy and lactation all result in alterations in the structure of the mammary gland, with cycles of growth and involution (Ali et al., 2011; Sternlicht et al., 2006).

Histologically, the mammary gland contains a rudimentary branching duct system, which lies in a fat pad (Figure 1.1). The rudiment undergoes a cyclical increase in ductal branching at the post-pubertal stage, resulting in extensive branching in the fat pad. The duct consists of a continuous layer of epithelial cells, which produce and release milk into the lumen, surrounded by a layer of contractile, myoepithelial cells. The myoepithelial cells lay down the basement membrane and play an important role in forcing milk in the lumen out through the ducts. During pregnancy, further side branching of ducts occurs and secretory acini develop from the terminal ductal alveoli (Russo and Russo, 1994; Sternlicht, 2006). There is considerable evidence for a role for ovarian steroid hormones in regulating mammary gland growth and development (Imagawa et al., 1990; Silberstein et al., 1994). In the absence of intact ovarian function, there is a complete failure of breast development (Imagawa et al., 1990; Silberstein et al., 1994). Further insight into the role of estrogen in mammary gland development has come from studies on mice where the gene for ER α has been knocked out (Bocchinfuso et al., 2000). The mammary gland in these ER α knockout mice comprise rudimentary ducts confined to the nipple area, which do not develop further despite elevated levels of circulating estrogens.

Immunohistochemical analysis reveals that ER α and ER β are expressed in mammary epithelial cells, these cells being the main site of estrogen action in the breast (Ricketts et al., 1991). There is evidence to suggest that the surrounding fibroblasts may also be involved in mediating estrogen action in the mammary gland development (Cunha et al., 1997).

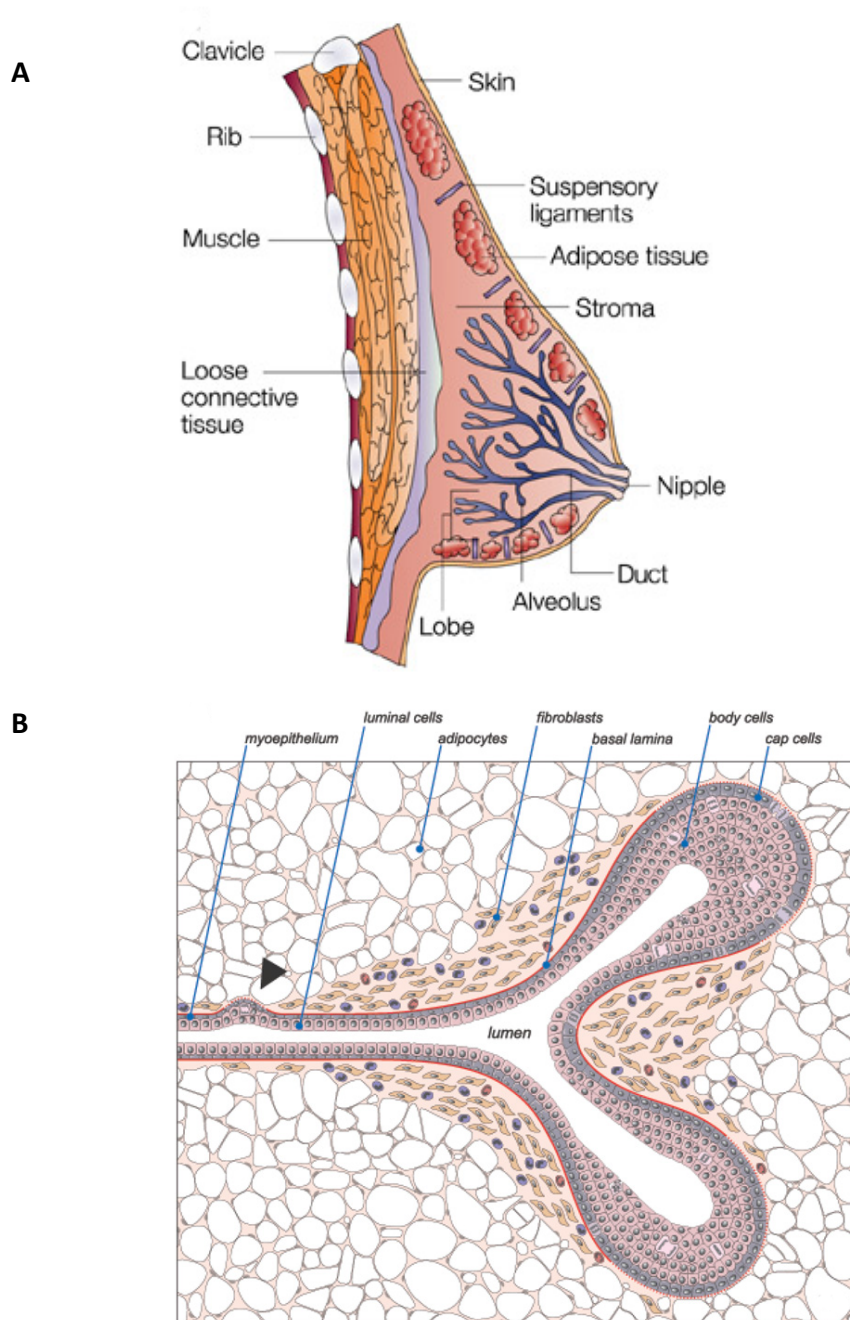


Figure 1.1. Morphology of the normal breast and luminal duct.

A. Structure of the normal human mammary gland. Each mammary gland approximately contains 15-20 lobes, which contain a network of branched ducts that drain into the nipple. B. Diagram showing major features of bifurcating luminal duct. Taken from Ali and Coombes, 2002 and Sternlicht et al, 2006.

The expression of ER α in both luminal epithelial cells and BCs, along with the histological observations that show a morphological similarity between luminal epithelial cells and BCs, support the idea that the majority of BCs are of luminal epithelial origin (Anderson et al., 1998). However, recent gene expression microarray studies have shown that a proportion of BCs express a gene expression signature normally associated with myoepithelial cells, indicating the possibility that a significant proportion of BCs arise from this basal cell type, rather than the epithelial cells, (Perou et al., 2000). This type of tumour has been termed “basal breast cancer” (Rakha et al., 2008).

The pathway to BC development is unclear. There is some evidence to show that BC development is a multi-step process (Beckmann et al., 1997). In this, the initial hyper-proliferative state is considered to progress through a pre-cancerous (non-invasive) stage called ductal carcinoma *in situ* (DCIS), which is bounded by the basement membrane, and then develop into invasive BC, in which the basement membrane has been breached, allowing malignant cells to metastasise to distant sites around the body (Lakhani, 1999).

1.4 Endocrine therapies for the treatment of breast cancer

The realisation that estrogens regulate BC growth has led to the development of endocrine therapies that act either by inhibiting estrogen biosynthesis or through the use of estrogen antagonists which compete with estrogen for binding to receptors, in order to inhibit receptor activation (Lewis and Jordan, 2005). It has long been thought that estrogen ablation in women with advanced BC is an effective therapy (Brown and Davidson, 2006). Historically, BC was treated either by surgical oophorectomy (surgical removal of ovaries) for pre-menopausal women or adrenalectomy (surgical removal of adrenal glands) and hypophysectomy (surgical removal of pituitary gland) in post-menopausal women, both strategies designed to reduce levels of circulating estrogens (Dellapasqua et al., 2005; Wells et al., 1978). These approaches have largely been abandoned because of the morbidity associated with the surgical procedure and because of the development of new drugs that achieve chemical ovarian ablation and aromatase inhibitors that inhibit the aromatase enzyme that is

required for estrogen biosynthesis (Gruber et al., 2002). Antiestrogens can be divided into two main classes based on their chemical structure and different functions in tissues (Figure 1.2). Non-steroidal antiestrogens (Type I) such as tamoxifen, raloxifene and idoxifene demonstrate estrogen like activities in some tissues and antiestrogenic activities in other tissues and are, therefore, often called partial antagonists, or selective estrogen receptor modulators (SERMs). The so-called pure antiestrogens (Type II) such as ICI 182, 780 (Faslodex or Fulvestrant), ICI 164, 384 and EM-800 demonstrate little or no agonistic activity (MacGregor and Jordan, 1998; Osborne and Schiff, 2011; Sommer and Fuqua, 2001). Tamoxifen, an antiestrogen that blocks the binding of estrogen to ER α , has been the mainstay of endocrine therapy for the past three decades. The Food and Drug Administration (FDA) approved tamoxifen in 1977 for the treatment of women with advanced BC and subsequently for adjuvant treatment of primary BC (Jordan, 1988; Osborne, 1998). After administration, tamoxifen undergoes metabolism in the gastrointestinal tract and in the liver to generate 4-hydroxytamoxifen (OHT), which has high affinity for ER α (Ali et al., 2011; Jordan, 2003).

Data from large-scale, randomized, adjuvant treatment BC trials have shown that tamoxifen given for 5 years immediately after surgery for early stage ER α -positive BC reduces mortality by 28% and reduces BC recurrence by nearly 50%, with no improvement in recurrence or mortality in ER α negative patients (EBCT, 1998; EBCTCG, 2005). Tamoxifen has also been shown to be effective for the treatment of women with metastatic BC, with tumour regression in 30% of women for one year and stabilisation of disease in a further 20% of women (Ali et al., 2011; Ali and Coombes, 2002). However, long-term use of tamoxifen has been linked with some side effects, which include a 2.5-fold increase in the incidence of endometrial cancer and a 2-fold increase in thromboembolism (Cuzick et al., 2003). In addition, a large proportion of women with advanced ER α positive disease do not respond to tamoxifen and of those that respond to tamoxifen, many eventually become resistant to treatment with this agent. The majority of resistant patients remain ER α positive and frequently respond to another hormonal therapy, for example pure antiestrogens or aromatase inhibitors, evidence of a continued role for ER α in endocrine resistance (Johnston and Dowsett, 2003). Evidence so far indicates that the agonistic activity of

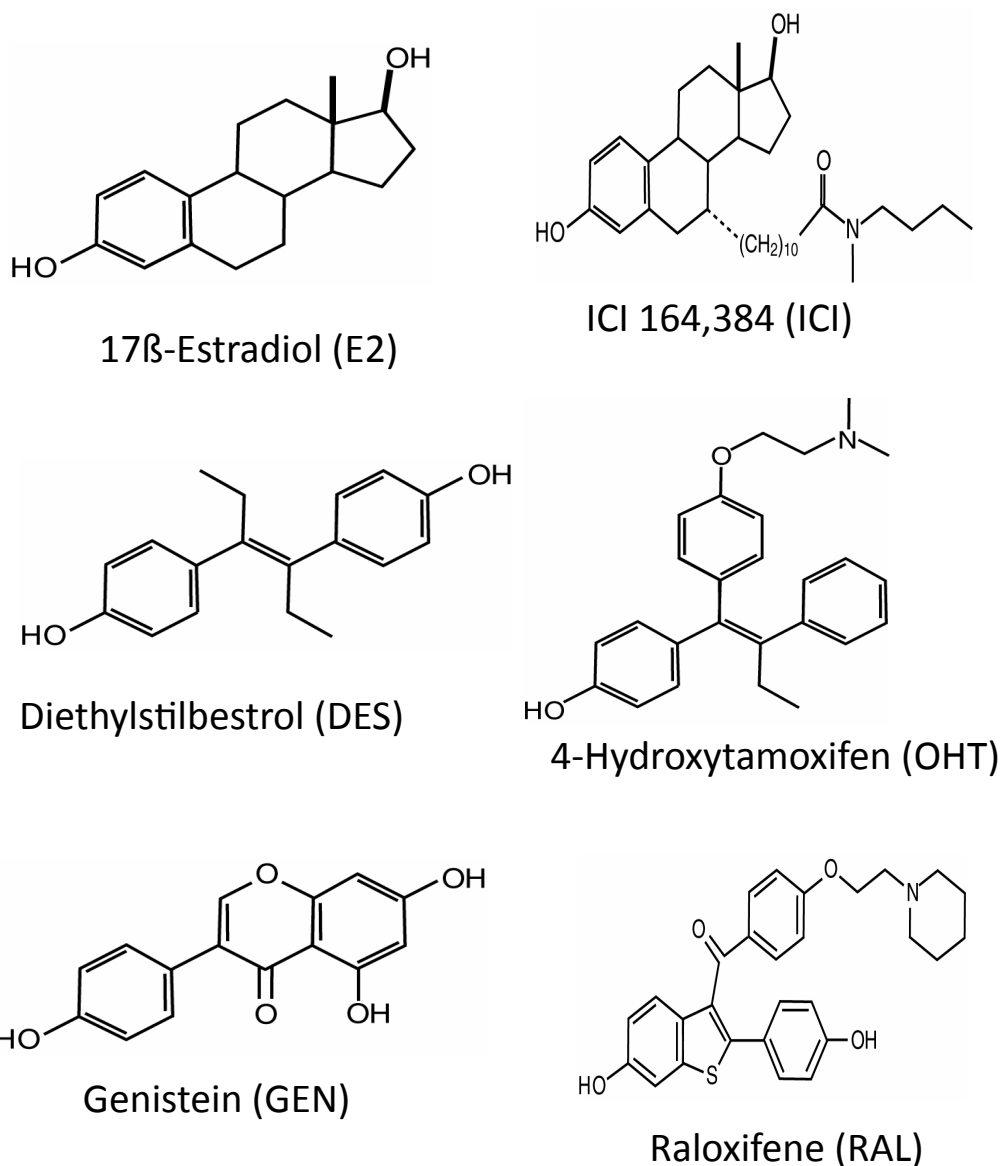


Figure 1.2. Chemical structure of estrogens and antiestrogens.

Shown are the chemical structures of circulating estrogens, non-steroidal antiestrogens, 4-Hydroxytamoxifen (OHT) and second generation SERMs, raloxifene. Fulvestrant (ICI 164,384) is a pure antiestrogen because it is devoid of agonistic activity and stimulates the degradation of ER α protein.

tamoxifen inhibits its efficacy against BC and that the acquisition of increased agonism in BC cells may play an important role in disease relapse (Ring and Dowsett, 2004). As a result, attention has been paid to the development of drugs that can block synthesis of estrogens or downregulate ER α , but lack estrogenic activity.

The pure antagonist ICI 164,384 and the derivative faslodex (ICI 182, 780 or fulvestrant) binds to ER α with 100 fold greater affinity than tamoxifen and inhibits ER α activity by a mechanism that involves blocking receptor dimerization and promoting nuclear export and degradation, resulting in abrogation of estrogen signalling with no agonist activity (Osborne et al., 2004; Riggins et al., 2007). Importantly, the majority of ER α expressing, tamoxifen resistant BCs continue to respond to fulvestrant. However, chronic exposure of BC cells to fulvestrant can also generate resistance (McClelland et al., 2001; Sommer et al., 2003). The results of recent clinical trials suggest that compared to tamoxifen, aromatase inhibitors are more efficacious in the treatment of post-menopausal women with ER α positive BC (Gradishar, 2004; Strasser-Weippl and Goss, 2005), although unlike tamoxifen, which is as effective in ER α positive BC in premenopausal women as in post-menopausal women, aromatase inhibitors appear to be ineffective for the treatment of premenopausal women (Gonzalez Martin et al., 2010).

Aromatase inhibitors work by inhibiting the function of the enzyme responsible for the conversion of androgens such as testosterone and androstenedione to 17 β -estradiol and estrone (Johnston and Dowsett, 2003; Smith and Dowsett, 2003; Strasser-Weippl and Goss, 2005). It is hypothesised that complete removal of the endogenous ligand for ER α might stop activation and, therefore, transcriptional activity in BC cells. The main source of circulating estrogens in premenopausal women comes from the ovaries. In post-menopausal women, estrogens are synthesised in a number of extragonadal sites where it functions locally, including the mesenchymal cells of adipose tissue, osteoblasts and chondrocytes of bone, the vascular endothelium, aortic smooth muscle cells, and numerous sites in the brain (Simpson and Dowsett, 2002). It has been estimated that estrogens levels in BCs of postmenopausal women are 20 times higher than levels in the plasma (Pasqualini et al., 1996), with aromatase expression being greatly elevated in breast tumours associated stroma (Ghosh et al.,

2011). In general, there are two types of aromatase inhibitors: type I (steroidal or irreversible) and type II (non-steroidal or reversible) (Brueggemeier, 1994). A number of aromatase inhibitors have been developed and used in clinical studies over the past 20 years (Buzdar and Howell, 2001; Goss and Strasser, 2001). The inhibitors in clinical use today are the third-generation, non-steroidal agents anastrozole and letrozole and the third-generation steroidal aromatase inhibitor exemestane (Johnston and Dowsett, 2003; Strasser-Weippl and Goss, 2005).

In postmenopausal women, third generation aromatase inhibitors suppress circulating estrogen levels by approximately 98% (Geisler et al., 2001; Geisler et al., 2002; Geisler et al., 1998; Geisler et al., 1996). In premenopausal women, the use of aromatase inhibitors causes incomplete estrogen suppression and more gonadal stimulation. In animal models, it has been reported that the use of aromatase inhibitors is connected to increased ovarian weight (Sinha et al., 1998). As a result, these inhibitors are used only in postmenopausal breast cancer women or premenopausal breast cancer women whose ovarian estrogen production has been ablated (Johnston and Dowsett, 2003).

1.5 Structure - function relationships and the estrogen receptor

1.5.1 The nuclear receptor superfamily

As indicated previously, ER α and ER β are members of the NR superfamily of ligand-regulated transcription factors, which in humans comprise 48 members. NRs mediate cellular responses to steroids, retinoids and thyroid hormones and are responsible for key aspects of eukaryotic development, differentiation, reproduction and metabolic homeostasis. Abnormal NR signaling causes metabolic, reproductive and proliferative diseases such as cancer, diabetes, obesity and infertility (Bain et al., 2007; Blumberg and Evans, 1998; Gronemeyer et al., 2004; Mangelsdorf et al., 1995). NRs can be classified into three groups based on their ligand binding- the so-called endocrine receptors, the orphan receptor family and the orphan receptor family (Figure 1.3) (Chawla et al., 2001).

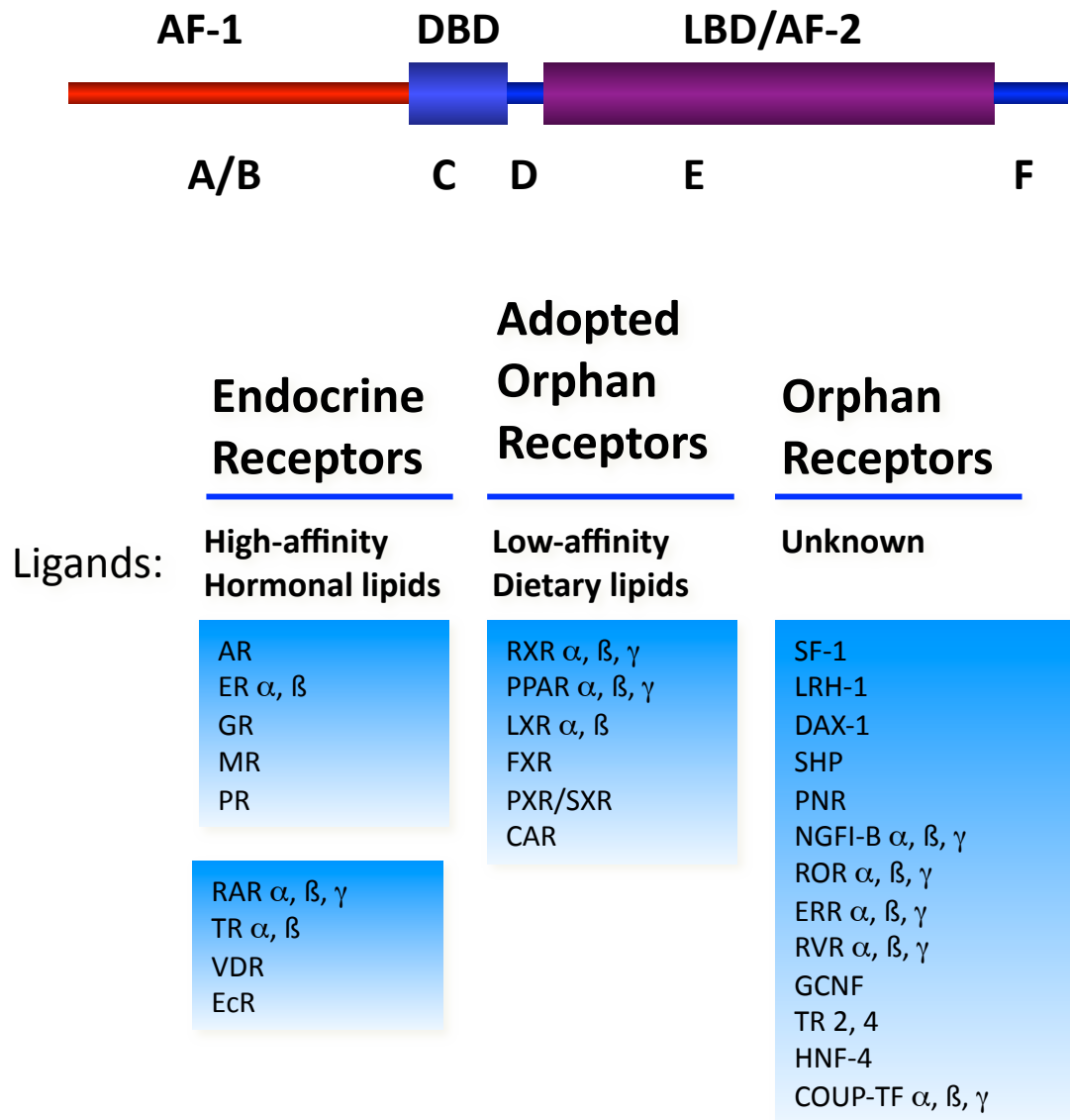


Figure 1.3. The nuclear receptor superfamily.

Shown is a schematic representation of the domain structure for a nuclear receptor, comprising six domains (A-F). Upon ligand binding, nuclear receptors stimulate gene expression through two transcription activation functions, AF-1 and AF-2. The DNA binding domain (DBD) and the ligand binding domain (LBD) are also represented. Also shown is the classification of human nuclear receptors, based on ligand binding.

The endocrine receptor family, which encompasses receptors that bind to hormonal lipids with high affinity, includes ER α and ER β , as well as the androgen glucocorticoid, mineralocorticoid and progesterone receptors. These receptors bind to palindromic DNA response element in target genes promoters as head to head homodimers (Khorasanizadeh and Rastinejad, 2001), although ER α and ER β can also bind DNA as heterodimers (Cowley et al., 1997). The endocrine receptor group also includes retinoic acid receptors (RAR α , β , γ), thyroid hormone receptors (TR α , TR β) and the vitamin D3 receptor, which bind to their responsive element as heterodimers with retinoid X receptors (Figure 1.4) (Chawla et al., 2001). The second group of NRs includes receptors that bind with lower affinity to ligands, often dietary lipids that may be present in target tissues in large amounts. This group includes the retinoid X receptors (RXR α , β , γ), peroxisome proliferator activated receptor (PPAR α , β , γ), Farnesoid X receptor (FXR) and liver X receptor (LXR α , β) (Chawla et al., 2001). The third groups of receptors have long been termed the orphan receptor family. The orphan receptors are so-called because no physiological ligands have as yet been identified for these NRs. Ligands for some receptors in this group have recently been identified. For example, liver receptor homolog 1 (LRH-1) binds phosphatidylinositols (Krylova et al., 2005; Lee et al., 2011; Ortlund et al., 2005) and phosphatidylcholines (Lee et al., 2011) and these ligands can regulate LRH-1 activity. It is possible that some members of the orphan receptor group do not have ligands and act in a constitutive manner, with their activities are regulated by post-translational modification such as phosphorylation, for example NGFI-B (Fahrner et al., 1990).

NRs share a common structure, with a C-terminal ligand binding domain (LBD). Ligand binding results in a conformational change in the LBD that allows the recruitment of transcriptional coregulator complexes to promote chromatin remodeling and chromatin modification that results in gene activation or gene repression, depending on the promoter context and nature of ligand that binds to the receptor. DNA binding is mediated by two zinc finger motifs located N-terminal to the LBD. Some, but not all NR also contain a region N-terminal to the DNA binding domain (DBD), which participates in gene regulation. Upon binding of ligand, NRs regulate the expression of genes by binding to DNA in promoter of genes (Green and Carroll, 2007; Perissi and Rosenfeld, 2005).

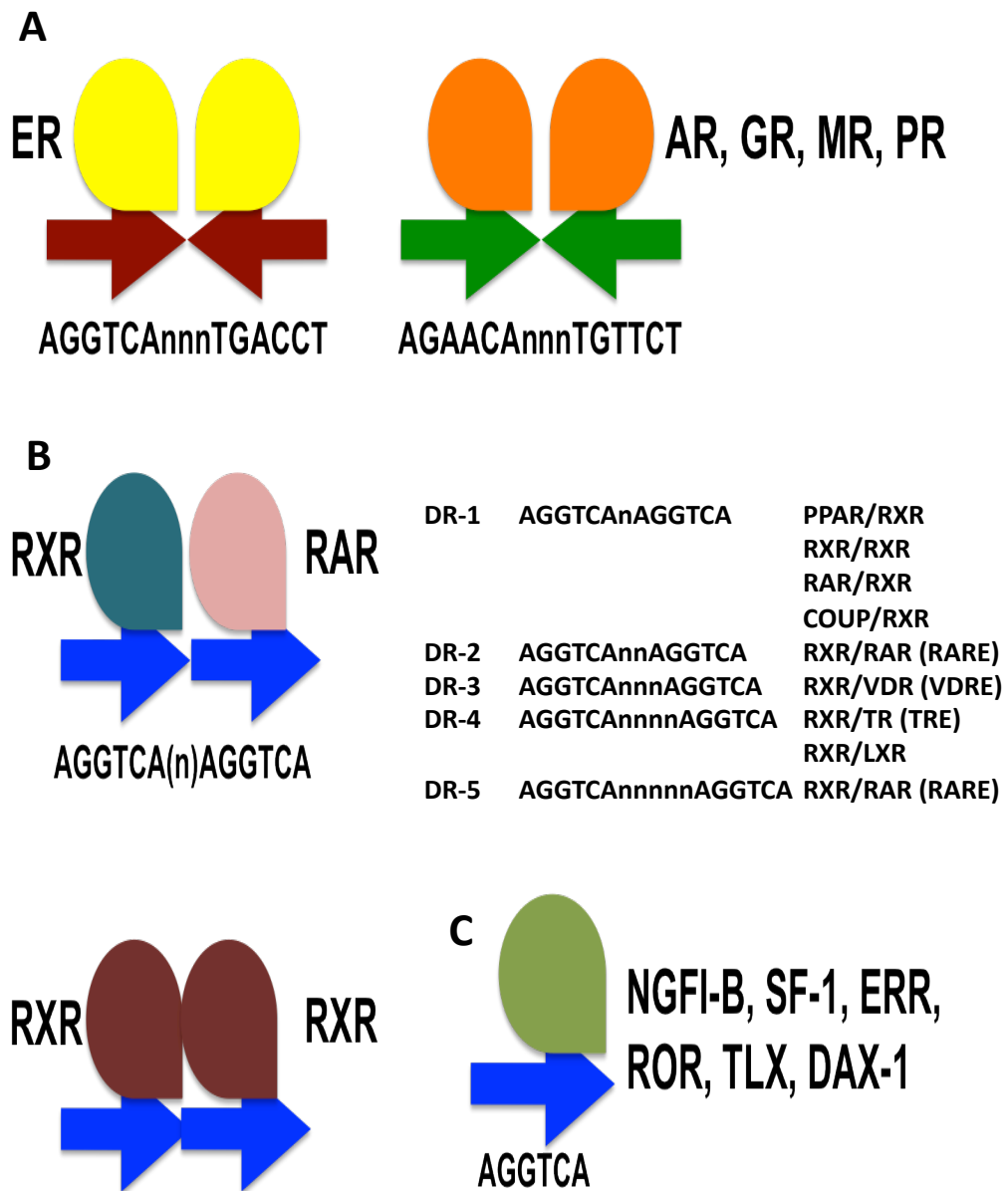


Figure 1.4. Different classes of DNA response elements used by nuclear receptors:

- Steroid receptors bind as homodimers. GR, PR, AR and MR bind to symmetrical repeat (palindromic) DNA response sequences, while ER binds to symmetrical sites based on the consensus AGGTCA (n_3) TGACCT
- RXR homo or heterodimers bind to direct repeats of the AGGTCA sequence. The number of nucleotides between their half sites specify the binding sites for various RXR heterodimers.
- Nerve growth receptor induced B (NGFI-B), SF-1 and other orphan receptors bind to DNA sequences containing AGGTCA flanked by specific 5' sequences.

The mechanisms of action of the DBD, LBD and coregulator recruitment are explained below with reference to ER, particularly ER α , which is perhaps the most intensively studied NR.

1.5.2 Estrogen Receptor structure and mechanisms of action

As described previously, the biological actions of estrogen are mediated by binding to two estrogen receptors, ER α (NR3A1) and ER β (NR3A2), encoded on chromosomes 6q25.1 and 14q23-2.1, respectively. Only ER α is important for mammary ductal growth, as very little growth was observed in ER α knockout mice, whilst ER β knockout mice showed normal development of mammary gland with regular ductal branching (Zilli et al., 2009). Similarly, gene knockout studies in mice have shown that the major phenotypes associated with estrogen action, including development of the mammary gland, uterus and in bone require ER α , with ER β playing only a minor role in these tissues (Couse and Korach, 1999; Curtis Hewitt et al., 2000; Korach, 1994).

The two ER subtypes have different tissue distributions, and although they bind with similar affinities to estrogen, they have different affinities and responsiveness to some SERMs. In BC cells, ER α appears to be the predominant regulator of estrogen responses (Speirs, 2003). At present, the role of ER β in BC is still unclear. However, some studies have suggested that ER β is anti-proliferative in the breast and that it may function to negatively regulate ER α activity (Hartman et al., 2009; Paruthiyil et al., 2004). By contrast, the importance of ER α in BC is quite clear, as the majority of BCs express ER α and only patients with ER α positive disease respond to hormonal therapies (Cuzick et al., 2010; Osborne, 1998). Studies of ER α function, particularly in model systems has established ER α as the key mediator of estrogen response in BC development and progression, by regulating the expression of genes that involved in proliferation and survival of BC cells (Ali et al., 2011; Green and Carroll, 2007).

The ER α and ER β genes are each encoded by eight exon genes spanning 140 kb and 40 kb, respectively (Ascenzi et al., 2006). As mentioned above, several splice variants for each gene have also been described, although the importance of these variants

remains unclear. The human ER α gene is transcribed into a 6.39 kb mRNA, and contains an 1800 bp open reading frame encoding a 595 amino acid polypeptide (Green et al Nature 1986). A comparison of the amino acid sequences of the human and chicken ER α proteins highlights the presence of six regions of amino acid sequence homology, named A through F that can be extended to other members of the NR superfamily (Krust et al 1986) (Figure 1.5). The blocks of amino acid sequence homology suggested that these regions encoded distinct functional domains, an idea that was confirmed through deletional and mutational analysis. Thus, the near central region C encodes the DBD, comprised of two zinc finger motifs, which mediate receptor dimerization and binding to specific DNA sequences known as estrogen response elements (ERE) in promoters of estrogen-regulated genes. Amino acids located at the C-terminal end of the first zinc finger, the so called P-box, determine the specificity of receptors for its response element (Green et al., 1988), while the second zinc finger mediates receptor dimerization between DBDs, with amino acid residues in the so-called D-box being critical for dimerization (Ascenzi et al., 2006; Schwabe et al., 1993) (Figure 1.6a). ER α and ER β bind to palindromic DNA response elements conforming to the consensus ERE sequence 5'-AGGTCANNNTGACCT-3' (N corresponds to any nucleotide), as homodimers or as ER α /ER β heterodimers (Green and Carroll, 2007; Nilsson and Gustafsson, 2011).

Region D, or the hinge region, is also seen to be highly divergent in ER α from different species, and across the NR superfamily, and has been implicated in co-regulatory protein binding. The hinge region includes both nuclear localisation signals (NLS) and also sites for post-translational modifications (Sentis et al., 2005).

The A/B region is highly divergent between ER α from different species and is poorly conserved between ER α and ER β , as well as within other NR. This region can vary considerably in length, from just a few amino acids in some NR, for example thyroid hormone receptors, to more than 500 amino acids in the human androgen receptor (AR) (Robinson-Rechavi et al., 2003; Tata, 2002). Deletion of the A/B region in ER α reduced ER α activation of reporter genes in a cell-specific manner, indicating that this region is required for transcriptional activation by ER α (Kumar et al., 1987). In the

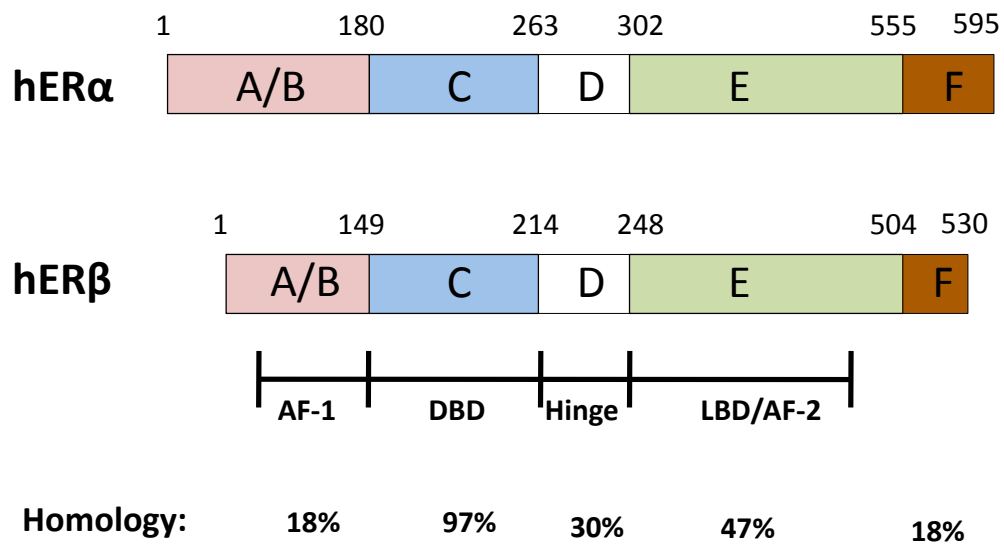


Figure 1.5. A diagrammatic representation of the structure of ER α and ER β .

Shown are the comparison of amino acid sequences of human ER α and ER β . Regions A/B encode ligand independent transcription activation function AF-1, region C contains the DNA binding domain (DBD) and nuclear localisation signal, D encodes the “hinge” region, E encodes the ligand binding domain (LBD) and ligand dependent transcription activation function AF-2 and E region play a modulatory role on both AF-1 and AF-2. (Zilli et al, 2009).

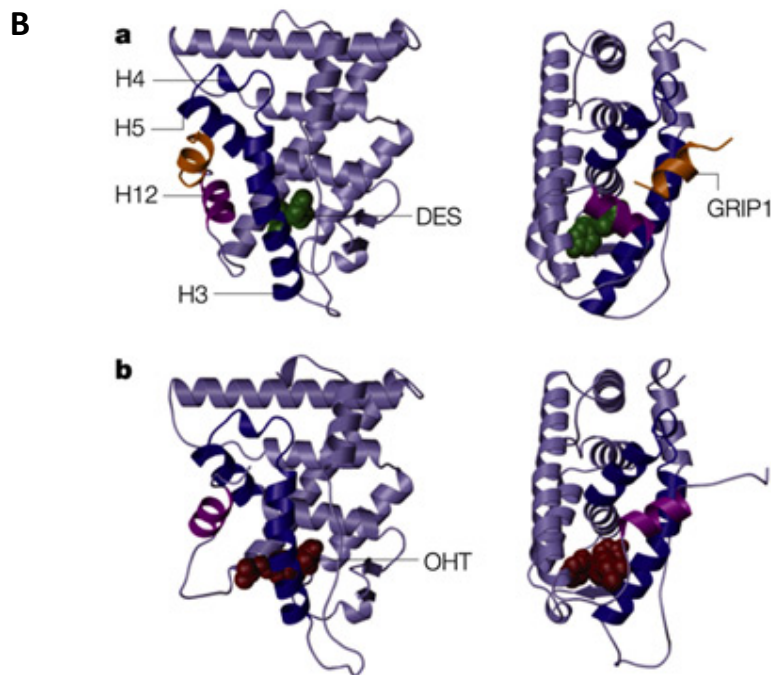
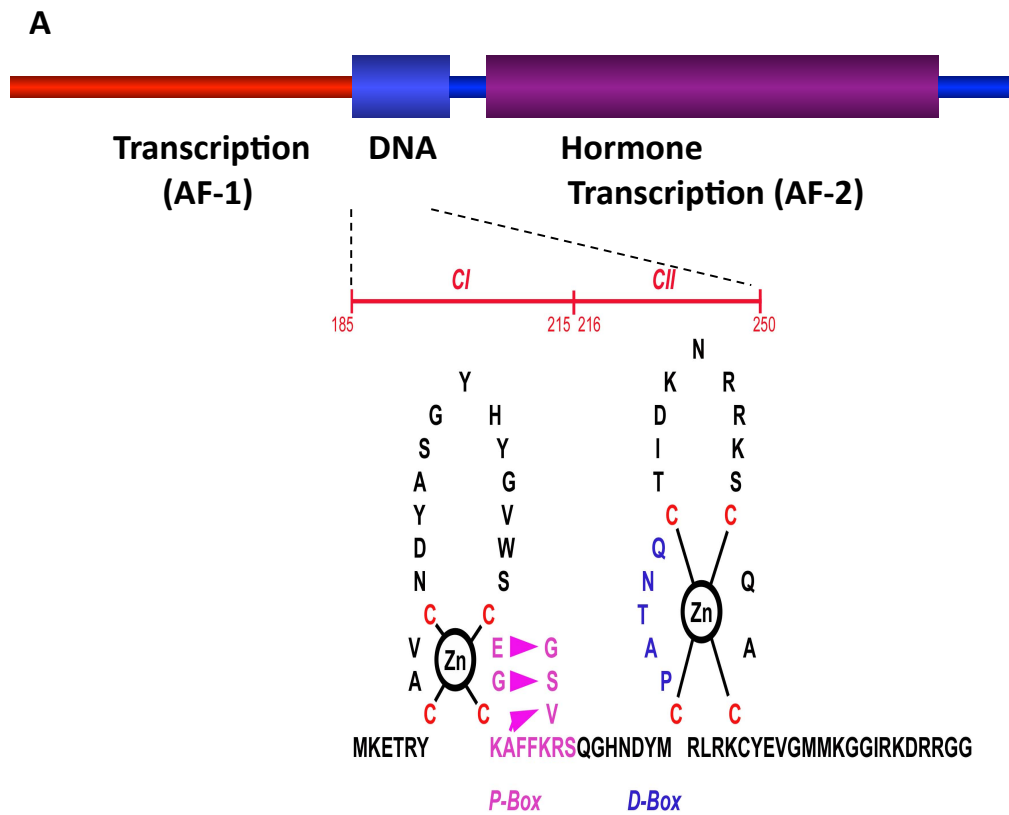


Figure 1.6. Structures of the ER α DBD and LBD.

- A. Schematic representation of ER α DBD: Amino acid residues shaping the P and D box are shown in red and blue color, respectively. The P-box at the C-terminus of the first zinc finger construct specific contacts with bases in the estrogen responsive elements. The D-box in the second zinc finger involves in receptor dimerization (Figure kindly provided by Prof. Simak Ali).
- B. Ribbon structure of three dimensional structure of ER α LBD bound to the estrogen agonist diethylstilbesterol (DES, green sphere), is shown in two views.
- a. showing the LBD structure comprised of 12 α - helices and 1 β -sheet (arrow). Helix-12 (purple) is positioned to create a contact between LXXLL motif from coactivator (GRIP1) and ER α LBD (gold ribbon). b. ER α LBD bound to 4-hydroxytamoxifen (OHT, red sphere) is shown. In this situation, positioning of the OHT side chain and conformational changes in the LBD stabilize helices 12 (H12). Taken from Ali and Coombes, 2002.

absence of the LBD this region, named activation function 1 (AF-1), in combination with the ER α DBD, or a heterologous DBD, can activate gene expression in an estrogen-independent or constitutive manner (Berry et al., 1990; Metzger et al., 1995; Tora et al., 1989). However, complete transcriptional activation by ER α and ER β requires functional synergy with a transcription activation function in the LBD (Ascenzi et al., 2006).

The carboxy-terminal E/F regions encode the LBD, which mediates ligand binding, receptor dimerization and transactivation of target gene expression through transcription activation function activation function 2 (AF-2) (Evans, 1988). The LBD is the second most conserved domain within ER α and ER β . Overall, the ER α LBD is organized in an antiparallel α -helical fold sandwich, a structure that was first described for the human unliganded RXR α LBD and subsequently found to be common to the LBDs of most NRs (Ruff et al., 2000). The ER α -LBD is comprised of 12 α -helices (H1-H12) folded into a three layered anti-parallel α -helical sandwich structure, with a central core layer of H4, H5, H6, H8 and H9 being sandwiched on one side by H1 and H3 and on the other side by H7, H10 and H11. This structural arrangement generates a wedge shaped structure that contains a ligand-binding pocket at the end of domain. The ligand-binding pocket is closed on one side by an anti-parallel β sheet and on the other by H12 (Figure 1.6 B panel a). Structural studies of the ER α -LBD have shown that upon ligand binding, the receptor undergoes structural changes leading to receptor dimerisation and coactivator recruitment.

The ER α -LBD structure has been determined in complex with estrogen or estrogen agonists, such as diethylstilbesterol (DES) and with estrogen antagonists, such as 4-hydroxytamoxifen (OHT) (Brzozowski et al., 1997; Shiau et al., 1998). The AF-2 interaction surface in the LBD is comprised of amino acids in helix H3, H4, H5 and H12. The orientation of H12 is highly altered according to the nature of ligand being bound to the receptors. When the ER α -LBD is bound by agonists, H12 is positioned over the ligand-binding pocket and creates the surface for recruitment and interaction of coactivators, whereas when the receptor is bound to OHT, H12 is moved from its position over the ligand-binding pocket and re-located in the hydrophobic groove

formed by H3, H4 and H5. In this position, H12 occludes the coactivator interaction surface (Figure 1.6 B panel a).

The interaction of many coactivators with NR-LBD is mediated by a short α -helix motif with the consensus sequence LXXLL (where L is leucine and X is any amino acid) (Feng et al., 1998; Heery et al., 1997). The LXXLL motif, also known as the NR box, has been shown to be important for interaction between receptors and coactivators, where multiple motifs can exist within a distinct NR interacting domain. The LXXLL motif is bound to the H3, H4, H5 hydrophobic grove on the LBD, with H12 also participating in this interaction, as seen for an LXXLL motif for the GRIP1 coactivator (Figure 1.6B panel a). The coactivator is held in place through hydrophobic interactions via its leucines and the hydrophobic groove of the receptor. In addition, lysine at the C-terminus of H3 and a glutamate in H12 are hydrogen bonded to peptide bonds in the LXXLL motif, forming a charge clamp that stabilizes the receptor and coactivator interaction. Interestingly, in the OHT bound LBD structure, H12 takes up the position occupied by the coactivator LXXLL motif, thereby preventing coactivator recruitment by the LBD (Figure 1.6B panel b). It is now considered that this inhibition of coactivator recruitment by the OHT-bound ER α is, at least in part, responsible for the antagonist action of tamoxifen. In addition, other antiestrogen, ICI 164,384 has also been shown to prevent coactivator recruitment to the ER α -LBD through positioning of ligand side chains in the coactivator binding groove (Pike et al., 2001).

Corepressor proteins, such as nuclear-receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), interact with unliganded retinoic acid receptor (RAR) and TR and with antagonist-bound steroid hormone receptors through a distinct LXX I/H IXXX I/L motif, known as the corepressor-nuclear-receptor (CoRNR) box (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). Interestingly, this motif is related to the coactivator LXXLL motif, and can be consider as a more extended α -helix when compared to the short LXXLL containing helix. The CoRNR box binds in the same pocket that is required for coactivator binding, but being a more extended α -helix, pushes H12 away from its normal binding position in the coactivator occupied NR-LBD (Perissi and Rosenfeld,

2005). The ligand dependent exchange between coactivators and corepressors is dependent upon the interacting motifs that can be occupied in the binding pocket.

1.6 Estrogen receptor phosphorylation and ER α activity

ER α phosphorylation (Figure 1.7) is an important element of non-genomic or rapid estrogen signaling, with several studies showing it can also affect transcription mediated ER α events (Riggins et al., 2007). Phosphorylation at several sites in ER α increases its activity. Estrogen induces serine 118 phosphorylation (Ser-118) (Joel et al., 1998), while other kinases such as CDK7, which is associated with TFIIH (Chen et al., 2000), MAPKs (ERK1 and ERK2) (Kato et al., 1995) and glycogen synthase kinase-3 (GSK3) (Medunjanin et al., 2005) may also phosphorylate the Ser-118 site, resulting in estrogen independent transactivation. Ser-167 in the AF1 domain of ER α is phosphorylated by ribosomal S6 kinase (RSK), ERK and AKT kinases (Ali and Coombes, 2002; Riggins et al., 2007). It has been shown that overexpression of EGFR or AKT increases Ser-167 phosphorylation and reduces tamoxifen sensitivity, while knockdown of AKT reduces Serine167 phosphorylation (Glaros et al., 2006). Collectively, these results suggest that increased growth factor signaling activities confer resistance to endocrine therapy.

Ser-104 and Ser-106 are located in the AF1 domain of ER α , and are subject to phosphorylation by cyclin A/ CDK2 complexes. Mutation of Ser-104 and Ser-106 to alanine stops cyclin A mediated ER α transcriptional activity. Conversely, cyclin A increases ER α transcriptional activity in the presence of tamoxifen (Rogatsky et al., 1999). Collectively, these results suggest that cyclin A/ CDK may play a role in endocrine resistance by inappropriate phosphorylation of Ser-104 and Ser-106. Indeed, it has been reported in several studies that higher levels of cyclin A are associated with poor prognosis for BC (Bukholm et al., 2001; Michalides et al., 2002). Protein kinase A (PKA) is involved in Ser-236 phosphorylation and stimulate ER α activity in a ligand independent manner (Chen et al., 1999b). Indeed, higher levels of cyclic-AMP binding protein has been found in endocrine resistant tumours (Miller et al., 1990). Interestingly, PKA can phosphorylate Ser-305 and prevent tamoxifen from inducing an inactive conformation of ER α (Michalides et al., 2004).

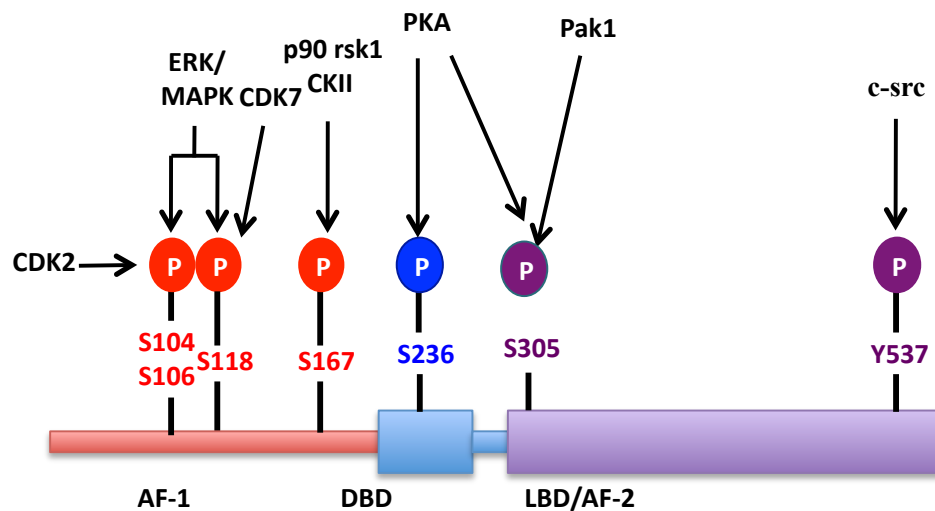


Figure 1.7. ER α phosphorylation sites and the kinases which target them.

Shown are the mapped ER α phosphorylation sites and the Protein kinases that have been shown to phosphorylate the different sites. Phosphorylation of serines 104, 106, 118 and 167 increase ER α activity. Serine 236 phosphorylation by PKA regulates ER α dimerisation. Phosphorylation within LBD regulates ER α activity, likely by altering ligand sensitivity.

Ser-305 can be phosphorylated by p21-activated kinase-1 (PAK1), which has shown to increase ER α transcriptional activity (Wang et al., 2002). PAK1 is a mediator of cell survival and migration, and is involved in tamoxifen resistance (Rayala et al., 2006). Rayala *et al* have shown that phosphorylation of Ser-118 is required for PAK1 mediated ER α transcriptional activity through Ser-305, meaning that these two sites may work together to regulate ER α activity (Rayala et al., 2006). Src family kinases can phosphorylate tyrosine-537 (Tyr-537), which is located in the AF2 domain of ER α . Mutation of Tyr-537 to either serine or alanine result in ligand independent ER α transcriptional activity, while mutation of Tyr-537 to phenylalanine increases basal ER α transcriptional activity (Arnold et al., 1995; Yudt et al., 1999).

1.7 Regulation of Gene Expression by ER α

Regulation of gene expression by ER α requires its recruitment to estrogen-regulated genes by direct binding to EREs or indirect binding through interaction with other transcription factors (Figure 1.8). The classical mechanism of ER α action involves ligand binding to the ligand-binding domain of the receptor followed by receptor dimerization and binding directly to EREs in the promoters of estrogen-regulated genes, thereby activating or repressing target gene transcription. (Carroll and Brown, 2006; Frasor et al., 2003; Heldring et al., 2007; Nilsson et al., 2001; O'Lone et al., 2004; Osborne and Schiff, 2005, 2011) (Figure 1.8).

1.7.1 ER α mediated gene regulation

ER α has been shown to alter gene expression at alternative regulatory DNA sequences, such as activator protein-1 (AP-1), specificity protein-1 (SP-1), cyclic AMP response elements (CREs) and upstream stimulatory factor sites, which directly bind c-jun/c-fos, c-jun/ATF2 and SP-1 (GC-rich SP-1 motif) transcription factors, respectively (Kushner et al., 2000; Sabbah et al., 1999; Saville et al., 2000; Xing and Archer, 1998). In these circumstances, ER α interacts with these transcription factors in order to stabilize their direct binding to their respective DNA recognition sequences and thereby stimulate transcription. This mechanism is referred to as a “non-classical” pathway for activation (Figure 1.8). The non-classical pathway regulates transcription

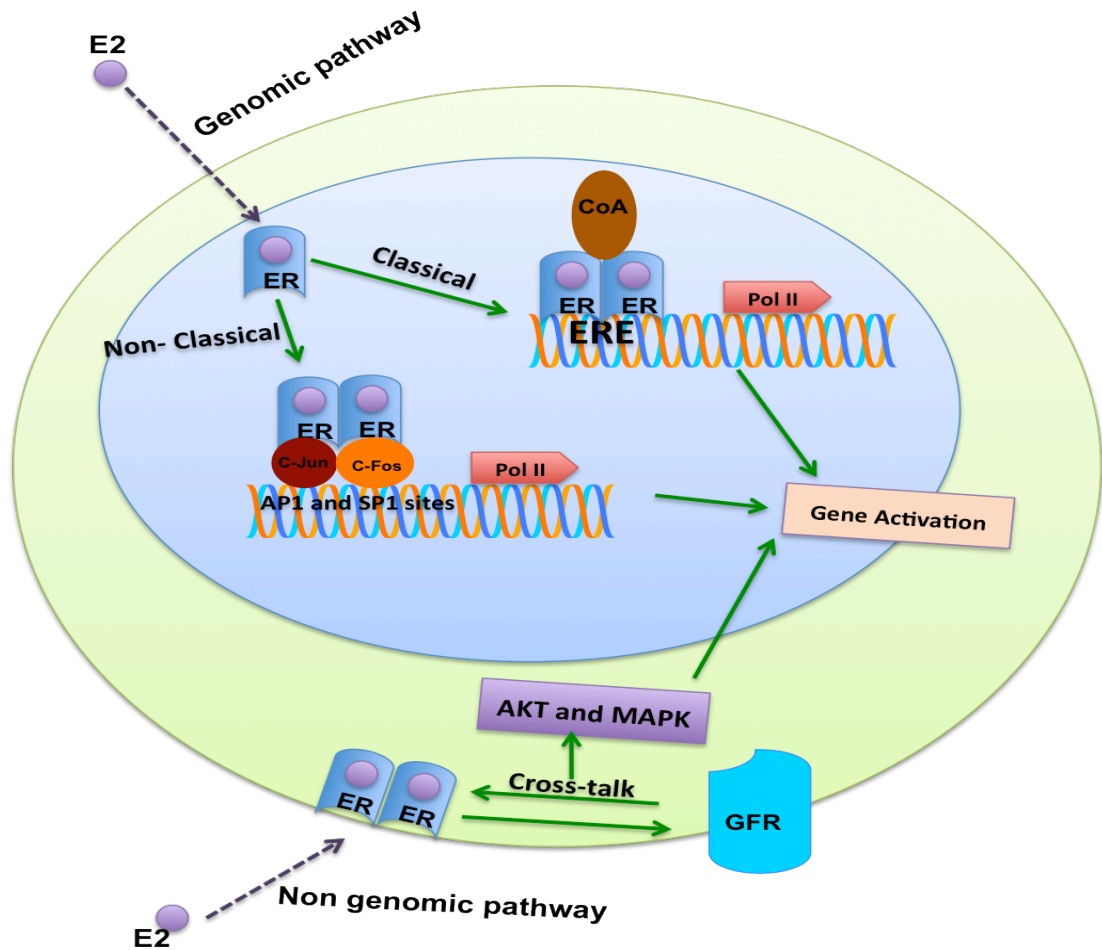


Figure 1.8. Estrogen receptor signaling pathways: Estrogen binds to ER in nucleus. Activated receptor bind to estrogen responsive elements (ERE) in target gene promoter as dimer and recruit coactivators to activate gene transcription (Classical pathway). Alternatively, activated receptor binds to AP-1 and SP-1 sites, which directly bind to c-jun and c-fos respectively and enhance gene transcription (Non-classical pathway). Estrogen can also regulate gene transcription by binding to membrane bound ER and cooperate with growth factor receptors such as EGFR and HER2 and activates the signal transduction pathways of MAPK and AKT, leading to activation of gene

of several genes involved in growth factor signal transduction pathways. Estrogen induced transcription of genes such as IGFR1, cyclin D1, myc and Bcl-2 are regulated by this pathway (Dong et al., 1999; Eeckhoute et al., 2006; Maor et al., 2006).

Recent studies using ChIP microarray (ChIP-chip) analysis in the MCF7 breast cancer cell line, in which DNA sequences to which ER α binds following estrogen treatment are profiled using genomic microarrays, show that the majority of ER α binding sites are located at considerably greater distances from transcription start sites than previously thought, often 50 kb or more away from these, with as little as 4% mapping to proximal promoter regions (Carroll et al., 2005; Carroll et al., 2006). ChIP-chip is a technique that combines chromatin immunoprecipitation with microarray that contain probes that cover extensive regions of the genome to define transcription factors binding sites. ChIP-Seq (a technique that combines chromatin immunoprecipitation with parallel high-throughput DNA sequencing) studies have similarly confirmed that 7% of the ER α binding sites map to promoter regions, with the majority of binding sites seen in these studies being located distally to transcription start sites (Welboren et al., 2009). The ChIP-chip studies have highlighted a previously unidentified ER α binding site 67 kb upstream of the c-Myc promoter and, for the cyclin D1 gene, emphasized the cell-type specific enhancer downstream of the coding region of the cyclin D1 gene (Carroll et al., 2005; Carroll et al., 2006; Eeckhoute et al., 2006). Together, these results highlight the significance of distal transcription factor binding sites in gene regulation. Indeed, recent studies using chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) analysis showed that most distal ER α binding sites are used with gene promoters through long-range chromatin interactions, suggesting that ER α acts through extensive chromatin looping, which bring genes and regulatory elements together for coordinated transcriptional regulation (Fullwood et al., 2009). ChIA-PET method exploits the proximity ligation concept to capture interacting DNA segments within DNA-protein complexes. Chromatin samples from cell cultures are cross-linked, sonicated, and chromatin immunoprecipitated. Separate aliquots of ChIP DNA are ligated to linkers, and proximity ligation is carried out. DNA is then restriction digested, purified on streptavidin-coated magnetic beads, and ligated to adapters for next-generation sequencing. Further studies have shown that a large number of estrogen responsive genes have forkhead factor (FoxA1) binding sites in close proximity to the mapped

EREs and at these EREs, FoxA1 is important for the recruitment of ER α to DNA (Carroll et al., 2005; Carroll et al., 2006). The importance of FoxA1 in this context is proved by siRNA-mediated knockdown of FoxA1, which results in an inhibition of the recruitment of ER α and estrogen mediated gene expression (Laganiere et al., 2005). These studies emphasize the importance of other transcription factors, in particular FoxA1, in the recruitment of ER α to EREs in estrogen responsive genes. These studies together with gene expression microarray analyses show that ER α regulates the expression of a large number of genes in breast cancer cells. An early gene expression microarray study carried out on 12, 000 genes showed that more than 400 genes demonstrated estrogen stimulation (Frasor et al., 2003; Frasor et al., 2004). These studies are in agreement with ChIP-chip results, which identified 3,665 ER α binding sites in MCF-7 cells (Carroll et al., 2006). Therefore, the estrogen-mediated growth of breast cancer cells appears to require the concerted action of a large number of genes. It is also important to note that these studies have also shown that in addition to increasing gene expression, ER α can repress the gene expression in breast cancer cells, for example expression of growth inhibitory genes and pro-apoptotic genes are repressed by estrogen.

1.8 Coactivator and corepressor proteins in ER α regulated gene expression

More than 300 coregulators that can modulate NR function have been described, but only 50-70 of these coactivators have been characterised (Lonard et al., 2007; Lonard and O'Malley, 2006). Key amongst the transcriptional co-regulators for ER α (Figure 1.9) are members of the p160 family, which stimulate ER α activity by interaction with the AF-2 domain, and which include the nuclear receptor co-activators, NCoA1 (also known as SRC-1), NCoA2 (TIF2, GRIP-1) and NCoA3 (AIB1, RAC3, SRC3) (McKenna and O'Malley, 2002a, b). Other key ER α coactivators include SWI/SNF complexes, CREB binding protein (CBP), p300/CBP Associated factor (PCAF) and the TRAP/DRIP/SMCC complex [thyroid-hormone-receptor-associated protein (TRAP), vitamin-D-receptor-interacting protein (DRIP), and SRB and mediator-protein-containing complex (SMCC)] (Nilsson et al., 2001). Numerous co-repressors have also been identified, with NCoR1 and the related NCoR2 (SMRT) being particularly important for NR function. (Chen and Evans, 1995; Glass and Rosenfeld, 2000).

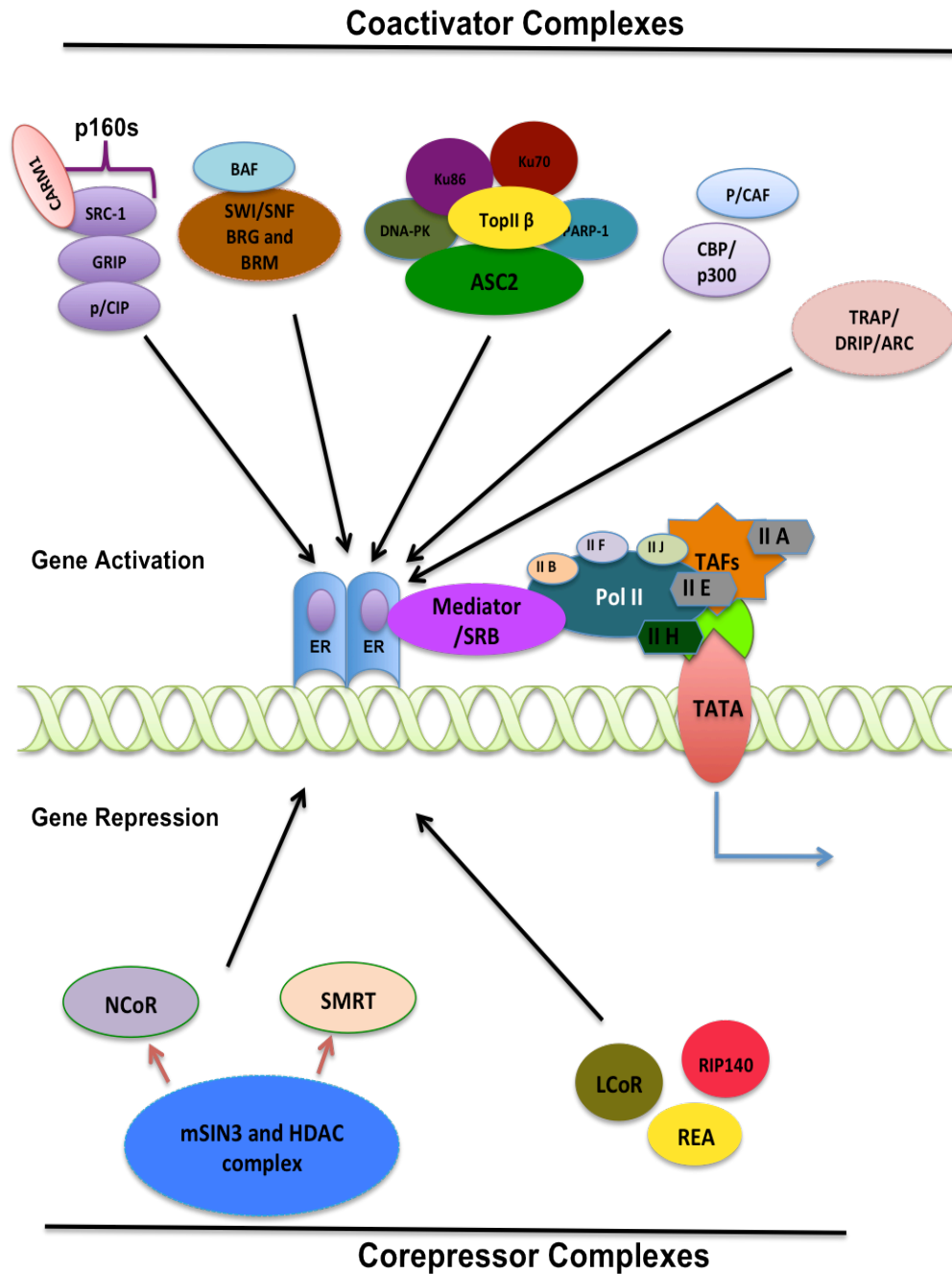


Figure 1.9 Coactivator and corepressor complexes in estrogen receptor regulated gene transcription.

Ligand activated estrogen receptor binds to specific sequences in gene promoters and recruits various coactivator complexes including p160 proteins, SWI/SNF proteins, DNA repair complexes, CBP/p300 and TRAP/DRIP proteins in a parallel or sequential manner to activate transcription. p160 proteins increase ER transcriptional activity, whereas SWI/SNF complex possess ATP- dependent chromatin remodeling activities. CBP, p300 and p/CAF proteins have histone acetyltransferase activities. Topoisomerase II beta and repair proteins cause sequence specific DSB and repair, which alters local chromatin and allows efficient binding of coactivators. Corepressors NCoR or SMRT recruit corepressor complexes mSin3 and HDACs proteins to repress gene transcription. LCoR, RIP140 and REA binds to promoter in ligand dependent manner

1.8.1 The p160/SRC coactivator family (SRC-1, SRC-2 and SRC-3)

The p160 family are well-characterised coactivators and are highly homologous proteins (McKenna et al., 1999). Critical to the functioning of these coactivators is the central ER interaction domain consisting of three equally spaced conserved LXXLL motifs, also known as the NR box (Xu et al., 1999). This motif represents the primary docking site in the AF-2 domain of estrogen receptor and is present in many coactivators (Nilsson et al., 2001). In addition, the p160 coactivators have been shown to bind within the AF-1 domain of ER α (Webb et al., 1998). RNA helicase proteins p68/p72, which is found in a complex containing SRC coactivators and the RNA coactivator steroid receptor RNA activator (SR), bind to AF-1 and AF-2 domain of ER α to increase the transcriptional activity (Endoh et al., 1999; Watanabe et al., 2001; Wortham et al., 2009). The p160/SRC family proteins contain two different transcription activation domains, AD-1 and AD-2. AD-1 domain is engaged in recruitment of CBP/p300 coactivators, while AD-2 is involved in the recruitment of protein methyl transferase enzyme known as co-activator associated arginine methyl transferase (CARM1) (Chen et al., 1999a). The C-termini of SRC-1 and SRC-3 exhibit intrinsic lysine acetyl transferase activity. The fact that the p160 coactivators interact with histone acetyl transferase (HAT) complexes consisting of CBP/p300 and p300/CBP associated factor (pCAF) indicate that p160 coactivators are involved in recruiting chromatin modifying enzyme to liganded estrogen receptor (Glass and Rosenfeld, 2000).

1.8.2 CBP/p300 coactivators

CBP/p300 are ubiquitously expressed proteins that are involved in many signalling pathways. These coactivators are generally viewed as general coactivators and possess histone acetyl transferase activity. They act as coactivators for different transcription factors, including nuclear receptors as well as directly interacting with components of the basal transcription machinery (Dilworth and Chambon, 2001). Several studies have reported that CBP/p300 can interact directly with nuclear receptors (Chakravarti et al., 1996; Kamei et al., 1996). Similar to SRC proteins, CBP/p300 can functionally interact with ER α to increase transcription activity (Kraus and Kadonaga, 1998). CBP and p300 may recruit other proteins with HAT activity,

such as p/CAF and GCN5L2 (hGCN5), which together enhance chromatin remodeling, thereby enhancing gene transcription (Girault et al., 2006).

1.8.3 SWI/SNF complexes

The SWI/SNF (switch/sucrose non-fermenting) complexes, originally identified in yeast, are large complexes that mediate ATP dependent chromatin remodelling to regulate cellular processes including transcription, replication, DNA repair and recombination (Trotter and Archer, 2007; Varga-Weisz, 2001). Human homologues SNF2 α (BRM1) or SNF2 β (BRG) and BAF57, a subunit of SWI/SNF, have been shown to interact with ER α in ligand dependent manner (Belandia et al., 2002; Bulynko and O'Malley, 2010; Chiba et al., 1994; Ichinose et al., 1997). In addition, functions of other coactivators are also dependent on the activity and recruitment of chromatin remodelling complexes. For example, BRG1 is required for SRC-1 and CBP mediated estrogen receptor coactivation (DiRenzo et al., 2000). In turn, SWI/SNF complexes require additional bridging molecules in order to target them to nuclear receptors. For example, interaction of BAF53, a subunit of SWI/SNF with Flightless-I (Fli-I) coactivator is important for recruitment of SWI/SNF to estrogen receptor target genes (Jeong et al., 2009; Lee et al., 2004). Sequential recruitment of p160 coactivator complex, Mediator complexes, and SWI/SNF complexes has been proposed to account for their combinatorial and complementary actions in mediating transcriptional activation (Jeong et al., 2009).

1.8.4 The TRAP/DRIP/SMCC complex

TRAP and DRIP binds to nuclear receptor, including ER α , in ligand dependent manner through direct interaction with TRAP220/DRIP205, a subunit of TRAP/DRIP complex (Burakov et al., 2000; Rachez et al., 2000; Rachez et al., 1998). As with other coactivators, TRAP220/DRIP205 interaction of nuclear receptor is mediated by LXXLL motifs. TRAP/DRIP subunits have also been found in the mediator complex, which is a complex of proteins that, together with suppressors of RNA polymerase II (SRB) associates with RNA polymerase II (Poll II) and are important for recruitment of Poll II to gene promoters (Rachez et al., 1998). TRAP/DRIP complex has been

identified in SRB and SMCC complex, which stimulate the activity of different classes of transcription factors, including p53 (Ito et al., 1999), indicating that TRAP/DRIP/SMCC complex play an important role in transcription initiation.

1.8.5 Coactivators with histone methyltransferase activity (HMT)

Histone methyltransferases are the second most common histone-modifying enzymes in the nuclear receptor coactivator family (Bulyanko and O'Malley, 2010). Arginine methyltransferases, coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1) can methylate histones and nuclear receptors and other coactivators (Lee and Stallcup, 2009a). SRC-3 has been methylated by CARM1, which leads to dissociation of CBP and CARM1 from SRC-3 and reduces SRC-3 stability. Methylation of SRC-3 by CARM1 regulates coactivator assembly or promotes disassembly to maintain the rapid assembly-disassembly cycle of transcription complexes on the promoter (Lee and Stallcup, 2009b). Methylation of a corepressor, RIP140 [also known as nuclear receptor interacting protein 1 (NRIP1)] by PRMT1 leads to suppression of corepressor activity of RIP140 by inhibiting interaction between RIP140 and histone deacetylase (HDAC) (Mostaqul Huq et al., 2006). Methylation of histone arginine 3(H4Arg3) by PRMT1 is important for successive acetylation of histone lysine 4 and 9 (H3Lys4 and 9) in ER α mediated pS2 gene activation (Wagner et al., 2006).

1.9 Transcriptional corepressors in estrogen receptor function

Many nuclear receptors repress gene expression in the unliganded state by recruiting transcriptional corepressors (Ali and Coombes, 2002; Glass and Rosenfeld, 2000; Xu et al., 1999). Gene expression microarray analysis has shown that a large number of estrogen-regulated genes are repressed by ER α (Frasor et al., 2003). Furthermore, tamoxifen bound ER α also represses the expression of some estrogen responsive genes (Frasor et al., 2004). Transcriptional repression by NR is mediated by the recruitment of transcriptional corepressors, in particular NCoR and SMRT, acting as molecular scaffolds for the recruitment of Histone deacetylases (HDACs) (Perissi et al., 2010b). NCoR/SMRT complex has also been associated with components of

SWI/SNF chromatin remodeling complex, including BRG1 and KAP-1 (Underhill et al., 2000), indicating that NCoR/SMRT can mediate chromatin remodeling to a repressive state. NCoR/SMRT recruitment, like coactivator, is mediated by LBD/AF-2 of nuclear receptors, in the absence of ligand. Ligand binding results in the dissociation of corepressors and the recruitment of co-activator complexes. Tamoxifen action appears to be mediated, at least in part, through its prevention of coactivator recruitment, but also through the recruitment of NCoR/SMRT (Ali and Coombes, 2002). As described previously (Section 1.5.2), corepressors bind to nuclear receptors through a core consensus motif LXX I/H IXXX I/L, the so-called corepressor-nuclear receptor box (CoRNR) (Perissi and Rosenfeld, 2005). Unlike NCoR/SMRT, other corepressors such as RIP140 and L-CoR are recruited through interaction with NR ligand binding domain through α -helical motifs having the consensus sequence LXXL motif, normally found in coactivators such as SRC1. Though there is no evidence to indicate that RIP140 and L-CoR are required for estrogen-responsive genes whose expression is stimulated by ER α , it is important to note that the majority of estrogen-regulated genes in breast cancer cells are those whose expression is repressed by estrogen. Another ER α corepressor, ZNF366, binds to the ER α DNA binding domain, and acts as a repressor by recruiting the corepressors CtBP and RIP140 to ER α regulated genes and exhibits direct interaction with HDACs (Lopez-Garcia et al., 2006). ZNF366 is interesting as its expression is considerably lower in breast cancer cells, compared to normal breast epithelial cells, suggesting that it acts to reduce ER α activity.

1.10 Transcriptional coactivators and corepressors in breast cancer

Both *in vitro* and *in vivo* data shows that coactivators and corepressors are essential for BC progression and for the response and resistance to endocrine therapies. In particular, amplified in breast cancer 1 (AIB1) has been seen to be overexpressed in BC cells (Anzick et al., 1997) and in breast tumours, when compared to normal tissue, with the overexpression being associated with high-grade tumours (Hudeliste et al., 2003). High levels of AIB1 have been associated with a greater chance of relapse and decreased overall survival (Lonard et al., 2007; Shou et al., 2004). In addition, overexpression of SRA, SRC-2/TIF2 and CBP in breast tumours compared to normal

tissues has been documented (Girault et al., 2006; Murphy et al., 2000). Further, transgenic mice overexpressing AIB1 develop mammary tumours, with 85% of the tumours being ER α positive (Torres-Arzayus et al., 2004; Torres-Arzayus et al., 2006), suggesting that AIB1 is an oncogene, at least in ER α positive BC. SRC-1 levels have also been associated with shorter disease-free survival in BC (Myers et al., 2004).

In the case of corepressors, strong expression of the repressor of ER activity (REA) in breast tumours associated with slower cancer progression (Girault et al., 2006). On the other hand, low levels of NCoR and SMRT have been reported to be associated with a shorter disease-free interval and a decreased rate of survival (Girault et al., 2003; Green et al., 2008). A role for these coregulators in tamoxifen resistance is also suggested by several *in vitro* studies. Long-term treatment of MCF-7 cells xenografts with tamoxifen results in the development of tamoxifen resistant tumours, characterised by low levels of NCoR (Lavinsky et al., 1998). Interestingly, in fibroblast obtained from mice in which the NCoR gene has been deleted, tamoxifen acted as an agonist (Jepsen et al., 2000) and siRNA mediated silencing of NCoR and SMRT resulted in tamoxifen mediated stimulation of MCF-7 cell growth (Keeton and Brown, 2005). Taken together, these findings suggest that the balance between coactivators and corepressors may dictate responses to endocrine treatments.

1.11 Mechanisms of resistance to endocrine therapy

Only about 50% of patients with ER α -positive BC respond to tamoxifen. Of those who respond, considerable proportions go on to relapse. Resistance to other anti-estrogens and to aromatase inhibitors is similarly observed (Ali and Coombes, 2002; Jordan and O'Malley, 2007; Musgrove and Sutherland, 2009; Normanno et al., 2005; Osborne and Schiff, 2011; Ring and Dowsett, 2004). De novo and acquired resistance to endocrine therapies is therefore a major clinical problem and has been the subject of intense study. A number of mechanisms have been proposed for the resistance of BC to endocrine therapy, the key ones being outlined below.

1.11.1 Loss of ER α expression/function and ER α mutations

Loss of ER α expression has been reported in 17-28% of patients with acquired resistance to tamoxifen (Gutierrez et al., 2005). Often, loss of ER α expression is accompanied by methylation of CpG islands in the ER α gene (Ottaviano et al., 1994). However, in the majority of cases of tamoxifen resistance, the tumours remain ER α positive and often tamoxifen resistant tumours will respond to aromatase inhibitors or to fulvestrant, implying that tumours with acquired resistance to tamoxifen still require ER α for their growth (Howell et al., 2004; Osborne et al., 2002). However, recent studies indicate that ER α gene amplification is a frequent feature in ER α positive breast tumours and has been associated with tamoxifen resistance. These ER α gene amplifications are associated with poor disease-free and overall survival (Nielsen et al., 2011).

Many studies have highlighted ER α splice variants, some of which, such as ER α lacking exon 5 sequences would encode a constitutively active and truncated ER α protein (Fuqua et al., 1991). ER α exon 5-deletion isoform (ER α Δ 5) devoid of most of the ligand-binding domain, but exhibit DNA binding and AF-1 domain. It has been shown in *in vitro* studies that ER α Δ 5 confer resistance to tamoxifen treatment. The ER α 36 splice variant predominantly localizes to the plasma membrane and cytoplasm and mediates non-genomic-signaling. ER α 36 lacks both transcriptional activation function domains (AF-1 and AF-2) but maintains the DNA binding domain. It has been reported that ER α 36 has been associated with tamoxifen resistance. In ER α 36 over-expressing cells, tamoxifen treatment fails to block the ER α 36 mediated activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway; instead, it promotes cell growth (Murphy and Watson, 2006; Riggins et al., 2008; Shi et al., 2009).

Mutations in the androgen receptor (AR) gene are commonly present in prostate cancer and are likely to be important determinants of prostate cancer progression and response to hormonal therapies (Brooke and Bevan, 2009). By contrast, very few ER α mutations have been observed in BC. A mutation of tyrosine 351 to asparagine has been identified in the ER α in tamoxifen resistant MCF-7 xenograft, which result in

tumour growth stimulated by tamoxifen (Wolf and Jordan, 1994). However, this mutation has not been found in human breast carcinomas (Johnston and Dowsett, 2003). However, several mutations that would result in amino acid substitutions in the ER α polypeptide sequence have been described in metastatic BC, one of which, resulting in substitution of tyrosine 537 in the LBD to asparagine is active in the absence of estrogen or in the presence of estrogen or tamoxifen (Zhang et al., 1997). Further, Fuqua et al reported a single amino acid substitution of lysine 303 to arginine mutation in 34% of hyperplastic breast lesions. Arginine at this position allows ER α to be more sensitive to very low concentrations of estrogen (Fuqua et al., 2000).

1.11.2 ER α coregulators and endocrine resistance

Coregulators such as coactivators and corepressors alter transcriptional activity of ER α . It is reported that increased transcriptional activities of activator protein 1 (AP1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) have been associated with endocrine resistance (Johnston et al., 1999; Schiff et al., 2000; Zhou et al., 2007). An increased expression of ER α coactivators, particularly, AIB1 has been associated with shorter disease free survival in patients undergoing tamoxifen treatment (Osborne et al., 2003). These data support the hypothesis that higher levels of ER α coactivators might augment the agonistic activity of tamoxifen and contribute to tamoxifen resistance (Normanno et al., 2005). PRMT1, an arginine methyl transferase, methylates ER α at R260 transiently, which leads to the formation of cytoplasmic complexes containing SRC kinase, PI3 kinase and focal adhesion kinase (FAK), which activate AKT pathway. However, it is not known whether this methylation event, which occurs frequently in BC, is responsible for endocrine resistance (Le Romancer et al., 2008). It has been proposed that another ER α coactivator proline-rich, glutamic acid-rich, and leucine-rich protein-1 (PELP1) is involved in tamoxifen resistance. PELP1 modulate ER α interaction with Src kinase, leading to enhanced mitogen-activated protein kinases (MAPK) activation and constitutive activation of AKT signaling. In addition, PELP1 interacts with epidermal growth factor receptor (EGFR) and involves in growth factor mediated ER α transactivation functions (Gururaj et al., 2006; Wong et al., 2002).

Increased expression of human epidermal growth factor receptor 2 (HER2), EGFR and insulin-like growth factor 1 receptor (IGF1R) can induce tamoxifen resistance by activating downstream signaling pathways including, MAPK/ERK, AKT/PI3 kinase and p38 MAPK pathways. It has been proposed that overexpression of HER2/ERBB2 is one of the main mechanisms of endocrine resistance (Arpino et al., 2008). Hurtado et al has shown that HER2 transcription can be regulated by paired-domain transcription factor (PAX2). ER α mediated repression of HER2 is dependent on competition between PAX2 and AIB1 for binding and regulation of ERBB2. Survival with BC following tamoxifen treatment increases when there are higher levels of PAX2 and lower levels of HER2, with loss of expression of PAX2 and more levels of AIB1 showing poor clinical outcome (Hurtado et al., 2008). Expression of ERBB2 can be suppressed by GATA4 and FOXP3 proteins, with increased expressions of GATA4 and forkhead box P3 (FOXP3) negatively regulate HER2 expression (Hua et al., 2009; Zuo et al., 2007).

1.11.3 Cell cycle regulators and endocrine resistance

Inappropriate expression of various cell cycle regulators confers resistance to endocrine therapy. Overexpression of c-Myc, cyclin D1, cyclin E1, while retinoblastoma (RB) inactivation, has been implicated in tamoxifen resistance. There is also evidence to suggest that overexpression cyclin E1 and RB inactivation reduces p27 expression and clinical response (Bosco et al., 2007; Butt et al., 2005; Caldon et al., 2006). Several microRNAs, such as mir-221 and mir-222 down-regulate p27 expression and confer resistance to tamoxifen, though the mechanism of resistance is unclear because these microRNAs also reduce ER α levels and are overexpressed in HER2/ERBB2 positive BCs (Miller et al., 2008; Zhao et al., 2008).

1.12 Transcriptional regulation and cycling of ER α binding

In eukaryotes, genomic DNA is packaged around histone proteins to form nucleosomes, which are further packaged into chromatin. Critical for gene regulation by transcription factors is chromatin remodelling at gene promoters. This includes ATP-dependent remodelling to alter nucleosome phasing and extensive modification of core histones, particularly involving lysine acetylation, and lysine and arginine

methylation (Strahl and Allis, 2000). Chromatin remodelling and modification is mediated by transcriptional coregulators that are recruited to gene promoters by transcription factors. As NR activities are often tightly regulated by ligand binding, coregulator recruitment has been particularly well studied for NRs, and especially for ER α (Glass and Rosenfeld, 2000; Green and Carroll, 2007; Lonard et al., 2007). Until recently, it was thought that such receptors initiate transcription as they remain bound to their DNA regulatory elements, where they are resident over long periods of time. However, it has now become very clear that proteins involved in transcriptional activation, DNA repair, chromatin modification, RNA splicing and processing, all show dynamic recruitment and release from DNA response elements (Hager et al., 2004). For example, Shang et al, showed for the first time by using ChIP experiments that ER α and its coregulators are recruited to gene promoters in a cyclical manner, so that they can efficiently initiate transcription (Shang et al., 2000).

The unliganded ER α appears to be maintained in an inactive state through association with the heat shock protein 90 (hsp90) complex (Zilli et al., 2009), although some studies have also shown that unliganded ER α cycles on and off the promoters of estrogen responsive genes, through a cyclical association and dissociation with transcriptional coregulator proteins. These events are unproductive for transcriptional initiation, but appear to be preparative for the initiation of productive transcription (Metivier et al, 2004). Estrogen binding by ER α causes a conformational change in the LBD, promoting dissociation of the hsp90 complex and post-translational modification, particularly phosphorylation (Perissi and Rosenfeld, 2005). An elegant study using ChIP has shown that upon estrogen treatment, ER α binds the cathepsin D (CTSD) gene promoter in a cyclical manner and recruits various coregulators, including members of the p160 family (SRC1, GRIP1 and AIB1), proteins with histone acetyl transferase activity (HAT), p300, CBP and pCAF and the DRIP complex in a cyclical manner. Finally, the basal transcription machinery is recruited to initiate gene transcription. Both ER α and coregulator recruitment return to basal levels 60 minutes following hormone treatment and load again onto CTSD promoter within 2 hours of estrogen treatment. The duration of each cycle is 40-60 minutes. This cyclical assembly and disassembly of ER α and coregulators correlates with cycles of transcription initiation on the CTSD promoter (Shang et al., 2000). A separate study by Metivier *et al* describes a comprehensive analysis of the coordinated

recruitment of up to 46 regulatory factors to another estrogen responsive gene promoter pS2 (TFF1). By performing sequential immunoprecipitations using a technique termed “re-ChIP”, that simultaneously measures the occupancy of a gene promoter by several regulatory factors, the authors discovered several transcriptional complexes assembled with ER α at the pS2 promoter. By synchronizing cells with the transcriptional inhibitor α -amanitin before treating cells with estrogen, Metivier *et al* were able to quantify the cyclical recruitment of ER α to the pS2 promoter. In addition, the authors show that three types of cycling occur in the presence of estrogen; an initial transcriptionally unproductive cycle that prepares the pS2 promoter for transcriptional activation, followed by two alternating transcriptionally productive cycles (Figure 1.10).

In the initial transcriptionally unproductive cycle, after estrogen treatment, ER α initiates the recruitment of chromatin remodeling complex, SWI/SNF complexes, to alter the local chromatin conformation so that it is permissive for transcriptional initiation. This complex repositions the nucleosome associated with the TATA box of the pS2 promoter, so that the TATA box remains outside the DNA occluded by the histone core. The additional recruitment of coregulators that have histone methyl transferase activity (HMT), CARM1 and PRMT1, and histone acetyl transferase activity (HAT), CBP, p300 and pCAF defines a transcriptionally permissive promoter. Transcription during transcriptionally productive cycles is initiated by ER α , which is responsible for recruiting various coregulators and the basal transcription machinery with Pol II. The kinetic associations of ER α and Pol II on the pS2 promoter show a periodicity of 40-60 minutes (Metivier et al., 2003). At the end of all transcriptionally productive cycles, SWI/SNF complexes, in association with HDAC are again recruited to the promoter to remodel local chromatin and restrict transcriptional engagement, with the ERE now located within the nucleosome core. This suggests that the cyclical recruitment of chromatin remodeling complexes may be important to reset the permissiveness of the promoter for transcription. The cyclical association of ER α on the pS2 promoter has been correlated with the proteasome-mediated degradation of ER α (Reid et al., 2003).

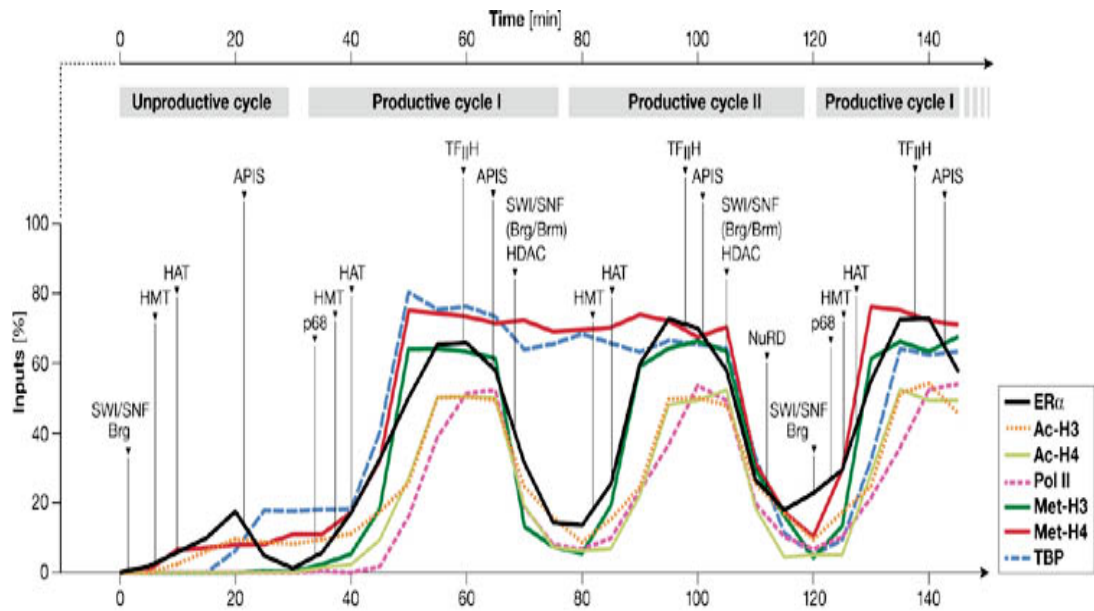


Figure 1.10 A model for transcriptional regulation and promoter cycling by ER α .

Kinetic ChIP analysis of the estrogen responsive gene pS2 showing cyclical recruitment of ER α and transcriptional coactivators and Pol II.

Taken from Metivier et al, 2006.

Reid *et al* showed that ER α mediated transcription and proteasome-mediated degradation of ER α are interlinked. In the absence of proteasome inhibitors, both unliganded and liganded ER α cyclically occupy the pS2 promoter every 20-45 minutes. However, by blocking the proteasomal pathway ER α loading and unloading on the pS2 promoter is slowed and transcription is inhibited. The authors further showed constant ubiquitination of ER α , making ER α transcriptionally inactive and tagging the protein for degradation. Antiestrogens, such as tamoxifen, compete with estrogen for ER α binding and induce a conformational change in which the recruitment of p160 coactivators is blocked (Brzozowski *et al.*, 1997). Shang *et al* observed that tamoxifen treatment induced ER α occupancy of the CTSD and pS2 promoters. However, the tamoxifen bound ER α complex did not recruit p160 coactivators such as AIB1, CBP and p300. In contrast, the recruitment of the nuclear receptor corepressors N-CoR and SMRT was observed on CTSD and pS2 promoters. These data demonstrate that in addition to inducing a conformational change in ER α that blocks coactivator recruitment, tamoxifen can induce the recruitment of ER α and corepressor complex to a gene promoter, suggesting tamoxifen and ER α complex may be actively involved in gene repression.

Fascinatingly, cycles of recruitment and release have also been observed for different NRs and for NF- κ B transcription factor, which suggests that the ordered and cyclic recruitment of several components within transcription complexes might be a common process for gene promoters that are regulated by inducible transcription factors (Perissi and Rosenfeld, 2005). The AR has been shown to regulate gene transcription by rapid and cyclic assembly of AR transcription complex on prostate-specific antigen (PSA) and kallikrein-2 promoters (Kang *et al.*, 2002). Similar results were observed for TR on the deiodinase, iodothyronine, type I gene (*dio1*) (Sharma and Fondell, 2002), for vitamin D receptor (VDR) on 24-hydroxylase gene (Kim *et al.*, 2005), and PPAR δ on pyruvate dehydrogenase 4 (PDK4) gene (Degenhardt *et al.*, 2009). All of these results show cyclical associations of nuclear receptors and coregulators on target gene promoters with a periodicity of 30 to 45 minutes. In addition, periodic inhibition of gene transcription is produced by clearing

transcription factors from gene promoters and the recruitment of HDACs and HMTs. Together, these studies demonstrate that transcription at the promoters of hormone responsive genes involves cycles of chromatin remodeling and Histone modification, requiring the orchestrated recruitment and dissociation of a large series of transcriptional coactivator and corepressor complexes, in addition to RNA polymerase II. The dynamic association and clearance of NR on inducible gene promoters is interesting because the kinetic processes of the cycling of NRs and their coregulators may signify a way to monitor the local gene environment and allow tight control over the expression of genes that are stimulated by hormonal treatment (Perissi and Rosenfeld, 2005).

1.13 DNA double strand break (DSB) in estrogen receptor regulated gene transcription

Two major pathways are involved in the repair of DSBs in mammalian cells: homologous recombination (HR) and nonhomologous end joining (NHEJ). NHEJ is an error-prone process, which repairs DSBs throughout the cell cycle, with the important phases being G1 and early S-phase of the cell cycle (Delacote and Lopez, 2008). NHEJ needs Ku heterodimer (Ku70/Ku80) proteins and DNA dependent protein kinase catalytic subunit (DNA-PKcs), DNA ligase IV, and X-ray repair cross-complementing protein 4 (XRCC4). Ku proteins bind with high affinity to DNA broken ends and recruit DNA-PKcs, which are then activated. Ku proteins then stabilise DNA-PKcs with DNA to join the broken DNA ends. DNA-PK is a serine/threonine protein kinase composed of a catalytic subunit and Ku70 and 80 acting as regulatory subunits (Collis et al., 2005; McKinnon and Caldecott, 2007). DNA-PK is the key component of the NHEJ pathway of DSB repair, as this recruits XRCC4/DNA ligase IV for ligation of the two DNA ends (Figure 1.13).

HR is an error free repair process and functions in the late S and G2 phases of the cell cycle. The process requires homologous undamaged DNA strands from sister chromatids as template for DNA polymerase to go past the break and requires RAD52 group of proteins (RAD50, RAD51, RAD52, and RAD54), RPA, XRCC2, XRCC3, and the breast cancer susceptibility protein (BRCA) (McKinnon and Caldecott, 2007).

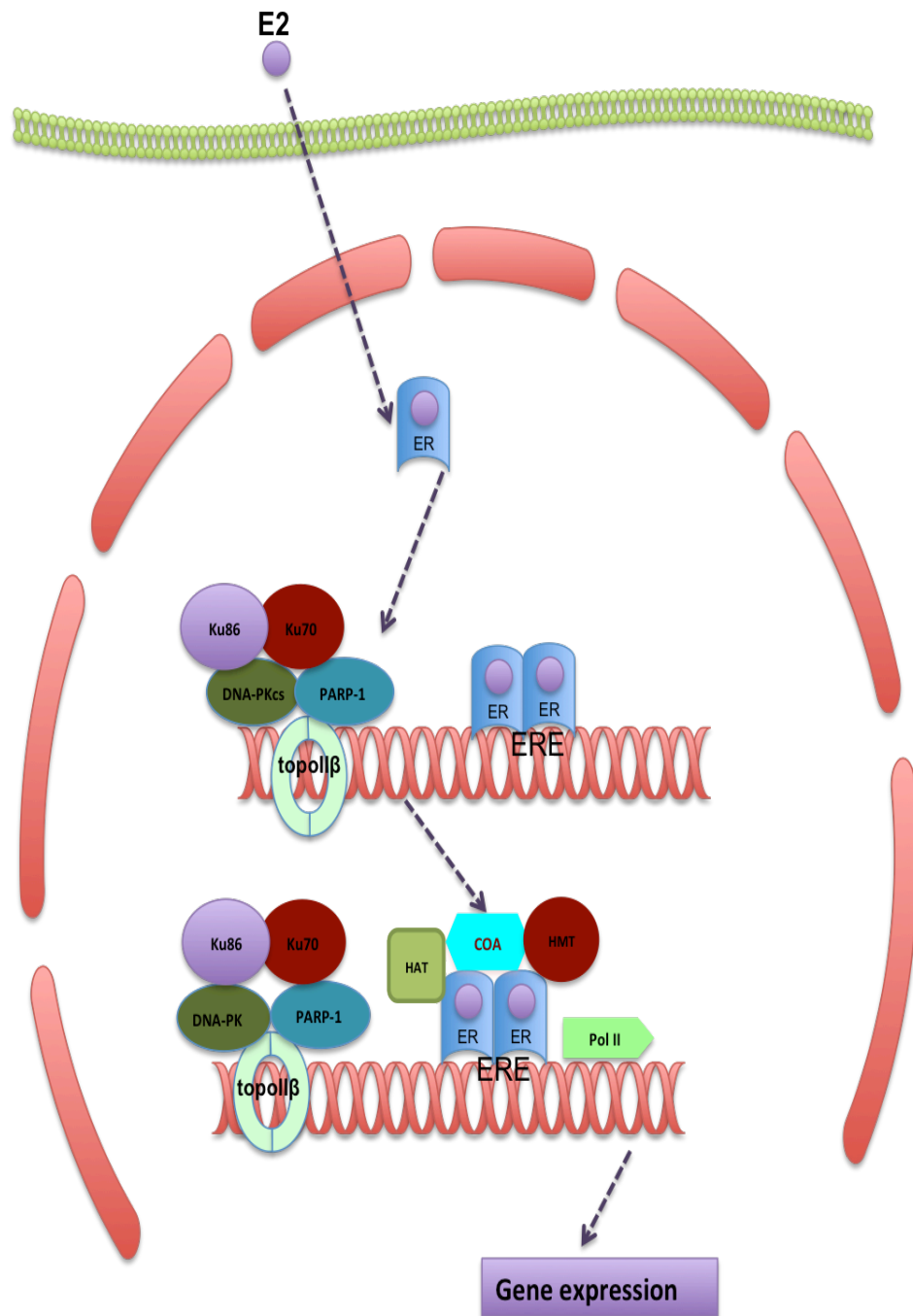
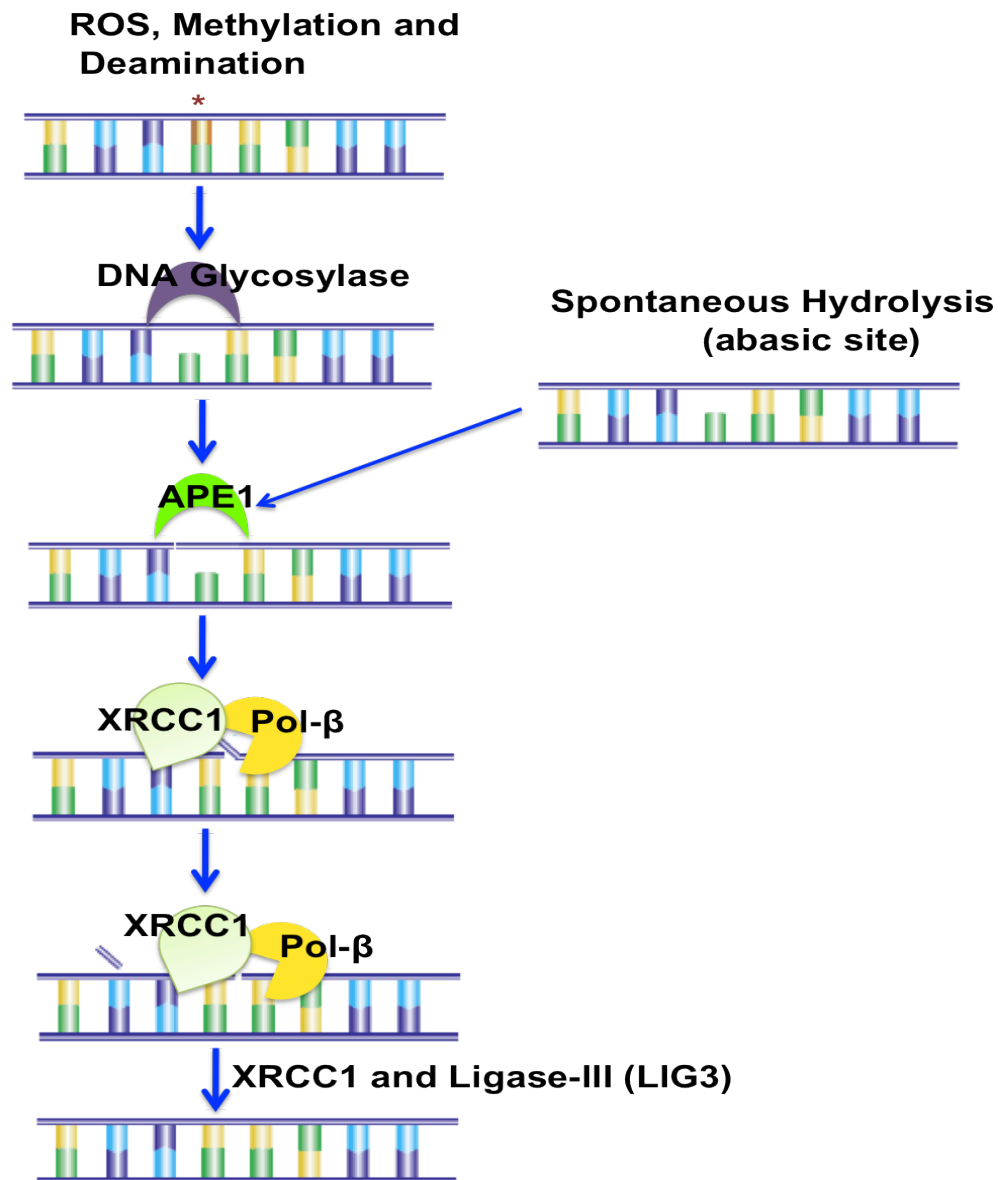


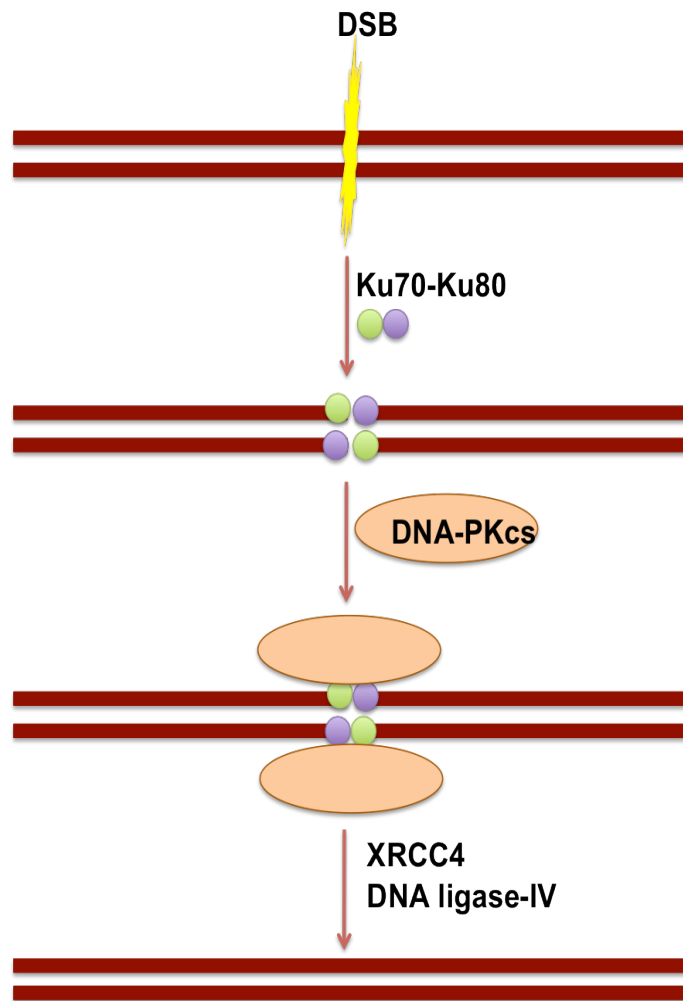
Figure 1.11. Estrogen induced DSBs.

In response to estrogen activation, estrogen bound ER recruits topoII β , NHEJ repair proteins DNA-PKcs, Ku70 and Ku86 and PARP-1 to target gene promoters to induce sequence specific DSB, followed by DNA repair by repair proteins. DSB relax local chromatin, leading to the recruitment of coregulators to the gene promoter and induction of gene transcription. Adapted from Ju et al, Science, 2006 and Susan P. Lees-Miller, Carcinogenesis, 2010.



1.12. Base excision repair process (BER).

DNA damage occurs by cellular metabolism, including damage resulting from reactive oxygen species (ROS), deamination, methylation, hydroxylation and the spontaneous hydrolysis of DNA bases. DNA glycosylases (e.g TDG or MBD4) cleave the damaged base from the sugar phosphate backbone, resulting in the exposure of an AP (Apyrimidinic/Apurinic) or abasic site in the DNA. Alternatively, AP sites also arise by spontaneous base hydrolysis. Abasic sites are recognized by the AP endonuclease (APE1), which nicks the phosphodiester backbone immediately 5' to the AP site, resulting in the formation of a 3' hydroxyl group and a transient 5' dRP (abasic Deoxyribose Phosphate). DNA polymerase β (Pol β) performs a one-nucleotide gap-filling reaction and removes the dRP residue via a lyase activity, and this is followed by sealing of the remaining nick by the XRCC1–ligase3 complex. Adapted from Jan H. J. Hoeijmakers, Nature, 2001.



1.13. DNA double-strand break repair through nonhomologous DNA end-joining (NHEJ).

The DNA broken ends are substrates for binding of the Ku70/80 heterodimer, which localizes DNA-PKcs to these ends. This promotes the juxtaposition of the broken ends. The broken ends may be processed further or, if no further processing is required, the other core components of nonhomologous DNA end-joining, XRCC4 and DNA ligase IV act to complete the end-to-end ligation. Adapted from Dik C. van Gent et al, Nature Genetics, 2001.

DNA DSBs in eukaryotes cause a rapid and widespread response in chromatin flanking the DNA break, as evidenced by phosphorylation of histone H2AX (γ H2AX) on the C-terminal Ser-139 residue (Rogakou et al., 1998). γ H2AX enhances the repair of the DNA break by either HR or NHEJ. Phosphorylated H2AX is seen within 1 minute of DNA damage. ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia mutated and Rad3 related) and DNA-PKcs have been shown to phosphorylate H2AX. Although there is evidence to suggest that these kinases can phosphorylate H2AX, ATM appears to be the major kinase associated with γ H2AX formation under normal physiological conditions (Hartlerode and Scully, 2009).

Recent evidence has revealed that induction of transcriptional activation by NRs and other transcriptional factors involves the recruitment of several proteins involved in DNA double strand break (DSB) and repair. In addition, proteins such as Ku70, Ku80, Topoisomerase II β (TOP2B), PARP1 and DNA-PK can be associated with NRs, including ER α , AR and the transcription factor USF1. Upon activation of transcription by NR, these proteins can bind to regulatory DNA sequences of target gene promoters to enhance transcription (Haffner et al., 2010; Ju et al., 2006; Lin et al., 2009; Mayeur et al., 2005; Wong et al., 2009). Interfering with the function of any of these proteins leads to decreased gene transcription, implying that DNA repair proteins play an important role in transcriptional regulation. Interestingly, Ju et al report that estrogen-dependent transcription of the pS2 gene in MCF-7 BC cells requires transient DSB in the promoter region. In addition, it was shown that the DSBs are generated by TOP2B. Reducing the levels of TOP2B, or blocking its catalytic activity considerably reduced DSB formation. Ju *et al* further demonstrated that DSBs are recognized by the DSB repair complex, including PARP-1, Ku70, Ku80 and DNA-PKcs (Ju et al., 2006), suggesting that non-homologous repair system involves in repairing TOP2B induced DSB (Figure 1.11).

In addition to the pS2 gene, Ju *et al* observed DSB dependent activation of the expression of genes regulated by other NRs, the AR regulated PSA gene, the RAR α regulated RAR β gene, the thyroid hormone receptor regulated Dio1 gene and the AP-1 regulated MMP12 genes, implying that DSB mediated gene transcription is a common mechanism for gene activation, at least for gene regulation by NR. In

agreement with these results, Williamson and Lees-Miller (Williamson and Lees-Miller, 2011) reported estrogen-induced ER α dependent formation of DSBs, as measured by γ H2AX foci, a sensitive marker that is phosphorylated within a minute of DSB formation (Kuo and Yang, 2008; Lobrich et al., 2010), on pS2 gene promoter in MCF-7 cells. It was further demonstrated that TOP2B is required for DSB formation on the pS2 promoter. In addition, Rad51 is colocalised at DSB sites, suggesting the involvement of homologous recombination repair (HRR) pathway in the repair of estrogen induced DNA breaks on the pS2 gene promoter.

Recently, Medunjanin *et al* demonstrated that upon estrogen stimulation in MELN BC cells, DNA-PK interacts with ER α and modulates ER α stability and activity by phosphorylating ER α at serine 118 (Medunjanin et al., 2010). It is now evident that efficient stimulation of ER α mediated gene transcription may require different forms of DNA damage, such as DNA cytosine deamination and DNA base alteration by local accumulation of reactive oxygen species (H₂O₂) at gene promoters (Metivier et al., 2008a; Perillo et al., 2008). It seems that these DNA damaging events recruit the appropriate DNA repair proteins, including those involved in base-excision repair (BER) machinery to target gene promoters in order to initiate transcriptional programmes (Kim et al., 2009; Metivier et al., 2008b; Perillo et al., 2008). DNA repair proteins such as apurinic/apyrimidinic endonuclease 1 (APE1) and flap endonuclease 1 (FEN-1) have been shown to interact with ER α and influences estrogen responsive gene expression (Curtis et al., 2009; Schultz-Norton et al., 2007). Since APE1 and FEN1 play an important roles in BER pathway (Figure 1.12) and are recruited to estrogen-responsive genes in breast cancer cells it appears that they could inspect DNA at the time of transcription and assist in repairing DNA damages. Indeed, increases in the repair of transcriptionally active genes compared to non-transcribed DNA regions, and selective repair of the DNA coding strand compared to the noncoding strand, indicate that transcription and DNA repair are functionally combined (Mellon et al., 1986; Mellon et al., 1987; Svejstrup, 2002). The interaction of ER α and DNA repair proteins could stabilise the transcription complex in allowing DNA repair and transcription to occur simultaneously. ER α has been shown to interact with free radical scavengers such as Cu/Zn superoxide dismutase (SOD1), thioredoxin (Trx), thioredoxin reductase (TrxR), protein disulfide isomerase (PDI).

Free radicals known to induce oxidative stress response in cells by damaging proteins and DNA. Each of the above mentioned free radical scavengers could modulate the expression of endogenous estrogen-responsive genes in MCF-7 breast cancer cells, with these proteins showing gene specific effect on estrogen responsiveness (Schultz-Norton et al., 2011).

Androgen treatment of LNCaP prostate cancer cells results in the recruitment of TOP2B to AR target genes as part of the mechanism described above for gene activation, with TOP2B recruitment inducing DSBs in target gene promoters, with γ H2AX foci forming around the DSBs. Further, TOP2B mediated DSBs have recently been shown to promote intrachromosomal rearrangements in which the AR regulated transmembrane protease serine 2 (TMPRSS2) gene is fused to members of the ETS gene family (ERG and ETV1) (Haffner et al., 2010). TMPRSS2-ERG and TMPRSS2-ETV1 fusions have been identified as prostate cancer specific rearrangements occurring in more than 50% of prostate cancers (Haffner et al., 2011). A separate mechanism for such rearrangements involves the AR dependent DSBs at specific TMPRSS2 and ERG rearrangement junction sites by the AR-dependent recruitment of activation-induced cytidine deaminase (AID) and LINE-1 repeat-encoded ORF2 endonuclease, which synergistically act to generate site specific DSBs at translocation loci that are repaired by NHEJ pathway (Lin et al., 2009). These findings are indicative of an important role for transcription factor induced DSB in human disease.

1.14 RNA based coactivator complexes

Steroid receptor RNA activator (SRA) is an exceptional NR coactivator in that it is an RNA (Lanz et al., 1999). Several laboratories have confirmed that SRA can coactivate a range of NRs including ER α and ER β , AR, GR, RAR α , PPAR γ , TR, and VDR. The recognition of RNA-binding domains within various coregulators, including SHARP (SMRT/HDAC1 associated repressor protein), peroxisome proliferator-activated receptor gamma, coactivator 1 (PGC-1), CoAA (coactivator activator), p68, p72, coactivator of activating protein-1 and estrogen receptor α and β (CAPER α , and β), has created much interest in understanding RNA-protein interactions in NR signaling. A number of coregulators with RNA recognition motif (RRM) influence transcript

splicing (CoAA, CAPER α , and β), at the same time others appear to target SRA and more directly influence NR transactivation (Colley et al., 2008). SHARP is a NR corepressor that associates with SRA *in vitro* and contains RNA recognition motifs (RRMs). These RRM motifs are required by SHARP to reduce SRA mediated estrogen-induced transactivation. Another ER α coregulator that binds SRA *in vitro* and copurifies with SRA from cell extracts is p72 (Watanabe et al., 2001). However, specific details of how each of these proteins interacts with SRA remain to be determined. Interestingly, p54^{nrb} (NONO) and PSF (SFPQ) proteins, which are RNA binding proteins and localized in sub-nuclear paraspeckles, have been shown to alter NR transactivation (Bond and Fox, 2009; Shav-Tal and Zipori, 2002).

1.15 DNA Methylation in estrogen receptor dependent gene activation

Genomic DNA in all prokaryotes and eukaryotes is subject to methylation, with methylation in prokaryotes being implicated mainly in “restriction”, a protective mechanism that enables foreign DNA to be distinguished from self DNA, allowing destruction of foreign DNA by specific endonucleases (Noyer-Weidner and Trautner, 1993; Wilson and Murray, 1991). DNA methylation on cytosines is an epigenetic mark found in vertebrate, plant, fungi and to a lesser extent, in insect genomes. Methylation, occurring mainly at CpG dinucleotides, can have profound effects on gene activity (Razin, 1998). Generally, methylation at CpGs in gene promoters is associated with stable gene silencing and is often associated with silencing of genes to prevent expression in inappropriate tissues. Proteins that bind to methylated CpG (MeCpG) binding proteins have also been identified (Boyes and Bird, 1991) and act as transcriptional repressors, recruiting histone deacetylase complexes to regions of CpG methylation, thereby maintaining a repressed chromatin state, whilst the transcriptional co-repressor NuRD complex contains DNA methyltransferases (DNMT) to facilitate methylation at repressed gene promoters (Fuks et al., 2000; Fuks et al., 2003; Nan et al., 1997). The patterns of DNA methylation are established and maintained by DNMT, which can act either on hemimethylated CpGs generated following DNA replication (DNMT1 or DNMT3A and 3B) or *de novo* on unmethylated DNA (DNMT3A and 3B). Maintenance methylation occurs during S

Phase, through a physical interaction between DNMT and the replication machinery (Araujo et al., 2001; Araujo et al., 1999).

The chemically unfavourable reaction to remove the methyl group from MeCpG, together with the lack of identified MeCpG demethylases have contributed to the general belief that DNA methylation is a stable mark that cannot be enzymatically removed, with multiple rounds of cell division without DNMT1 mediated remethylation being required to completely erase the epigenetic mark from DNA (Bird, 2002; Wolffe, 1998). However, there are potential examples of rapid, DNA replication-independent DNA demethylation. In terminally differentiated cells, hormones can trigger changes in chromatin structure and DNA demethylation at promoters of specific genes. A single dose of estradiol given to immature chickens leads to extensive demethylation of vitellogenin promoter regions and the activation of hepatic vitellogenin gene expression. The changes in methylation begin between 10 and 24 hours after estrogen treatment, with profound changes observed between 3 and 13 days after treatment (Jost et al., 1991; Wilks et al., 1982). Similarly, in hepatoma cells, glucocorticoids induce rapid DNA demethylation of the promoter region and the activation of the aminotransferase gene (Thomassin et al., 2001). In both cases, the local demethylation of target gene promoters creates priming and a developmental gene memory for subsequent hormone responses. However, the mechanisms underlying these instances of rapid demethylation are unclear. Few possible mechanisms have been put forward for active DNA demethylation, due to the energetically unfavourable nature of possible enzymatic reactions, although the methyl CpG binding protein MBD2b was shown to be a DNA demethylase, which acts by correcting methylated cytosine to cytosine (Bhattacharya et al., 1999). In this case, however, other laboratories have significantly failed to demonstrate similar demethylase activity for MBD2b. A recent study identified growth arrest and DNA-damage-inducible, alpha (GADD45 α) in a screen to look for proteins that may be involved in DNA demethylation. GADD45 α , a nuclear protein that maintains stability of the genome plays a role in DNA repair and suppression of cell growth was proposed to increase replication independent active DNA demethylation by a DNA repair mediated process (Barreto et al., 2007), although this finding has also been disputed by other workers (Jin et al., 2008).

While studying the mechanisms of the rapid estrogen-stimulated demethylation in the chicken oviduct, Jost and colleagues purified a DNA “demethylase” activity, which they subsequently named 5-Methyl cytosine DNA glycosylase (5-MCDG). Jost and colleagues reported that ER associates with 5-MCDG and potentiates the DNA glycosylase activity of 5-MCDG (Jost et al., 2002). Other reports by Jost and colleagues in human kidney cells demonstrated the 5-MCDG-mediated demethylation of an enhancer region composed of tandem repeats of retinoid receptor binding sites (Zhu et al., 2001). However, the identity of human 5-MCDG remains elusive, although Zhu et al have shown that the methyl CpG binding protein MBD4 is a human 5-MCDG (Zhu et al., 2000). Despite this, other studies have found no evidence of 5-MCDG activity for MBD4 (Hendrich et al., 1999). In the plant *Arabidopsis thaliana*, DEMETER (DME), a DNA glycosylase, is required to activate the MEA gene. MEA is an Arabidopsis polycomb group gene that is imprinted in endosperm tissue. It has been shown that DME is responsible for maternal allele-specific demethylation of the MEA gene in the endosperm. DME can remove 5-methylcytosine *in vitro* and *in vivo* (Gehring et al., 2006). Gong *et al* have developed a novel genetic system in Arabidopsis to find mutations in repressors of silencing (ROS) (Gong et al., 2002). They isolated the ROS1 gene and showed that it encodes a nuclear protein containing an endonuclease III domain that has bifunctional DNA glycosylase/lyase activity against methylated but not unmethylated DNA. These results show that ROS1 prevents DNA hypermethylation by demethylating the target promoter DNA. Together, these findings provide strong evidence supporting a base excision repair mechanism involving DNA glycosylases for active DNA demethylation, at least in plants.

The mechanisms underlying rapid DNA demethylation in animal cells, therefore, remain unclear. Further, the importance of DNA methylation/demethylation in the regulation of estrogen-responsive gene expression, outwith epigenetically silenced estrogen-responsive genes is still undefined. TDG, T:G mismatch-specific DNA glycosylase, also known as thymine DNA glycosylase, has been shown to act as a coactivator for the retinoid receptors (Um et al., 1998). TDG recognises U:G or T:G mismatches generated by spontaneous deamination of cytosines and methylcytosines, respectively. The T:G or U:G mismatches are then excised by TDG, generating abasic

sites that are repaired by the sequential recruitment of AP endonuclease, DNA polymerase β and DNA ligase (Figure 1.12). Previous work from our laboratory demonstrated that TDG could act as a coactivator for ER α through a direct interaction (Chen et al., 2003). ChIP further demonstrated that TDG is recruited to the promoters of estrogen-responsive genes in an estrogen-dependent manner. Further, RNAi-mediated down-regulation of TDG resulted in a reduction in the expression of estrogen-regulated genes and TDG was seen to cooperate with the coactivators SRC1 and AIB1 in regulating the expression of estrogen-responsive genes (Lucey et al., 2005). Taken together, these findings are indicative of an important role for TDG in regulating ER α activity in BC cells and indicate that TDG is a multifunctional protein that could be involved in both DNA repair and in transcriptional regulation.

GR mediated rapid demethylation of the TAT gene was followed by the demonstration that estrogen addition can induce rapid DNA demethylation at the pS2 gene promoter (Kangaspeka et al., 2008; Metivier et al., 2008a). Moreover, several cycles of DNA demethylation and remethylation at the pS2 gene promoter were observed over a 180 minute period, accompanied by cycles of meCpG binding protein recruitment. Moreover, *in vitro*, under conditions of limiting S-adenosylmethionine the demethylation was mediated by DNMT 3A and 3B and TDG, which is surprising because DNMTs are methyltransferases that mediate DNA methylation (Metivier et al., 2008a). The latter report indicates that DNMT3A/B deaminate methylcytosines in the pS2 gene promoter to thymines. The resulting T:G mismatches are repaired by the base excision repair pathway by TDG. It is puzzling in this respect, however, that a methylcytosine deaminase activity has previously not been described for DNMTs, despite intensive study of these proteins. Another example of rapid methylation/demethylation in a hormonally regulated promoter has been demonstrated by the cytochrome P450 27B1 (CYP27B1), an enzyme involved in vitamin D biosynthesis (Kim et al., 2009). CYP27B1 gene expression is maintained by parathyroid hormone (PTH) and vitamin D hormones. PTH stimulates CYP27B1 transcription, while vitamin D represses transcription through binding to the nuclear receptor VDR and by inducing rapid methylation of CpG sites in the CYP27B1 promoter. This methylation step needs DNMT1 and DNMT3B. On the other hand, treatment with PTH increases active CpG demethylation of the CYP27B1 promoter.

Purification of a VDR interacting repressor (VDIR) associated complex demonstrated the presence of both DNMT1 and DNMT3B and the DNA glycosylase methyl-CpG binding domain protein 4 (MBD4) in the complex. This result further suggests that DNA methylation/demethylation plays an important role in hormonal regulation of gene transcription.

Whilst the possible importance of DNMT3 in methylcytosine deamination remains controversial, a group of well-defined cytosine deaminases have been described. Cytidine deamination was first described in the case of mRNAs in which cytosines were substituted by uracil (Benne et al., 1986)) and was subsequently reported for the Apolipoprotein B gene, where conversion of a cytosine in the apolipoprotein B mRNA to uracil results in a glutamic acid residue (CAA) being replaced by a stop codon (UAA), leading to large (100 kDa) and small (48 kDa) forms of apolipoprotein B (Chen et al., 1990; Powell et al., 1987). Subsequent studies identified apolipoprotein B mRNA editing catalytic polypeptide 1 (APOBEC1) as the enzyme responsible for RNA editing of this gene (Teng et al., 1993). Further characterisation of APOBEC1 has led to the identification and cloning of APOBEC1-related genes, named APOBEC2, AID and APOBEC3. In primates including man, there are 8 closely linked APOBEC3 genes (named APOBEC3A-H), whilst there is only one rodent APOBEC3 gene. *In vitro* studies have shown that the APOBEC genes edit RNA or single-stranded DNA (Harris et al., 2002). However, AID has been implicated in somatic hypermutation that aids antibody diversity, in which deoxycytidine is deaminated to uracil, with subsequent base excision-mediated repair by uracil DNA glycosylase (related to TDG) allowing transversions (Di Noia and Neuberger, 2007; Neuberger et al., 2003). Finally, it has been shown *in vitro*, that AID and APOBEC1 can deaminate 5-meC to result in T and that these enzymes are expressed in oocytes and germ cells and that the resulting mismatch can be repaired by mismatch DNA glycosylases such as TDG and MBD4 (Morgan et al., 2005). Hence, APOBEC genes provide an attractive alternative mechanism for deamination of methylcytosines, which coupled to base excision repair of the resulting T:G mismatch by TDG, could provide a mechanism for active DNA demethylation in animal cells. As this thesis is related to importance of AID/APOBEC genes in regulating breast cancer cells growth, and mechanisms by which it regulates the cell growth, APOBEC family is introduced in greater detail below.

1.16 The AID/APOBEC family of cytidine deaminases

APOBECs are a family of zinc-dependant cytidine deaminases that include in humans AID, APOBEC1, APOBEC2 and a series of seven APOBEC3 genes, which are APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G and APOBEC3H, whereas in mice only one mouse APOBEC3 (mA3) present (Goila-Gaur and Strebel, 2008). APOBEC4 is a new member of the APOBEC family to which no function has as yet been assigned (Rogozin et al., 2005). The AID/APOBEC family of proteins catalyze deamination of cytidine to uracil on RNA and DNA and are implicated in lipid metabolism, inhibition of retrotransposons, retroviruses, DNA viruses and antibody diversification (Aguiar and Peterlin, 2008; Bransteitter et al., 2009; Conticello, 2008; Conticello et al., 2007; Cullen, 2006; Harris and Liddament, 2004). APOBEC1 was the first protein to be discovered in APOBEC family, hence, all other proteins named after APOBEC1, which was shown to edit apolipoprotein B mRNA (Conticello, 2008; Holmes et al., 2007b).

Table 1.1. Zinc-dependant deaminases in different species (adapted from Conticello *et al*, 2007).

Enzyme	Substrate	Organisms
AID and APOBECs	Cytosine in DNA/mRNA	Vertebrates
Cytosine deaminase	Cytosine/methylcytosine	Bacteria, archea, yeast
Cytidine deaminase	Cytidine/deoxycytidine	Bacteria, archea, yeast, plants, metazoa
Riboflavin deaminase	Riboflavin synthesis	Bacteria, archea, fungi, plants
Guanine deaminase	Guanine	Some bacteria, (archea)
dCMP deaminase	dCMP	(Archea, bacteria), yeast, plants, metazoa, virus
TadA/Tad2p-3p/ADAT2-3 Tad1p/ADAT1	Adenosine-34 in tRNA Adenosine-37 in tRNA ^{Ala}	Bacteria, yeast, metazoa Yeast, metazoa
ADAR1-2-3	Adenine in mRNA	Metazoa

1.17 Evolution of the AID/APOBEC family

Genome sequencing has shown that the AID/APOBEC family is limited to jawed vertebrates. Nevertheless, the AID/APOBEC family proteins are related to an extensively expressed superfamily of zinc-dependant deaminases that act on pyrimidines and purines (Conticello et al., 2007). Amongst different deaminases, the tRNA adenosine deaminases (Tad/ADAT2) edit adenosine to inosine at the anticodon of tRNAs in both eukaryotes and prokaryotes and AID/APOBEC genes are believed to be derived from these tRNA deaminases (Conticello, 2008). In addition to having structural and functional resemblance to AID/APOBEC, ADAT2 from trypanosomes appear to be capable of deamination of cytidine in DNA (Rubio et al., 2007). An AID gene has been identified in cartilaginous fish and bony fish. The origin of APOBEC proteins can be put at around 500 million years ago, at the time of divergence of the cartilaginous fish from teleost/tetrapod lineage (Conticello, 2008; Conticello et al., 2007; Conticello et al., 2005). It appears likely that the APOBEC2 gene might have evolved as a result of retrotranspositional events as the gene structure of APOBEC2 is different from that of the other APOBEC genes, with few amino acids in first exon of APOBEC2 that have no similarity to any other gene sequences. This suggest that AID and APOBEC2 were the only two members of the AID/APOBEC family before APOBEC1 and APOBEC3 evolved from AID by gene duplication in mammals around 300-400 million years ago (Conticello, 2008; Conticello et al., 2007; Conticello et al., 2005). In most mammals, the APOBEC1 locus is believed to have developed from the AID locus by inverted duplication on the same chromosome. APOBEC1 has an extended coding sequence located at the 3' end, which is the main difference between AID/APOBEC genes (Conticello, 2008). APOBEC3 genes are believed to have originated after the divergence of the placental mammals from the marsupials around 170 million years ago and went through a swift expansion in primates, where seven APOBEC3s (A to H) have been identified, compared with one APOBEC3 gene in rodents, two APOBEC3 genes in pigs and three APOBEC3 genes in sheep and cattle (LaRue et al BMC Molecular Biology 2008). The fast evolution of the APOBEC3 gene family is believed to be the result of selective pressure on APOBEC3 arising from target agents such as retrovirus and retrotransposons, which are capable of neutralizing APOBEC3 mediated retroviral replication inhibition

(Conticello et al., 2005; Jarmuz et al., 2002; Wedekind et al., 2003). APOBEC4 might have originated before the divergence of amphibia and reptiles.

1.18 Domain organization and structural characteristics of AID/APOBEC proteins

The AID/APOBEC proteins have a characteristic domain structure (Figure 1.14A). Similar to all zinc dependant deaminases, the important structural characteristics of AID/APOBECs is the domain responsible for their catalytic activity, which consists of a short α -helical domain, followed by a catalytic domain, then a short linker peptide and finally a pseudocatalytic domain (Jarmuz et al., 2002). APOBEC1 and AID are situated on chromosome 12 and have one catalytically active zinc-dependant cytidine deaminase domain (CD). APOBEC2 is located on chromosome 6 and also has one CD. APOBEC3 genes are located on chromosome 22 in a tandem arrangement. APOBEC3A and APOBEC3C genes also encode a single CD (Holmes et al., 2007b). However, the APOBEC3B, APOBEC3F, APOBEC3DE and APOBEC3G genes encode two CDs. Current findings indicate that in APOBEC3 proteins containing two CDs, normally only one domain is catalytically active and the other domain is engaged in nucleic acid binding and viral encapsidation (Goila-Gaur and Strebel, 2008). The N-terminal domain of APOBEC3F and APOBEC3G is enzymatically inactive, whereas both N- and C-terminal domains of APOBEC3B are potentially enzymatically active, although this continues to be a topic of further investigation because some studies have failed to demonstrate catalytic activity for the N-terminal CD in APOBEC3B (Bogerd et al., 2007; Hakata and Landau, 2006; Prochnow et al., 2009). In the case of mouse APOBEC3 the catalytically active and inactive CDs are swapped, the N-terminal domain being more catalytically active than the C-terminal domain (Hakata and Landau, 2006).

The catalytic domains of APOBEC proteins contain a conserved amino acid sequence motif having the sequence H-X-Glu-X₂₃₋₂₈-Pro-Cys-X₂₋₄-Cys, where X denotes any amino acid. The histidine and two cysteines coordinate a Zn²⁺ ion and the glutamine is believed to be involved in proton shuttling during the cytidine deamination reaction (Harris and Liddament, 2004).

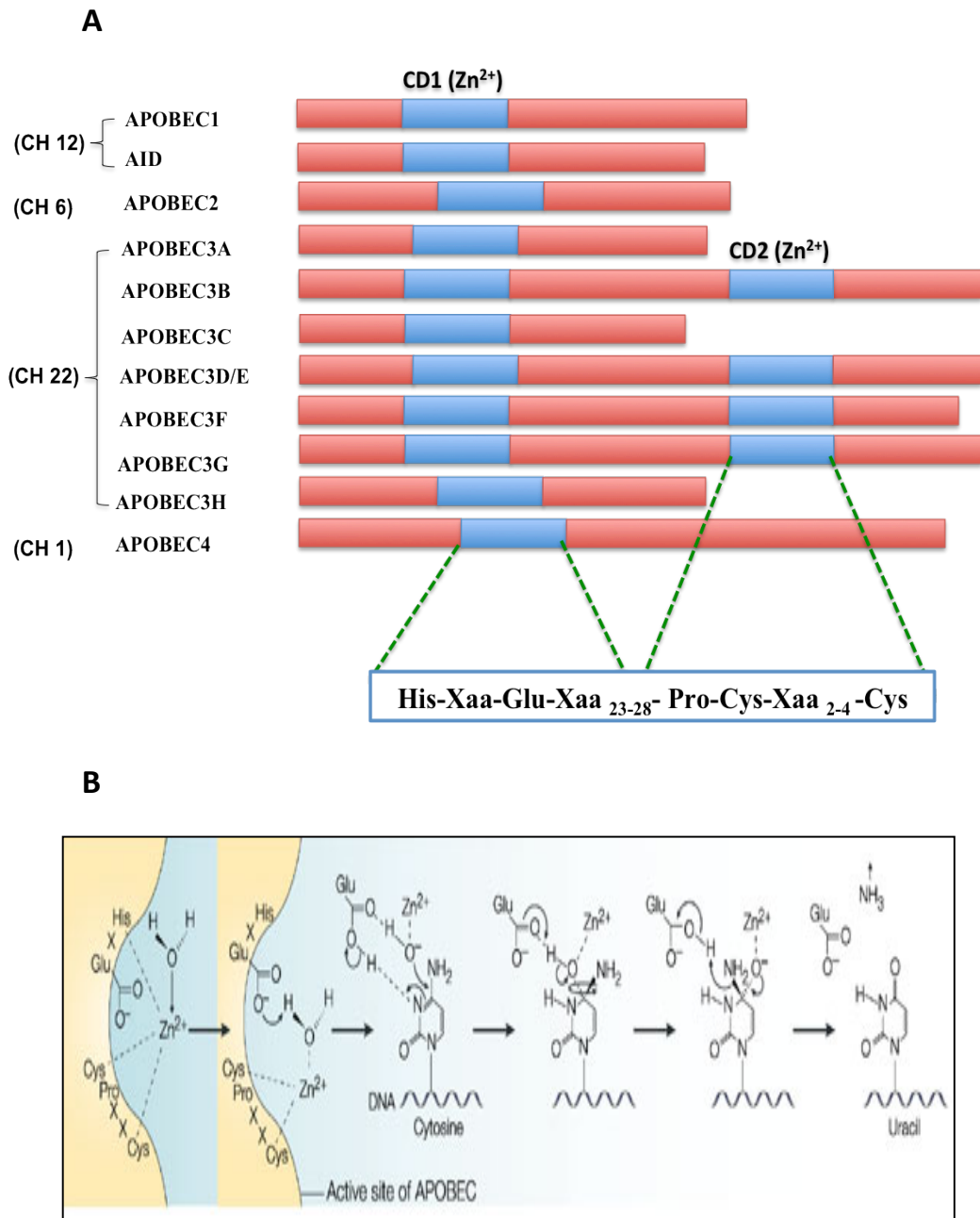


Figure 1.14. Schematic representation of the human APOBEC family of proteins. A. Domain organization of human APOBEC proteins. Activation- induced deaminase (AID) and APOBEC1 genes which has only one cytidine deaminase domain (CD1) are located on chromosome 12 and separated by about 1MB. APOBEC2 is located on chromosome 2. APOBEC3 proteins are located on chromosome 22 in clusters. APOBEC3B, APOBEC3D/E, APOBEC3F and APOBEC3G have two cytidine deaminase domains. Catalytic domain is characterized by the conserved amino acid sequence motif “His-X-Glu-X₂₃₋₂₈-Pro-Cys-X₂₋₄-Cys”. The cysteine and histidine sequence within the motif coordinate Zn ion. B. A potential mechanism of APOBEC mediated cytosine deamination. Hydroxide ion (OH⁻) is produced when glutamic acid and Zinc react with water. Glutamic acid add proton to N3 of the cytosine pyrimidine ring, which leads to destabilization of N3-C4 double bond and hydroxide ion (OH⁻) attack C4. Finally, uracil is formed. Adapted from Harris and Liddament, 2004 and Holmes et al, 2007

High-resolution crystal structures for APOBEC2 and the APOBEC3G C-terminal CD have been described (Chen et al., 2008; Holden et al., 2008; Prochnow et al., 2007) and show that the APOBEC CDs are similar in structure to other zinc-dependant deaminases, with a well-defined core structure of five α -helices, in which α -helices 2 and 3 hold the histidine and the cysteine residues in place and shape the catalytic pocket (Figure 1.16). The five β -strands forms the backbone of the structure. AID/APOBECs forms dimerization/oligomerization, however, but AID and APOBEC3G multimerization is not important for the catalytic activity (Conticello, 2008).

1.19 Mechanism of AID/APOBEC deamination and DNA binding

The mechanisms of cytidine deamination by AID, APOBEC1 and APOBEC3s are very similar (Harris and Liddament, 2004). Figure 1.14B illustrates a model for the potential mechanism for APOBEC mediated cytosine deamination. The cytidine deaminase mechanism was predicted on the basis of structural studies from bacterial and yeast cytidine deaminases (Johansson et al., 2002; Ko et al., 2003). The histidine and two cysteine residues in the catalytic active site coordinate a single zinc ion (Zn^{2+}) and glutamine residue, which mediates proton shuttling during deamination process. One molecule of water interacts with glutamic acid and Zinc in order to generate hydroxide ion (OH^-). Glutamic acid protonates position N3 of cytosine pyrimidine ring to destabilize the double bond of N3-C4 of cytosine pyrimidine ring and hydroxide ion attacks C4 of cytosine. This results in formation of uracil from cytidine and ammonia is the by-product of this reaction (Harris and Liddament, 2004).

APOBEC1 was the first protein to be described to have RNA editing activity (Teng et al., 1993). APOBEC1, along with apobec complementation factor (ACF) edit specific site (C^{666}) of apolipoprotein B transcript containing > 14000 nucleotides long to create a truncated apolipoprotein B 48 (Chen et al., 1987; Teng et al., 1993). Both full length and truncated form of apolipoprotein B protein plays an important role in lipid metabolism (Anant and Davidson, 2001; Chen et al., 1987). The cis acting and trans acting factors regulate RNA editing function of APOBEC1. Approximately 30 nucleotide flanking the edited base (C^{666}) contains sequence information for the

editing process and other sequences located distal 5' and 3' to edited base important for efficiency of catalysis (Blanc and Davidson, 2003). The cis acting factor includes 11 nucleotides termed mooring sequences, situated 4-6 nucleotide downstream of the edited base and consensus binding sequences for APOBEC1 (UUUN (A/U) U), which located 3 nucleotide downstream of the edited base (Blanc and Davidson, 2010). Maris et al has shown that ACF recognise important nucleotides in the mooring sequences and target the cytidine to be edited by APOBEC1 for deamination (Maris et al., 2005).

It has been shown that different APOBEC proteins preferentially deaminate specific cytidines inside the trinucleotide sequence motif in DNA. AID, which deaminates cytidines in both single and double stranded DNA, prefers cytidines in the hot spot motif of WRC (where R = A or G; W = A or T). Further, it can also frequently target cytidine outside this motif (Pham et al., 2003; Shen et al., 2009; Shen and Storb, 2004). APOBEC3G, which edit viral DNA has extremely strong preference for CCC trinucleotide motifs, where it edits the third cytidine on a single strand DNA and it never edits other cytidines outside the CCC context (Chelico et al., 2006). Moreover, It has been discovered that APOBEC3G edit cytidines in 3' to 5' direction. The deamination of cytidines by APOBEC3G preferentially happens close to the 5'- side of the single strand DNA. It was proposed that deamination by APOBEC3G occurs processively and that it may involve jumping and sliding mechanism on the DNA (Chelico et al., 2009; Chelico et al., 2008). In the case of APOBEC3F, which also edit viral DNA, targets cytidines in TTC trinucleotide context (Langlois et al., 2005). Qin Yu *et al* characterized APOBEC3B and APOBEC3C for their DNA sequences preference and shown that APOBEC3B, similar to APOBEC3G, is highly active against the third cytidine of the CCCA nucleotide sequences. APOBEC3B can also deaminate cytidine in the context of CCA, CCT and CCG trinucleotide sequence, while APOBEC3C has weak deamination activity against CCCA, CCA, CCT and CCG sequences (Yu et al., 2004).

1.20 APOBEC proteins and anti- retroviral activity

Amongst APOBEC proteins, APOBEC3G was first shown to have strong anti-HIV-1 activity (Sheehy et al., 2002). APOBEC3G deaminates cytidine (dC) to uridine (dU) in the retroviral minus strand (nascent viral reverse transcript or cDNA) after reverse transcription. APOBEC3G creates uracil bases in the place of cytidines. These mutations convert guanosine (G) to adenine (A) in the positive strand of viral DNA (Figure 1.15), which in turn leads to more G to A mutations in the viral genome and affect the viability of the virus (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). Human APOBEC3G can inhibit both human and mouse retrovirus but mouse APOBEC3 can only inhibit human HIV-1 not murine leukemia virus (MLV) (Mangeat et al., 2003; Mariani et al., 2003). However, HIV-1 is able to inhibit the antiretroviral function of APOBEC3G by expressing virion infectivity factor (VIF) protein (Gabuzda et al., 1992). VIF causes APOBEC3G degradation by activating polyubiquitylation and proteasomal pathways and may also inhibit APOBEC3G protein translation (Marin et al., 2003; Stopak et al., 2003).

Other APOBECs family proteins that are located close to the APOBEC3G gene on chromosome 22 are also found to strongly inhibit retroviral infection. For example, studies have shown that APOBEC3B and APOBEC3F can significantly reduce retroviral infection (Bishop et al., 2004a). Specifically, APOBEC3F has been shown to inhibit VIF deficient HIV (HIV Δ VIF), while APOBEC3B inhibited both wild type HIV-1 and HIV Δ VIF (Bishop et al., 2004a; Liddament et al., 2004). Whilst APOBEC3DE can weakly inhibit HIV-1 in VIF sensitive manner, the expression profile of this protein is similar to that of APOBEC3G (Dang et al., 2006). Other proteins, such as APOBEC3C and APOBEC3A also have weak activity against both wild type HIV-1 and HIV Δ VIF (Bishop et al., 2004b; Bourara et al., 2007). Finally, APOBEC3H is seen to be ineffective against viral replication and the expression of this protein is found to be low in primates (OhAinle et al., 2006). Originally it was hypothesized that cytidine deamination was the main mechanism of antiviral activity for APOBEC proteins. Recent studies have observed that APOBEC3G and APOBEC3F can inhibit the growth of virus in the absence of DNA editing (Holmes et al., 2007a; Newman et al., 2005).

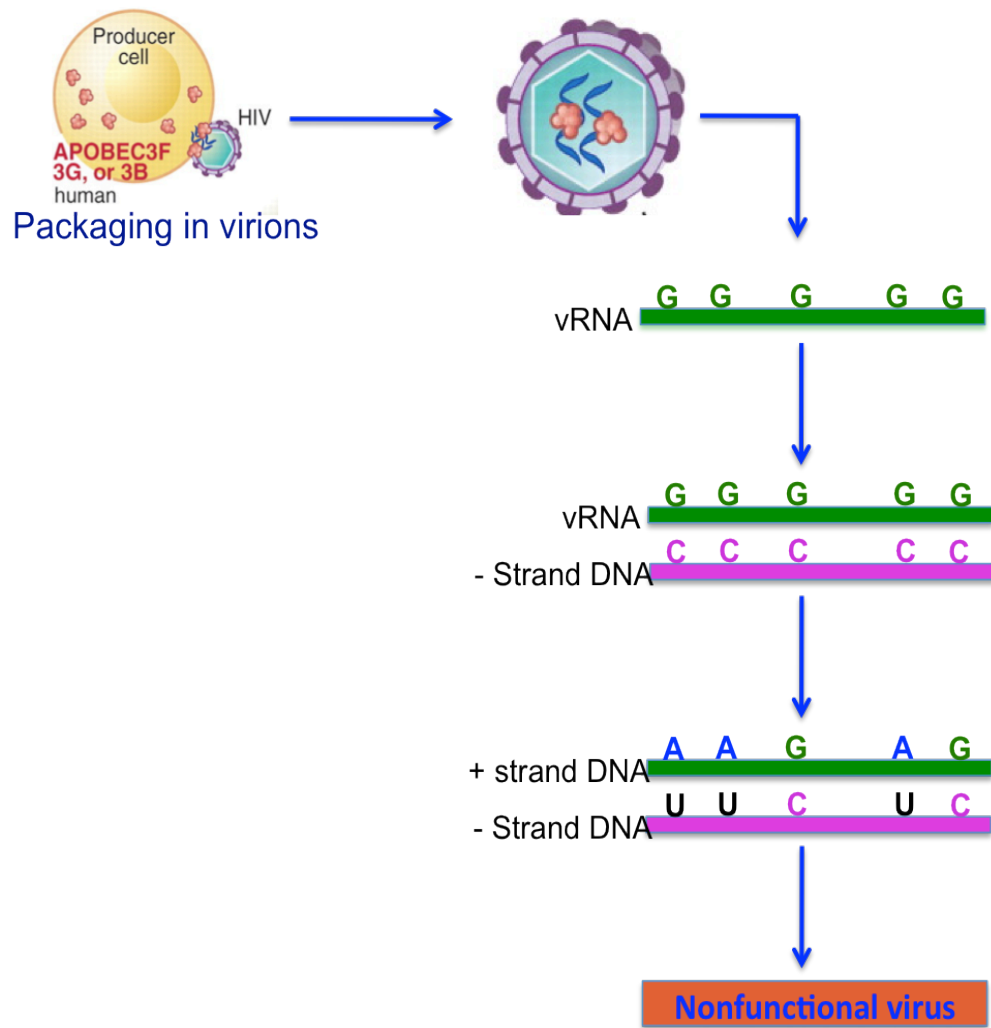


Figure 1.15. Antiviral mechanism of APOBEC proteins.

In HIV virus producing cells APOBEC3G, 3F or 3B are packaged into shed virions. In target cells, APOBEC3G, APOBEC3F and APOBEC3B deaminate cytosine on the minus strand DNA, which leads to G to A hypermutation in the plus strand DNA, making the viral genome become non-functional.

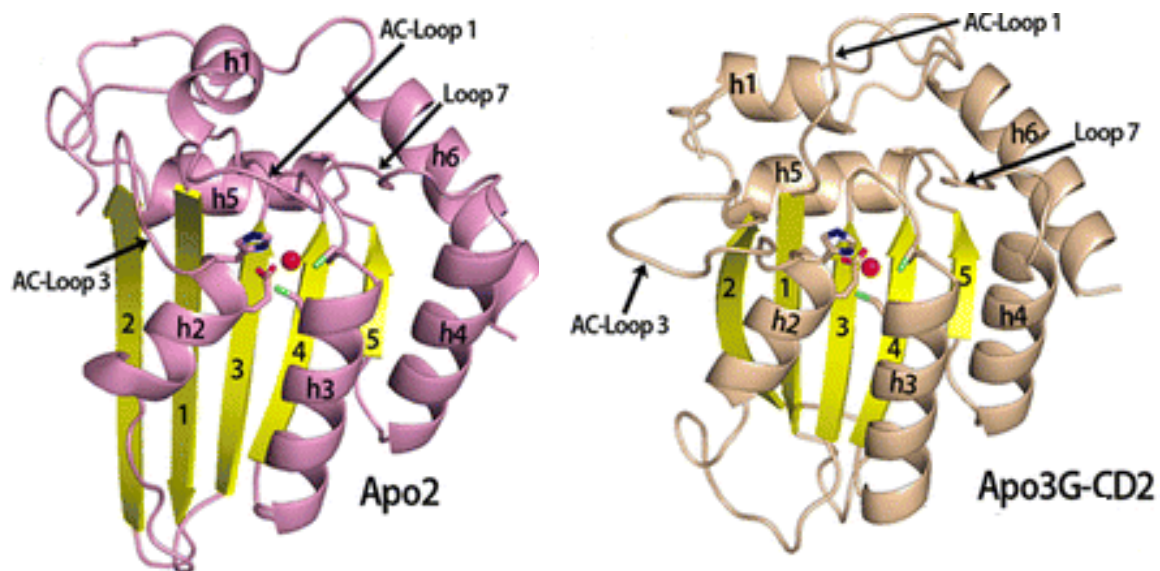


Figure 1.16. Three-dimensional structure of APOBEC2 and APOBEC3G-CD2.

The catalytic pocket is formed by α helices 2 and 3, which hold cysteine and histidine residues. The zinc ion is shown as a red sphere. β strands providing the molecules scaffold are shown in yellow. Amino acids within the Loop 7 region may provide specificity for deamination of cytidine in the context of different sequence motifs.

This may be achieved by affecting viral nucleoprotein complexes, changing the kinetics of viral reverse transcription and also affecting interactions between viral and cellular proteins (Aguiar and Peterlin, 2008).

1.21 Intracellular localization and expressions of AID/APOBEC proteins

Cell specific expression of AID/APOBECs is important in order to avoid the non-specific editing of RNA and DNA. Table 1.2 shows the intracellular localization and the expression profiles of various APOBECs in different cell types and tissues. For example, Koning *et al* determined the APOBEC3 expression pattern in human tissues and lymphoid cells and found that APOBEC3 family members are expressed at higher levels in both B and T cells (Koning et al., 2009). Adam Jarmuz and Qin Yu *et al* examined the expression of various APOBECs in different tissues and cancer cell lines and found that APOBEC3B was highly expressed in peripheral blood leukocytes, colon cancer, chronic myelogenous leukemia cells and at lower levels in cervical cancer, lung cancer, melanoma and burkitt's lymphoma. APOBEC3B and APOBEC3C are expressed more in the small intestine and heart, while APOBEC3B is expressed in human embryonic carcinoma cell lines (Jarmuz et al., 2002; Yu et al., 2004). APOBEC3C and APOBEC3G are highly expressed in colon cancer and at a lesser extent in lymphoma and leukemia (Jarmuz et al., 2002). As the various APOBECs family members have different expression profiles in different cell and tissue types it is important to note the fact that these proteins may have different physiological functions other than RNA and DNA editing.

Nuclear localisation of proteins may often be regulated by their size. Proteins, which have the size of less than 20-30 kDa, can diffuse into nucleus freely and maintain their levels equally in both cytoplasm and nucleus (Gorlich, 1998). AID and APOBEC1 are 24 and 27 kDa in size and should freely enter the nucleus. Nevertheless, it was found that APOBEC1 and AID are predominantly located in the cytoplasm and can shuttle between the cytoplasm and the nucleus (Brar et al., 2004; Chester et al., 2003; Ito et al., 2004; Yang and Smith, 1997). In the case of AID a strong nuclear export signal (NES) was found at the C-terminus and a weak nuclear localization signal (NLS) was found at the N-terminus region (Brar, Watson et al. 2004, Ito, Nagaoka et al. 2004).

Table 1.2 Human AID/APOBEC family proteins

Name	Genomic location	Exons	Deaminase domains	Expression profile	Cellular localization	Known editing function	Physiological target	References
AID	12p13	5	1	Activated B cells, oocytes, ovaries, embryonic germ cells and testis	Mainly cytoplasmic, function in nucleus	DNA deaminase	Ig gene diversification and gene translocation	(Harris et al., 2002b; Lin et al., 2009; Muramatsu et al., 1999)
APOBEC1	12p13.1	5	1	Gastrointestinal tissue (small intestine)	Cytoplasmic and nuclear, acts in nucleus	RNA, DNA deaminase	Apolipoprotein B mRNA	(Navaratnam et al., 1993; Teng et al., 1993)
APOBEC2	6p21	3	1	Skeletal muscle and heart	Cytoplasmic and nuclear	Unknown	Unknown	(Liao et al., 1999)
APOBEC3A	22q13.1	5	1	Keratinocytes, lung, blood (monocytes)	Cytoplasmic and nuclear	DNA deaminase	Retrotransposons, adeno-associated virus	(Jarmuz et al., 2002; Madsen et al., 1999; Refsland et al., 2010)
APOBEC3B	22q13.1	8	2	Intestine, uterus, cancer cell lines, keratinocytes, mammary gland, heart, small intestine human embryonic carcinoma cells, lung, other tissues	Mainly nuclear	DNA deaminase	Retrovirus, retrotransposons, HBV	(Jarmuz et al., 2002; Madsen et al., 1999; Yu et al., 2004)
APOBEC3C	22q13.1	4	1	Cancer cell lines, heart, small intestine, lymphoid organs, lung and other tissues	Cytoplasmic and nuclear	DNA deaminase	Retrovirus, retrotransposons, HBV	Jarmuz, Chester et al. 2002(Refsland et al., 2010; Yu et al., 2004)
APOBEC3DE	22q13.1	7	2	Thyroid, spleen, blood and other tissues	Unknown	DNA deaminase	Retrovirus	Jarmuz, Chester et al. 2002(Conticello et al., 2005; Dang et al., 2006; Wedekind et al., 2003)
APOBEC3F	22q13.1	8	2	Ovary, testis, lymphoid organs, prostate, possibly coexpressed with APOBEC3G	Cytoplasmic	DNA deaminase	Retrovirus, retrotransposons, HBV	Jarmuz, Chester et al. 2002(Conticello, 2008; Dang et al., 2006; Refsland et al., 2010)
APOBEC3G	22q13.1	8	2	Activated T cells, lymphoid organs, testis, ovary and renal carcinoma, tissues and cell lines, other cancer cell lines	Cytoplasmic	DNA deaminase	Retrovirus, retrotransposons, HBV	(Jarmuz et al., 2002; Refsland et al., 2010)
APOBEC3H	22q13.1	5	1	Lymphoid organs, thyroid, placenta, lung, blood	Cytoplasmic and nuclear	DNA deaminase	Retrovirus	(Conticello, 2008; Muckenfuss et al., 2006; Refsland et al., 2010)
APOBEC4	1q25.3	2	1	Testis	Unknown	Unknown	Unknown	(Rogozin et al., 2005)

Like AID, APOBEC1 has a NLS region in the N-terminus and a NES in the C-terminus region (Yang and Smith 1997; Chester, Somasekaram et al. 2003). APOBEC3B is predominantly localised in the nucleus, whereas APOBEC3A, APOBEC3C and APOBEC3H are localised in the cytoplasm and nucleus. Finally, APOBEC3G and APOBEC3F are localised to the cytoplasm (Muckenfuss et al., 2006).

Wichroski *et al* has shown that APOBEC3F and APOBEC3G are localised to mRNA processing bodies in the cytoplasm (P-bodies). P-bodies are found in the cytoplasm, where non-translating mRNAs are subject to storage or degradation. Wichroski *et al* further showed that APOBEC3G can interact with P-body proteins, which work in RNAi mediated gene silencing (AGO2), translational suppression (RCK/p54), cap dependent translation (eIF4E and eIF4E-T), and decapping of mRNA (Wichroski et al., 2006). The fact that interaction between APOBEC3G and P-body proteins is RNA dependent and suggests that APOBEC3G is localised to large multiprotein ribonuclear protein complex in the cell. These properties may provide a new role for APOBEC3s as regulator of microRNA (Conticello, 2008). Remarkably, APOBEC3B that has around 59% sequence similarity to APOBEC3G is not seen to be localised to P-bodies in the cytoplasm (Wichroski, Robb et al. 2006).

1.22 Regulation of AID/APOBEC proteins

The AID/APOBEC family proteins potentially have mutagenic activity and, as a result tight regulation of their expression and activity is important. As far as AID function is concerned, two types of regulatory mechanism have been proposed to avoid inappropriate DNA damage. One regulates the availability of AID to the appropriate cell type, and the other controls the accessibility of DNA sequences to be edited by AID (de Yebenes and Ramiro, 2006). Different cellular factors can regulate expression levels, localisation and activity of APOBEC3 proteins. It was reported that the transcriptional activity of APOBEC3G can be regulated by Sp1 and Sp3 transcription factors (Muckenfuss et al., 2007). In this study, the authors found that a 180bp region downstream of the transcription start site mediates full promoter

activity and that Sp1 and Sp3 recognise GC-box motifs on the promoter, in order to stimulate transcriptional activity of APOBEC3G.

Stopak *et al* studied the cytokine regulation of APOBEC3G expression in peripheral blood lymphocytes (PBLs) and found that IL-2, IL-7 and IL-15 enhances APOBEC3G expression in PBLs (Stopak et al., 2007). Interestingly, other cytokines, such as IL-4, IL-6, IL-9, IL-12, IFN- α , IFN- β , IFN- γ and TNF- α did not enhance APOBEC3G expression in PBLs (Stopak et al., 2007). Increased levels of APOBEC3G expression by IL-2, IL-7 and IL-15 is mediated by the JAK/MAPK pathways. T-lymphocytes pretreated with JAK/MAPK inhibitors do not increase APOBEC3G expression (Stopak et al., 2007). It has been reported that both APOBEC3A and APOBEC3B are up-regulated by phorbol myristate (PMA) (Rasmussen and Celis, 1993; Rose et al., 2005).

In addition, PMA has been shown to induce the expression of APOBEC3G by 20 fold in T-lymphocytes, and this enhancement is mediated by protein kinase C α / β 1 isozyme (PKC), mitogen activated protein kinase (MEK) and extracellular signal regulated kinase (ERK) (Rose et al., 2004). Both levels of APOBEC3G mRNA and protein are increased by PMA treatment. Interestingly, the basal levels of APOBEC3G also seems to depend on PKC α / β 1/MEK/ERK pathways, as presence of these inhibitors reduce the basal levels of APOBEC3G (Rose et al., 2004).

Specific factors have been reported to regulate the expression and functions of APOBEC1 (Dance et al., 2002; Nakamuta et al., 1995). It has been reported that the tissue specific expression of APOBEC1 in mice is determined by alternative mRNA splicing and differential promoter utilisation (Nakamuta et al., 1995). AID is also expressed in inducible manner, and normally the expression is limited to germinal center B cells. Recently it has been reported that AID expression is increased in a cell division dependant manner and the increase is temporally and functionally linked to COX-2/PGE2 pathway (Lee et al., 2010).

Multiple signalling pathways have been shown to regulate AID expression. IL-4 and anti-CD40 signals have been shown to act synergistically to activate AID expression

by binding of STAT6 and NF- κ B to the proximal promoter (Blackledge et al., 2010; Dedeoglu et al., 2004; Zhou et al., 2003). Sayegh and colleagues have shown that E-box element in the AID gene positively or negatively regulate AID transcription by binding the helix-loop-helix transcription factor (HLH) E47, which induces AID transcription and binding of inhibitor of DNA binding HLH protein ID3, which reduce AID transcription (Sayegh et al., 2003). The Paired Box gene (PAX5) has been shown to enhance AID expression, whereas the inhibitor of HLH DNA binding protein ID2 reduced AID expression. Hence, the balance between PAX-5 and E-box binding proteins plays major role in the regulation of AID expression. The levels of AID/APOBEC proteins transcription and its activity in cells may be regulated by combination of ubiquitous and tissue specific expression of transcriptional coactivators and corepressors (Aguiar and Peterlin, 2008; de Yébenes and Ramiro, 2006).

It has been suggested that AID/APOBECs editing activity can be modulated by post-translational modification, particularly phosphorylation. Chen et al. has proved that the apolipoprotein B mRNA editing activity of APOBEC1 is regulated by phosphorylation at serine 47 and serine 72. When these sites were mutated, APOBEC1 editing activity was reduced (Chen et al., 2001). Targeting of AID to its editing site is enhanced by interaction with replication protein (RPA) (Chaudhuri et al., 2004), which is ssDNA binding protein involved in DNA repair, recombination and replication. The interaction of AID and RPA depends on AID phosphorylation by protein kinase A (PKA) at serine 38 and threonine 27 in activated B cells (Basu et al., 2005). In activated B cells, RPA binds with phosphorylated AID to enhance AID activity on transcribed dsDNA containing somatic hypermutation (SMH) and class switch recombination (CSR) target sequences (Basu et al., 2005; Pasqualucci et al., 2006). In addition, AID target preferences can be altered by mutS homolog 6 (MSH6), implying that mismatch-repair protein may also contribute to AID targeting (Li et al., 2006).

It has been recently published that estrogen directly activate AID transcription and function in mouse splenic B cells, whereas tamoxifen treatment reduces AID protein expression. ER α can directly bind on AID promoter to increase AID expression, enhance CSR, augment mutational frequency in Ig and non-Ig genes and increase c-

myc translocation. Different APOBEC3 proteins such as APOBEC3B, APOBEC3F and APOBEC3H were also found to be increased by estrogen treatment in various tissues and cell lines (Pauklin et al., 2009). Progesterone has been shown to reduce AID mRNA levels by 5 fold (Pauklin et al., 2009) , possibly by binding directly to AID promoter (Pauklin and Petersen-Mahrt, 2009). Very recently Mai *et al* suggest that estrogen induced AID expression is through activation of homeobox transcription factor HOXC4 in activated B cells (Mai et al., 2010). The author further showed that ER α does not directly activate AID transcription in B cells, instead this binds directly to EREs in the HOXC4 gene promoter to upregulate HOXC4 expression, which in turn mediates AID expression by binding to and activating the AID promoter (Mai et al., 2010). Together, these findings have highlighted that nuclear receptors such as ER α and PR play an important role in altering the expression and editing activity of AID/APOBEC family proteins.

1.23 Cytosine deamination and epigenetic modification

Epigenetic information can be preserved and transmitted in the form of any of the three molecules, DNA, RNA and protein (Jablonka and Raz, 2009). Eukaryotic chromatin holds a large amount of information needed for the growth and development of a multicellular organism. This information is not only stored in DNA sequences, but also resides epigenetically through DNA methylation and post-translational modification of histone proteins (Jenuwein and Allis, 2001). The presence or absence of methylation marks on DNA and histone proteins can alter gene expression; hence, various cell types can be described by their epigenetic and gene expression profiles (Wu and Zhang, 2010). Studies in the past have highlighted that during embryogenesis gametes have more levels of DNA methylation, with global demethylation occurs shortly after fertilisation, and finally methylation patterns are re-established in somatic cells (Morgan et al., 2005). The enzymes that mediate DNA methylation have been well documented, but the mechanisms involved in DNA demethylation have not been so well studied. As mentioned earlier, cytidine deaminases such as AID/APOBEC proteins play a major role in various biological processes such as retroviral defence, antibody diversification and DNA/RNA editing. In addition to this, it has been shown *in vitro* and E.coli assay that both AID and

APOBEC1 can deaminate methylated cytosine (5meC) to thymine in single stranded DNA, suggesting that both could act as a DNA demethylase (Morgan et al., 2004).

AID was shown to express in primordial germ cell (PGCs) and in the early embryo, although its expression in PGCs has been recently challenged (Hajkova et al., 2010). Studies on DNA methylation levels of male and female PGCs isolated from AID-null embryos reveal that global methylation levels increase by 4% in male embryos and 13% in female embryos respectively, suggesting that AID may have DNA demethylation effects (Popp et al., 2010). This study provides direct evidence for deamination dependent cytosine demethylation in mammalian somatic cells. Nevertheless, a study by Rai *et al* reported that AID and APOBEC2a/b could demethylate methylated DNA duplex in zebrafish embryos (Rai et al., 2008). When the methylated DNA fragment was injected into single cell embryos the expression levels of AID, APOBEC2a and APOBEC2b were increased significantly. In addition, combined over-expression of AID and T:G mismatch repair protein MBD4 causes efficient demethylation of methylated DNA fragments. In this study, it was suggested that AID deaminated 5meC to thymine, causing a T:G mismatch, which can be repaired by MBD4, a T:G mismatch DNA glycosylase. Interestingly, the authors further suggest that Gadd45a, a DNA repair protein, serves as a scaffold protein to enhance the interaction between AID and MBD4 on methylated DNA.

Additional evidence suggesting that AID can change epigenetic information through DNA demethylation comes from a studies of reprogramming of interspecies heterokaryons made by fusion of human fibroblasts with mouse embryonic stem cells (Bhutani et al., 2010). Activation of pluripotency genes such as OCT4 and NANOG, which are not expressed in somatic cells because of DNA methylation, is very important for cellular reprogramming towards pluripotency. In this study, it has been suggested that AID binds to promoter region of human OCT4 and NANOG gene, where demethylation and activation of gene expression take places independent of cell division and DNA replication (Figure 1.17).

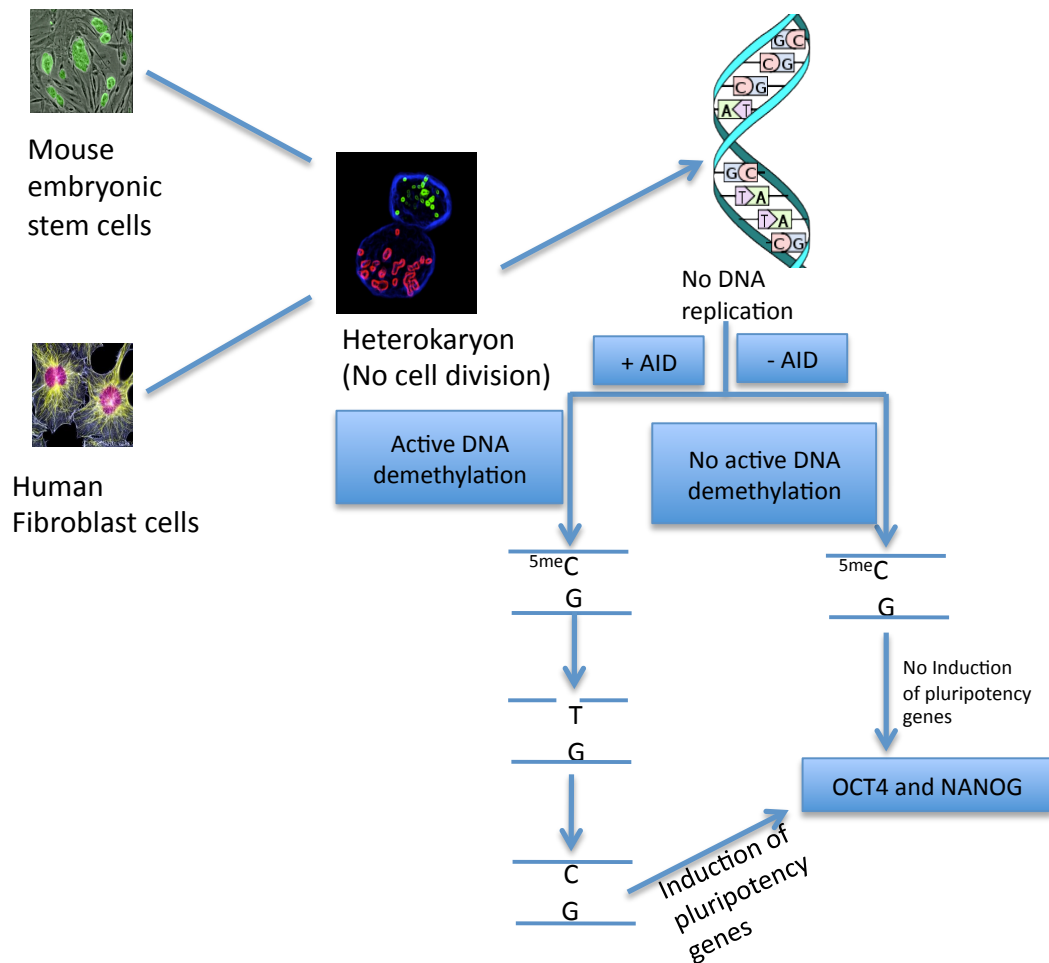


Figure1.17 Model for AID-dependent active DNA demethylation.

AID-mediated reprogramming and DNA demethylation in heterokaryons occurs in the absence of cell division and DNA replication. The deamination of 5-mC by AID produces thymidine, which can then be removed by a T-G mismatch-specific DNA glycosylase enzyme, and leads to the expression of pluripotency genes Oct4 and Nanog.

TET proteins, a group of Fe(II)/2-oxoglutarate-dependent dioxygenases, have recently been discovered as 5-methyl cytosine (5mC) hydroxylases that oxidize 5mCs to produce 5-hydroxyl methyl cytosine (5hmC) (Ito et al., 2010; Tahiliani et al., 2009). 5hmC may mediate passive DNA demethylation by excluding DNMT1, which maintains symmetric CpG methylation during DNA replication (Valinluck and Sowers, 2007). Fascinatingly, Tet1 knockdown in mouse embryonic stem cells (ESCs) causes Nanog promoter hypermethylation and a failure of ESC self-renewal (Ito et al., 2010). 5hmC has also been hypothesized as a possible intermediate for active DNA demethylation (Hajkova, 2010; Hajkova et al., 2010; Mohr et al., 2011; Wu and Zhang, 2010). It has been recently shown that overexpression of TET1 protein in human cells reactivates a methylation plasmid reporter and promotes active DNA demethylation of both exogenous non-replicable methylated reporter plasmids and endogenous gene promoters of ribosomal DNA (rDNA), fibroblast growth factor 1 (FGF1), and neuroligin-3 (NLGN3) (Guo et al., 2011). The authors further examined whether the AID/APOBEC family proteins might play a role in 5hmC demethylation. Indeed, overexpression of mouse APOBEC1, human APOBEC2, APOBEC3A, APOBEC3C, APOBEC3E and AID, but not APOBEC3B, APOBEC3G showed an increase in 5hmC demethylation. In addition, overexpression of inducers of 5hmC demethylation, including mouse APOBEC1, APOBEC3A, and APOBEC3C, seemed to produce higher levels of 5-hydroxyluracil (5hmU), which can be removed by 5hmU glycosylases and repaired by the BER pathway with unmethylated cytosines. Cortellino *et al* found that a complete TDG knockout in mice is embryonic lethal and that TDG null cells feature hypermethylated DNA at specific CpG islands (Cortellino et al., 2011). The author further found that TDG could interact with AID and GADD45a. Furthermore, 5mC and 5hmC can be deaminated by AID to thymine and 5hmU, which was rapidly repaired by TDG.

Several recent studies have examined DNA modifications of the immunoglobulin (Ig) gene locus during CSR and SHM (Li et al., 2004; Nambu et al., 2003; Wang et al., 2006). In this, as increased accessibility of AID to the target Ig locus is seen and could be achieved by chromatin modifications that have been associated with targeting transcription, replication and repair processes. It has been suggested that modifications of histone H3 and H4 are important for sequential V(D)J recombination (Chowdhury and Sen, 2001), and acetylation of H3 and H4 have been implicated in

targeting variable regions for SHM (Nambu et al., 2003). Hyperacetylation of H3 in switch region of Ig locus is associated with induction of CSR and germline transcription; suggesting that this modification may come first to mark the Ig locus for AID to start the recombination process (Nambu et al., 2003; Wang et al., 2006). Fascinatingly, AID dependent increase in acetylation of H4 in switch region of Ig locus is associated with CSR, implying that this histone modification might be a downstream effect of AID and may have role in recruiting repair protein to double strand break sites (Wang et al., 2006). Taken together, these studies ascertain a role for AID/APOBEC family proteins in DNA demethylation and histone modifications, thus emphasizing the potential of AID/APOBEC proteins in modulating epigenetic information.

1.24 AID/APOBEC proteins and cancer

It is very well understood that spontaneous deamination of cytosine and 5-methyl cytosine is a possible contributor of somatic and germline mutations. Nevertheless, the AID/APOBEC family members belongs to the cytidine deaminase family which increases the possibility that some of the mutations in somatic and germline cells may point to mistargeted deamination of cytosine in DNA by these members (Neuberger et al., 2003). In addition, it has been reported that AID/APOBECs are expressed in different cancer tissues and cell lines (Harris et al., 2002; Jarmuz et al., 2002; Muramatsu et al., 1999; Okazaki et al., 2007).

1.24.1 Abnormal RNA editing of nuclear protein

Over-expression of APOBEC1 in transgenic mice and rabbit develops hepatic dysplasia and hepatocellular carcinoma (Yamanaka et al., 1995). Aberrant C to U RNA editing of tumour suppressor NAT1 protein had been observed within hepatocytes of APOBEC-1 transgenic animals (Yamanaka et al., 1997). These alterations introduce multiple stop codons within the coding region of NAT1 protein, which leads to reduced NAT1 levels in transgenic animals. Another example of aberrant C to U RNA editing by APOBEC1 had been documented in neurofibromatosis type 1 (NF1) protein (Skuse et al., 1996). NF1 has tumour

suppressor function, altered editing in NF1 transcript results in translational termination and inactivation of tumour suppressor function. Skuse and colleagues have shown C to U editing of NF1 transcript in a subset of peripheral nerve sheath tumours, and observed that increased levels of C to U editing had been associated with an aggressive malignant phenotype (Cichowski and Jacks, 2001; Skuse et al., 1996).

1.24.2 Stabilisation of proto-oncogene mRNA

It has been shown that APOBEC1 stabilizes RNA targets that contain AU rich motifs in 3' untranslated region (UTR). APOBEC1 binds to 3'UTR of mRNAs encoding IL-12 and c-myc and increases its stability (Anant and Davidson, 2000). These discoveries imply that altered expression of APOBEC1 may modify the stability of RNAs that are involved in cell growth regulation and proliferation, and may lead to cancer development. For example, APOBEC1 can stabilize COX2 mRNA in intestinal stem cells and may be involved by increasing the levels of COX-2 mRNA (Anant et al., 2004).

1.24.3 AID in cancer

Among the APOBEC family members, AID has a unique function. Activated B lymphocytes trigger antibody diversification by somatic hypermutations (SHM) and class switch recombination (CSR) of immunoglobulin (Ig) genes upon antigen binding to neutralize invading antigen molecules effectively. Both SHM and CSR involve DNA strand breaks and AID expression is essential for this process (Marusawa, 2008; Okazaki et al., 2007). In addition, it is very well known that targets of AID have not been restricted to the Ig genes but also to the non-Ig genes. Therefore, It is essential that AID expression and activity is limited to the target genes, as deregulation of AID leads to abnormal SHM and CSR, which play a dangerous role in lymphoma generation (Martin and Scharff, 2002).

Okazaki *et al*, (2003) first provided the direct correlation between AID overexpression and tumourigenesis in AID transgenic mice. Constitutive and

ubiquitous expression of AID in transgenic mice developed T-cell lymphomas, lung microadenomas, hepatocellular carcinoma, melanomas, and sarcomas (Okazaki et al., 2003). Further, genes for T-cell receptor (TCR), CD4, CD5, Pim1 and c-myc were massively mutated in T-cell lymphomas, proposing that deregulated expression of AID in transgenic mice induce T-cell lymphomas by introducing point mutations in non-Ig genes, including oncogenes (Okazaki et al., 2003). Furthermore, overexpression of AID induces c-myc/IgH translocations in activated B cells and burkitt lymphomas (Ramiro et al., 2004; Robbiani et al., 2008). It has been estimated that approximately 25% of non-Ig genes expression analysis, including Bcl6 and Cd83, showed AID mediated mutations in germinal B cells (Liu et al., 2008). These findings suggest that the deregulated expression of AID can cause tumourigenesis in both lymphoid and non-lymphoid organs by accumulation of mutations in tumour related genes.

Recently it has been shown that estrogen induces AID mRNA and protein expression in activated B cells and cancer cell lines. Further, estrogen treatment stimulates, Ig class switching, somatic hypermutation and potential oncogenic c-myc/IgH translocations (Pauklin et al., 2009). As AID is expressed in breast tissue, estrogen may target AID to non-Ig loci. Because tumour suppressor proteins BRCA1 and BRAC2 are frequently found to be mutated in breast and ovarian cancer it may not be surprising that estrogen may target AID to these genes promoters to cause mutation (Maul and Gearhart, 2009).

While APOBEC3 proteins are expressed in various cancers there are currently no experimental findings that substantiate their involvement in cancer initiation and progression. This is a key area that needs to be developed.

1.25 APOBECs and miRNA/RNA binding protein

RNA binding proteins plays a very important role in RNA metabolism that includes splicing, mRNA stability, polyadenylation, mRNA localisation and translation, thereby regulating gene expression mammals (Lunde et al., 2007). CUGBP2, a RNA binding protein, has been shown to be a components of holoenzyme, which

includes APOBEC1 as core enzyme, ACF, cofactor of APOBEC1 and interact with AU rich regions encoded in apolipoprotein mRNA, immediately upstream of the edited cytidine base (Anant et al., 2001). In this study, it was suggested that CUGBP2 inhibits the RNA editing function of APOBEC1 by either interacting with APOBEC1 in the cytoplasm, to block nuclear entry or by disturbing the functional interaction between APOBEC1, ACF and apolipoprotein mRNA in nucleus.

Yeast two-hybrid screens have discovered several other RNA binding proteins, which includes heterogeneous nuclear ribonucleoprotein C-1 (hnRNAPC-1), apolipoprotein-binding protein (ABBP1) and GRY-RBP, which interact with APOBEC1 and apolipoprotein RNA (Blanc et al., 2001; Greeve et al., 1998; Lau et al., 1997). ABBP1 is a RNA binding protein that is a splice variant of human RNP-A/B protein. ABBP1 positively regulates APOBEC1 editing activity as immunodepletion of ABBP1 reduces apolipoprotein RNA editing and transfection of antisense gene expression constructs reduces endogenous apolipoprotein RNA editing in HepG2 cells transfected with APOBEC1 (Lau et al., 1997). Greeve *et al* indentified hnRNPC-1 as an APOBEC1 binding protein that binds apolipoprotein RNA and inhibits the RNA editing activity of APOBEC1 (Greeve et al., 1998). 7SK is a small nuclear RNA (snRNA) that interacts with the transcription elongation factor P-TEFb to control cell growth, transcription and cell differentiation (He et al., 2006). 7SK has been shown to interact with APOBEC3C to reduce deaminase activity and acts to sequester APOBEC3C in the nucleolus; thereby it prevents unwanted mutations of genomic DNA by APOBEC3C (He et al., 2006). This demonstrates that small nuclear RNAs may regulate other APOBEC3s during deamination to safeguard cells from deleterious mutations.

Using tandem affinity purification coupled with mass spectrometry, Sarah *et al* discovered that APOBEC3G, which can interact with several RNA binding proteins, is localised in P-bodies and stress granules in the cytoplasm (Gallois-Montbrun et al., 2007). RNA binding proteins that interact with APOBEC3G are Ago1, Ago2, Mov10, GW182 and poly(A)-binding protein 1(PABP1) (Gallois-Montbrun et al., 2007). The author further reports that APOBEC3G, APOBEC3B, APOBEC3C and APOBEC3F can inhibit various micro RNAs such as mir-16, mir-10b and mir-25 to enhance the

expression luciferase or GFP reporter gene, and that this activity is independent of cytidine deaminase activity (Gallois-Montbrun et al., 2007). This report suggests that in addition to the antiviral effect of APOBRC3G, the protein may have additional roles in maintaining mRNA stability by preventing miRNA function. It has been shown that dead end protein (DND1), which is important for germ cell survival, interacts with mouse APOBEC3 (Bhattacharya et al., 2008). DND1 is a RNA binding protein that binds 3'UTR of mRNAs to prevent binding of miRNAs to same mRNA, thereby prevent miRNA mediated repression of protein expression (Bhattacharya et al., 2008). Collectively, these results suggest that APOBEC proteins may play a key role in RNA metabolism.

1.26 Study aims

Previous work from our laboratory has shown that cytosine deaminases in the apolipoprotein B mRNA editing enzyme, catalytic subunit-like (APOBEC) family stimulate ER α transcriptional activity. We performed gene reporter assays for APOBEC3 genes (3A, 3B, 3C, 3F and 3G), APOBEC1, AID and mouse APOBEC3, and found only APOBEC3B acted as a coactivator of ER α . APOBEC3B increased the transcriptional activity of ER α by three fold, while the other APOBECs tested showed no increase in ER α transcriptional activity (Dr R.Thomas and Prof S.Ali unpublished data). The importance of APOBEC3B in breast cancer cell growth, and the mechanisms for APOBEC3B mediated ER α transcriptional activities have not been studied. The project main aim is to find out the importance of APOBEC3B in regulating ER α activity in breast cancer cells, and to determine the effect of APOBEC3B on the regulation of estrogen-responsive genes. Further work has been carried out to determine the mechanisms by which APOBEC3B regulates ER α activity. Our hypothesis is that APOBEC3B may be involved in active DNA demethylation, DNA double strand breaks mediated gene transcription, and/or RNA metabolism (mRNA processing) in breast cancer cells. Together, these studies aim to define the importance of APOBEC3B in regulating estrogen-mediated breast cancer cell growth, and identify the mechanisms of APOBEC3B action in the breast.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and materials

General chemicals and reagents were obtained from Sigma Aldrich (Gillingham, UK), VWR International Ltd (Lutterworth, UK), BDH/Merck (Dorset, UK) and Fisher Scientific (Loughborough, UK). Molecular biology reagents such as DNA size markers (ladders) and restriction enzymes were obtained from Fermentas (York, UK) and Invitrogen (Paisley, UK). AnalR or molecular biology grade reagents were used, unless otherwise stated. ReddyMixTM PCR master mix was obtained from ABgene (Epsom, UK). SYBR Green Real-time PCR master mix was obtained from Applied Biosystems (Warrington, UK). TaqMan Real-time PCR reagents and assays were obtained from Applied Biosystems (Warrington, UK). Oligonucleotides were synthesised by Invitrogen (Paisley, UK) or Eurofins (Germany). Kits for large and small scale DNA and RNA preparation were obtained from QIAGEN Ltd (Crawley, UK). FuGENE[®] HD and FuGENE[®] 6 transfection reagents and protease inhibitor cocktail tablets were obtained from Roche Diagnostics Ltd. (Burgess Hill, UK). The Dual-GloTM Luciferase Assay System was purchased from Promega (Southampton, UK). LipofectamineTM RNAiMAX transfection reagent was obtained from Invitrogen (Paisley, UK). siRNA's were obtained from Dharmacon (Thermo Fisher Scientific UK Ltd, Leicestershire, UK). Standard solutions were prepared by dissolving the appropriate reagents in double distilled, de-ionised water (ddH₂O). Solutions were autoclaved and stored at room temperature, unless otherwise stated.

2.1.2 Cell culture reagents

Cells were maintained at 37°C in a humidified air atmosphere supplied with 5% CO₂. Cell culture manipulations were carried out in a NuAIRE class II, ducted laminar flow safety cabinet (NUAIRE DH Autoflow, Oxon, UK). Dulbecco's Modified Eagle's Medium (DMEM), L-Glutamine–Penicillin–Streptomycin solution (200mM L-glutamine, 10,000 units/ml penicillin, and 10mg/ml streptomycin in 0.9% sodium

chloride) (PSG), 0.02% EDTA solution and 10X trypsin solution were supplied by Sigma-Aldrich (Poole, UK). Phenol red-free DMEM and Opti-MEM were purchased from Gibco-Invitrogen Corporation (Paisley, UK). Fetal calf serum (FCS) and dextran-coated, charcoal-treated FCS (DSS) were purchased from First Link (Birmingham, UK). Stock solutions of 17 β -estradiol (E2, Sigma-Aldrich), 4-Hydroxytamoxifen (Sigma-Aldrich), and ICI 182,780 (Tocris Bioscience, Bristol, UK) were prepared by dissolving solid in ethanol at a concentration of 1 mM and stored at -20°C.

2.1.3 Sundries

Microcentrifuge tubes were purchased from Triple Red (Oxon, UK). Saran Wrap was obtained from The Dow Chemical Company (London, UK) and Whatman filter paper from Whatman International Ltd (Kent, UK). Tissue culture (Corning) and microbiological grade plastics were obtained from Appleton Woods, Birmingham, UK. HybondTM ECL nitrocellulose membrane was obtained from GE Healthcare Life Science (Buckinghamshire, UK). Kodak BioMax light Film was supplied by Sigma-Aldrich (Sigma, Poole, UK).

2.1.4 General stock solutions

Solutions were made in distilled de-ionised water (ddH₂O) by dissolving the appropriate quantity of reagent. The solution was then autoclaved and stored at room temperature, unless otherwise stated.

Table 2.1 General stock solutions

Stock solutions	
TE Buffer	10mM Tris-HCl, 1mM Na EDTA, pH 8.0
Sodium ethylenediaminetetraacetate (Na EDTA)	0.5M Na-EDTA, pH 8.0
10% (w/v) Lauryl sodium dodecyl sulphate (SDS)	10g SDS in 100ml ddH ₂ O, filter sterilized
X10 phosphate buffered saline (PBS)	80g NaCl, 2g KCl, 14.4g Na ₂ HPO ₄ , 2.4g KH ₂ PO ₄ in 1L ddH ₂ O, pH adjusted to 7.4, sterilized by autoclaving
X10 TBE	108g Tris-base; 55g Boric acid; 9.3g Na-EDTA in 1L ddH ₂ O, sterilized by autoclaving
Sodium Acetate	3M Sodium Acetate, pH 5.2
DNA digestion buffer	100mM NaCl, 10mM Tris-HCl pH 8, 25 mM Na-EDTA pH 8, 0.5% (w/v) SDS, 0.1mg/ml Proteinase K. Proteinase K, which is labile, was added fresh before each use.
Tween 20	10% Tween 20 in PBS
10% APS (Ammonium Persulfate)	1g in 10ml of ddH ₂ O, aliquoted and stored at -20°C

2.1.5 Media and solutions for bacterial work

Bacterial media was prepared using ddH₂O. Medium was autoclaved and stored at 4°C. Selection antibiotics were added fresh prior to inoculation with bacteria. L-Broth and L-Broth agar concentrates were obtained from Merck (Germany).

Table 2.2 Bacterial plates and media

Reagent	Recipe
L-Broth (LB) (Luria-Bertani)	LB capsules (16g bacto-tryptone, 10g bacto-yeast extract, 5g NaCl) in a total of 1 litre of double distilled deionized (ddH ₂ O), adjusted to pH 7.0 with NaOH. Autoclaved to sterilize and stored at 4°C.
LB Agar	16g Tryptone, 10g yeast extract-B, 5g NaCl and 15g of Agar-B. Capsules were added to 1 litre of ddH ₂ O
LB plates	Solid LB agar media was melted in a microwave and then cooled to 50°C prior to the addition of the selection antibiotics. The LB-agar was then poured into Sterilin Petri dishes, allowed to set and stored at 4°C.
Ampicillin	Ampicillin (sodium salt) solid was dissolved in 10ml ddH ₂ O to a final concentration of 100mg/ml and then filter sterilized. Ampicillin was used at a final concentration of 100µg/ml.
S.O.C medium	2% Tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl ₂ , 10mM MgSO ₄ , 20mM glucose. Purchased from Invitrogen (Paisley, UK).

2.1.6 Cell lines

All cell lines were purchased from the American Tissue Type Culture Collection (ATCC) and the European Collection of Cell Cultures (ECACC). Cell lines were tested every 3-6 months to ensure mycoplasma negativity. ER, PR and ERBB2 status (Kao et al., 2009) is given in Table 2.3.

Table 2.3 Cell lines used in this study

CELL LINE	TUMOUR TYPE	SOURCE	ER STATUS	PR STATUS	ERBB2/HER2 STATUS
MCF7	Metastatic adenocarcinoma	Pleural effusion	+	+	-
ZR-75-1	Invasive ductal carcinoma	Ascites fluid	+	-	-
T47D	Invasive ductal carcinoma	Pleural effusion	+	+	-
BT474	Invasive ductal carcinoma	Primary tumour	+	+	+
BT-20	Adenocarcinoma	Primary tumour	-	-	-
BT483	Invasive ductal carcinoma	Primary tumour	+	+	-
SKBR3	Adenocarcinoma	Pleural effusion	-	-	+
MDA-MB-231	Metastatic adenocarcinoma	Pleural effusion	-	-	-
MDA-MB-436	Adenocarcinoma	Pleural effusion	-	-	-
MDA-MB-453	Metastatic adenocarcinoma	Pleural effusion	-	-	+
CAL51	Adenocarcinoma	Pleural effusion	-	-	-
COS-1	SV-40 transformed African Green Monkey kidney cells.				
U2OS	Human osteosarcoma				

2.1.7 DNA size markers

The size of DNA fragments was determined with reference to standard DNA size markers. The markers used included: Low Range or High Range, ready to use, MassRuler™ DNA ladder (Fermentas, UK). Low range fragment size (bp): 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80. High range fragment size (bp): 10 000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500.

2.1.8 Protein size markers

The molecular weights of the proteins following western blotting were estimated by comparison with the PageRuler™ Fermentas Prestained Protein Ladder (MW 250, 130, 100, 70, 55, 35, 25, 15, 10 kDa).

2.1.9 General Equipment

For harvesting bacterial cultures, disposable 30ml centrifuge tubes (Sarsdedt, UK) were used in a Sorval SS34 rotor on a Sorval RC-5B refrigerated centrifuge (Du Pont Instruments UK Ltd, Stevenage, UK). Volumes less than 2ml were harvested in a bench top micro-centrifuge (Sorval Pico or Sorval Fresco, Sorval, Leicester, UK).

Samples were incubated at constant temperature in water baths (Techne, Bibby scientific Ltd, Staffordshire, UK) or incubator ovens (LEEC, Nottingham, UK; Fissions scientific Instruments, Loughborough, UK), which were also used for growth of bacteria on LB agar plates. Bacterial cultures were grown in a shaking incubator (New Brunswick Scientific Company Incorporated, Edison, NJ, USA). Samples were mixed using a Vortex-Genie 2 (Scientific Industries, London, UK). Polymerase Chain Reaction (PCR) was carried out using a GeneAmp PCR system 9700 (Applied Biosystems, Chesire, UK).

Real-time PCR was carried out using an Applied Biosystems 7900HT Fast Real-Time PCR System. Spectrophotometric measurements were carried out on a Shimadzu UV-

1201 spectrophotometer (Shimadzu Corporation, Tokyo, Japan) and Nanodrop ND-1000 (Labtech International, UK). Horizontal agarose gel electrophoresis tanks were purchased from Thistle Scientific, UK. Western Blotting vertical gel electrophoresis tanks and transfer apparatus were from Hoeffer (GE Healthcare Life Science, Buckinghamshire, UK) and Bio-Rad (Hemel Hempstead, UK). Power packs were from Bio-Rad (UK).

2.2 Methods

2.2.1 Cell culture

Cell lines were routinely maintained in DMEM supplemented with 10% FCS and antibiotics (Penicillin (100U/ml), Streptomycin (100µg/ml) and L-Glutamine (2mM), in a humidified atmosphere supplied with 5% CO₂. For experiments in which the effects of estrogen and anti-estrogens were determined, cells were cultured in phenol red-free DMEM (DMEM-PR) supplemented with 5% DSS for three days before the experiments. 17β-estradiol was added to a final concentration of 10nM. Anti-estrogens were used at a final concentration of 100 nM.

Cells were grown to approximately 80% confluency, at which point they were passaged by removing the medium and washing with pre-warmed PBS. In order to detach adherent cells, 2 ml of trypsin (1X trypsin in 0.02% EDTA) was added and the cells were incubated in the incubator at 37°C for 5 minutes. The flasks were gently tapped to dislodge trypsinised cells and, when detachment was complete, the trypsin was inactivated by the addition of 20 ml DMEM containing 10% FCS. Cells were centrifuged and re-suspended in fresh media and transferred to new flasks. The cell lines were routinely maintained in a logarithmic phase of growth in T150 tissue culture flasks.

In order to maintain cell stocks, cell aliquots were cryo-frozen on a regular basis. For this, cells at approximately 80% confluency were trypsinised from the culture flask and spun at 1,200 rpm in a bench top centrifuge, to form a cell pellet. The pellet was re-suspended in enough freezing medium (90% FCS, 10% DMSO) to make cell

suspension of 2×10^6 cells/ml. The cell suspension was then aliquoted in 1ml volumes into 2 ml cryovials and placed in a -80°C freezer for 24 hours before being placed in a liquid nitrogen storage dewar for long term storage.

For recovery from liquid nitrogen storage, frozen cells were rapidly thawed at 37°C in a water bath, and transferred a drop at a time into a centrifuge tube containing pre-warmed culture media. The cell suspension was centrifuged at 1200 rpm for 5 minutes. On removing the supernatant the cells were re-suspended in 5 ml of media and placed in a T25 tissue culture flask, which was placed in the incubator set at 37°C with humidified 5% CO_2 .

2.2.2 Cell counting and cell viability measurement

Following trypsinisation, cells were re-suspended in an appropriate volume of culture medium. Ten microlitres of the harvested cell suspension was mixed with an equal volume of trypan blue 0.4% (Invitrogen, UK), in order to assess viability. Ten microlitres of the trypan blue/cell suspension mixture was loaded into a haemocytometer chamber (Sigma, UK). The chamber was viewed using a 10x objective on an inverted phase contrast microscope (Zeiss, Germany). Using a hand tally counter, the number of cells in 16 squares was counted. Only viable phase bright cells, which had not taken up the dye, were included in the count. The cell count (cells/ml) was calculated using the formula, average number of cells (from 4 corner square) $\times$ dilution factor $\times 10^4/\text{ml}$.

2.2.3 Sulphorhodamine B (SRB) growth assay

For experiments in which the effects of ligands were determined, cells were cultured in DMEM-PR, supplemented with 5% double charcoal stripped serum (DSS) for 3 days before the addition of ligands. Cells were then seeded at 5000 cells/well in flat-bottomed 96-well plates, in media (200 μl /well) containing 5% DSS. After overnight incubation of cells in the tissue culture incubator, estrogen was added as appropriate. Cell growth was assessed over a five-day period using the sulphorhodamine B (SRB)

assay, a colorimetric assay that measures cell number by staining for basic amino acids, as described (Skehan et al., 1990). In brief, the cells were fixed by adding 100µl ice-cold 40% trichloroacetic acid (TCA) to each well and incubated at room temperature for 1 hour. The wells were then washed five times in distilled water and the cells stained by the addition of 100µl of 0.4% SRB (Sigma, UK) prepared in 1% acetic acid, for 30 minutes. Cells were washed five times with 1% acetic acid and allowed to air-dry. The dye content of each well was then solubilised by the addition of 100µl of 10mM Tris-base and completed by incubation on a plate shaker for 10 minutes at room temperature. The absorbance of the solubilised dye was read at 492nm using a Tecan Infinite M200 plate reader (TECAN UK Ltd, Reading, UK).

2.2.4 DNA preparation

2.2.4.1 Transformation of bacterial competent cells with plasmid DNA

All plasmids were transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen, UK). Cells were thawed on ice and 50µl gently aliquoted into pre-chilled 1.5 ml microcentrifuge tubes. Ten nanograms of plasmid DNA was added to the cells and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 45 seconds and subsequently cooled on ice for 2 minutes. Six hundred microlitres of S.O.C medium (Invitrogen, UK) was added to the transformed cells and these allowed to recover by incubation with shaking at 37°C for 1 hour at 200 rpm. Cells were spread on pre-warmed LB-agar ampicillin plates. Plates were incubated face side down in an incubator overnight at 37°C.

2.2.4.2 Screening of transformed clones

In order to select transformed cells containing the desired insert, up to 10 colonies were picked from LB-agar plates and Wizard®*Plus* SV spin column minipreps (Promega, Hants, UK) was carried out as described below. Appropriate restriction endonuclease digestion was carried out and the fragments were resolved by agarose gel electrophoresis in order to identify clones containing the insert. Positive clones

were subjected to DNA sequencing, using appropriate primers to confirm the identity of the insert.

2.2.4.3 Plasmid DNA (mini preps)

The Wizard® Plus SV Minipreps DNA Purification System (Promega, UK) was used to purify small- scale (5-10 µg) plasmid DNA from 2 ml bacterial cultures. A single bacterial colony was inoculated in 5ml of LB medium containing appropriate antibiotics in a 7ml bijou tube (Sterelin UK). The culture was incubated for 12-16 hours at 37°C in a shaking incubator (Certomat BS-1; B Braun Biotech International). One and a half millilitres of this culture was transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for 5 minutes to pellet the cells. The cell pellet was re-suspended in 250µl of cell re-suspension solution (50mM Tris HCl pH 7.5, 10mM Na-EDTA pH 8.0, 100µg/ml RNase A) and lysed by the addition of 250µl of cell lysis solution (200mM NaOH, 1%SDS), followed by mixing by inversion and incubation at room temperature for 5 minutes. Ten microlitres of alkaline protease solution was added and tubes were mixed and incubated at room temperature for an additional 5 minutes. The reaction was neutralized by adding 350 µl of neutralization / denaturation solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid, pH 4.2), mixed and incubated at room temperature for 10 minutes. The bacterial lysates were then centrifuged at 13,000 rpm for 10 minutes to precipitate unwanted cellular components. Cleared lysates were transferred to Wizard Plus SV Miniprep Spin Columns by decanting and centrifuged at 13,000 rpm for one minute. The flow through was discarded and the spin column was reinserted. Columns were washed with wash buffer (60mM potassium acetate, 10mM Tris HCl pH 7.5, 60% ethanol). The DNA was eluted with 100µl of nuclease free water into clean 1.5ml microcentrifuge tubes by centrifugation at 13, 000 rpm for one minute.

2.2.4.4 Plasmid DNA preparation (maxiprep)

Table 2.4 Reagents for plasmid DNA maxiprep

Reagents	
Buffer P1	50 mM Tris-HCl, pH 8.0; 10 mM EDTA pH 8.0; 100µg/ml RNase
Buffer P2	0.2 M NaOH; 1% SDS
Buffer P3	3.0 M Potassium acetate, pH 5.5
Buffer QBT	0.75 M NaCl; 50mM MOPS pH 7.0; 15% isopropanol, 0.15% Triton X-100
Buffer QC	1.0 M NaCl; 50 mM MOPS pH 7.0; 15% isopropanol
Buffer QF	1.25 M NaCl; 50 mM Tris-HCl, pH 7.5; 15% isopropanol
TE buffer	10 mM Tris-HCl; 0.1 mM EDTA pH 8.0

The QIAGEN-tip 500 Plasmid maxi-prep kit was used for the extraction and purification of plasmids. A sterile pipette tip or a disposable plastic bacterial inoculation loop was used to inoculate 5ml of LB containing ampicillin (100µg/ml) with a single bacterial colony containing the desired plasmid construct, in a 7 ml bijou tube. This starter culture was then incubated at 37°C for 6-8 hours in a shaking incubator (Certomat BS-1; B Braun Biotech International). After incubation, 1ml of starter culture was added to 200ml of LB broth containing ampicillin, in a large sterile conical flask. The culture was then incubated at 37°C in a shaker (Certomat BS-1; B Braun Biotech International) (~ 300 rpm) for 12-16 hours. The starter culture was used to prepare glycerol stocks. These were prepared by adding 1 volume of 50% glycerol to the culture and then stored at -80°C. The cultured bacteria were harvested by centrifugation in a Sorvall RC-5B refrigerated centrifuge using an A-15C rotor (DuPont, Herts, UK) at 4°C for 15 minutes at 6000 rpm. The bacterial pellets were re-

suspended in 10ml of Buffer P1 and the cells lysed by the addition of 10ml Buffer P2 at room temperature for 5 minutes. Ten millilitres of chilled Buffer P3 was added to precipitate unwanted components and the solution was incubated on ice for 20 minutes. The mixture was filtered through 2 layers of butter muslin and then centrifuged at 13,000rpm for 30 minutes at 4°C. The supernatant was added to Qiagen-tip500 columns, previously equilibrated with 10 ml of Buffer QBT. The supernatant was then loaded into the QIAGEN-tip and allowed to drip through by gravity flow. Each column was then washed twice using 30ml of Buffer QC and the DNA eluted with 15 ml of Buffer QF.

Eluted plasmid DNA was precipitated with 10.5ml of isopropanol and pelleted by centrifugation at 11,000 rpm for 30 minutes at 4°C. The DNA pellet was re-suspended in 400µl of TE buffer and the dissolved DNA transferred to a 1.5ml microcentrifuge tube. 40µl of sodium acetate (3M, pH 5.2, stored at 4°C) and 1ml of 100% ethanol were added and the tubes stored at -20°C for 30 minutes, to precipitate the DNA. The DNA was then pelleted by centrifugation at 13,000 rpm for 5 minutes at room temperature. The supernatant was carefully removed and the DNA pellet washed twice with 70% ethanol. The ethanol was carefully removed and the DNA was vacuum dried and then re-suspended in appropriate volume of TE Buffer to make 1µg/µl concentration and stored at -20°C.

2.2.4.5 Phenol extraction of DNA

An equal volume of phenol (equilibrated with 0.1 M Tris pH 8.0) was added to the DNA to be extracted, vortexed and centrifuged for 5 minutes at 13,000 rpm to separate the aqueous and organic phases. The aqueous phase, containing the DNA was transferred to a 1.5ml microcentrifuge tube, ethanol precipitated as described above and dissolved in the appropriate volume of TE buffer to give a final concentration of 1µg/µl.

2.2.5 Spectrophotometry

Nucleic acid concentrations were determined using the nano-drop ND1000 spectrophotometer (Lab tech International, East Sussex). An optical density (OD) of 1 at 260nm corresponds to a concentration of 50µg/ml for double-stranded DNA and 40µg/ml for RNA. An estimate of nucleic acid purity was obtained by measuring the absorption at an OD of 260nm and at 280nm. The ratio between these two readings (OD_{260nm}/OD_{280nm}) for pure nucleic acid should be 1.8 for DNA and 2.0 for RNA. Readings significantly less than this indicate contamination with protein or phenol.

2.2.6 Agarose gel electrophoresis of DNA

DNA was resolved on a 1-2% agarose gel prepared in 1X TBE buffer using a horizontal gel electrophoresis apparatus (Thistle Scientific, UK). Agarose gels contained 0.25µl/ml ethidium bromide (Sigma, UK) or 0.1µl/ml SYBR Safe™ DNA gel stain (Invitrogen, UK). DNA was visualised using an UVipro Platinum Gel Doc System ultraviolet transilluminator (UVItec, UK). MassRuler™ DNA ladders were obtained from Fermentas (York, UK).

2.2.7 Automated sequencing of DNA

Automated DNA sequencing was performed using the DNA Sequencing Service, Genetics Core Facility, Imperial College London, Hammersmith Campus, which uses, an ABI3700 DNA sequencer and ABI Big Dye chemistry version 3 terminator reagents (Applied Biosystems, Cheshire, UK) and templates and primers supplied by the user. In this system, the DNA template is labelled with four fluorescent dyes, one for each nucleotide, using a PCR primer extension method. The fragments are then electrophoresed through a denaturing acrylamide gel to size separate the fluorescent dyes attached to each fragment. The dyes emit light at a different wavelengths upon excitation and this is collected and separated by a spectrograph onto a cooled, charged couple device. Computer software then collates this data and presents this as a virtual representation of a DNA sequencing chromatogram. Plasmid DNA, at a concentration

of 200-500ng per 3Kb, together with a sequencing primer (3.2pmol), in a total volume of 10µl was provided for sequencing. Sequence chromatograms were analysed using Vector NTI software.

2.2.8 RNA preparation

2.2.8.1 Extraction of total RNA

For RNA preparation from cultured cells, cell culture medium was completely removed and the cells collected by scraping into RLT buffer (containing guanidine isothiocyanate and β -mercaptoethanol) from the RNeasy Mini Preparation kit (QIAGEN Ltd, UK). Cells were homogenized by centrifuging at 13,000 rpm for 2 minutes through a QIAshredder spin column (QIAGEN Ltd, UK). Following homogenisation, one volume of 70% ethanol was added to the homogenized lysate and mixed well. The sample was then applied to an RNeasy mini spin column sitting in a 2 ml collection tube. The sample was then centrifuged at 13000 rpm for 30 seconds, and the flow through discarded. The remainder of the sample was then applied to the column and the centrifugation step repeated. Three hundred and fifty microlitres of buffer RW1 was then pipetted into the column and centrifuged for 30 seconds at 13000 rpm. DNase I incubation mix (70 µl Buffer RDD plus 10 µl DNase stock solution) was then added into the RNeasy mini spin column and incubated at room temperature for 15 minutes. A further 350µl of buffer RW1 was then added into the column and centrifuged at 13000 rpm for 30 seconds to wash the column. The flow through was discarded. The RNeasy column was then transferred to a new 2ml collection tube and 500µl of buffer RPE was added. The sample was centrifuged again at 13000 rpm for 30 seconds to wash the column. Another 500 µl of buffer RPE was added and the sample was centrifuged at the same speed for 2 minutes to dry the column. To accomplish the drying step, the column was transferred to new 2 ml collection tube and centrifuged again for 2 minutes. The column was then transferred to a sterile 1.5 ml microcentrifuge tube and 50µl of RNase-free water added to the column which was incubated for 5 minutes. The column was centrifuged for one minute at 13000 rpm to elute the RNA. The RNA concentration and purity were determined by measuring spectrophotometric absorption at 260nm using the nano-

drop ND1000 spectrophotometer (Lab tech International, East Sussex).

2.2.9 cDNA preparation by Reverse Transcription

cDNAs were prepared from total RNA using thin-walled PCR tubes, with the reagents listed below added in the following order:

- 4µl 5x RT buffer (250mM Tris-HCl, 200mM KCl, 30mM MgCl₂), containing 50mM Dithiothreitol (DTT) (Fermentas, Helena Biosciences Europe, UK) pre-warmed to 37°C for 30 minutes
- 2µl 10mM dNTP stock (Fermentas, Helena Biosciences Europe, UK)
- 1 µl random hexamers (200ng) (Fermentas, Helena Biosciences Europe, UK)
- 2 µg RNA
- Sterile water to a total volume of 19µl
- 1 µl Reverse transcriptase (200U/µl) (Fermentas, Helena Biosciences Europe, UK)

The reaction was incubated at 42°C for 1 hour in a thermal cycler and heat inactivated at 95°C for 5 minutes. The cDNA was then diluted 1:5 with sterile water and 2µl of cDNA subsequently used as template for each PCR and real time PCR reaction.

2.2.10 Polymerase chain reaction (PCR)

Twenty five microlitres PCR reactions were set up in 0.2 ml thin-walled PCR tubes, containing 12.5 µl of PCR master mix (2x PCR ReddyMixTM; Abgene, UK)) along with 2.5 µl of forward and reverse primers (10pmol/µl), 2 µl cDNA and sterile water to make up a total volume of 25 µl. The PCR mixture was centrifuged briefly, prior to thermal cycling on a GeneAmp 9700 PCR machine (Applied Biosystems, UK). PCR was carried out over 22-40 amplification cycles using the following amplification profile:

- Initial Denaturing 94°C, 5 minutes, 1 cycle
- Denaturing 94°C, 30 seconds
- Annealing X°C, 30 seconds (X= annealing temperature)
- Extension 72°C, 1 minute
- Final Extension 72°C, 4 minutes, 1 cycle

PCR ReddyMix™ components:

- 0.625 units Thermoprime Plus DNA polymerase
- 75mM Tris-HCl (pH 8.8)
- 20mM (NH₄)₂SO₄
- 2.5mM MgCl₂
- 0.01% (v/v) Tween 20
- 0.2mM each of dATP, dCTP, dGTP and dTTP

Table 2.5 Oligonucleotides (PCR and DNA sequencing primers)

Primer	Sequence
GAPDH-Forward	5'-TCCCATCACCATCTTCCA-3'
GAPDH-Reverse	5'-CATCACGCCACAGTTTCC-3'
APOBEC3A-Forward	5'-AAGGGACAAGCACATGGAAG-3'
APOBEC3A-Reverse	5'-GCTCCACTTCGTAGCACAGG-3'
APOBEC3B-Forward	5'-GGACAGGGACAAGCGTATCT-3'
APOBEC3B-Reverse	5'-GAGGATGGGTTCGTTTCAA-3'
APOBEC3C-Forward	5'-AAGGACGCTGTAAGCAGGAA-3'
APOBEC3C-Reverse	5'-AAGCACAGCCAAGTTTCGTT-3'
APOBEC3D-Forward	5'-GTCAGGCTGGTCTCGAACTC-3'
APOBEC3D-Reverse	5'-TGCTGTGGTTTCACTTCCTG-3'
APOBEC3G-Forward	5'-AAAACCAGAAGCTTGGAGCA-3'
APOBEC3G-Reverse	5'-GACGGTATTCCGACGAGAAA-3'
pCH-Apobec3B (cytidine deaminase domain 1 mutant,	5'-ATGGTCGGAGCTACACTTGG-3'

C97A/C100A)- Forward sequencing primer	
pCH-Apobec3B (cytidine deaminase domain 1 mutant, C97A/C100A)- Reverse sequencing primer	5'-CAAAGAAGGAACCAGGTCCA-3'
pCH-Apobec3B (cytidine deaminase domain 1 mutant, C284A/C289A)- Forward sequencing primer	5'-TGAAGGTCAGCAATTCATGC-3'
pCH-Apobec3B (cytidine deaminase domain 1 mutant, C284A/C289A)- Reverse sequencing primer	5'-TATAAGGAGGCGCTGCAAAT-3'
pS2 ERE- ChIP primer- Forward	5'-TATGAATCACTTCTGCAGTGAG-3'
pS2 ERE- ChIP primer- Reverse	5'-GAGCGTTAGATAACATTTGCC-3'
pS2 control- ChIP primer-Forward	5'-GTGATTCTCCTGACTTAACC-3'
pS2 control- ChIP primer-Reverse	5'-TGGCGCAGTGGCTCACGCTG-3'
GREB1-ChIP primer- Forward	5'-GGGTGAAATGAAGTGGCATGTG-3'
GREB1-ChIP primer- Reverse	5'-GAACAAAACAGAGCAAGGCCAAA-3'
pCR4-TOPO-PCR primer-Forward	5'-CTTTATGCTTCCGGCTCGTA-3'

pCR4-TOPO-PCR primer-Reverse	5'-GTTTTCCCAGTCACGACGTT-3'
pCR4-TOPO- sequencing primer	5'-TGTGGAATTGTGAGCGGATA-3'
pS2-pyrosequencing PCR primer-Forward	5'-GAGGATTTTAGGGTTTTTTTAGGTAAATGT-3'
pS2-pyrosequencing PCR primer-Reverse	Biotin-5'-CAAAACCCAAATCACCTTATTCTC-3'
pS2-pyrosequencing- sequencing primer	5'-TTTTTTAAGTAAATAGAGTT-3'
pS2-pyrosequencing PCR primer-Forward	5'-CCCCC AAAATAAATACTATACTCACTA-3'
pS2-pyrosequencing PCR primer-Reverse	Biotin-5'-TAGAAGGAATGGGTTTTATGAGT-3'
pS2-pyrosequencing- sequencing primer	5'-AATAACCCAAAAAAACTT-3'
pS2-pyrosequencing PCR primer-Forward	5'-TTAGTGGAGATTATTGTTTTAGAGGA-3'
pS2-pyrosequencing PCR primer-Reverse	Biotin 5'-AACCATTACCTCCTCTCTACTCCA-3'
pS2-pyrosequencing- sequencing primer	5'-AGATTATTGTTTTAGAGGAT-3'
pS2-MBD pulldown downstream ERE primer-Forward	5'-GTCAGGTTGGAGGAGACTCCCATGGC-3'
pS2-MBD pulldown downstream ERE primer-Reverse	5'-GAGCGTTAGATAACATTTGGCTAAGG-3'
pS2-MBD pulldown ERE primer-Forward	5'-GCTTAGGCCTAGACGGAATG-3'
pS2-MBD pulldown ERE primer-Reverse	5'-CTCCCGCCAGGGTAAATACT-3'
pS2-MBD pulldown	5'-AAACAGAGCCTGCCCTATAAAATCC-3'

TSS primer-Forward	
pS2-MBD pulldown TSS primer-Revere	5'-TGCCTCCTCTCTGCTCCAAA-3'
pS2- ERE-Forward	5'-TTCATGAGCTCCTTCCCTTC-3'
pS2- ERE-Reverse	5'-AGGTTGGAGGAGACTCCCAT-3'
pS2- flanking DNA break site-Forward	5'-TTAAGTGATCCGCCTGCTTT-3'
pS2- flanking DNA break site-Reverse	5'-TTTCCGGCCATCTCTCACTA-3'
NaCHII-Forward	5'-AGAGTGGGCTTTGGTTTCCT-3'
NacHII-Reverse	5'-CCATCAGAAGCCAAAATGCT-3'
L13a-Forward	5'-CACGTTCTCTTGGACAGCAA-3'
L13a-Reverse	5'-TTAAGGCTCCGCTGAGAGAG-3'

2.2.11 Quantitative real-time PCR

Real-time PCR was carried out on an ABI 7900HT fast Real-time PCR system using SYBR[®] Green PCR Master Mix (Applied Biosystems, UK), according to manufacturer's protocols. Each sample was analysed in triplicate and reactions set up in a Microamp[™] fast optical 96-well reaction plate sealed using the MicroAmp[™] Optical Adhesive Film (Applied Biosystems, UK).

Each reaction consisted of:

- 10µl of SYBR[®] Green PCR Master Mix
- 1µl of forward primer (10µM)
- 1µl of reverse primer (10µM)
- 5.5µl of nuclease free water
- DNA

Amplification for SYBR green reactions consisted of the following profile:

- Denaturing 95°C - 10 minutes
 - Annealing 95°C - 15 seconds
 - Extension 60°C - 1 minute
- 40 cycles

TaqMan Real-time RT-PCR was also carried out according to manufacturer's protocols on an Applied Biosystems (ABI) 7900HT fast Real-time PCR system using Assay-on Demand primers (Applied Biosystems). The assay identification numbers are given in Table 2.6. In brief, each PCR reaction was carried out in triplicate, in a MicroampTM fast optical 96-well reaction plate (ABI, UK). Each reaction mixture consisted of the following components:

- 10 µl TaqMan fast universal PCR master mix (ABI, UK);
- 1 µl primer (Custom TaqMan Gene Expression Assays, ABI, UK)
- 2 µl cDNA
- 7 µl RNase free water

The thermal cycle profile was:

- Stage 1 (cycle x 1) 95°C for 20 seconds
- Stage 2 (cycle x 40) 95°C for 3 seconds and 60°C for 30 seconds.

The amount of target gene was normalized to GAPDH and relative to the vehicle control using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 2.6 TaqMan Real-time PCR primers

Gene	ABI Primer code
ER α	Hs00174860_m1 ESR1
pS2	Hs00170216_m1 TFF1
Cathepsin D	Hs00157201_m1 CTSD
GREB1	Hs00536409_m1 GREB1
PR	Hs00172183_m1 PGR
GAPDH	Hs99999905_m1 GAPDH
APOBEC3B	Hs0035890_m1APOBEC3B
RPLPO	Hs99999902-m1RPLPO

2.2.12 SDS-Polyacrylamide gel electrophoresis (Western blotting)

Cell lysates were denatured at 100°C for 5 minutes prior to gel loading. Ten to twenty microlitres of protein lysate and 5 μ l of protein marker (Fermentas, UK) were separated on a SDS-polyacrylamide resolving gel with a 5% stacking gel. The percentage of resolving gel was determined by the molecular weight of the protein of interest. The Amersham mini-gel system was used (Amersham Biosciences, UK) for electrophoresis. Electrophoresis was carried out at 60V for 30 minutes and then at 100V for 1 to 2 hours until the dye front reached the bottom of the resolving gel. Separated proteins on resolving gels were transferred onto Hybond ECL Nitrocellulose membrane (GE Healthcare, UK) by using a power supply (Bio-Rad Laboratories, UK) at 100V for 90 minutes. The membrane was then incubated in blocking buffer for 1 hour at room temperature to reduce non-specific binding of primary antibody. The primary antibodies were diluted in blocking buffer and incubated with the membrane overnight at 4°C with gentle shaking. The membrane was then washed three times in PBST (15 minutes each wash on a rocking platform) and then incubated with the HRP-conjugated secondary antibody diluted in blocking buffer for 1 hour. The membrane was then washed again three times in PBST. For visualisation, SuperSignal West Pico Chemiluminescent Substrate (Perbio Science, UK) was added to the membrane followed by autoradiography using Hyperfilm ECL

(GE Healthcare, UK). Films were developed using a Konica SRX-1001A X-ray developer.

For stripping and re-probing a blot using a new antibody, the membrane was incubated in 10 ml of stripping buffer for a minimum 4 hours or overnight and then washed for 30 minutes in PBS. Probing with primary and secondary antibodies was carried out as above.

Table 2.7 SDS-PAGE Acrylamide gel solutions

	7% Resolving Gel	10% Resolving Gel	15% Resolving Gel	Stacking Gel
Lower Buffer (4X)	5ml	5ml	5ml	-
Upper Buffer (10X)	-	-	-	0.5ml
30% Acrylamide: 0.8% Bis acrylamide stock solution (37.5:1)	4.7ml	6.7ml	10.7ml	0.5ml
ddH ₂ O	10.3ml	8.3ml	5.3ml	4ml
10%(w/V) APS	200µl	200µl	200µl	50µl
TEMED	20µl	20µl	20µl	5µl

Table 2.8 SDS-PAGE buffer solutions

Reagents	
Upper buffer (Stacking gel buffer, 10X)	1.25M Tris; 1% SDS; pH 6.8
Lower Buffer (Resolving gel buffer, 4X)	1.5M Tris; 0.4% SDS; pH 8.8
SDS-PAGE running buffer (x10)	0.25M Tris; 1.9M Glycine; 1% SDS; pH 8.3
Transfer buffer	0.25M Tris; 1.9M Glycine; 20% Methanol
Blocking buffer	5% dried skimmed milk in wash buffer
Stripping buffer	15g glycine; 10ml 10% Tween-20; 83ml 1M HCl
Harsh stripping buffer	For 100 ml: 20 ml 10% SDS 12.5 ml 0.5 M Tris-HCl pH 6.8 67.5 ml ultra pure water Add 0.8ml β -mercaptoethanol under the fume hood
Wash buffer (PBST)	0.1% Tween 20 in PBS
2X Sample buffer	125mM Tris base, 4% SDS, 0.2% Bromophenol blue, 200mM DTT and 20% Glycerol, pH6.8

Table 2.9 Antibodies used in this study

Antibody	Cat No	Make	Dilution
ER α (6F11)	CP-E614	Vector	1:2000
Rabbit APOBEC3B	Gift from Dr. N. Navaratnam (MRC Clinical Sciences Centre)		1:3000
β -Actin	AB6276	Abcam	1:50000
PR	SC538	Santacruz	1:200
Lamin A/C	SC7292	Santacruz	1:500
pS2	SC28925	Santacruz	1:500
Cathepsin D	AB6313	Abcam	1:3000
HA	H9658	Sigma	1:5000
ER α (HC20)	SC543	Santacruz	1:500
Anti-mouse IgG HRP	P0447	DAKO	1:2000
Anti-Rabbit IgG HRP	P0448	DAKO	1:2000
γ H2AX (p139)	AB18311	Abcam	1:250
p54 ^{nrb} (NONO)	A300-582A	Bethyl	1:1000
PSF (SFPQ)	A301-320A	Bethyl	1:1000
PSPC1	SAB4200068	Sigma	1:1000
Biotin antibody	B7653	Sigma	5 μ g
Topoisomerase II β (TOP2B)	SC-13059	Santacruz	1:200

2.2.13 Site-directed mutagenesis

pCH-3B (APOBEC3B-HA) mutants were made by site-directed mutagenesis. Site-directed mutagenesis is a technique whereby single or multiple bases in a DNA sequence can be altered. The method was performed on double-stranded plasmid DNA using a kit from Stratagene (Agilent Technologies UK Limited, Cheshire, UK). Two complimentary oligonucleotides containing the desired mutation, flanked on either side by approximately 15 unmodified nucleotides were designed and synthesized (MWG-Biotech, Ebersberg, Germany).

Sample preparation for mutagenesis

5µl	10x reaction buffer
5µl	10ng/µl dsDNA template (Plasmid)
12.5µl	Forward oligonucleotide primer (10ng/µl)
12.5µl	Reverse oligonucleotide primer (10ng/µl)
1µl	dNTP mix (10mM stock)
to 49µl with sterile water	
Add	1µl native Pfu DNA polymerase (2.5U/µl)

Each reaction was placed in a GeneAmp 9700 PCR machine and cycled according to the following parameters:

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	10 – 18	95°C	30 seconds
		55°C	1 minute
		68°C	2 minutes/kb of plasmid

Segment 2 parameters were adjusted in accordance with the type of mutation required:

Point mutations	12 cycles
Single amino acid changes	16 cycles
Multiple amino acid deletions or insertions	18 cycles

Following completion of thermo-cycling, 1µl of *Dpn I* restriction endonuclease (10Units/µl) was added directly to each amplification reaction mixed and incubated at 37°C for 1 hour. *Dpn I* restriction endonuclease (target sequence 5' G^{m6}ATC 3') is specific for methylated and hemi-methylated DNA and is used to digest the parental (non-mutated, wild-type) DNA template and to select for mutation-containing synthesized DNA. DNA isolated from most *Escherichia coli* strains is dam (DNA adenine methylase) methylated and therefore susceptible to *Dpn I* digestion.

Table 2.10 List of mutagenesis primers used for generating pCH-3B mutants

pCH-Apobec3B (cytidine deaminase domain 1 mutant, C97A/C100A)	Forward primer: 5'GTATCCTGGACCCCCgcCCCGGACgcTGTGGCGAA GCTGGCC-3' Reverse primer: 5'- GGCCAGCTTCGCCACAgcGTCCGGGgcGGGGGTCCA GGATAC-3'
pCH-Apobec3B (cytidine deaminase domain 2 mutant, C284A/C289A)	Forward primer: 5'CATCTCCTGGAGCCCCgcCTTCTCCTGGGGCgccGC CGGGGAAGTGCGT-3' Reverse primer: 5'ACGCACTTCCCCGGCggcGCCCCAGGAGAAGgcGG GGCTCCAGGAGATG-3'

Note: lower case denotes nucleotides to be mutated

2.2.14 Transient transfection of mammalian cell lines for luciferase reporter assay

2.2.14.1 Transient transfection of cells

Cells were grown to 80-100% confluency in DMEM phenol red free media (DMEM-PR), supplemented with 5% DSS. At this point cells were harvested by trypsinisation, counted and seeded into the wells of a 24 well plate at a density of 5×10^4 cells/well in 500µl of the same medium. Cells were transfected using FuGENE[®] HD transfection reagent according to manufacturer's instructions (Roche, UK).

Each well was transfected with a transfection mixture of:

- 100ng of the firefly luciferase reporter plasmid DNA
- 100ng of the renilla luciferase reporter plasmid DNA (pRL-TK)
- 1-50ng of expression plasmid or control vector (pcDNA3.1 or pSG5) DNA
- Bluescribe carrier plasmid DNA (BSM) to make up to a total of 400ng DNA

The DNA was diluted with Opti-MEM to get a final DNA concentration of 0.02µg/µl. FuGENE[®] HD was added to the diluted DNA at a ratio of 2.5 µl reagent per 1µg of DNA and incubated for 15 minutes at room temperature. After incubation, 50µl of the DNA- FuGENE[®] mixture was added drop wise to the cells in each well. After five hours incubation, the medium was replaced with DMEM-PR supplemented with 5% DSS containing antibiotics. Twenty-four hours following transfection firefly and renilla luciferase activities were determined using the DualGlo luciferase assay kit, according to manufacturer's instructions (Promega, UK). For determining luciferase activities, the culture medium was removed from the wells, cells were washed twice with PBS, followed by the addition of 50µl of passive lysis buffer (PLB, Promega, UK) to each well. Lysis was completed by incubation on a plate shaker for 20 minutes at room temperature. Lysates were then transferred to optical quality 96 well plates, to which 50µl of the Firefly luciferase reagent was added and the resulting light emission read using a Tecan infiniteM200 plate reader. Following

this step, 50µl of Stop-Glo buffer was added to detect Renilla luciferase activity, the plate was read again. Firefly luciferase readings were subsequently normalized against the control Renilla luciferase, such that the renilla luciferase activity served to control for transfection efficiency. For experiments in which ligand was added, cells were maintained in DMEM without phenol red supplemented with double stripped serum for at least three days prior to the experiment. Ligand stocks were dissolved in absolute ethanol and stored at -20°C until needed. Ligands were added directly to the media at the appropriate final concentration and cells were collected 24 hours after treatment.

2.2.15 Transient transfection of mammalian cell lines for protein extraction

Cells were seeded at a density of 3.5×10^5 cells in 6 well plates containing DMEM with 10% FCS and kept in the cell culture incubator overnight. Next day, prior to transfection the culture medium was replaced with DMEM without serum. In a sterile microcentrifuge tube, 3µl of FuGENE[®] 6 transfection reagent was diluted in serum free DMEM and incubated for 5 minutes. One microgram of expression plasmid was added to the diluted FuGENE[®] 6 reagent (3 µl FuGENE[®] 6 per 1µg DNA), mixed and incubated for 20 minutes at room temperature to form a DNA-FuGENE[®] 6 complex. After incubation, the DNA-FuGENE[®] 6 complex was added to cells drop-wise. Cells were incubated for 5 hours and the medium was replaced with fresh DMEM containing 10%FCS. The cells were incubated for 48 hours before making cell lysates using hot lysis buffer (see 2.2.16.1) For experiments in which estrogen and antiestrogens were added, cells were maintained in DMEM phenol red free media (DMEM-PR) containing 5% DSS for at least three days prior to the seeding in 6-well plates in DMEM-PR containing 5% DSS. Ligands were added directly to the media at the appropriate final concentration and cells were collected 24 hours after treatment. For transfection of cells in 10 cm dishes, 2×10^6 cells were seeded and 10 µg of expression plasmid was used for transfection using FuGENE[®] 6 in the ratio of 3:1.

2.2.16 Preparation of protein lysates

2.2.16.1 “Hot lysis” buffer

Transfected cells in 10 cm dishes or 6 well plates were washed twice with ice-cold PBS. The PBS was removed by aspiration and 300 µl (for 10 cm dishes) and 100 µl (for 6 well plate) of 2X SDS-PAGE sample buffer (0.12M Tris-HCl, pH6.8, 4% w/v SDS, 20% w/v glycerol, 0.2M DTT, 0.008% w/v bromophenol blue) heated to 100°C, was added to the cells. Cells were scraped into 1.5ml microcentrifuge tubes, using a rubber policeman. The samples were heated at 100°C for 10 minutes, cooled on ice, and stored at -80°C.

2.2.16.2 Radioimmune Precipitation Buffer (RIPA)

For co-immunoprecipitation studies, whole cell lysates were prepared in RIPA buffer (150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris-HCl pH 7.5), supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF) and complete protease inhibitor tablets (Roche Applied Science). Transfected cells were washed twice with ice-cold PBS. The PBS was removed by aspiration and 1 ml of ice-cold RIPA buffer was added to the cells, which were scraped using a rubber policeman, into 1.5ml microcentrifuge tubes previously chilled on ice and incubated at 4°C for 30-60 minutes, with vortexing every 5 minutes. The lysate was centrifuged at 13,000 rpm for 10 minutes. The lysates were then used immediately or stored at -80°C until required.

2.2.17 Determination of Protein Concentrations

The concentration of the protein lysates was determined using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, UK). The BCA assay is based on the reduction of Cu^{2+} ions from cupric sulfate to Cu^{1+} by peptide bonds in protein. The reduction of Cu^{2+} is directly proportional to the amount of protein present in the solution. Next, chelation of two molecules of bicinchoninic acid with one molecule of Cu^{1+} occur, forming a purple-coloured reaction product, which can be read at 562 nm.

A standard curve was generated using serial dilutions (1-30 μ g) of bovine serum albumin (BSA). The BCA reagent A and B were diluted 50:1 and 200 μ l of diluted BCA reagent was mixed with 10 μ l of lysates and BSA standards in 96 well plate (Corning, USA). The samples were mixed and incubated at room temperature for 30 minutes. The optical density (OD) was measured at 562 nm. The OD of the standard was plotted and the protein concentration of the samples was determined from the standard curve.

2.2.18 siRNA transfection

Cells cultured for 3 days in DMEM-PR supplemented with 5% DSS were transfected with double-stranded RNA oligonucleotides using the reverse transfection method. For this, the double-stranded RNA oligonucleotides, purchased as ON-TARGETplus SMARTpool siRNA or Non-targeting siRNA (Dharmacon, USA) were mixed with Lipofectamine RNAiMAX (Invitrogen, UK), according to the manufacturer's protocol.

2.2.18.1 siRNA transfection of cells in 100mm culture dishes

On the day of transfection, the siRNA -transfection reagent complex was prepared by diluting 800pmol of siRNA in 500 μ l of DMEM-PR in a 10cm dish. This was followed by the addition of 40 μ l lipofectamine RNAiMAX, mixed gently and incubated for 20 minutes at room temperature. 2.25 X 10⁶ cells in DMEM-PR containing 5% DSS and 2mM L-Glutamine without antibiotics were seeded on the top of siRNA-Lipofectamine complexes. Cells were mixed and incubated for 24 hours, following which fresh DMEM-PR containing 5% DSS and 2mM L-Glutamine with antibiotics, and estrogen or anti-estrogens were added, as appropriate. Cells were incubated for 24 hours before making RNA and 48 hours before making protein lysates.

2.2.18.2 siRNA transfection of cells in 6 well plates

On the day of transfection the siRNA- transfection reagent complex was prepared by diluting 100pmol of siRNA in 250µl of DMEM-PR in each well of a 6 well plate. This was followed by the addition of 5µl lipofectamine RNAiMAX, mixed gently and incubated for 20 minutes at room temperature. Three hundred and fifty thousand cells in DMEM-PR containing 5% DSS and 2mM L-Glutamine without antibiotics were seeded on the top of siRNA-Lipofectamine complexes. Cells were mixed and incubated for 24 hours, following which fresh DMEM-PR containing 5% DSS and 2mM L-Glutamine with antibiotics. 17β-estradiol (10nM), 4-hydroxytamoxifen (100nM) or ICI182, 780 (100nM) were added as appropriate. An equal volume of ethanol was added to the no ligand controls. Cells were incubated for 24 hours before making RNA and 48 hours before making protein lysates.

2.2.18.3 siRNA transfection of cells in 96 well plates

On the day of transfection, the siRNA - transfection reagent complex was prepared by diluting 100pmol of siRNA in 250µl of DMEM serum free medium in a 24 well plate. This was followed by the addition of 5µl lipofectamine RNAiMAX, mixed gently and incubated for 20 minutes at room temperature. Ten microlitres of the siRNA-Lipofectamine complex was added to each well of a 96-well plate. Five thousand cells in DMEM-PR containing 5% DSS and 2mM L-Glutamine without antibiotics were seeded on top of the siRNA-Lipofectamine complexes. Cells were mixed and incubated for 24 hours, following which fresh DMEM-PR, supplemented with 5% DSS and 2mM L-glutamine with antibiotics. 17β-estradiol (10nM), 4-hydroxytamoxifen (100nM) or ICI182, 780 (100nM) were added as appropriate. An equal volume of ethanol was added to the no ligand controls. Cells were incubated in CO₂ incubator and fixed for cell number estimation using the SRB growth assay on 1, 3 and 5 days following transfection.

2.2.19 Transient transfection for immunofluorescence staining

Square glass cover slips were sterilised by autoclaving and individually placed into 6 well plates using sterile forceps (VWR, UK). Trypsinised cells were seeded onto the cover slips in 2 ml DMEM containing 10% FCS and antibiotics. After overnight incubation, transient transfections were carried out using the FuGENE[®] HD transfection reagent (Roche, East Sussex, UK). A total 2µg of plasmid DNA per well was diluted in 100µl of Opti-MEM. 5µl of FuGENE[®] HD transfection reagent was added to the diluted plasmid DNA, mixed and incubated for 20 minutes at room temperature. One hundred microlitres of the transfection mixture was added to each well. Five hours after the transfection, the media was replaced with fresh cell culture media. Twenty four hours post-transfection the cell monolayers were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature, prior to immunofluorescent staining.

2.2.19.1 Immunofluorescent staining of transfected cells

Following fixation, cells were permeabilised by the addition of 0.2% (v/v) Triton X-100 in PBS for 10 minutes at room temperature, followed by two washes in blocking buffer (3% (v/v) FCS, 1% (w/v) BSA in PBS). Cells were blocked in blocking buffer for 30 minutes on a shaking platform (Innova 2000 platform shaker, New Brunswick Scientific, NJ, USA) at room temperature to prevent non-specific binding by primary antibodies. Cells were incubated with primary antibody diluted in blocking buffer for 1 hour at room temperature, on a platform shaker. Cells were washed three times in blocking buffer for 5 minutes with shaking. Cells were subsequently incubated with Alexa Fluor 488 or Alexa Fluor 555 secondary antibody (Invitrogen, UK), for 1 hour at room temperature with shaking. After washing five times with blocking buffer and PBS, cover slips were incubated with TO-PRO[®]-3 stain (Invitrogen, UK) for 5 minutes at room temperature with shaking, followed by washing once in PBS. Cover slips were mounted onto glass microscope slides using Vectashield mountant containing DAPI nuclear stain (Vector Laboratories, UK). In order to prevent drying out, colourless nail varnish was used to seal the edges of the cover slips. Slides were

placed in slide boxes, covered in aluminium foil to prevent exposure to light and stored at 4°C. Slides were visualized using a confocal microscope (Zeiss, Germany).

2.2.20 Co-immunoprecipitations

Whole cell lysates using RIPA buffer (2mg) were pre-cleared with 100µl of protein A or G sepharose beads (Sigma-Aldrich, UK) for 2 hours at 4°C. Lysates were then centrifuged at 3,000 rpm for 3 minutes at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and the beads discarded. One hundred microlitres of protein A or G sepharose bead were incubated with 2-5µg of primary antibody for 2 hours at 4°C with rotation. The beads were then centrifuged and washed twice with RIPA buffer. The antibody bound beads were added to the pre-cleared lysates and incubated overnight with rotation at 4°C. Lysates were then centrifuged at 3000 rpm for 3 minutes at 4°C and the supernatant discarded. The beads were washed five times in RIPA Buffer and re-suspended in 2X SDS-PAGE sample buffer containing 200mM DDT and subject to protein gel electrophoresis and immunoblotting using the appropriate antibodies.

2.2.21 Preparation of genomic DNA

Two million cells were seeded in 10cm dishes and after overnight incubation at 37°C in a 5% CO₂ incubator, cells were scrapped into 1.5ml microcentrifuge tubes and centrifuged for 5 minutes at 2,000 rpm. The resulting cell pellet was re-suspended in 600µl DNA digestion buffer.

DNA digestion buffer:

- 100 mM NaCl
- 10 mM Tris-HCl, pH 8
- 25 mM Na-EDTA, pH 8
- 0.5% (w/v) SDS
- 0.1mg/ml proteinase K (proteinase K is labile, was added fresh with each use)

Cells were incubated in digestion buffer with shaking at 50°C for 12-16 hours. Digested samples were extracted in an equal volume of phenol/chloroform/isoamyl alcohol, followed by centrifugation at 5,000 rpm for 5 minutes. The aqueous layer was transferred into another microcentrifuge tube and 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol were added to precipitate the genomic DNA. The tubes were centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the DNA pellet washed twice with 70% Ethanol. The pellet was air dried and dissolved in TE buffer. The concentration of DNA and purity were measured by spectrophotometry using the NanoDrop spectrophotometer.

2.2.22 ChIP (Chromatin Immunoprecipitation)

Fifty million MCF-7 cells were seeded in 15cm dishes and incubated at 37°C in a 5% CO₂ incubator overnight. The following day, ligands were added for the appropriate time, followed by two washes in ice-cold PBS and fixation by adding freshly made 1% formaldehyde in 50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA. Cells were fixed in this solution for 10 minutes at room temperature, after which 1/20 volume of 2.5M glycine was added to quench unreacted formaldehyde reagent. Cells were then washed twice with ice-cold PBS and scraped into 2ml microcentrifuge tubes. Cells were centrifuged at 2,000 rpm for 5 minutes at 4°C and the supernatant discarded. The pellet was re-suspended in 1ml of Lysis buffer 1 (LB1) and placed on a rotating wheel at 4°C for 10 minutes. The tubes were then centrifuged at 2000 rpm for 5 minutes at 4°C and the resulting pellet re-suspended in lysis buffer 2 (LB2). The tubes were then placed on a rotating wheel at 4°C for 10 minutes. Tubes were then centrifuged at 2000 rpm for 5 minutes at 4°C and the resulting pellet re-suspended in lysis buffer 3 (LB3). Cells were sonicated in 1.5ml microcentrifuge tubes in a volume not more than 300µl and the sonicated lysates centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatants were collected and 10% Triton X-100 was added to the supernatants to obtain a final concentration of 1%.

One millilitre of sonicated lysate was used for immunoprecipitation and 50µl of lysate was used as the input sample. One hundred microlitres of paramagnetic beads (Invitrogen, Dynabeads) were added to a 1.5ml microfuge tube and the beads were washed three times by adding 1 ml of block solution (0.5% BSA (w/v) in PBS) to beads. The beads were collected by using moulded ceramic magnets shaped as tube holders. Supernatants were removed by aspiration and the paramagnetic beads re-suspended in 250µl of block solution. Two micrograms of antibody was added to re-suspended beads and incubated overnight at 4°C. After incubation, beads were washed once and then resuspended in 100µl of blocking solution. One millilitre of lysate was added to antibody bound beads and the beads incubated overnight at 4°C. After incubation, tubes were placed in magnetic tube holders to collect beads and the supernatant removed. The beads were washed six times using 1 ml of RIPA buffer for each wash. After washing with RIPA buffer, beads were finally washed with 1 ml TBS (20 mM Tris-HCl pH 7.6, 150 mM NaCl). Tubes were placed in magnetic tube holders and the supernatant was removed. 200µl of elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (w/v) SDS) was added. The beads were mixed for 15 minutes on a shaker and the supernatant collected and transferred to a fresh microcentrifuge tube. Reverse cross-linking was performed at 65°C for a minimum of 6-18 hours. In order to purify the reverse cross-linked DNA, 200µl of TE buffer was added to each sample, followed by 4µl of 10mg/ml RNaseA. The sample was mixed and incubated at 37°C for 30 minutes. Then, 4µl of 20mg/ml proteinase K was added to each sample and the tubes incubated at 50°C for 2 hours. Four hundred microlitres of phenol: chloroform: isoamyl alcohol was added to each tube and the whole solution was transferred to 2ml phase-lock gel tubes (5 Prime, UK), mixed and centrifuged at 13,000 rpm for 5 minutes. The aqueous layer was transferred to a new microcentrifuge tube. Sixteen microlitres of 5M NaCl (200mM final concentration), 1 µl of glycoblue DNA carrier and 800µl of 100% ethanol were added to each tube and incubated for 30 minutes at -80°C. The tubes were centrifuged for 10 minutes at 4°C to harvest the DNA and the pellet washed with 80% ethanol and centrifuged at high speed for 5 minutes. The resulting washed DNA pellet was air dried and re-suspended in 30µl of 10mM Tris-HCl pH 8.0. Two microlitres of re-suspended DNA was used for quantitative real-time PCR.

Lysis buffer 1 (LB1)

- 50 mM HEPES-KOH, pH 7.5
- 140 mM NaCl
- 1 mM Na-EDTA
- 10% Glycerol
- 0.5% Igepal CA-630
- 0.25% Triton X-100

Lysis buffer 2 (LB2)

- 10 mM Tris-HCl, pH 8.0
- 200 mM NaCl
- 1 mM Na-EDTA
- 0.5 mM EGTA

Lysis buffer 3 (LB3)

- 10 mM Tris-HCl, pH 8.0
- 100 mM NaCl
- 1 mM EDTA
- 0.5 mM EGTA
- 0.1% sodium deoxycholate
- 0.5% N-lauroylsarcosine

RIPA buffer:

- 50 mM HEPES-KOH, pH 7.5
- 500 mM LiCl
- 1 mM EDTA
- 1% Igepal CA-630
- 0.7% sodium deoxycholate

2.2.23 DNA-break labelling and ChIP

Detection of transient DNA strand break formation was performed as described (Ju et al., 2006). Briefly, ligand treated or untreated cells were fixed with Streck Tissue Fixative (STF, Streck Laboratories), which does not cause any DNA damage (Ju et al., 2006) for 10 minutes at 37°C. The cells were harvested, washed twice with ice-cold PBS and centrifuged at 2,000 rpm for 5 minutes. The pellet was re-suspended in Buffer A (0.25% Triton X-100, 10mM EDTA, 10mM HEPES [pH6.5]) and placed on a rotating wheel for 10 minutes. Cells were centrifuged at 2,000 rpm for 5 minutes and the pellet was then suspended in Buffer B (200mM NaCl, 1mM EDTA, 10mM HEPES pH6.5), and permeabilized with Buffer C (100mM Tris-HCl pH7.4, 50mM EDTA, 1% Triton X-100) for one hour at 4°C. Subsequently, the nuclei were sequentially washed with ice-cold PBS, deionized water and 1x terminal deoxynucleotide transferase (TdT; Promega) reaction Buffer. The DNA breaks were labelled with biotin-11-dUTP using 50U of TdT for 1 hour at 37°C and residual biotin-dUTP was washed away with Buffer D (100mM Tris-Cl pH7.4, 150mM NaCl). Nuclei were refixed in 1% formaldehyde in 50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA and chromatin immunoprecipitation was performed using an anti-biotin antibody (Sigma-Aldrich, UK), as described above. Figure 2.1 shows a diagram of the protocol.

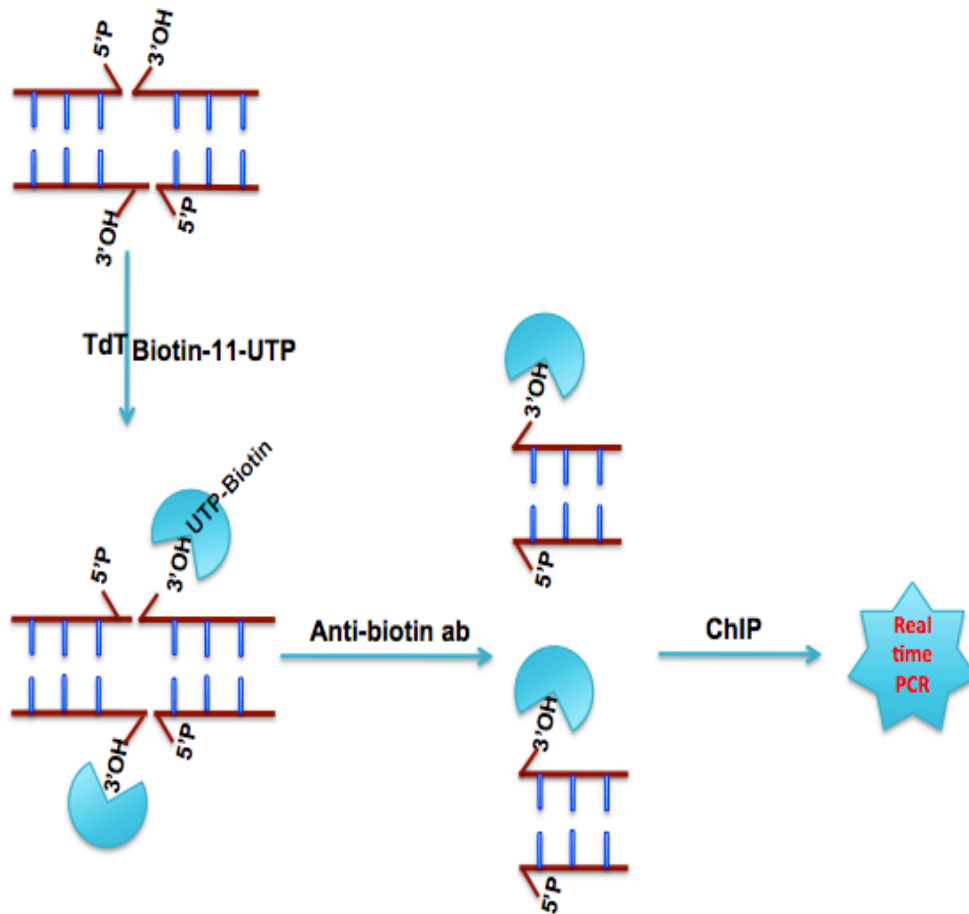


Figure 2.1 DNA break labeling ChIP analysis. A diagrammatic summary of the method used to label DSB sites. Following estrogen treatment, the purified genomic DNA is biotinylated by the addition of biotin-11-UTP by terminal deoxynucleotidyl transferase (TdT). Biotin labeled DNA is purified using a biotin antibody and real-time PCR is done to determine out amounts of DNA breaks in the vicinity of the amplified region of interest

2.2.24 Bisulfite modification of genomic DNA

The bisulfite conversion of genomic DNA was performed using a Qiagen EpiTect kit according to manufacturers instructions (Qiagen, Crawley, UK). The method involves converting unmethylated cytosine to uracil by deamination while the methylated cytosines are left unchanged. In brief, 1µg of genomic DNA was mixed with the bisulfite reaction mix and DNA protection buffer. The bisulfite reaction components were placed in a thermal cycler. The following thermal cycle conditions were used for bisulfite conversion.

Table 2.11 Bisulfite conversion PCR cycle conditions

Step	Time (mins)	Temperature
Denaturation	5	95°C
Incubation	25	60°C
Denaturation	5	95°C
Incubation	85	60°C
Denaturation	5	95°C
Incubation	175	60°C
Hold	Indefinite	20°C

The bisulfite converted DNA was purified using the column provided with the kit and the DNA was dissolved in RNase and DNase free water. Figure 2.1 shows schematic diagram of the protocol. Figure 2.1 shows schematic diagram of the steps in the bisulphite conversion and PCR amplification.

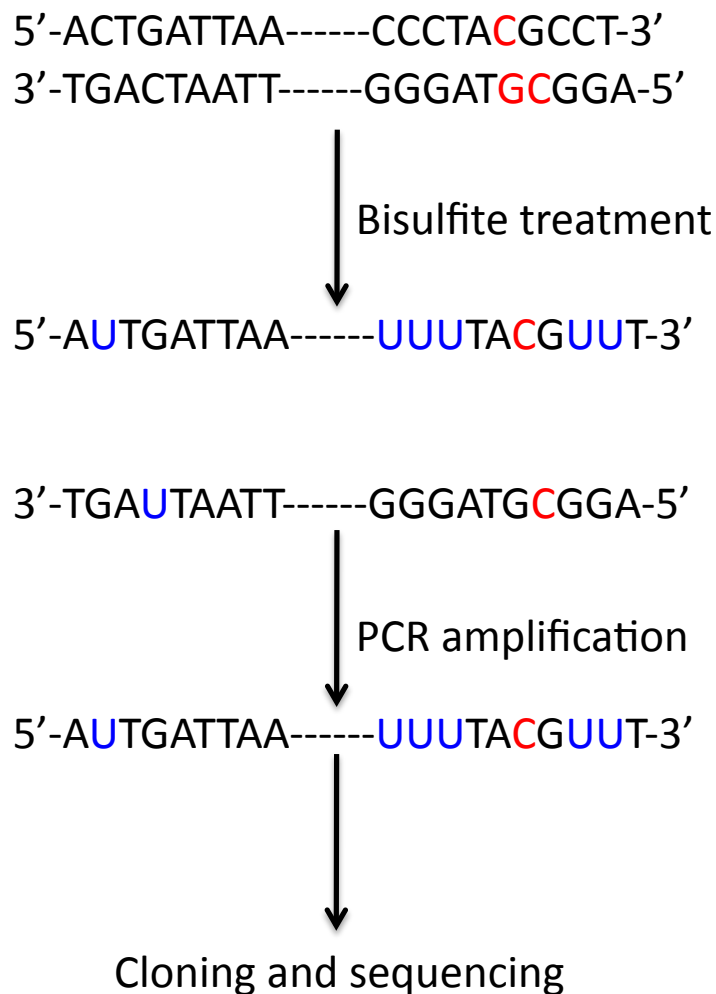


Figure 2.2 Bisulfite conversion of genomic DNA for identification of methylated cytosines. Bisulfite treatment of DNA converts unmethylated cytosine (blue) to uracil while methylated cytosine (red) is resistant to conversion. PCR amplification of the sequence of interest converts uracil to thymine and the PCR product is cloned and sequenced for methylation analysis.

2.2.25 DNA methylation analysis by pyrosequencing

Quantitative DNA methylation analysis of the bisulfite treated DNA was performed by pyrosequencing (Figure 2.3). Regions of interest were amplified using 2µl of bisulfite-treated genomic DNA and 10 pmol of forward and reverse primers, one of them being biotinylated. PCR reaction conditions were HotStar *Taq* buffer supplemented with 3 mM MgCl₂, 200 mM dNTPs, and 2.0 U of HotStar *Taq* DNA polymerase (Qiagen, Crawley, UK) in a 25µl volume.

The PCR thermal cycle program:

Denaturing	95°C - 15 minutes
45 cycles of	
Denaturing	95°C - 30 seconds
Annealing	56°C - 30 seconds
Extension	72°C - 30 seconds

PCR products were purified and rendered single-stranded on a Pyrosequencing workstation (Biotage, Qiagen, Crawley, UK). PCR products were diluted in 40µl of ddH₂O and incubated for 10 minutes at room temperature with 40µl of binding buffer (10 mM Tris, 2 mM NaCl, 1 mM EDTA and 0.1% Tween 20, pH 7.6, adjusted with 1 mM HCl) containing 2µl of streptavidin-coated sepharose beads (GE Healthcare, UK). The binding mix was aspirated, and the template was successively washed with 70% ethanol, denatured with 0.2 mM NaOH, and washed with washing buffer (10 mM Tris, pH 7.6, adjusted with 4M acetic acid). Beads were released into 12µl of annealing buffer (20 mM Tris and 2 mM magnesium acetate, pH 7.6, adjusted with 4M acetic acid) containing 10 pmol of sequencing primer. Primers were annealed to the target by incubation at 80°C for 2 minutes. Quantitative DNA methylation analysis was performed on a Pyromark MD system with the PyroGold SQA reagent kit (Qiagen, UK), and results were analyzed using the Q-CpG software.

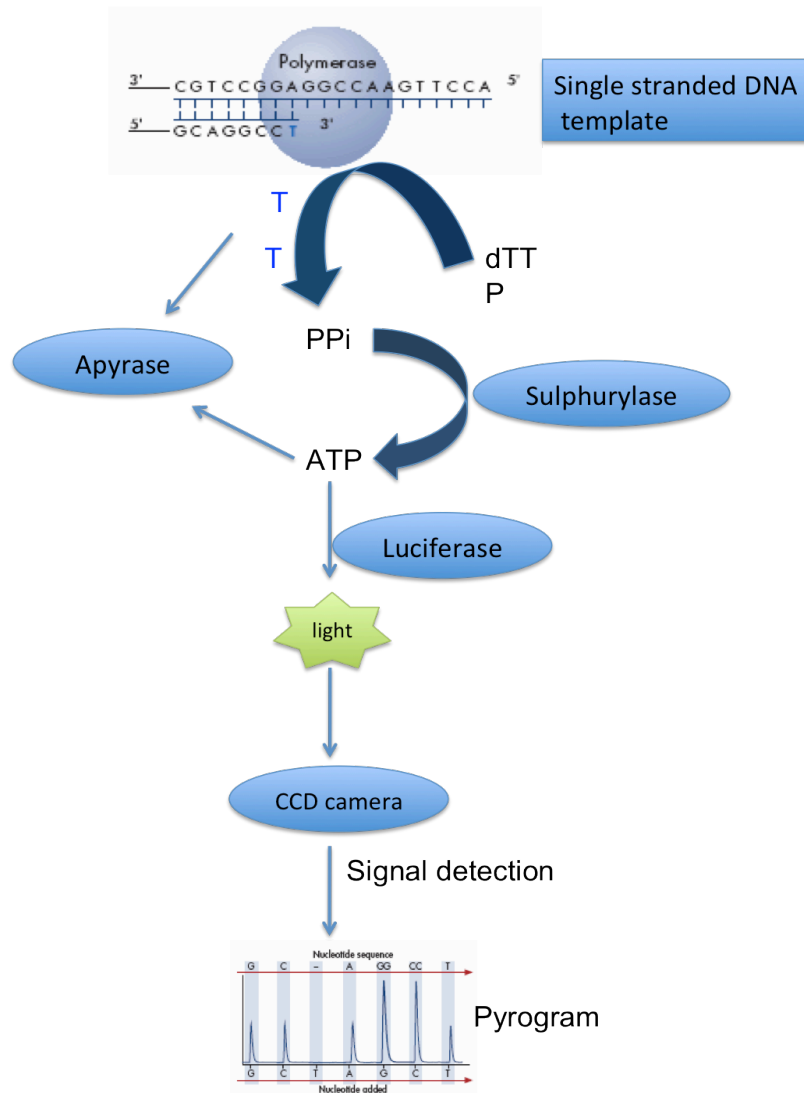


Figure 2.3 Principles of pyrosequencing methodology for DNA sequencing. The primer for the sequencing analysis is annealed to a single-stranded DNA template, and incubated with DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the luciferin substrates. The four nucleotides (A, T, C and G) are added by a pyrosequencing machine, one at a time, to the pyrosequencing reaction. For every successful nucleotide incorporation pyrophosphate (PPi) is released. The ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). The signal light produced by the luciferase-mediated reaction in presence of ATP is detected by a CCD camera and seen as a peak in a Pyrogram. The height of each peak is proportional to the number of nucleotides incorporated. The Apyrase enzyme continuously degrades ATP and unincorporated dNTPs. When degradation is complete, another nucleotide is added. The nucleotide sequence of the complementary DNA strand is inferred from the signal peaks of the pyrogram. Methylation of CpG sites are analyzed using the Q-CpG software (SW 1.0).

2.2.26 Preparation of GST and GST–MBD fusion protein

E. coli (Rosetta) bacteria were cultured overnight at 37°C in LB broth containing 100µg/ml ampicillin. Fifty millilitres of overnight culture was used to inoculate 500ml of LB broth at 37°C and allowed to grow until OD_{600nm} of between 0.6-0.8, was reached. Protein expression was induced by the addition of 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture incubated for an additional 4 hours at 37°C. Bacterial cells were collected by centrifugation and GST-MBD fusion protein was purified using B-PER GST Fusion Protein Purification kit (Pierce, UK), according to manufacturer's instructions. Purified protein was aliquoted; protein concentration was determined using the BCA protein assay kit and purity was confirmed by Coomassie staining following SDS-PAGE. The purified GST-MBD protein was stored at -80°C until required.

2.2.27 GST–MBD pull-down experiment

Twenty micrograms of genomic DNA was sonicated in a 400µl volume of Pull-down buffer (20mM Tris pH 8, 100mM NaCl, 1mM EDTA and 2mM DTT) to an approximate size of 200-500bp. Five micrograms of sonicated DNA was incubated with 5 µg of GST-MBD fusion protein in a volume of 600µl Pull-down buffer for 2-3 hours at 4°C, with constant rotation. Methylated DNA and GST-MBD complexes were separated by the addition of 50% slurry of GST agarose beads equilibrated in Pull-down buffer, with a further incubation of one hour at 4°C. After incubation, the beads were washed four times in Pull-down buffer and the methylated DNA was eluted from the complex by adding 700µl of 5M Guanidine hydrochloride, 30% isopropanol and 20mM HEPES pH 7.4. The eluate was purified by using a Qiaquick PCR Purification kit (Qiagen). The purified DNA was used in quantitative real-time PCR. Sonicated DNA was used as Input to normalise the DNA pulled down in the GST-MBD assay.

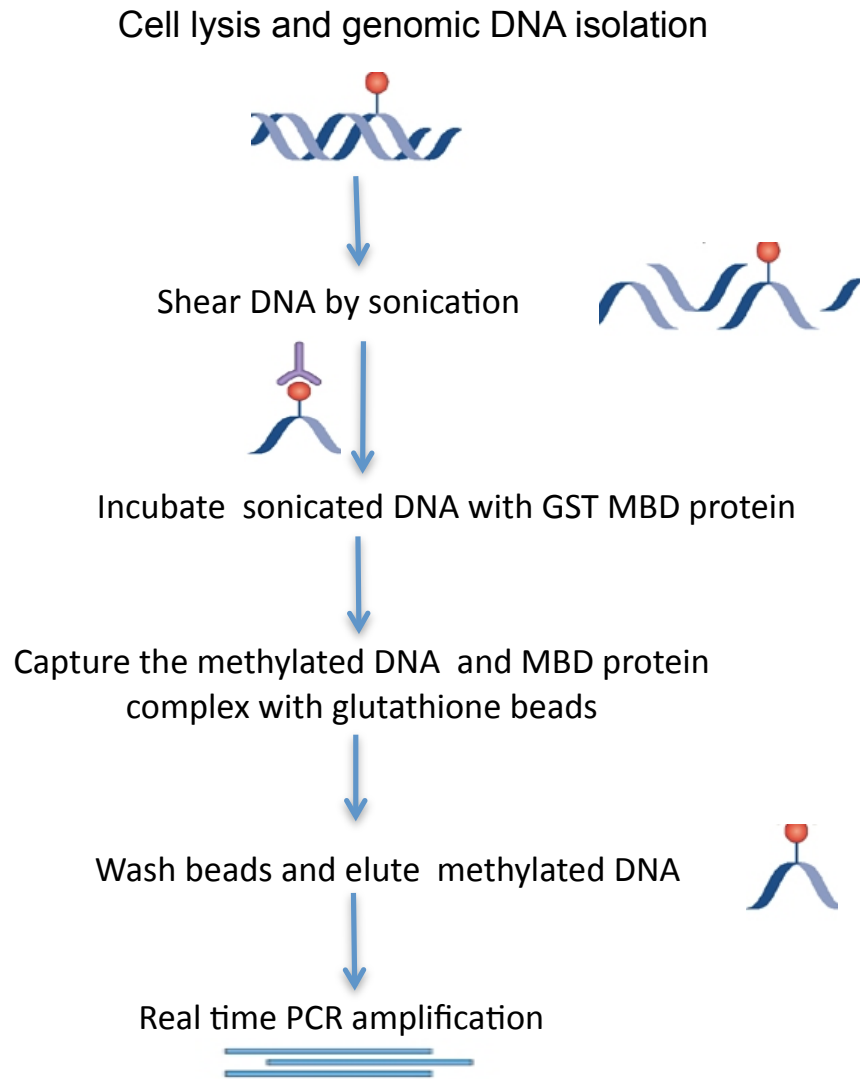


Figure 2.4 Scheme for pulldown of genomic DNA methylated at CpG dinucleotides. Genomic DNA prepared from cells is subjected to sonication to obtain fragments sizes of 200-1000bp. The fragmented DNA is incubated with GST fused to the methyl CpG binding domain of MeCpG2. Glutathione beads are added to pulldown GST-MBD and bound methylated DNA. The pulldowns are washed extensively and DNA eluted for subsequent analysis by PCR.

3. Results

3.1 Expression of AID/APOBEC family proteins in breast cancer cells

As described in the introduction, preliminary studies from our laboratory have shown that APOBEC3B can act as a co-activator for ER α and ER β in reporter gene assays, but not for other steroid receptors, or for non-steroid receptors in the nuclear receptor superfamily. By contrast some other members of the AID/APOBEC family which do not stimulate ER α or ER β activity, act as co-activators for other steroid receptors (Thomas et al., unpublished). AID/APOBEC family members have been shown to widely express in cancer tissues and cell lines (Harris et al., 2002; Jarmuz et al., 2002; Okazaki et al., 2007). APOBEC3B has been shown to abundantly expressed in colorectal adenocarcinoma and chronic myelogenous leukaemia cell lines and to a lesser extent in lung carcinoma, melanoma and cervical carcinoma cell lines (Jarmuz et al., 2002). In order to determine if AID/APOBEC genes may be involved in gene regulation in breast cancer, RT-PCR was carried out to determine if they, and in particular APOBEC3B, are expressed in breast cancer cell lines. In a panel of ER α positive and negative breast cancer cell lines (RNA kindly provided by Dr Ross Thomas, Imperial College) APOBEC3B expression was observed in all of the breast cancer cell lines examined, with the exception of the ER α negative SkBr3 cells (Figure 3.1). Deletion polymorphism for APOBEC3B has been observed in breast cancer tissue and in some breast cancer cell lines, including SkBr3 (Komatsu et al., 2008), but not the other cell lines examined here, which explains the absence of APOBEC3B expression in SkBr3 cells. Expression of APOBEC3G was also observed in most of the cell lines, with expression of the other APOBEC3 genes and AID being more variable among the breast cancer cell lines. Hence a number of APOBEC3 genes are expressed in breast cancer cell lines, with APOBEC3B being expressed in almost all breast cancer lines examined, demonstrating that APOBEC3B is widely expressed in breast cancer cell lines, with its expression being independent of ER α expression.

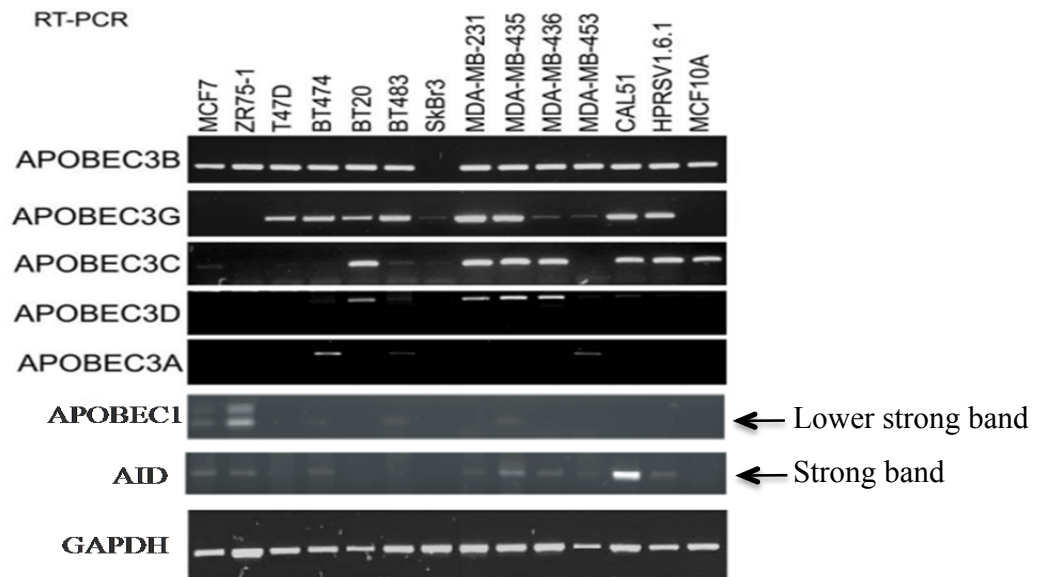


Figure 3.1. RT-PCR analysis of endogenous expression of AID/APOBEC family proteins in breast cancer cell lines. RT-PCR of total RNA prepared from various breast cancer cell lines was carried out using primers specific for members of the AID/APOBEC family, as shown. Note that gene expression microarray analysis has now indicated that the MDA-MB-435 cell line does not represent breast cancer, but is likely a melanoma line (Rae et al., 2007).

3.2 Over-expression of APOBEC3B in MCF-7 breast cancer cell stimulates its growth

APOBEC3B is the sole member of the APOBEC/AID family that stimulated ER α activity in reporter gene assays, in which ER α and APOBECs were transiently transfected. In order to determine if APOBEC3B also regulates the activity of endogenous ER α and if it is required for the growth of estrogen-dependent breast cancer cell lines, APOBEC3B was overexpressed in MCF-7 cells. MCF-7 cells are the classic model cell line for ER α -positive breast cancer, requiring estrogen for growth, with growth being inhibited by anti-estrogens (Brooks et al., 1973; El Etreby and Liang, 1998; Jensen et al., 2003; Levenson and Jordan, 1997; Lippman and Bolan, 1975). Further, the expression of several estrogen-responsive genes has been intensively studied in MCF-7 cells, whilst extensive gene expression profiling and global ER α binding sites determination has been carried out (Carroll et al., 2006; Garza et al., 2009; Metivier et al., 2003; Welboren et al., 2007; Welboren et al., 2009). Moreover, expression analysis showed that APOBEC3B is the only member of the APOBEC3 gene family strongly expressed in MCF-7 cells, with low level expression of APOBEC3C, APOBEC1 and AID observed in MCF-7 cells.

In order to determine if APOBEC3B regulates breast cancer cell growth, MCF-7 cells were transiently transfected with HA-tagged APOBEC3B. Over-expression of APOBEC3B stimulated MCF-7 cell growth (Figure 3.2A), whereas another member of the APOBEC3 gene family that encodes an APOBEC containing 2 cytosine deaminase domains, as does APOBEC3B, but which does not stimulate ER α activity in reporter gene assays, did not affect MCF-7 cell growth.

As described in section 1.18, the APOBEC3B gene encodes a protein with two catalytically active cytidine deaminase domains, CD1 and CD2. Reporter gene assays showed that mutation of cysteine residues in these domains prevent stimulation of ER α activity by APOBEC3B in reporter gene assays (Thomas et al., unpublished). The growth of MCF-7 cells transfected with APOBEC3B was stimulated (Figure 3.3B), in agreement with the results shown in Figure 3.2A. CD1 or CD2 mutation prevented stimulation of MCF-7 cell growth, indicating that the enzymatic activity is required for stimulation of MCF-7 cell growth by APOBEC3B. Growth of the ER α -

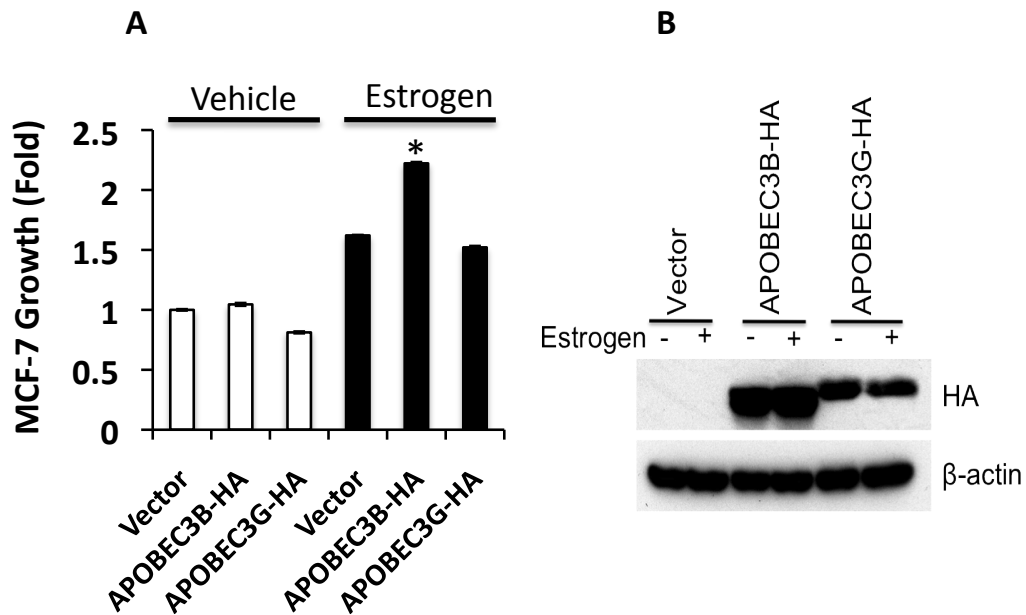


Figure 3.2. Over-expression of APOBEC3B stimulates MCF-7 cell growth.

A. MCF-7 cells cultured in DMEM lacking phenol red and containing 10% dextran-coated charcoal-stripped serum were plated in 96 well plates. Twenty four hours later cells were transiently transfected with HA-tagged APOBEC3B or APOBEC3G using Fugene 6 transfection reagent. Twenty four hours post transfection cells were treated with 10nM estrogen prepared in ethanol. An equal volume of ethanol was added to the vehicle control. Cell growth was estimated after 5 days using the SRB assay. Growth is shown relative to the vehicle-treated vector control. The bar graph represents the means and the error bars show the standard error of the mean (s.e.m) for four replicates. The asterisk denotes statistically significant difference ($P < 0.05$) relative to the vector control, determined using the unpaired t-test. **B.** Western blot analysis of protein lysates prepared from MCF-7 cells following transfection with HA-tagged APOBEC3B or APOBEC3G was carried out using an HA antibody. Immunoblotting for β -actin was used as a protein loading control.

positive and estrogen-responsive T47D cells was similarly stimulated by APOBEC3B, but not by the CD domain mutants (Figure 3.3C), indicating that the effects of APOBEC3B are not restricted to MCF-7 cells.

As described in Chapter 1, ER α controls breast cancer cell growth upon binding estrogen by regulating the expression of estrogen-responsive genes. To determine if APOBEC3B regulates the expression of endogenous estrogen-responsive genes, MCF-7 cells were transfected with APOBEC3B and the CD domain mutants. Western blotting for ER α showed that APOBEC3B over-expression did not affect ER α levels (Figure 3.4). Interestingly, expression of the estrogen-regulated cathepsin D (CTSD) and pS2 (TFF1) genes was increased in cells transfected with APOBEC3B. CTSD and pS2 expression was not elevated following transfection with APOBEC3B CD1 or CD1/2 mutants, and appeared to be slightly lower than the expression for the vector control. However, transfection with the APOBEC3B CD2 mutant showed similar levels of expression of CTSD and pS2 compared to vector alone transfected cells, suggesting that the CD1 domain is particularly important for the co-activator function of APOBEC3B, a result which is in agreement with reporter gene assays that showed the CD1 domain of APOBEC3B is more important for co-activation of ER α than the CD2 domain (Thomas et al, unpublished). Clearly, however, both domains are required for stimulation of ER α activity, since the CD2 mutant did not considerably stimulate the expression of estrogen-responsive genes and did not stimulate MCF-7 or T47D cell growth.

3.3 RNAi-mediated knockdown of APOBEC3B expression inhibits the growth of ER α positive breast cancer cells and the expression of estrogen-regulated genes

APOBEC3B over-expression stimulated breast cancer cell growth and expression of estrogen-responsive genes. To determine if endogenous APOBEC3B plays a role in the regulation of ER α activity and breast cancer cell growth, MCF-7 cells were transfected with APOBEC3B siRNA. APOBEC3B knockdown inhibited MCF-7 cell growth (Figure 3.5A) and similarly inhibited the growth of the T47D and ZR-75-1 ER α -positive breast cancer cell lines (Figure 3.5B, C). Growth of the ER α -negative

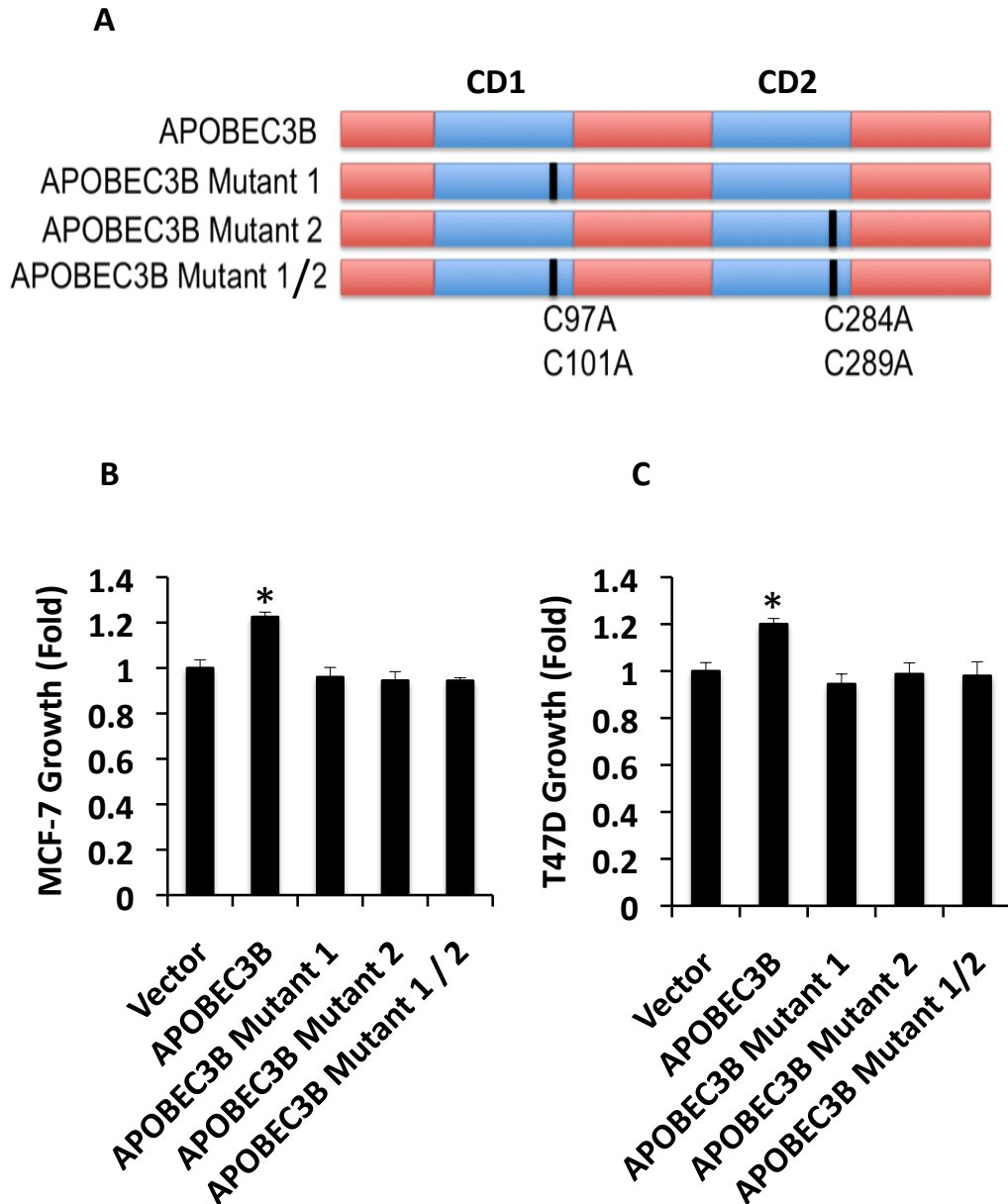


Figure 3.3. Mutation of the critical cysteine residues in the cytosine deaminase domains prevents stimulation of MCF-7 and T47D growth by APOBEC3B.

A. Domain organization of APOBEC3B, blue color represent catalytic domains 1 and 2 and the black bar shows the positions of the cysteine to alanine amino acid substitutions within each domain. **B and C.** MCF-7 and T47D cells were transiently transfected with APOBEC3B or cytosine deaminase domain mutants as in figure 3.3. Twenty four hours post transfection medium was replaced with fresh medium containing 10% FCS and cell numbers were estimated after 5 days using the SRB assay. Growth is shown relative to the vector control. The bar graph shows as the means for six replicates. Error bars represent the s.e.m. Asterisks denote statistically significant difference ($P < 0.05$) relative to vector control, determined using the unpaired t-test.

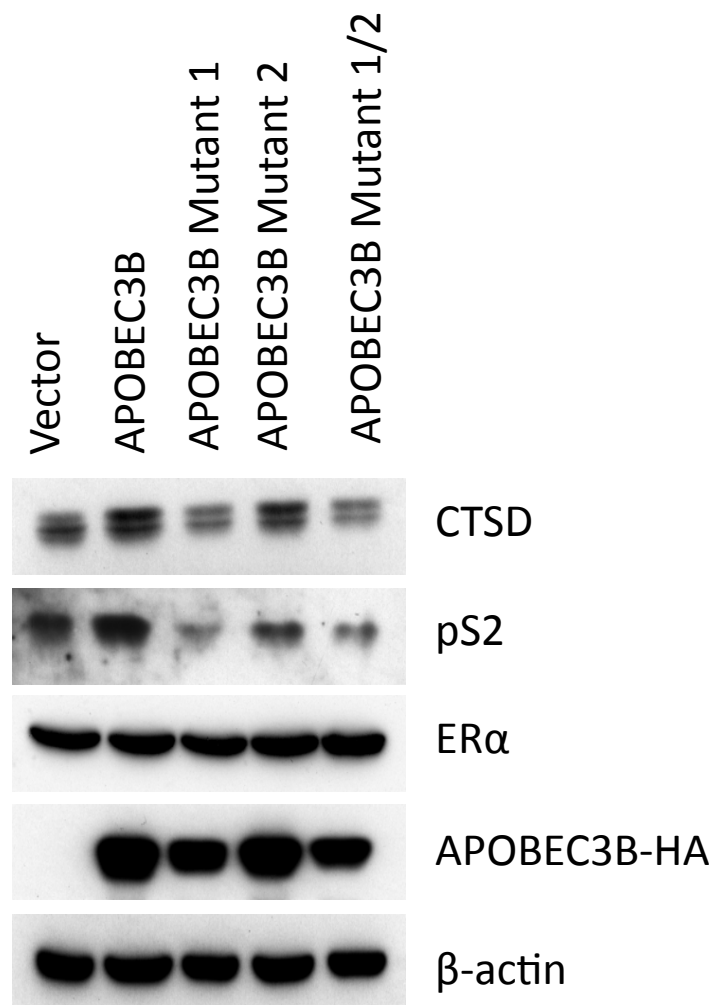


Figure 3.4. Effect of over-expression of APOBEC3B or cytosine deaminase domain mutants on the expression of estrogen regulated genes in MCF-7 cells.

MCF-7 cells cultured in DMEM medium containing 10% FCS were plated in 6 well plates. Twenty four hours later cells were transiently transfected with HA-tagged APOBEC3B or the cytosine deaminase domain 1 and 2 mutants (shown in Fig. 3.4). Cell lysates were prepared 24 hours later, using hot lysis buffer. Immunoblotting was carried out using antibodies to cathepsin D (CTSD), pS2, ERα and β-actin, as shown. Immunoblotting using an HA antibody was used for detection of transfected APOBEC3B.

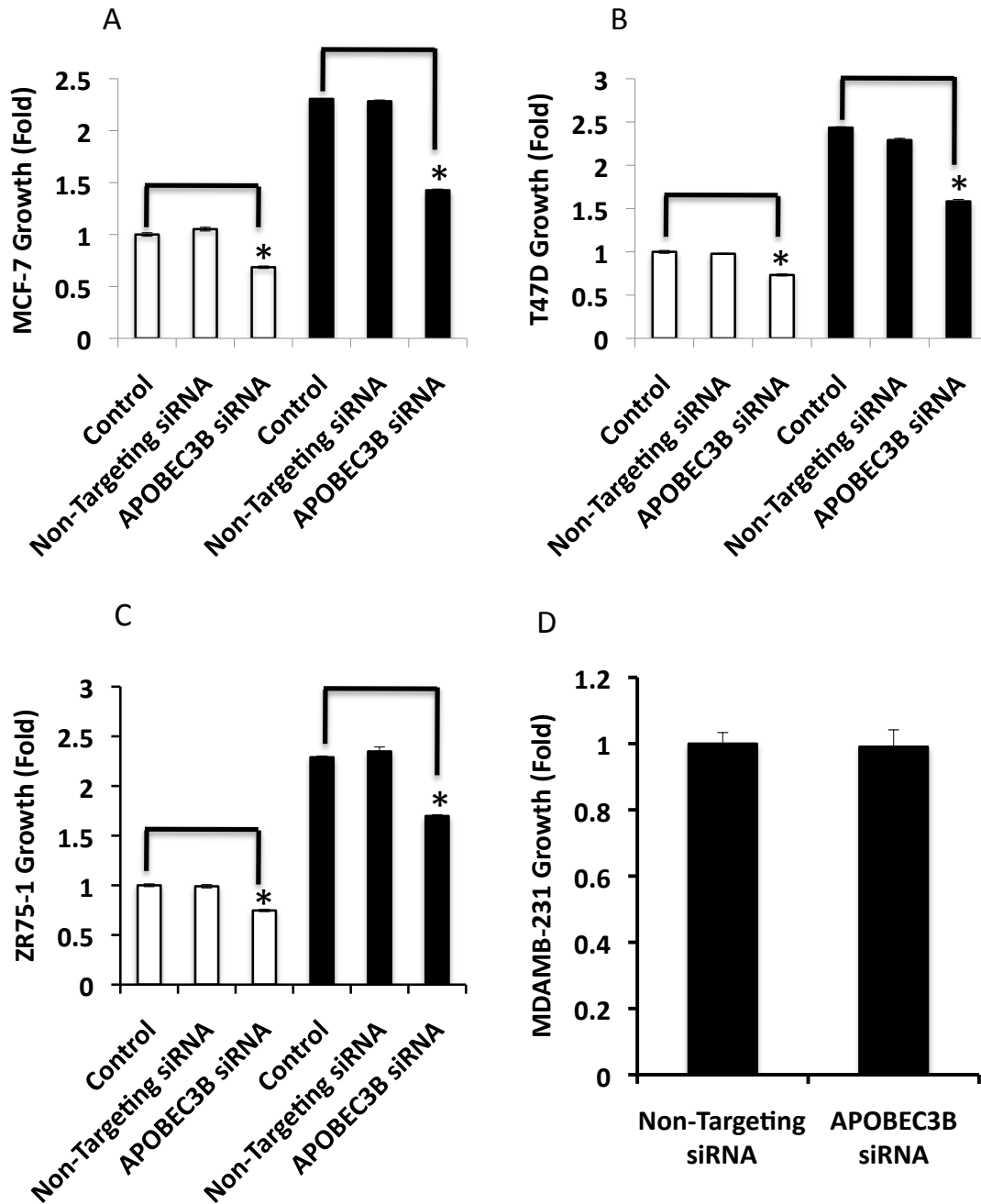


Figure 3.5. APOBEC3B siRNA inhibits breast cancer cell growth.

A-C. MCF-7, T47D and ZR75-1 cells were transfected with Non-Targeting siRNA or with APOBEC3B siRNA using the lipofectamine RNAiMAX reagent by the reverse transfection method. Control denotes transfection with reagent alone. Non-targeting siRNA was characterized by genome-wide microarray analysis by supplier and found to have least targeting of known genes in the human genome. Twenty four hours post transfection cells were treated with 10nM estrogen and cell growth was estimated after 5 days using the SRB assay. **D.** MDA-MB-231 ER-negative breast cancer cells were similarly transfected and growth was determined using the SRB assay after 5 days. The bar charts show the results of 4 replicates, with error bars depicting the s.e.m. Asterisks show statistically significant differences ($p < 0.05$), determined using the unpaired t-test. Cells were grown in the absence of estrogen (white bars) or in the presence of estrogen (10nM) (filled bars).

MDA-MB-231 breast cancer cells was not affected by APOBEC3B siRNA, suggesting that APOBEC3B is important for ER α action in breast cancer.

To further investigate whether APOBEC3B is required for expression of endogenous estrogen-responsive genes in breast cancer cells, total RNA and protein lysates were prepared from MCF-7 cells following transfection with the APOBEC3B siRNA. Quantitative Real-time RT-PCR (qRT-PCR) for APOBEC3B showed 80-90% reduction in APOBEC3B expression following transfection with the APOBEC3B siRNA (Figure 3.6A). APOBEC3B siRNA did not affect ER α expression in MCF-7 cells (Figure 3.6B). However, estrogen stimulated expression of GREB1, CTSD, PR and pS2 genes was significantly reduced (Figure 3.6C-F). In agreement with the qRT-PCR results, immunoblotting for APOBEC3B showed no detectable APOBEC3B following transfection with the APOBEC3B siRNA (Figure 3.8). In addition, immunoblotting for pS2, CTSD and PR showed reduced expression of these genes in cells transfected with APOBEC3B siRNA, mirroring the qRT-PCR results. GREB1, CTSD, PR and pS2 expression was similarly reduced in T47D cells following APOBEC3B siRNA (Figure 3.7C-F), although the reduction in GREB1 and PR expression did not reach statistical significance in T47D cells. Furthermore, APOBEC3B siRNA led to a reduction in ER α levels, although the reduction did not reach significance (Figure 3.7B). Together, these results show that APOBEC3B is required for the ER α -mediated expression of estrogen-induced gene expression in breast cancer cells.

3.4 APOBEC3B interact with ER α *in vivo* in an estrogen-independent manner

To determine if APOBEC3B interacts directly with ER α whole cell lysates were prepared from COS-1 cells transiently co-transfected with HA-tagged APOBEC3B and ER α , in the absence and presence of estrogen for 4 hours. Immunoprecipitations (IP) were performed on whole cells lysates using ER α and HA antibodies. IP using the ER α antibody, followed by immunoblotting using the HA antibody, showed that APOBEC3B co-immunoprecipitates with ER α in an estrogen independent manner (Figure 3.9A).

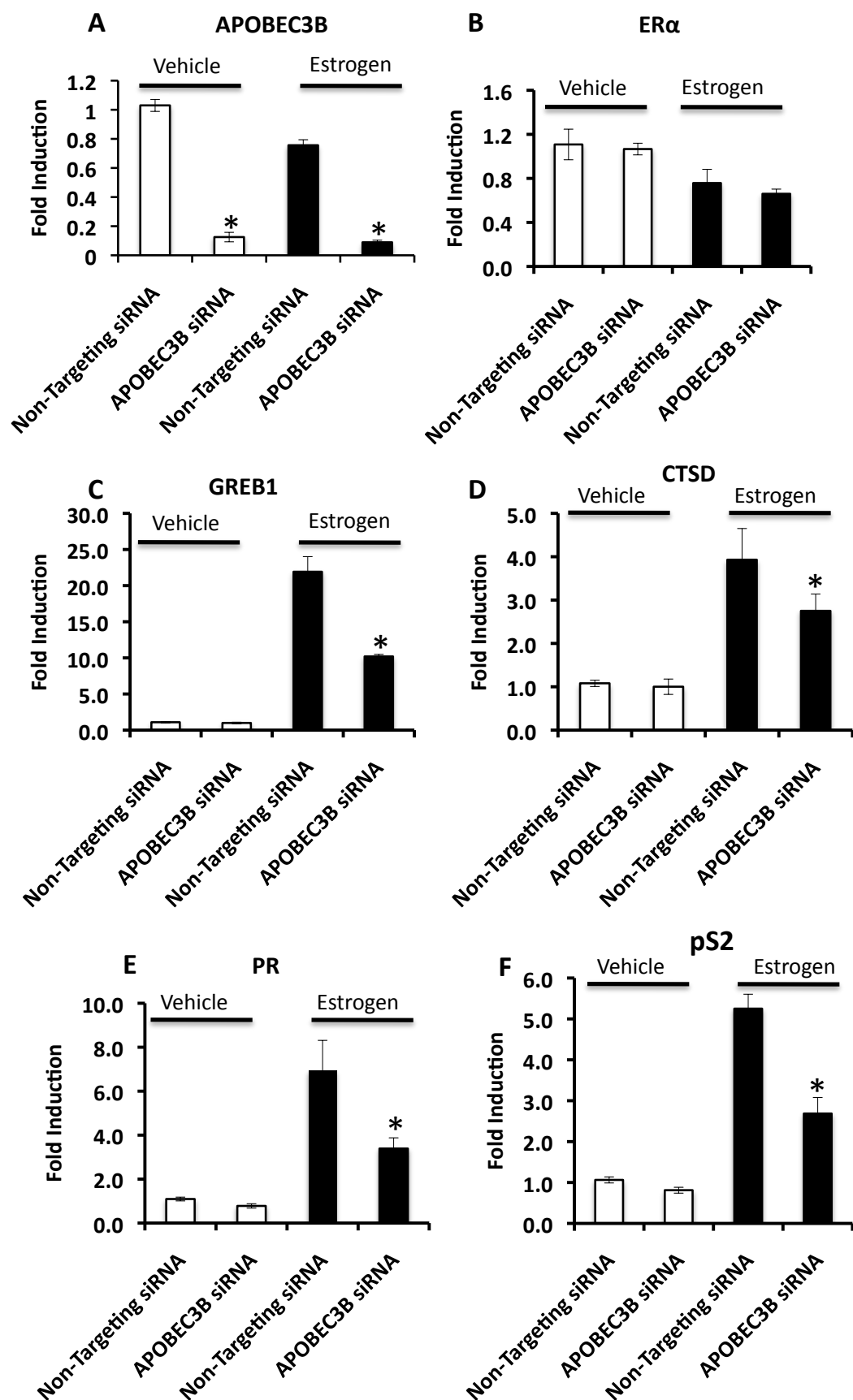


Figure 3.6. APOBEC3B siRNA inhibits the the expression of estrogen regulated genes in MCF-7 cells. MCF-7 cells cultured in DMEM lacking phenol red and containing 10% dextran-coated charcoal-stripped serum were transfected with APOBEC3B or Non-Targeting siRNA using lipofectamine RNAiMAX with the reverse transfection method. Non-targeting siRNA was characterized by genome-wide microarray analysis by supplier and found to have least targeting of known genes in the human genome. Forty eight hours post transfection media were supplemented with 10nM estrogen or an equal volume of ethanol (vehicle) and total RNA prepared after a further 24 hours. Shown are the results of real-time RT-PCR for three replicates, following normalisation for GAPDH. Asterisks denote statistically significant differences ($P < 0.05$) relative to the respective non-targeting siRNA control, determined using the unpaired t-test.

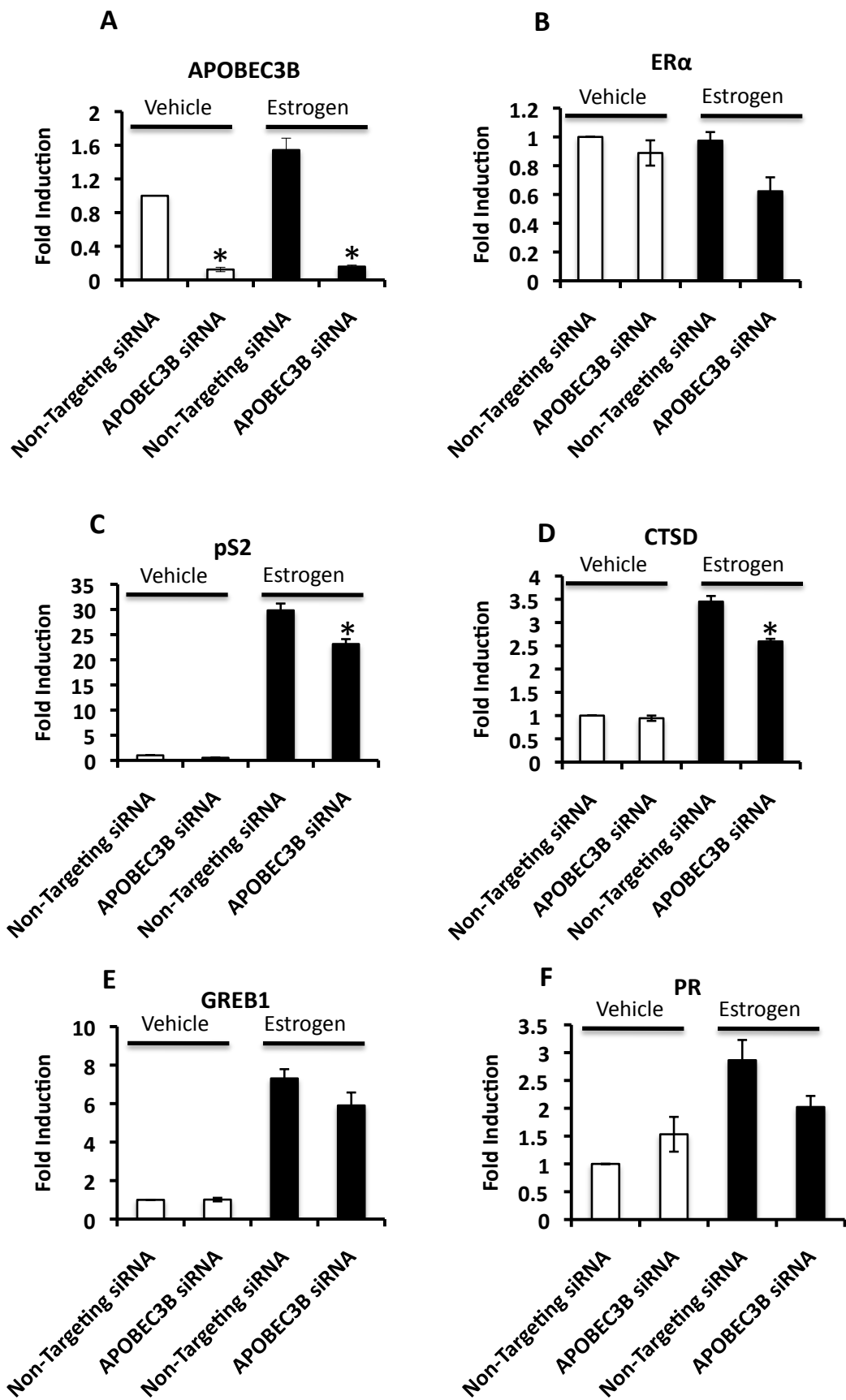


Figure 3.7. APOBEC3B siRNA inhibits the the expression of estrogen regulated genes in T47D cells. T47D cells cultured in DMEM lacking phenol red and containing 10% dextran-coated charcoal-stripped serum were transfected with APOBEC3B or Non-Targeting siRNA using lipofectamine RNAiMAX with the reverse transfection method. Forty eight hours post transfection media were supplemented with 10nM estrogen or an equal volume of ethanol (vehicle) and total RNA prepared after a further 24hrs. Shown are the results of three independent replicates, following normalisation for GAPDH. Asterisks denote statistically significant differences ($P < 0.05$) relative to the respective Non-Targeting siRNA control, determined using the unpaired t-test. Non-targeting siRNA was characterized by genome-wide microarray analysis by supplier and found to have least targeting of known genes in the human genome.

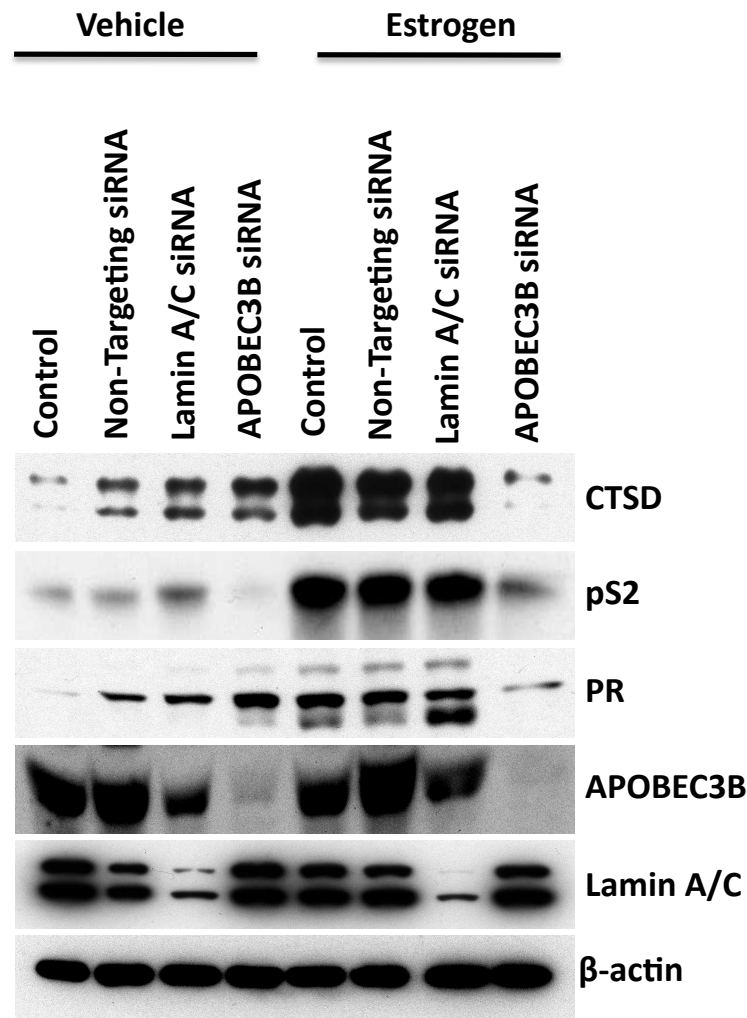


Figure 3.8. Immunoblot analysis shows that APOBEC3B siRNA reduces the expression of estrogen responsive genes in MCF-7 cells. MCF-7 cells cultured in DMEM lacking phenol red and containing 10% dextran-coated charcoal-stripped serum were transfected with APOBEC3B siRNA, Lamin A/C siRNA or Non-Targeting siRNA as in Fig. 3.8. Control refers to transfection with reagent alone. Forty eight hours post-transfection the culture medium was supplemented with 10nM estrogen. Cell lysates were prepared in hot lysis buffer after a further 24 hrs. Lysates were resolved by SDS-PAGE and immunoblotted, as shown.

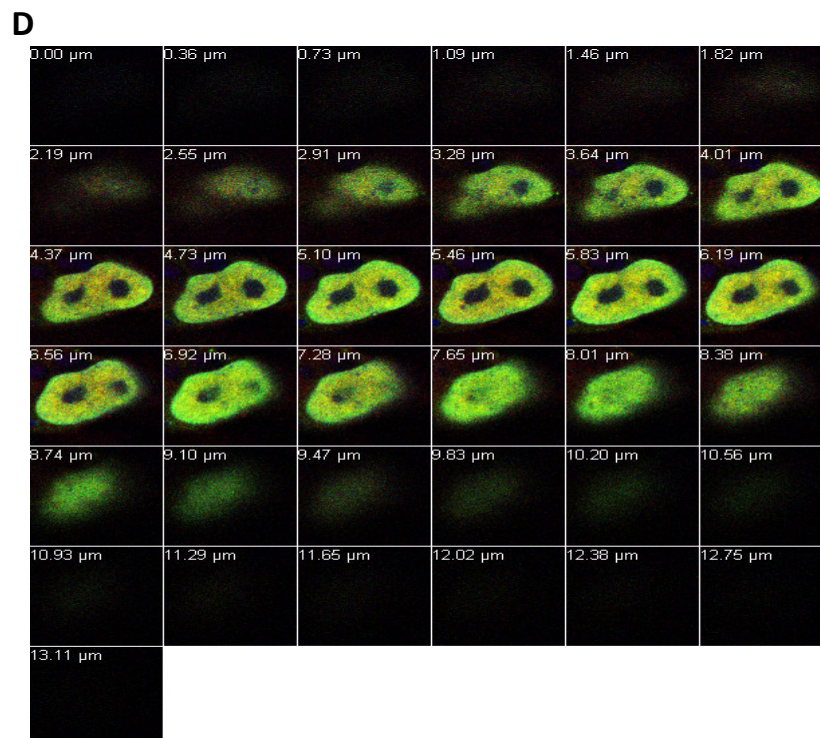
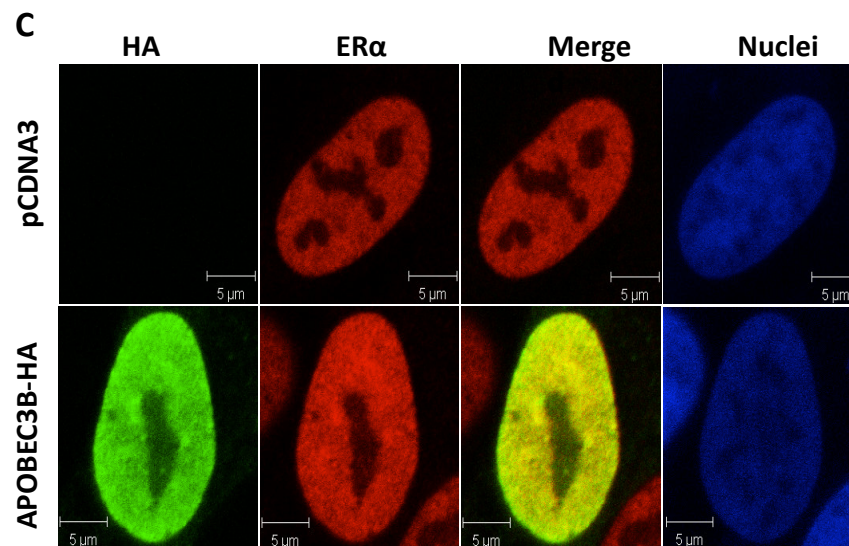
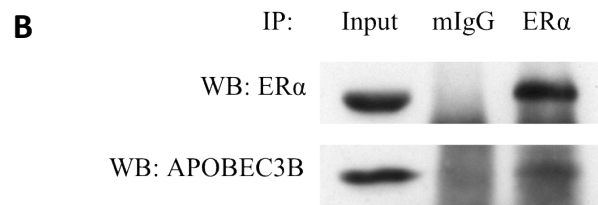
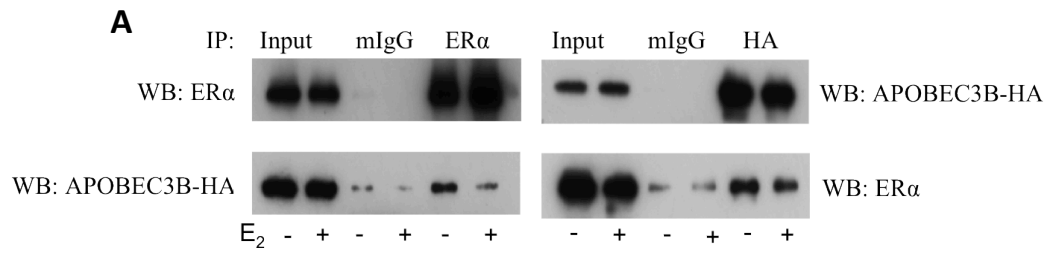


Figure 3.9. APOBEC3B interact with ER α *in vivo*. **A.** COS-1 cells were transfected with ER α and APOBEC3B-HA. Five hours post transfection medium was replaced with fresh medium. Twenty four hours post-transfection cells were treated with 10nM estrogen for 4hrs and whole cell lysates were prepared and immunoprecipitated with mouse IgG or with HA or ER α antibodies, followed by immunoblotting for HA or ER α . Input represents 1% of the total volume of lysate used in the immunoprecipitation reactions. **B.** Whole lysates prepared from MCF7 cells were immunoprecipitated using an ER α antibody, followed by immunoblotting for ER α and APOBEC3B. Input represents 1% of the total volume of lysate used in the immunoprecipitation reactions. **C.** U2OS cells were transiently transfected with ER and HA-tagged APOBEC3B, as shown. Twenty four hours following transfection cells were fixed in 4% paraformaldehyde and then incubated with ER α (red) and HA (green) antibodies. The cells were subsequently incubated with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse secondary antibodies and TO-PRO-3 for the nuclei counterstaining. Images were acquired using a Zeiss LSM510 confocal microscope. **D.** Z-stacks showing colocalization of APOBEC3B and ER α . Numbers in white represent the depth of the Z section in μm . Merged figure showing colocalization (yellow color) of APOBEC3B and ER α in U2OS cells.

Similar results were obtained following IP using the HA antibody, indicating that ER α and APOBEC3B interact *in vivo* in an estrogen independent manner. To further confirm the interaction between ER α and APOBEC3B, whole cell lysates were prepared from MCF-7 cells cultured in DMEM containing 10% FCS. IP with an ER α antibody, followed by immunoblotting for APOBEC3B demonstrated interaction of endogenous ER α and APOBEC3B in MCF-7 cells (Figure 3.9B).

Immunofluorescence staining was undertaken to visualise the interaction between APOBEC3B and ER α and to determine if APOBEC3B co-localises with ER α . Immunofluorescence staining was performed in U2OS cells transiently cotransfected with HA tagged APOBEC3B and ER α . ER α and APOBEC3B-HA were both present in the nucleus. Further, sectioning of the cells indicates that APOBEC3B-HA and ER α were colocalized within the nucleus (Figure 3.9C and D).

3.5 APOBEC3B is recruited to the promoters of estrogen responsive genes in a dynamic manner

Since ER α and APOBEC3B interact and APOBEC3B acts as an ER α co-activator, I determined whether APOBEC3B is recruited to promoters of ER α target genes. Chromatin immunoprecipitation (ChIP) was performed over a time-course of estrogen treatment in MCF-7 cells. MCF-7 cells were maintained in estrogen-depleted medium for 72 hours, after which estrogen was added to a final concentration of 10 nM and cells were fixed at 10 minutes intervals over a 3 hour period, since ChIP studies have shown cyclical recruitment and dissociation of a large number of ER α coactivators over this period following E2 addition (Metivier et al., 2003; Shang et al., 2000). ChIP was performed using ER α or APOBEC3B antibodies or with rabbit IgG as a control. Real-time PCR was performed to determine relative levels of pS2 and GREB1 promoter occupancy by ER α and APOBEC3B. In the absence of estrogen treatment ER α was detected at both the pS2 and GREB1 promoters but at very low levels, in agreement with previous reports. ER α recruitment was markedly enhanced by estrogen treatment.

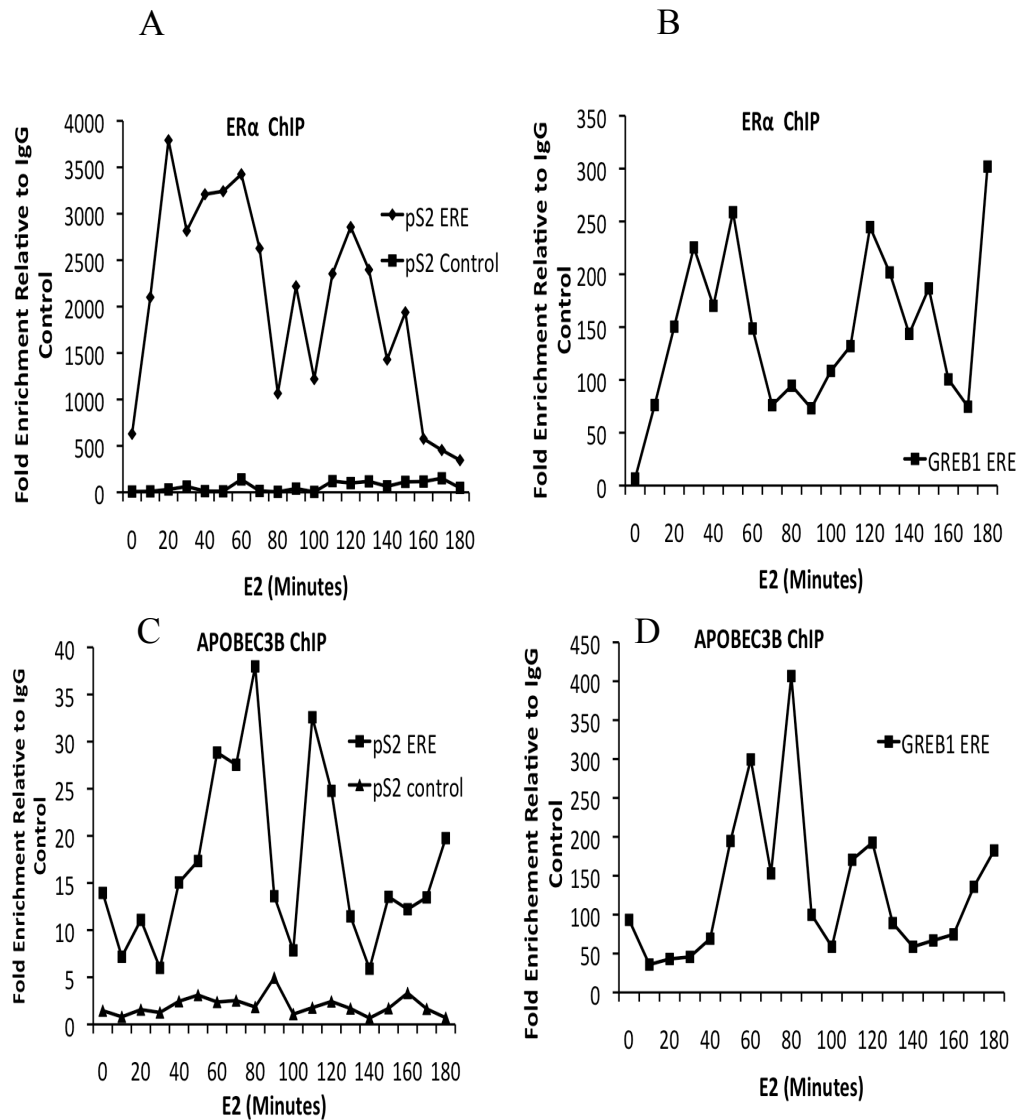


Figure 3.10. Chromatin immunoprecipitation showing dynamic occupancy of ERα and APOBEC3B at estrogen regulated genes promoters. MCF-7 cells were plated in 15cm culture dishes in estrogen-free medium. After 24 hours, estrogen was added to a final concentration of 10 nM and cells were crosslinked by the addition of 1% formaldehyde for 10 minutes at which point glycine was added. Cells were lysed and sonicated followed by immunoprecipitation with ERα and APOBEC3B antibodies. Immunoprecipitated DNA was reverse cross-linked and purified by phenol-chloroform extraction. The purified DNA was used for quantitative RT-PCR using primers for the promoter proximal pS2 ERE region, pS2 control and the region encoding the ERE in the GREB1 gene. Real-time PCR was also carried out using primers for a region of the pS2 promoter to which ERα is not recruited. Results are shown as as mean values of two replicates.

The measured level of pS2 promoter occupied by ER α increased 2000 fold within 10 minutes following the addition of estrogen (Figure 3.10A). Maximal induction of promoter occupancy of greater than 3000 fold was detected at 20 minutes (peaks at 20-60 minutes) following addition of estrogen and this returned almost to baseline by 80 minutes. The second round of ER α binding on the pS2 promoter was seen between 110-160 minutes. Real-time PCR using primers that amplify a region of the GREB1 promoter encoding an ERE showed similar cyclical recruitment/loss of ER α at the GREB1 promoter (Figure 3.10B). As observed for ER α the recruitment of APOBEC3B to the pS2 and GREB1 promoters similarly showed cyclical recruitment and loss (Figure 3.10C and 3.10D). The level of APOBEC3B occupancy at the promoters increased over 40-60 minutes, reaching a peak at 80 minutes and returning to baseline levels by 90 minutes, with a second cycle between 110-140 minutes (Figure 3.10C and 3.10D). Interestingly, however, highest levels of APOBEC3B at the pS2 and GREB1 promoters coincided with the lowest levels of ER α .

3.6 The pS2 gene promoter DNA is rapidly methylated and demethylated in a temporal fashion

In mammals, DNA methylation occurs predominantly in the context of CpG dinucleotides. Epigenetic maintenance of gene silencing is provided by cytosine methylation at CpG dinucleotides. Dynamic reprogramming of DNA methylation patterns is considered to play an important role during development and differentiation in vertebrates. Demethylation of 5-methylcytosine may occur by an active mechanism (enzyme mediated) or a passive mechanism involving DNA replication. Various mechanisms have been proposed for active DNA demethylation, which include, enzymatic removal of the methyl group from 5-methyl cytosine, direct removal of 5-methyl cytosine, followed by base excision repair (BER), enzymatic deamination of 5-methyl cytosine to thymine (T) followed by BER of T:G mismatch and enzymatic conversion of 5-methyl cytosine to 5-hydroxy methyl cytosine followed by BER (Wu and Zhang, 2010). Although these mechanisms have been confirmed experimentally, the mechanisms of DNA demethylation remain unclear and controversial. It has been shown in MCF-7 cells that upon estrogen treatment pS2 gene promoter undergoes methylation/demethylation cycle; with the timing of

methylation/demethylation coincide with the cyclical recruitment of ER α to pS2 promoter (Kangaspeska et al., 2008). Similarly, MDA-MB-231 cell stably expressing ER α showed cyclical methylation/demethylation during transcriptional cycling of pS2 gene promoter on estrogen treatment (Metivier et al., 2008a). Further, It has been shown in MDA-MB-231 cells that DNMT3A and DNMT3B are involved in both methylation and demethylation of pS2 promoter, although others have challenged this mechanism (Wu and Zhang, 2010). So the mechanism for active DNA demethylation is still not known clearly. Since APOBEC3B is a member of the cytidine deaminase enzymes, we hypothesized that APOBEC3B may be involved in estrogen induced cyclical methylation/demethylation in MCF-7 cells by deaminating 5-methyl cytosine, causing T:G mismatch which can be repaired by BER.

In order to determine if the pS2 gene promoter undergoes cyclical methylation/demethylation upon estrogen treatment in MCF-7 cells methylated DNA immunoprecipitation assay (GST-MBD pull-down assay) was carried out. In brief, MCF-7 cells treated with 10nM estrogen were collected at 10 minutes intervals over a three hour period. Genomic DNA was isolated and fragmented by sonication to an average size of approximately 300-500 bp, and DNA containing symmetrically methylated cytosine was selectively isolated by incubation with glutathione S-transferase (GST)-MBD fusion protein. The pS2 promoter is initially unmethylated (Figure 3.11), with three cycles of DNA methylation and demethylation observed over the three hours following addition of estrogen, with peaks of methylation at 20-30 minutes, between 80-90 minutes and between 130-150 minutes. The observed result is showing a small difference to published results in MCF-7 cells (Kangaspeska et al., 2008), where two broad cycles of methylation observed between 20-50 minutes and between 120-150 minutes. Further, in order to confirm the cyclical methylation/demethylation observed by GST-MBD pull-down assay, pyrosequencing analysis was carried out to determine DNA methylation changes at individual CpGs in the pS2 promoter. In brief, genomic DNA was treated with sodium bisulphite to convert unmethylated cytosine to uridine. Methylated cytosine is resistant to this conversion. Bisulphite converted DNA was PCR amplified with specific sets of primers, and PCR products were sequenced using a pyrosequencer. Although there was evidence of methylation at several CpG sites located between -400 to + 20 of the

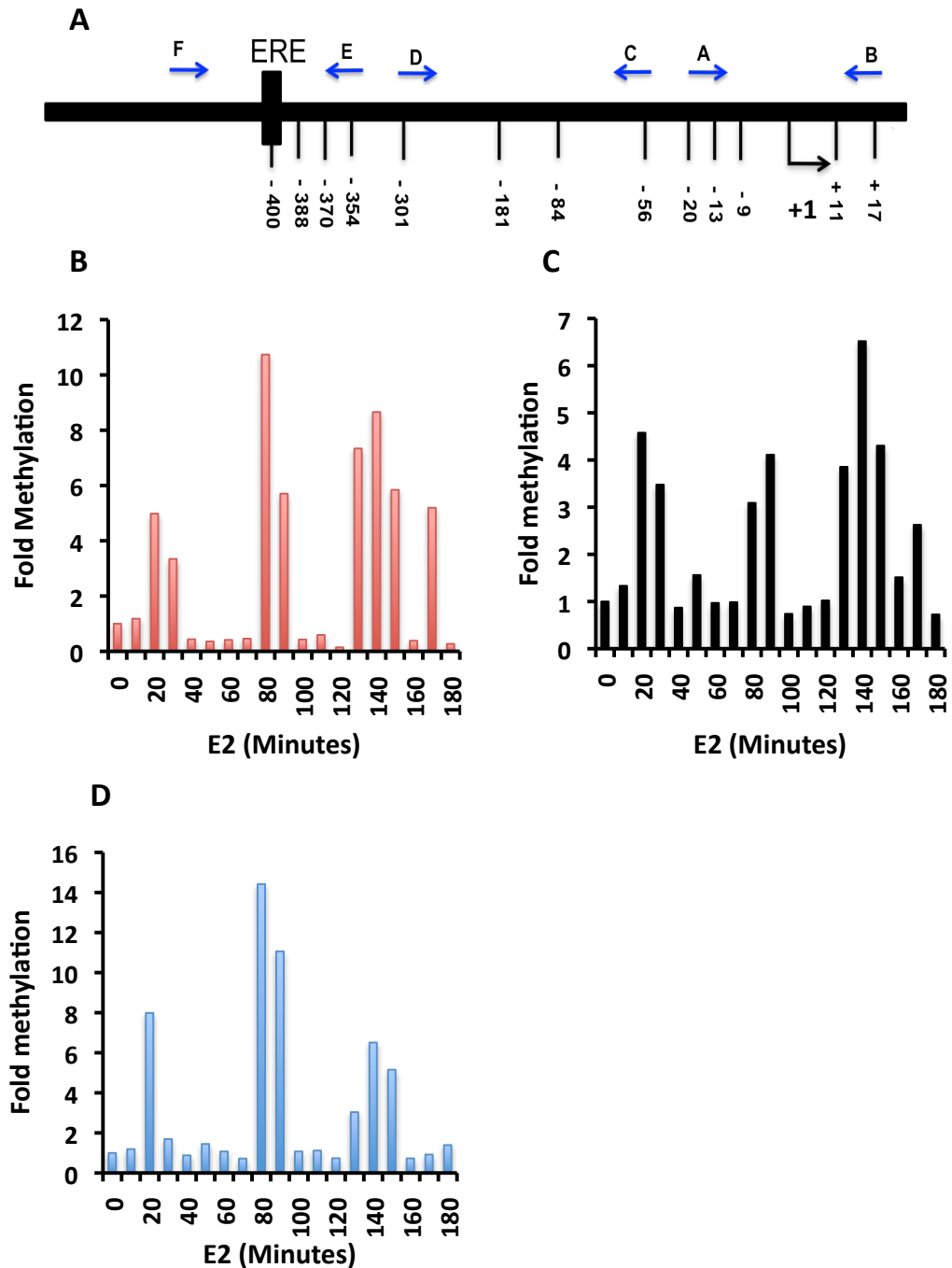


Figure 3.11. Estrogen induces cyclical methylation and demethylation at the pS2 promoter. **A.** Shown is a representation of the pS2 promoter gene promoter. The positions of CpG dinucleotides are marked by vertical lines, and horizontal (blue) arrows indicate positions of the different PCR primers used for amplifying the different regions of the pS2 promoter, with transcription initiation site denoted as +1. The position of the ERE is also indicated. **B-D.** MCF-7 cells were cultured for 72hrs in estrogen-free medium. Estrogen was then added and cells collected at 10 minutes intervals and genomic DNA was prepared. After fragmentation by sonication, DNA fragments containing methylated CpG's were selectively isolated using the GST-MBD pulldown method outlined in Fig. 3.11. Real-time PCR was carried out with different primer pairs A/B (part B), C/D (part C) and E/F (part D) spanning the pS2 promoter for quantitation of methylation levels. Results are shown as mean values of two replicates.

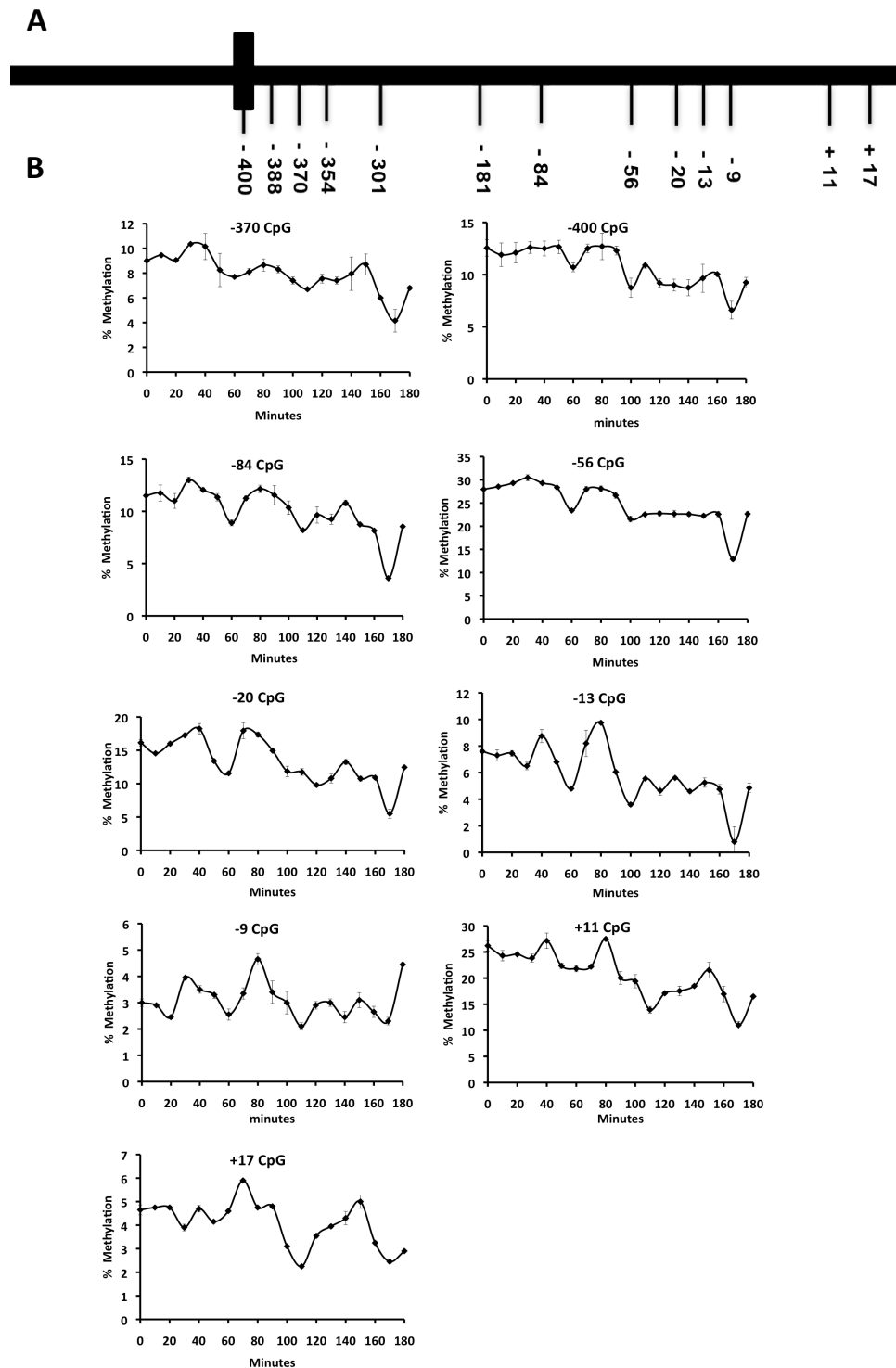


Figure 3.12. Pyrosequencing analysis shows only low DNA methylation at the pS2 gene promoter. **A.** Shown is the pS2 promoter region with positions of CpG dinucleotides indicated by vertical lines. **B.** MCF-7 cells were cultured for 72 hrs in estrogen-free medium, followed by the addition of estrogen to a final concentration of 10nM and genomic DNA was prepared at 10 minute intervals following estrogen addition. Genomic DNA was treated with sodium bisulfite to convert unmethylated cytosine. Methylation status of individual CpG sites was determined by pyrosequencing. Results are shown as mean values of three replicates, with error bars showing s.e.m.

pS2 promoter, the methylation levels were generally low (between 5-10%). Only 2 sites, at -56 and +11 were methylated at higher levels, around 25-30% methylation being observed (Figure 3.12). Moreover, there was little change in methylation at any of the sites, in contrast to the results of the GST-MBD pulldown. The reason for the discrepancy in results with the two different techniques is not clear. It may be that in the pS2 promoter GST-MBD pull-down assay detects methylation changes away from the pS2 promoter, since the pulldown is carried out using sonicated DNA, giving average DNA fragment lengths of 300-500 bp. It may be necessary therefore to examine DNA sequences further along the pS2 promoter or within the pS2 gene.

3.7 APOBEC3B is not involved in DNA demethylation at the pS2 gene promoter

As mentioned above, we hypothesised that APOBEC3B may be involved in estrogen mediated DNA demethylation. If APOBEC3B were involved in DNA demethylation, the DNA methylation levels of estrogen-regulated genes should be elevated following RNAi for APOBEC3B. To test this, I performed APOBEC3B knockdown experiments in MCF-7 cells cultured in estrogen-depleted medium for three days. Forty-eight hours after transfection, estrogen was added and cells were harvested at various times following estrogen addition for genomic DNA preparation. Genomic DNA was treated with sodium bisulfite to convert unmethylated cytosine to uridine and PCR was carried out to amplify the sequencing region in pS2 promoter, as described above. The PCR products were cloned into the pCR4-TOPO vector and at least 24 colonies were selected randomly and sequenced to determine DNA methylation levels. The pS2 promoter showed evidence of cyclical methylation and demethylation in non-targeting siRNA transfected cells, with few clones showing methylation at 20 and 50 minutes, respectively (Figure 3.13A). Although DNA methylation level was higher in the APOBEC3B knockdown at 0 minutes, compared to the non-targeting siRNA control, methylation levels at 20 minutes were considerably less compared to control, with more methylation being observed at 40 minutes of estrogen treatment and less at 50 and 60 minutes, respectively (Figure 3.13B). If APOBEC3B were important for DNA demethylation we would have expected to see more methylation across the time course, together with loss of the cyclical DNA methylation/demethylation, which was not observed. Collectively, this result indicates that APOBEC3B may not be important. Figure 3.13

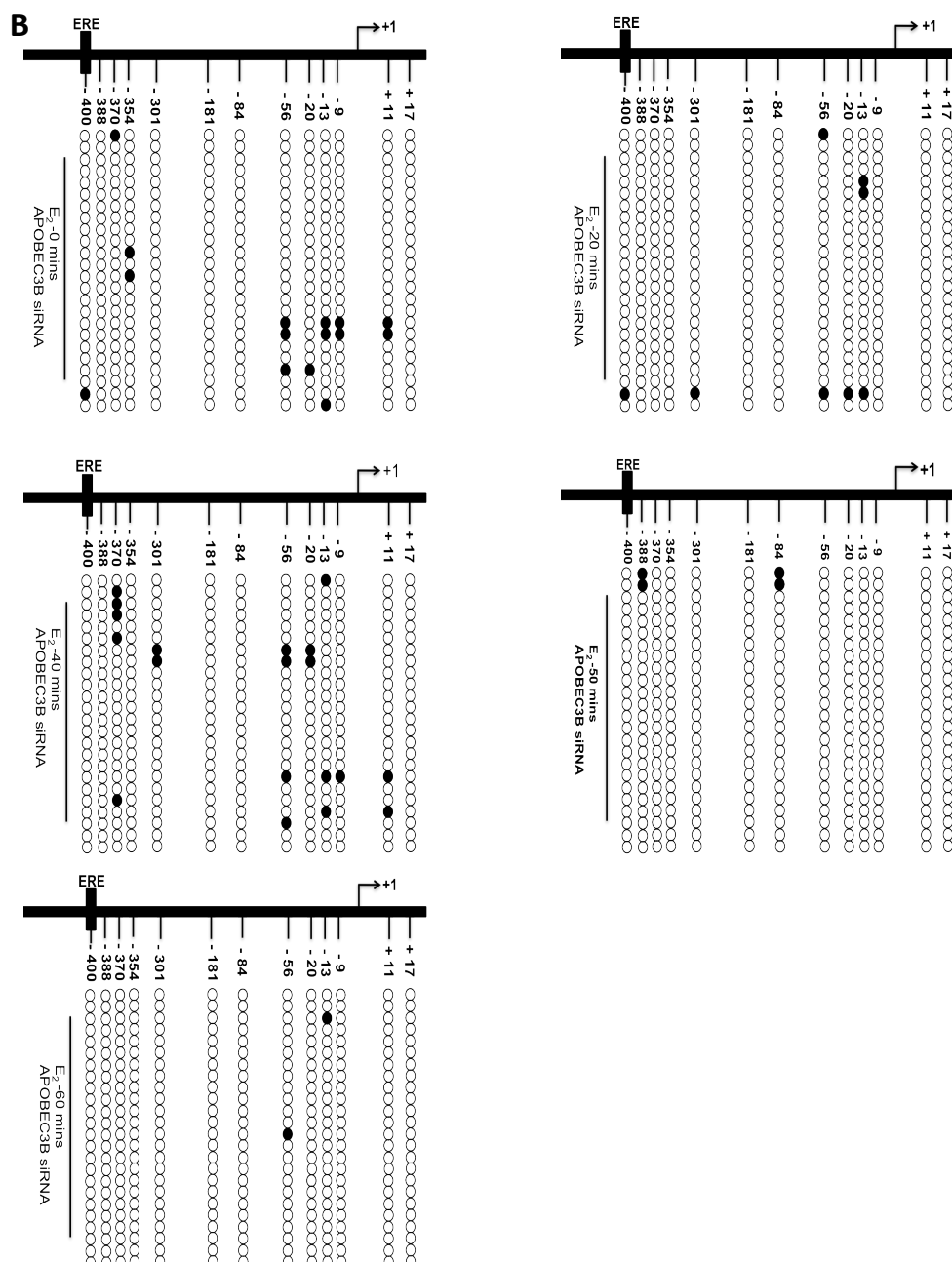


Figure 3.13. Bisulfite treatment and cloning of pS2 promoter sequences shows only a small increase in DNA methylation in MCF-7 cells following siRNA mediated knockdown of APOBEC3B. A and B. MCF-7 cells cultured in estrogen-depleted medium were transfected with APOBEC3B siRNA or Non-Targeting siRNA. Non-targeting siRNA was characterized by genome-wide microarray analysis by supplier and found to have least targeting of known genes in the human genome. Forty eight hours post transfection 10nM estrogen was added and genomic DNA was prepared at various time points, as indicated. Genomic DNA was treated with sodium bisulfite to convert unmethylated cytosines to uracils. Following amplification, PCR products were cloned into a pCR4-TOPO vector. Twenty four colonies were randomly picked for sequencing. White circles show unmethylated sites and black circles represent methylated sites for each colony. The positions of CpG dinucleotides are marked by vertical lines, with the transcription initiation site denoted as +1.

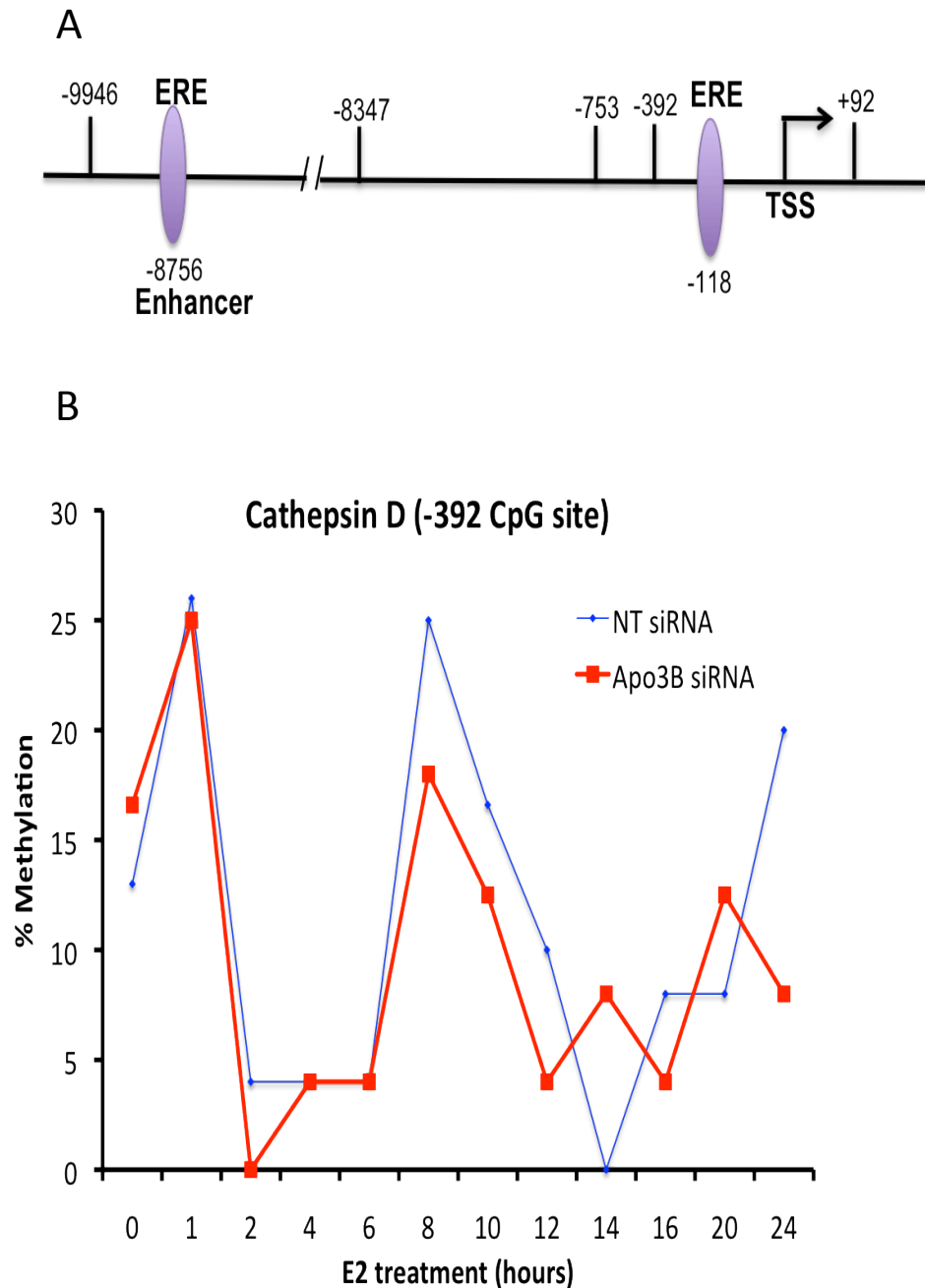


Figure 3.14 Dynamic changes in the methylation of cathepsin D promoter. **A.** Diagram showing the cathepsin D promoter, indicating transcription start site (TSS; arrow), ERE and enhancer regions. **B.** MCF-7 cells were cultured for 72 hrs in estrogen-free media. Forty eight hours post transfection 10nM estrogen was added and genomic DNA was prepared at various time points, as indicated. Genomic DNA was treated with sodium bisulfite to convert unmethylated cytosines to uracils. Following amplification, PCR products were cloned into a pCR4-TOPO vector. The percentage of methylation was calculated from 24 randomly picked colonies. Only -392 CpG site out of 30 CpG sites analysed shows cyclical methylation and demethylation upon estrogen treatment.

for estrogen induced DNA demethylation. Moreover, the extent of DNA methylation at the pS2 promoter was extremely low under all conditions, indicating that the pS2 promoter is not highly methylated in MCF-7 cells.

These studies were extended to the CTSD gene. Bisulphite sequencing results showed that -392 CpG site of the CTSD gene (Figure 3.14A) showed cyclical methylation/demethylation, with peak methylation showing at 1, 8 and 24 hours of estrogen treatment (Figure 3.14B). The extent of methylation at this site was greater than that seen for any of the sites in the pS2 gene. However, APOBEC3B knockdown did not increase or affect the cycling of methylation/demethylation, suggesting that APOBEC3B is not involved in rapid DNA demethylation at promoters of estrogen-regulated genes.

3.8 Estrogen treatment generates DNA strand breaks in the pS2 gene promoter

It is becoming increasingly evident that efficient initiation of transcriptional programs may involve DNA strand break mediated by several proteins usually linked to DNA damage recognition and repair, as exemplified by the finding that ER α promotes transient double strand DNA breaks (DSBs) at the pS2 gene promoter during transcriptional activation (Ju et al., 2006). These authors further showed that TOP2B is important for generating DSB at a specific site in the pS2 gene promoter. Promoter DNA is tightly wrapped around core histone proteins, forming compact nucleosomes that comprise chromatin, which creates obstacles to transcription factor binding to regulatory DNA sequences. Introducing DSBs at specific sites is proposed to allow local chromatin relaxation or a DSB repair intermediate, an abasic site, which is proposed to introduce substantial torsional flexibility, allowing transcription factor access to promoter DNA, which is needed to initiate gene expression. From the ChIP data presented above it is clear that APOBEC3B is recruited to promoters of estrogen-regulated genes and plays an important role in the regulation of gene expression by ER α . In order to further examine the potential mechanisms by which APOBEC3B might promote ER α regulated gene expression we investigated whether APOBEC3B

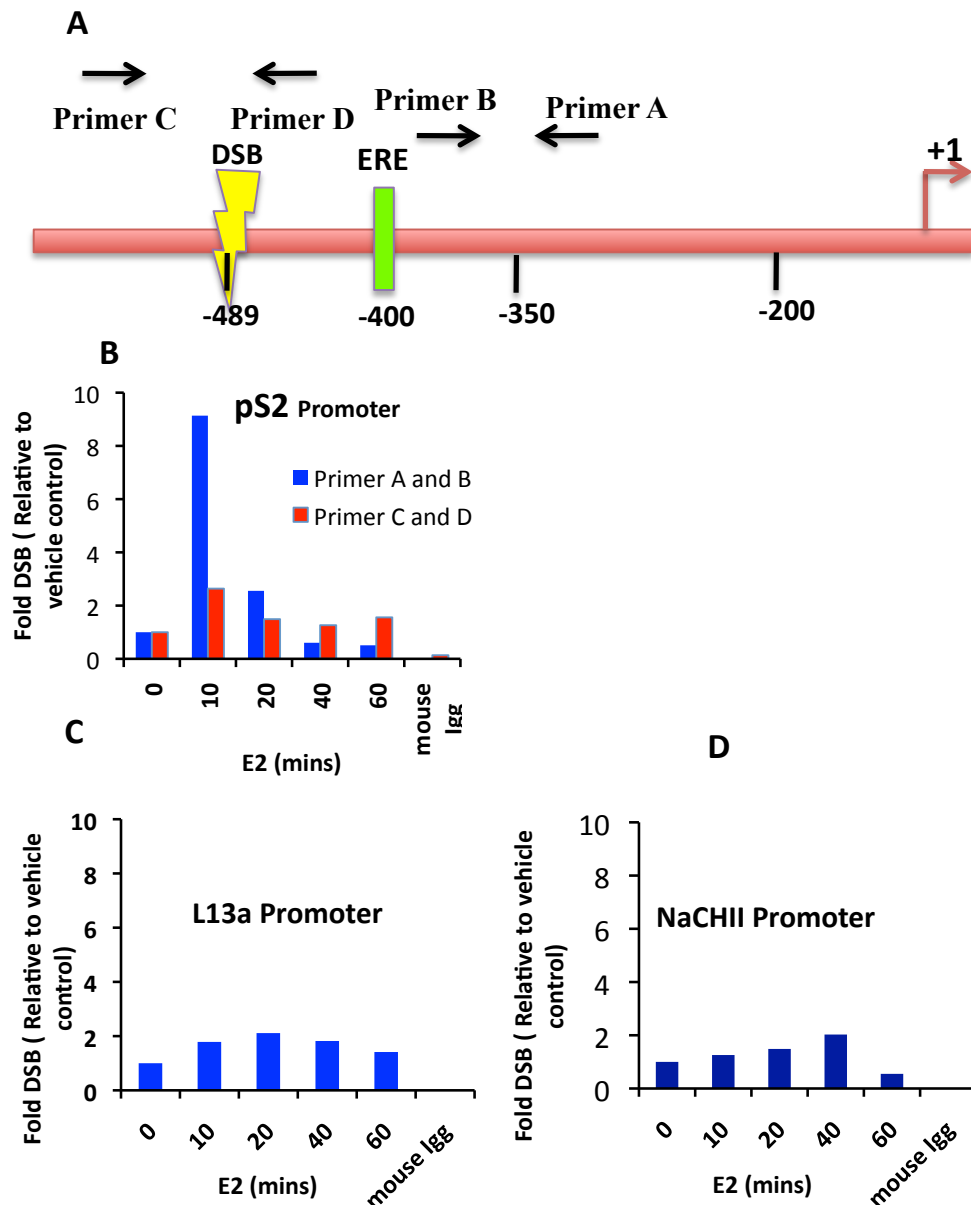


Figure 3.15. Estrogen treatment induces DNA double strand breaks at the pS2 gene promoter in MCF-7 cells. **A.** Shown is a representation of the pS2 promoter, with the site of the previously identified DNA double strand break (DSB) site (Ju et al 2006) is highlighted at position -489. The green bar shows the position of the estrogen responsive element (ERE). Transcription initiation site is indicated as +1. **B-D.** MCF-7 cells cultured in estrogen-free medium for 72 hours were plated in 15cm culture dishes, again in estrogen-free medium. After 24 hours, estrogen was added to a final concentration of 10nM and the cells were fixed, lysed and nuclei were prepared. The DNA breaks were labeled with biotin-11-UTP. The biotin labeled nuclei were refixed and ChIP was carried out using anti-biotin antibody. Real-time PCR was carried out for immunoprecipitated DNA using primer pairs A/B and C/D. Real-time PCR was also carried out for the expressed, but estrogen-independent L13a gene and for the NaChII gene, which is not expressed in MCF-7 cells. Results are shown as mean values of two replicates.

might be involved in chromatin relaxation by deaminating cytosines to uracil, with subsequent base excision mediated repair by uracil DNA glycosylase(s).

A method for identifying DNA breaks, in which genomic DNA is labeled at breakpoints with biotin-11-deoxyuridine triphosphate (biotin-11-dUTP) by incubation with terminal deoxynucleotide transferase (TdT), followed by ChIP using a biotin antibody, was used. MCF-7 cells were treated with estrogen and cells were collected at various time intervals. Following biotinylation, biotin incorporated DNA was purified by using a biotin antibody. qPCR was carried out using specific primer pairs that amplify a region downstream of a DSB site and primers that amplify a region around a cleavage site previously identified in the pS2 promoter and which is required for estrogen induced expression of the pS2 gene (Ju et al, 2006). Addition of estrogen to MCF-7 cells resulted in a 9-fold increase in biotinylated DNA in the pS2 promoter within 10 min (Figure 3.15B). The estrogen induced DNA break is transient as levels of biotinylated DNA was only 3 fold at 20 minutes and back to levels similar to those observed for the 0 min control. By contrast, qPCR performed using primers that encompass the mapped DSB site showed only a small increase in DSB. By contrast, no significant increased enhancement of biotin incorporation was detected either at the promoter region of the ribosomal L13a gene (Figure 3.15C), which is expressed in MCF-7 cells in an estrogen-independent fashion, or at the promoter of the neuronal-specific sodium channel II (NacHII) (Figure 3.15D), which is not expressed in MCF-7 cells, suggesting that estrogen induced DNA breaks are specific to estrogen regulated genes. Collectively these results confirm the previous findings that estrogen induces DNA breaks at the pS2 gene promoter in transient manner, as has been described previously (Ju et al., 2006).

3.9 Estrogen treatment induces γ H2AX foci in MCF-7 cells

Phosphorylation of γ H2AX at serine 139 is stimulated by DSB formation and can be readily detected by immunofluorescence staining. To further evaluate estrogen stimulation of MCF-7 cells on DSB formation, the cells were treated with estrogen for 10 minutes and the cells were scored for phosphorylated γ H2AX (phospho S139).

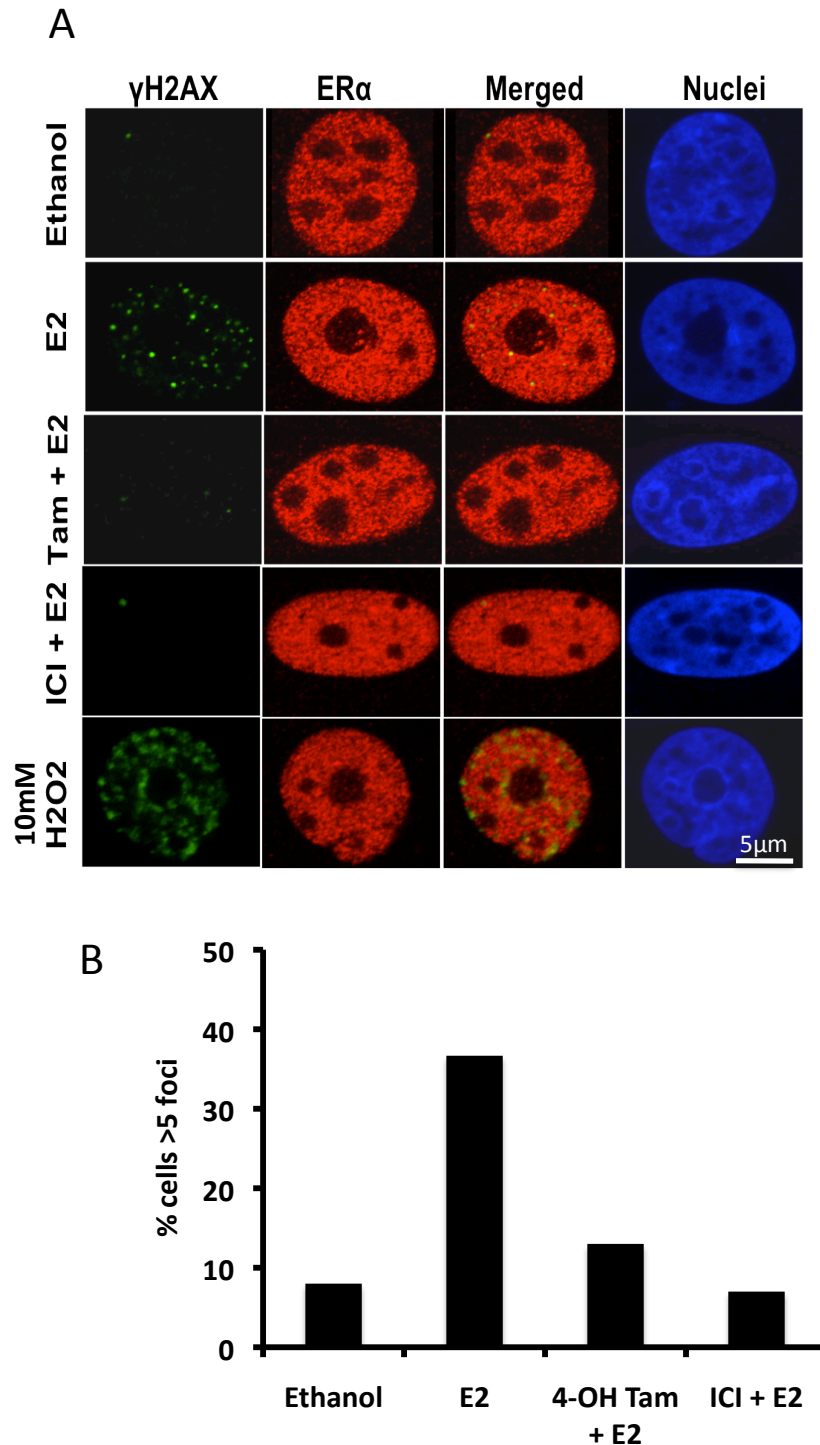


Figure 3.16. Estrogen induces γ H2AX foci in MCF-7 cells. **A.** MCF-7 cells cultured in estrogen-free medium were plated on glass slides. Cells were treated with vehicle control (ethanol) or 10nM estrogen for 10 minutes, 100nM 4-Hydroxy tamoxifen (4-OH Tam) or ICI182,780 (ICI) were added, as shown. Hydrogen peroxide addition for 45 minutes served as a control for γ H2AX phosphorylation, and fixed in 4% paraformaldehyde. Cells were stained with γ H2AX and ER α antibodies and TO-PRO-3 for nuclei staining. Images representative of the staining observed are shown. **B.** Percentage of cells that showed > 5 foci after estrogen treatment.

Interestingly, estrogen treatment resulted in a strong stimulation of γ H2AX phosphorylation, with 35% of the cells having ≥ 5 γ H2AX foci, as determined by immunofluorescence (Figure 3.16). Addition of the anti-estrogens tamoxifen or ICI blocked the estrogen stimulation of γ H2AX phosphorylation, indicating that estrogen induction of γ H2AX foci is mediated by ER α . Further, tamoxifen and ICI did not stimulate γ H2AX phosphorylation.

3.10 APOBEC3B expression induces γ H2AX foci in MCF-7 cells

As mentioned before, APOBEC3B belongs to cytosine deamination family. Cytosine deamination results in generation of uracil lesions that are repaired by the base excision repair pathway, a process that is commenced by cellular uracil-DNA glycosylases (UDG/UNG or TDG). Removal of uracil residues by these enzymes produces abasic sites that are subsequently processed by the apurinic/apyrimidinic endonuclease 1 (APE1). To assess whether APOBEC3B is involved in DNA break formation, MCF-7 cells were transiently transfected with plasmids encoding wild type HA tagged APOBEC3B and catalytic site mutants and cells were immunostained with the γ H2AX (phospho S139) antibody. Transfection of APOBEC3B strongly stimulated phospho- γ H2AX (Figure 3.17B and C). By contrast, the cytosine deaminase domain mutants stimulated γ H2AX phosphorylation to a considerably lower extent. During the course of these experiments, I also analyzed the effect of APOBEC3B expression on γ H2AX formation in U2OS ER α negative cell line. Interestingly, APOBEC3B transfection did not induce γ H2AX phosphorylation in U2OS cells (Figure 3.17D), suggesting a potential role of ER α in APOBEC3B induced γ H2AX formation. Together, these data demonstrate that APOBEC3B induces DNA breaks in ER α positive breast cancer cells and that the catalytic activity of APOBEC3B is important for γ H2AX formation.

To assess if APOBEC3B is involved in regulating the estrogen induced DNA breaks at the pS2 promoter, MCF-7 cells were transiently transfected with wild type APOBEC3B or APOBEC3B mutant 1 and DNA break labeling and ChIP assay was carried out in the presence and absence of estrogen treatment for 10 minutes. Interestingly, in the absence of estrogen treatment APOBEC3B expression stimulated

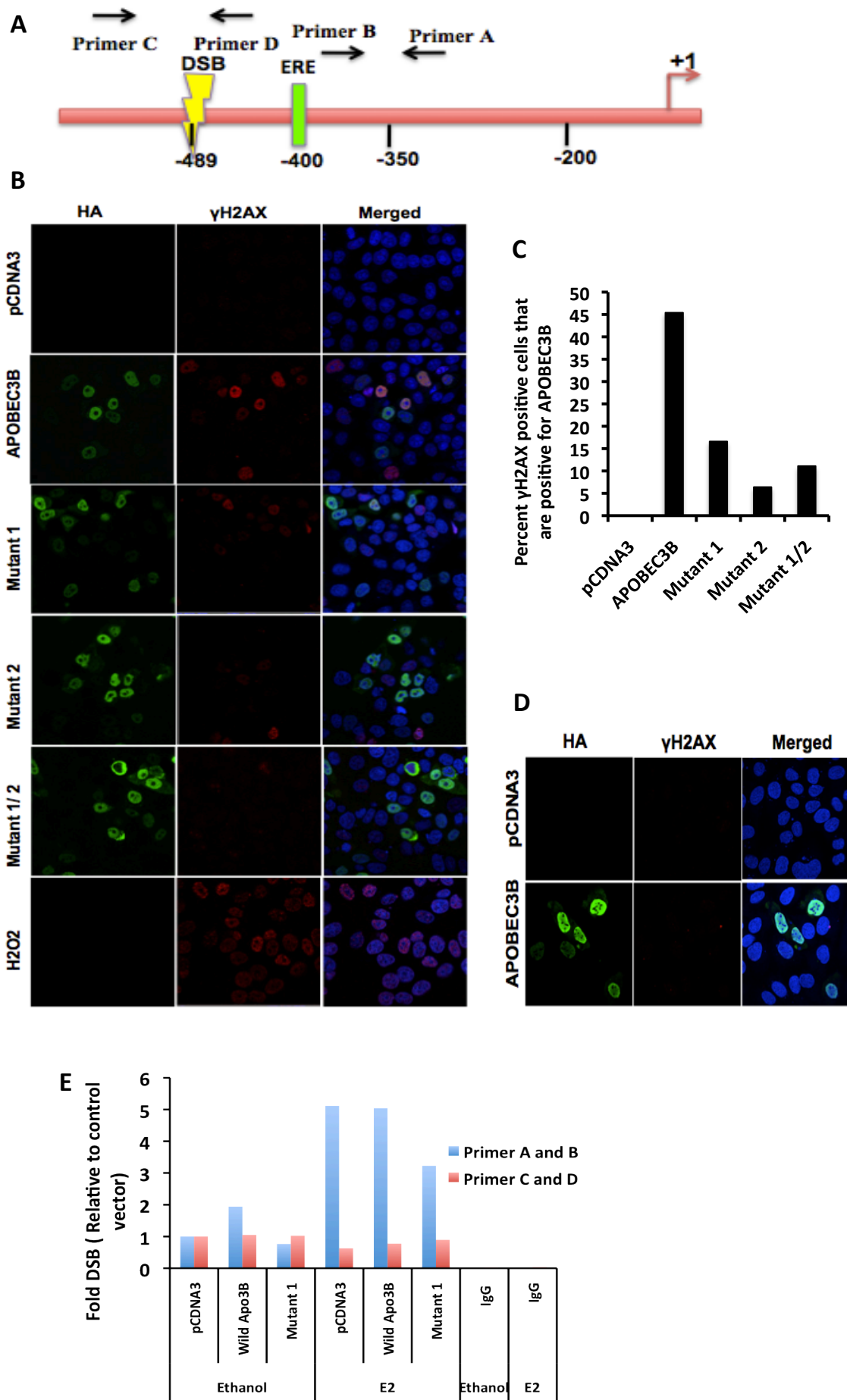


Figure 3.17. APOBEC3B over-expression induces DNA breaks in MCF-7 cells. **A.** Shown is the pS2 promoter, with the DSB and ERE sites highlighted. **B.** MCF7 cells were transiently transfected with plasmids expressing HA tagged APOBEC3B, APOBEC3B mutant1, mutant 2, mutant 1/2 or the pCDNA3 vector control. Twenty four hours post transfection cells were fixed in 4% paraformaldehyde, stained with HA (green) and γ H2AX (red) antibodies, and analyzed by confocal microscopy. **C.** Bar graph represents the percentage of γ H2AX positive cells that express transfected APOBEC3B-HA. **D.** U2OS cells transiently transfected with HA tagged APOBEC3B or pCDNA3 were analysed as in B. **E.** Transiently transfected MCF-7 cells were treated with estrogen or ethanol for 10 minutes. Following biotin labeling of the cells, immunoprecipitations were carried out, as before. The Real-time PCR results of two experiments are shown.

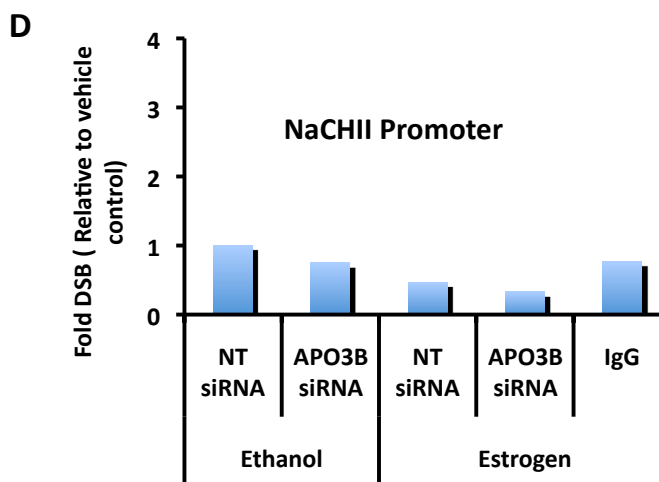
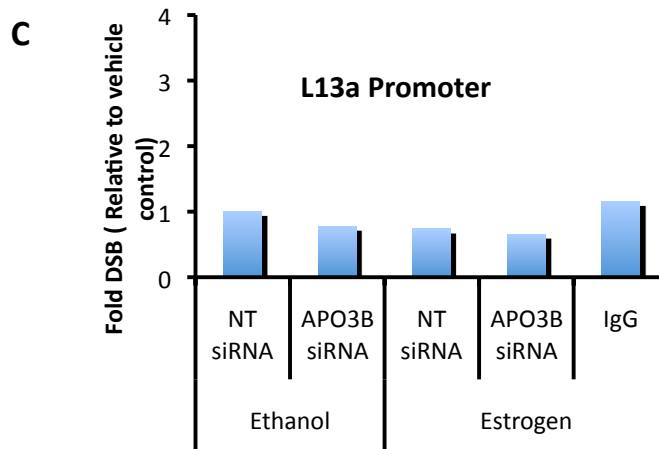
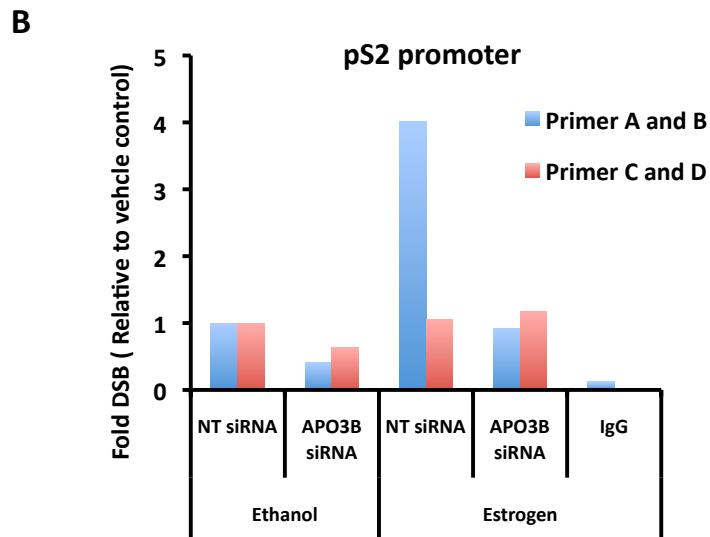
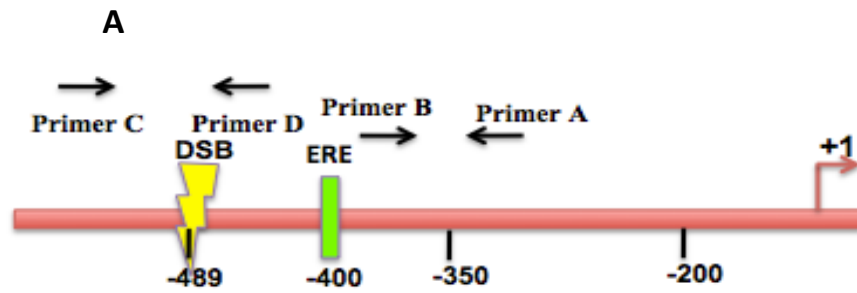


Figure 3.18. APOBEC3B knockdown reduces estrogen induced DNA double strand breaks in MCF- 7 cells. **A.** Diagrammatic view of the pS2 promoter is shown. **B-D.** Real-time PCR analysis of DNA immunoprecipitated from MCF-7 cells following transfection with non-targeting (NT) siRNA or APOBEC3B (Apo3B) siRNA is shown. Non-targeting siRNA was characterized by genome-wide microarray analysis by supplier and found to have least targeting of known genes in the human genome. The cells were treated with estrogen or ethanol for 10 minutes prior to fixation. The real-time PCR results of two experiments are shown.

biotin incorporation by the vector control, but mutant APOBEC3B failed to stimulate biotin incorporation (Figure 3.17E). Estrogen addition stimulated DNA strand breaks, which were not increased following APOBEC3B transfection. However, strand breaks were significantly inhibited by the APOBEC3B mutant, suggestive of a role for APOBEC3B in estrogen-stimulated DNA strand breaks at the pS2 promoter. Moreover, APOBEC3B siRNA resulted in complete inhibition of estrogen stimulated DNA strand breaks (Figure 3.18), suggesting that APOBEC3B is required for the estrogen stimulated DNA breaks at the pS2 gene promoter.

3.11 APOBEC3B may present at the break site within the pS2 promoter

Ju et al (2006) reported that estrogen induced DSB at the pS2 gene promoter is mediated by topoisomerase II β enzyme (TopoII β), with the TopoII β catalyzing the DNA strand break, with PARP1, DNA-PK, ku86 and ku70 being recruited to the break site to promote strand break repair. To determine if APOBEC3B is recruited to the DNA break site at the pS2 gene promoter, sequential ChIP experiment was performed in which biotin labeled chromatin from estrogen treated cells was divided in two aliquots, one of which was immunoprecipitated with the biotin antibody, the second with IgG. The immune complexes were released from beads and re-immunoprecipitated (re-IP) with biotin, ER α , APOBEC3B and TopoII β antibodies. Supernatants collected from the biotin IP were also subjected to re-IP for biotin, ER α , APOBEC3B and TopoII β . As expected, biotin antibody immunoprecipitated the pS2 promoter following estrogen treatment, demonstrating that estrogen treatment induces DNA breaks. Performing re-IP for ER α , APOBEC3B and TopoII β following primary immunoprecipitation for biotin showed that ER α , TopoII β and APOBEC3B are co-immunoprecipitated with biotin, which reveals the presence of ER α , TopoII β and APOBEC3B in the region of the DNA strand break within the pS2 promoter region (Figure 3.19B). However, the amount of APOBEC3B being co-immunoprecipitated with biotin was significantly less than the ER α and TopoII β being co-immunoprecipitated with biotin (Figure 3.19B).

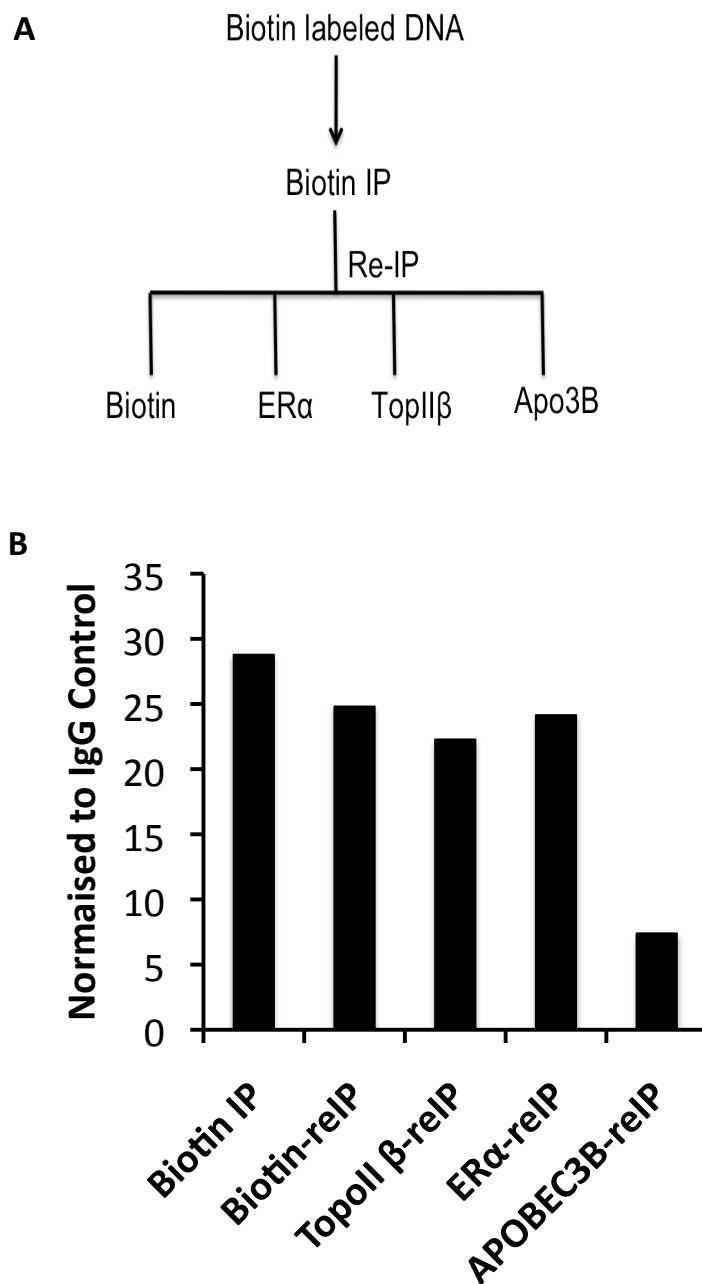


Figure 3.19. Corecruitment of ER α , TopoisomeraseII β (TopII β) and APOBEC3B (Apo3B) on pS2 promoter DNA break site. MCF-7 cells were treated with estrogen for 10 minutes. Cells were fixed, lysed and nuclei were prepared. The DNA breaks were labeled with biotin-11-UTP. The biotin labeled nuclei were refixed and immunoprecipitation was carried out using a biotin antibody. Eluted immune complexes were subjected to re-IP with antibodies for biotin, ER α , Topoisomerase II β (TopII β) and APOBEC3B (Apo3B) (re-IP). A similar procedure was followed for mouse IgG control. Immunoprecipitated DNA was quantified by real-time PCR using primers for the pS2 gene directed against a region of the gene encompassing the estrogen responsive element (ERE).

3.12 APOBEC3B interact with RNA splicing factor p54^{nrb} (NONO) and PSF

Identification of APOBEC3B interacting proteins would provide further insight into the mechanisms by which APOBEC3B regulates ER α activity. In order to identify APOBEC3B interacting proteins, a yeast 2-hybrid screen was carried out under contract with Hybrigenics plc, in which the human APOBEC3B cDNA was fused in frame with the bacterial LexA DNA binding domain and screened using a human placental cDNA expression library in yeast. Co-expression of the LexA fused protein of interest (bait) with an expression cDNA fused to a transcription activation domain, usually the yeast GAL4 transcription factor derived transcription activation domain, allows functional reconstitution of a transcription factor and consequent activation of the expression of a selection marker gene incorporating a DNA binding site for the fusion bait protein, in this case LexA. Screening of cDNA libraries by Hybrigenics is performed by mating of cells pre-transformed with the LexA fusion (sex type a), with cells pre-transformed with the cDNA expression library (sex type α). The selectable marker gene was a lexA regulated His3 gene, expressed in yeast cells deficient in the His3 gene, so that growth on medium lacking histidine requires the addition of histidine in the medium or functional reconstitution of a transcription activator by interaction of the lexA fusion protein with the interacting protein fused to a transcription activation domain. The human placenta library was chosen because of the large numbers of different genes expressed in placenta. Screening of 123 million clones resulted positives, which were validated by Hybrigenics plc by retransformation to identify 78 positives (Table 3.1). Based on bioinformatics analysis, including a likelihood score for proteins that are frequently identified in yeast 2-hybrid screens and which may represent false positives, as well as the number of independent clones identified, ranked β -catenin (CTNNB1) as a very high confidence APOBEC3B interacting protein, as well as NONO and SAFB as high confidence interactions, whilst a third gene, SMARCE1 was given a good confidence score.

Protein interaction databases allow interrogation of the protein of interest for previously described interactions to build interaction maps. Using the STRING protein interaction database (<http://string.embl.de>) (Jensen et al 2009), interaction

Table 3.1 Results of Yeast 2-hybrid screening of Human APOBEC3B (1-382) vs Human Placenta cDNA expression												
Clone Name	Type Seq	Contig(s) Name	Gene Name (Best Match)	GID	Global PBS	Start	Stop	Frame	Orientation	UTR Inclusion	% Id 5p/3p	
PLA RP4 hgx2185v1 pB27 A-43	5p 3p	36740290	Homo sapiens - CTNBN1	GID: 148233337	A	312	1704	IF	Sense		99.4 / 97.7	
PLA RP4 hgx2185v1 pB27 A-46	5p 3p	36740290	Homo sapiens - CTNBN1	GID: 148233337	A	486	1502	IF	Sense		99.7 / 99.2	
PLA RP4 hgx2185v1 pB27 A-51	5p 3p	36740290	Homo sapiens - CTNBN1	GID: 148233337	A	486	1502	IF	Sense		99.7 / 99.8	
PLA RP4 hgx2185v1 pB27 A-59	5p 3p	36740290	Homo sapiens - CTNBN1	GID: 148233337	A	486	1502	IF	Sense		99.2 / 86.1	
PLA RP4 hgx2185v1 pB27 A-52	5p 3p	36740290	Homo sapiens - CTNBN1	GID: 148233337	A	510	1501	IF	Sense		99.4 / 99.5	
PLA RP4 hgx2185v1 pB27 A-75	5p 3p	36740290	Homo sapiens - CTNBN1	GID: 148233337	A	510	1501	IF	Sense		99.8 / 99.5	
PLA RP4 hgx2185v1 pB27 A-15	5p 3p	36740355	Homo sapiens - CYP19A1	GID: 189181656	N/A	-120	988	OOF1	Sense		99.3 / 99.7	
PLA RP4 hgx2185v1 pB27 A-21	5p 3p	36740355	Homo sapiens - CYP19A1	GID: 189181656	N/A	-120	988	OOF1	Sense		99.4 / 99.7	
PLA RP4 hgx2185v1 pB27 A-84	5p 3p	36740261	Homo sapiens - FAM156B	GID: 153791170	D	84	446	IF	Sense		99.7 / 99.7	
PLA RP4 hgx2185v1 pB27 A-66	5p 3p	36740269	Homo sapiens - FBXL20	GID: 14249619	D	900	1257	IF	Sense		100.0 / 94.9	
PLA RP4 hgx2185v1 pB27 A-76	5p 3p	36740306 / 36740339	Homo sapiens - FES	GID: 219842229	N/A	1701	371	??	AntiSense		73.0 / 99.2	
PLA RP4 hgx2185v1 pB27 A-17	5p 3p	36740349 / 36740339	Homo sapiens - FES	GID: 219842229	N/A	1701	371	??	AntiSense		98.1 / 98.1	
PLA RP4 hgx2185v1 pB27 A-33	5p 3p	36740349 / 36740339	Homo sapiens - FES	GID: 219842229	N/A	1701	371	??	AntiSense		96.2 / 97.9	
PLA RP4 hgx2185v1 pB27 A-38	5p 3p	36740349 / 36740339	Homo sapiens - FES	GID: 219842229	N/A	1701	371	??	AntiSense		98.3 / 98.3	
PLA RP4 hgx2185v1 pB27 A-50	5p 3p	36740349 / 36740339	Homo sapiens - FES	GID: 219842229	N/A	1701	371	??	AntiSense		69.7 / 99.4	
PLA RP4 hgx2185v1 pB27 A-87	5p 3p	36740349 / 36740339	Homo sapiens - FES	GID: 219842229	N/A	1701	371	??	AntiSense		95.4 / 99.6	
PLA RP4 hgx2185v1 pB27 A-44	5p 3p	36740349 / 36740354	Homo sapiens - FES	GID: 219842229	N/A	1701	371	??	AntiSense		88.8 / 95.9	
PLA RP4 hgx2185v1 pB27 A-39	5p 3p	36740299	Homo sapiens - HEY-L	GID: 105990530	F	15	646	IF	Sense		88.7 / 98.4	
PLA RP4 hgx2185v1 pB27 A-42	5p 3p	36740299	Homo sapiens - HEY-L	GID: 105990530	F	15	646	IF	Sense		99.4 / 99.4	
PLA RP4 hgx2185v1 pB27 A-11	5p 3p	36740351 / 36740345	Homo sapiens - HEY1	GID: 105990527	E	-118	567	IF	Sense		94.1 / 88.1	
PLA RP4 hgx2185v1 pB27 A-29	5p 3p	36740351	Homo sapiens - HEY1	GID: 105990527	E	-118	567	IF	Sense		99.4 / 99.9	
PLA RP4 hgx2185v1 pB27 A-58	5p 3p	36740323 / 36740351	Homo sapiens - HEY1	GID: 105990527	E	-40	855	IF	Sense		93.0 / 85.2	
PLA RP4 hgx2185v1 pB27 A-72	5p 3p	36740351 / 36740345	Homo sapiens - HEY1	GID: 105990527	E	-40	855	IF	Sense		99.9 / 98.0	
PLA RP4 hgx2185v1 pB27 A-3	5p 3p	36740351	Homo sapiens - HEY1	GID: 105990527	E	129	852	IF	Sense		99.4 / 99.4	
PLA RP4 hgx2185v1 pB27 A-79	5p 3p	36740298 / 36740280	Homo sapiens - HNRNPA2B1	GID: 156151374	C	-154	882	IF	Sense		94.2 / 99.0	
PLA RP4 hgx2185v1 pB27 A-53	5p 3p	36740280	Homo sapiens - HNRNPA2B1	GID: 156151374	C	-118	879	IF	Sense		99.6 / 98.9	
PLA RP4 hgx2185v1 pB27 A-45	5p 3p	36740348 / 36740337	Homo sapiens - HYPE	GID: 42794619	D	699	1272	IF	Sense		99.3 / 96.6	
PLA RP4 hgx2185v1 pB27 A-8	3p	36740376	Homo sapiens - KIAA0431	GID: 54792091	N/A	No Data	693	??	Sense		80.5	
PLA RP4 hgx2185v1 pB27 A-26	5p 3p	36740325	Homo sapiens - KLF11	GID: 40548370	D	771	1795	IF	Sense		98.6 / 99.4	
PLA RP4 hgx2185v1 pB27 A-85	5p 3p	36740259	Homo sapiens - LAM5A	GID: 21264601	N/A	1883	1333	??	AntiSense		99.5 / 99.1	
PLA RP4 hgx2185v1 pB27 A-57	5p 3p	36740275	Homo sapiens - LOC100134439	GID: 169217049	D	-145	465	IF	Sense		97.8 / 95.6	
PLA RP4 hgx2185v1 pB27 A-49	5p 3p	36740283	Homo sapiens - MB01	GID: 156105677	N/A	-435	338	OOF1	Sense		72.6 / 72.6	
PLA RP4 hgx2185v1 pB27 A-92	5p 3p	36740249	Homo sapiens - NONO	GID: 224028247	B	120	671	IF	Sense		99.8 / 99.6	
PLA RP4 hgx2185v1 pB27 A-5	5p 3p	36740249	Homo sapiens - NONO	GID: 224028247	B	336	834	IF	Sense		100.0 / 99.8	
PLA RP4 hgx2185v1 pB27 A-7	5p 3p	36740249	Homo sapiens - NONO	GID: 224028247	B	336	834	IF	Sense		100.0 / 99.8	
PLA RP4 hgx2185v1 pB27 A-22	5p 3p	36740249	Homo sapiens - NONO	GID: 224028247	B	336	834	IF	Sense		100.0 / 98.6	
PLA RP4 hgx2185v1 pB27 A-27	5p 3p	36740249	Homo sapiens - NONO	GID: 224028247	B	336	834	IF	Sense		100.0 / 100.0	
PLA RP4 hgx2185v1 pB27 A-63	5p 3p	36740249	Homo sapiens - NONO	GID: 224028247	B	336	834	IF	Sense		99.8 / 98.5	
PLA RP4 hgx2185v1 pB27 A-89	5p 3p	36740249	Homo sapiens - NONO	GID: 224028247	B	336	834	IF	Sense		100.0 / 100.0	
PLA RP4 hgx2185v1 pB27 A-18	5p 3p	36740333	Homo sapiens - PABPN1	GID: 169808407	D	495	969	IF	Sense		100.0 / 100.0	
PLA RP4 hgx2185v1 pB27 A-9	5p 3p	36740362	Homo sapiens - PEG3	GID: 226053184	E	225	1370	IF	Sense		99.7 / 99.7	
PLA RP4 hgx2185v1 pB27 A-17	5p 3p	36740286	Homo sapiens - PIAS1	GID: 20987516	E	44	452	IF	Sense		99.8 / 99.3	
PLA RP4 hgx2185v1 pB27 A-14	5p 3p	36740330	Homo sapiens - PIPO	GID: 23397647	N/A	2533	3023	OOF1	Sense		100.0 / 100.0	
PLA RP4 hgx2185v1 pB27 A-19	5p 3p	36740373 / 36740364	Homo sapiens - RPC155	GID: 39725937	D	3264	3835	IF	Sense		100.0 / 84.0	
PLA RP4 hgx2185v1 pB27 A-6	5p 3p	36740366 / 36740247	Homo sapiens - RREB1	GID: 208609943	E	267	1135	IF	Sense		99.6 / 95.9	
PLA RP4 hgx2185v1 pB27 A-36	5p 3p	36740366 / 36740360	Homo sapiens - RREB1	GID: 208609943	E	267	1135	IF	Sense		98.0 / 97.9	
PLA RP4 hgx2185v1 pB27 A-23	5p 3p	36740366	Homo sapiens - RREB1	GID: 208609943	E	267	1135	IF	Sense		97.9 / 99.2	
PLA RP4 hgx2185v1 pB27 A-24	5p 3p	36740366	Homo sapiens - RREB1	GID: 208609943	E	267	1135	IF	Sense		98.5 / 98.9	
PLA RP4 hgx2185v1 pB27 A-41	5p 3p	36740302	Homo sapiens - SAFB	GID: 219518969	B	891	2206	IF	Sense		98.5 / 99.5	
PLA RP4 hgx2185v1 pB27 A-16	5p 3p	36740302	Homo sapiens - SAFB	GID: 219518969	B	1416	2252	IF	Sense		99.4 / 97.3	
PLA RP4 hgx2185v1 pB27 A-48	5p 3p	36740302 / 36740332	Homo sapiens - SAFB	GID: 219518969	B	1503	2224	IF	Sense		99.7 / 87.0	
PLA RP4 hgx2185v1 pB27 A-65	5p 3p	36740271	Homo sapiens - SMARCE1	GID: 45827732	C	27	901	IF	Sense		100.0 / 98.9	
PLA RP4 hgx2185v1 pB27 A-25	5p 3p	36740271	Homo sapiens - SMARCE1	GID: 45827732	C	39	890	IF	Sense		99.3 / 99.5	
PLA RP4 hgx2185v1 pB27 A-40	5p 3p	36740308	Homo sapiens - SNRP70	GID: 57634537	F	45	588	IF	Sense		97.0 / 97.0	
PLA RP4 hgx2185v1 pB27 A-55	5p 3p	36740308	Homo sapiens - SNRP70	GID: 57634537	F	45	588	IF	Sense		97.0 / 96.4	
PLA RP4 hgx2185v1 pB27 A-60	5p 3p	36740308	Homo sapiens - SNRP70	GID: 57634537	F	45	588	IF	Sense		97.0 / 96.6	
PLA RP4 hgx2185v1 pB27 A-64	5p 3p	36740308	Homo sapiens - SNRP70	GID: 57634537	F	75	595	IF	Sense		96.8 / 96.8	
PLA RP4 hgx2185v1 pB27 A-71	5p 3p	36740308	Homo sapiens - SNRP70	GID: 57634537	F	75	595	IF	Sense		96.8 / 96.3	
PLA RP4 hgx2185v1 pB27 A-28	5p 3p	36740319	Homo sapiens - SNRP70	GID: 57634537	F	90	669	IF	Sense		99.0 / 96.3	
PLA RP4 hgx2185v1 pB27 A-30	5p 3p	36740319	Homo sapiens - SNRP70	GID: 57634537	F	99	457	IF	Sense		100.0 / 100.0	
PLA RP4 hgx2185v1 pB27 A-32	5p 3p	36740319	Homo sapiens - SNRP70	GID: 57634537	F	99	457	IF	Sense		100.0 / 100.0	
PLA RP4 hgx2185v1 pB27 A-83	5p 3p	36740271 / 36740285	Homo sapiens - Smarce1 var6	GID: 169793994	E	-184	691	IF	Sense		95.0 / 97.4	
PLA RP4 hgx2185v1 pB27 A-77	5p 3p	36740267	Homo sapiens - TAF1	GID: 187761328	E	3633	4663	IF	Sense		99.0 / 99.0	
PLA RP4 hgx2185v1 pB27 A-80	5p 3p	36740266 / 36740263	Homo sapiens - TBX5	GID: 31652233	D	120	1820	IF	Sense		98.5 / 92.8	
PLA RP4 hgx2185v1 pB27 A-91	5p 3p	36740266 / 36740263	Homo sapiens - TBX5	GID: 31652233	D	120	1820	IF	Sense		99.4 / 98.4	
PLA RP4 hgx2185v1 pB27 A-4	5p 3p	36740372 / 36740369	Homo sapiens - TDRD3	GID: 225703107	D	1032	2243	IF	Sense		99.6 / 84.2	
PLA RP4 hgx2185v1 pB27 A-93	5p 3p	36740372 / 36740369	Homo sapiens - TDRD3	GID: 225703107	D	1032	2243	IF	Sense		99.8 / 89.0	
PLA RP4 hgx2185v1 pB27 A-13	5p 3p	36740335	Homo sapiens - TSNAH	GID: 20302159	E	108	1228	IF	Sense		99.0 / 99.4	
PLA RP4 hgx2185v1 pB27 A-69	5p 3p	36740358 / 36740317	Homo sapiens - USP9X	GID: 145309310	D	6891	7927	IF	Sense		99.6 / 86.9	
PLA RP4 hgx2185v1 pB27 A-10	5p 3p	36740257	Homo sapiens - WDR32	GID: 110815799	N/A	7028	7751	OOF2	Sense	3p	99.0 / 99.5	
PLA RP4 hgx2185v1 pB27 A-54	5p 3p	36740277	Homo sapiens - ZBTB38	GID: 148276989	E	1104	2203	IF	Sense		99.3 / 99.8	
PLA RP4 hgx2185v1 pB27 A-35	5p 3p	36740277	Homo sapiens - ZBTB38	GID: 148276989	E	1242	2280	IF	Sense		99.5 / 98.4	
PLA RP4 hgx2185v1 pB27 A-20	5p 3p	36740328	Homo sapiens - ZBTB7A	GID: 40549452	N/A	800	224	??	AntiSense		99.8 / 100.0	
PLA RP4 hgx2185v1 pB27 A-81	5p 3p	36740297 / 36740288	Homo sapiens - ZF	GID: 88900494	E	552	No Data	IF	Sense		97.5 / 76.2	
PLA RP4 hgx2185v1 pB27 A-12	5p 3p	36740308 / 36740374	Homo sapiens - hu1-70K-like protein	GID: 37530	F	240	728	IF	Sense		100.0 / 94.7	
PLA RP4 hgx2185v1 pB27 A-56	5p 3p	36740308 / 36740327	Homo sapiens - unknown	GID: 21756352	N/A	271	783	OOF1	Sense		99.6 / 84.3	

Summary of PBS categories

A : Very high confidence in the interaction

B : High confidence in the interaction

C : Good confidence in the interaction

D : Moderate confidence in the interaction

This category is the most difficult to interpret because it mixes two classes of interactions :

* False-positive interactions

* Interactions hardly detectable by the Y2H technique (low representation of the mRNA in the library, prey folding, prey toxicity in yeast...)

E : Interactions involving highly connected prey domains, warning of non-specific interaction. The threshold for high connectivity is 10 for

screens with Human, Mouse, Drosophila and Arabidopsis and 6 for all other organisms. They can be classified in different categories:

* Prey proteins that are known to be highly connected due to their biological function

* Proteins with a prey interacting domain that contains a known protein interaction motif or a biochemically promiscuous motif

F : Experimentally proven technical artifacts

N/A : The PBS is a score that is automatically computed through algorithms and cannot be attributed for the following reasons :

* All the fragments of the same reference CDS are antisense

* The 5p sequence is missing

* All the fragments of the same reference CDS are either all OOF1 or all OOF2

* All the fragments of the same reference CDS lie in the 5' or 3' UTR

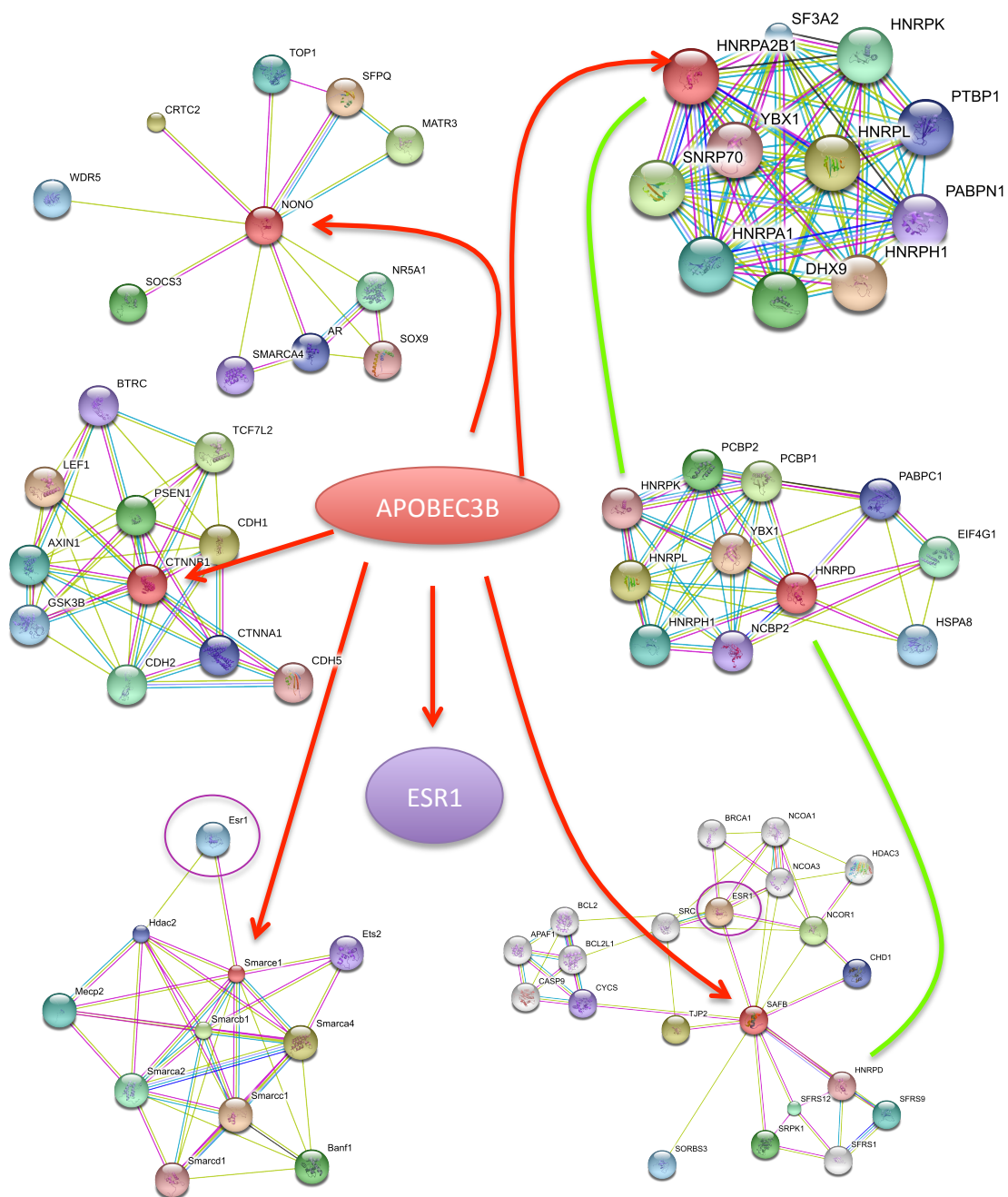


Figure 3.20. Interaction pathways involving proteins that interact with APOBEC3B in a yeast 2-hybrid screen. Shown are protein interaction maps for CTNNB1, NONO, HNRNPA2B1, SAFB and SMARCE1, taken from the STRING protein interaction database (<http://string.embl.de>) (Jensen et al 2009). Red arrows show APOBEC3B interacting partners. The bold green lines show link between SAFB and HNRNPA2B1. Open purple circles show the networks already linked to ER α (ESR1) function.

pathways for APOBEC3B were built (Figure 3.20). Interestingly, these highlight pathways involved in gene regulation and RNA processing. Furthermore, several of the pathways highlighted previously described interactions with ER α or with other nuclear receptors. This includes β -catenin (CTNNB1), which is an intracellular transducer of the Wnt/ β -catenin pathway that functions as a transcriptional activator and regulator of cell adhesion and migration. The Wnt/ β -catenin signaling pathway is an essential pathway that controls cell proliferation, differentiation and migration, making this pathway a strong regulator of cancer initiation (MacDonald et al., 2009; Moon et al., 2004). It has been shown that ER α physically interacts with β -catenin in an estrogen independent manner. However, the interaction between ER α and β -catenin is significantly increased upon estrogen treatment. In addition, β -catenin enhances ERE dependent reporter gene activity in MCF-7 cells and is recruited to promoters of estrogen responsive genes in an estrogen dependent manner (Kouzmenko et al., 2004). Hence, there is considerable evidence linking β -catenin to regulation of ER α activity. The yeast 2-hybrid screening suggest that regulation of ER α activity by APOBEC3B involves the Wnt/ β -catenin signaling pathway.

SMARCE1 (BAF57) is part of the SWI/SNF chromatin remodelling complex required for regulation of gene expression by nuclear receptors, including ER α (Trotter and Archer, 2008; Wilson and Roberts, 2011). Several studies have shown that BAF57 interacts directly with ER α and is required for ER α function in breast cancer cells (Belandia et al., 2002; Garcia-Pedrero et al., 2006). This raises the possibility that APOBEC3B may play a role in chromatin remodeling through interaction with the BAF57 containing SWI/SNF complex.

SAFB, also known as HAP, HET, SAF-B1, SAFB1, identified as a protein that binds to the ERE in the promoter of the estrogen regulated hsp27 gene (Oesterreich et al., 1997) is a nuclear matrix- or scaffold-associated factor. S/MAR regions are located near boundaries of active genes and are believed to play a role in gene regulation through interaction with RNA polymerase II and with splicing factors (Nayler et al., 1998). In the case of ER α , SAFB has been shown to repress expression of estrogen-regulated genes through recruitment of histone deacetylase complexes (Dobrzycka et al., 2003; Jiang et al., 2006; Townson et al., 2004). Interestingly, SAFB also interacts

with the heterogeneous nuclear ribonucleoprotein HNRNPD, which has interacts with HNRNPK containing complexes that include HNRNPA2B1 (Arao et al., 2000), another APOBEC3B interacting protein identified in the yeast 2-hybrid screen. These interactions indicate that APOBEC3B has a role in gene regulation through a mechanism involving RNA processing.

The NONO protein interacts with SMARCA4, also known as BAF190, BRG1, SNF2, SWI2 and SNF2 β . SMARCA4 is part of the SWI/SNF complex along with SMARCE1. Furthermore, NONO has been implicated in the regulation of AR, PR and LRH-1 function, where it appears to act as a transcriptional corepressor (Dong et al., 2007; Dong et al., 2009; Sewer et al., 2002). However, Kuwahara et al have shown that NONO, as well as PSPC1 and PSF, two proteins that interact strongly with NONO, activate AR mediated transcription in sertoli cells of the testis (Kuwahara et al., 2006). These proteins are members of the mammalian Drosophila melanogaster behavior, human splicing family (DBHS) that interact with each other and share >50% sequence homology within two N-terminal RNA recognition motifs (RRM) and a C-terminal coiled-coil domain (Bond and Fox, 2009; Shav-Tal and Zipori, 2002). The expression of NONO is higher in prostate cancer tissue compared to normal prostate tissue and there is a positive association between AR and NONO expression in prostate cancer (Ishiguro et al., 2003). Interestingly, there is also a strong correlation between ER α , PR expression and NONO expression in breast tumours and breast cancer cell lines. NONO expression is absent or expressed at lower levels in tumours that do not express ER α and PR (Pavao et al., 2001; Traish et al., 1997), suggestive of a role for NONO in ER α -positive breast cancer. In addition to its role in transcription, PSF and NONO play a role in RNA metabolism such as pre-mRNA processing, RNA splicing, transcription termination and nuclear retention of defective RNA (Bond and Fox, 2009; Kaneko et al., 2007). NONO and PSF are involved in nuclear retention of adenine deaminase mediated A to I edited mRNA (Zhang and Carmichael, 2001).

Immunofluorescence and co-immunoprecipitation was carried out in order to validate the findings of the yeast 2-hybrid screen. For this, I decided to concentrate on NONO because of its previously described role in regulating nuclear receptor function and

identified role in sequestering mRNA following RNA editing, which may provide a link with the deamination function of APOBEC3B. Transient transfection of U2OS cells with HA-APOBEC3B or ER α followed by immunoprecipitation with a NONO antibody showed co-immunoprecipitation of ER α and APOBEC3B (Figure 3.21A, B). NONO has previously been shown to interact with PSF and PSPC1 (Kuwahara et al., 2006). In agreement with published results both proteins co-immunoprecipitated with NONO. Conversely, IP with ER α and APOBEC3B-HA antibodies, followed by immunoblotting for NONO, PSF and PSPC-1, showed that ER α and APOBEC3B co-immunoprecipitate NONO, PSF and PSPC-1 (Figure 3.21C and D).

Immunofluorescence staining was undertaken to determine if APOBEC3B and NONO co-localise in cells. Immunofluorescence staining was performed in U2OS cells transiently transfected with APOBEC3B-HA. Staining for endogenous NONO showed its expression in the nucleus of the cell. APOBEC3B and NONO were found to be colocalized within the nucleus of the cell (Figure 3.21E and F). To address whether endogenously expressed ER α interact with NONO, PSF and PSPC-1, whole cell lysates prepared from MCF-7 cells were immunoprecipitated with an ER α antibody, followed by western blotting with APOBEC3B, NONO, PSF and PSPC-1 antibodies. As previously shown, APOBEC3B co-immunoprecipitated with ER α . In addition, NONO, PSF and PSPC-1 were detected in the ER α immunoprecipitate (Figure 3.22A). Collectively, these results suggest that ER α and APOBEC3B can interact with NONO and its binding partners, PSF and PSPC-1, and suggest that these proteins may present in a complex in cells.

3.13 APOBEC3B increases ER α transcriptional activity in synergy with p54^{nrb} (NONO)

To assess the functional significance of APOBEC3B and NONO interaction on ER α transcriptional activity, a reporter gene assay was carried out. COS-1 cells were transiently transfected with the ERE-3-TATA-luc firefly luciferase reporter gene, the RLTK renilla luciferase reporter, together with ER α or in combination with ER α and

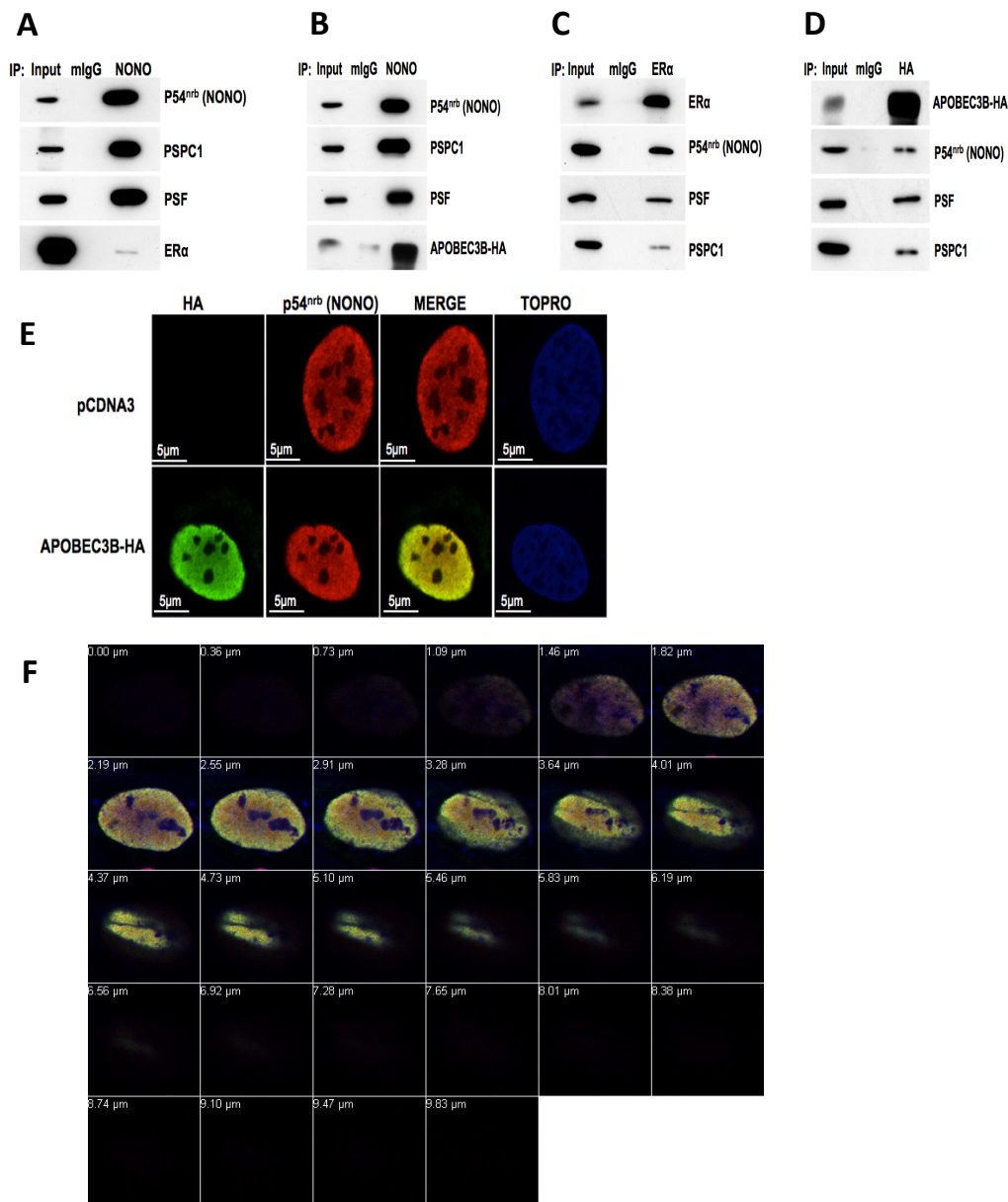


Figure 3.21. APOBEC3B interacts with p54^{nrB} (NONO). **A-D.** U2OS cells were transfected with ERα (**A**, **C**) or APOBEC3B-HA (**B**, **D**). 24hrs post-transfection whole cell lysates were prepared and immunoprecipitated with mouse IgG or with NONO (**A**, **B**), ERα (**C**) or HA (**D**) antibodies. Immunoblotting was carried out using antibodies for NONO, PSPC1, PSF, ER and HA, as shown. Input represents 1% of the total volume of lysate used in the immunoprecipitation. **E.** U2OS cells were transiently transfected with HA-tagged APOBEC3B. The cells were fixed after 24 hours in 4% paraformaldehyde and then stained with NONO (red) and HA (green) antibodies. The cells were subsequently incubated with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse secondary antibodies and TO-PRO-3 for the nuclear counterstain. Images were acquired using a Zeiss LSM510 confocal microscope. **F.** Z-stacks showing colocalization of APOBEC3B and NONO. Numbers in white represent the depth of the Z section in μm. Merged figure showing colocalization (yellow color) of APOBEC3B and NONO in U2OS cells.

APOBEC3B or ER α and NONO or ER α and PSF or in combination with ER α , APOBEC3B and NONO or ER α , NONO and PSF. In agreement with previous findings from our lab, co-transfection of APOBEC3B with ER α stimulated ER α transcriptional activity. However, no increase in transcriptional activity of ER α was observed for cells co-transfected with NONO and ER α or PSF and ER α . Interestingly, co-transfecting ER α , APOBEC3B and NONO in combination resulted in synergism, with a six-fold increase in reporter activity compared to the vector control, whilst cotransfecting ER α , APOBEC3B and PSF in combination did not show synergistic effect (Figure 3.22B). However, synergism was not found when cells were cotransfected with APOBEC3B, NONO and PSF, with the reporter activity observed being similar to that observed for co-transfection of APOBEC3B and ER α , suggesting that PSF does not participate in the synergistic action of APOBEC3B and NONO. Taken together, this result provides evidence that NONO may play a role in APOBEC3B effect on ER α transcriptional response.

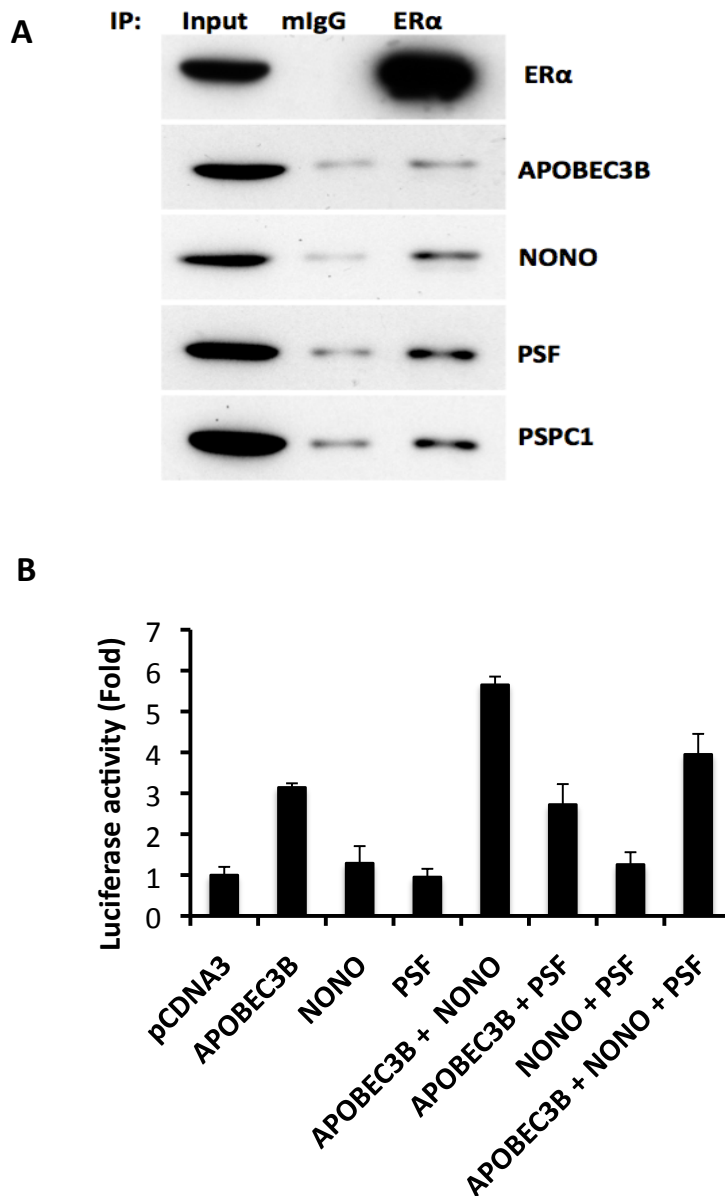


Figure 3.22. Endogenous ER α and APOBEC3B interact with NONO. **A.** whole cell lysates prepared from MCF-7 cells were immunoprecipitated with mouse IgG or ER α antibody, followed by immunoblotting for ER α , APOBEC3B, NONO, PSF and PSPC1. Input represents 1% of the total volume of lysate used in the immunoprecipitation. **B.** COS-1 cells were co-transfected with an estrogen-responsive luciferase reporter, together with ER α , APOBEC3B and p54 (NONO), as indicated. The mean Firefly luciferase activities of three replicates are shown, following correction for transfection efficiency against renilla luciferase activity. The activity of ER α in the presence of pCDNA3 control vector was taken as 1, with other activities being shown relative to this. Error bars represent the standard errors of the mean of three replicates.

4. Discussion

4.1 APOBEC3B is an ER α coactivator in breast cancer cells

As chromatin is repressive for gene transcription, transcription factor access and initiation of transcription is a complex process, requiring extensive chromatin remodelling and modification of core histones, and occurs through the ordered recruitment of a series of transcriptional coregulators to gene promoters. NRs comprise one of the largest classes of transcriptional regulators in higher eukaryotes. As such, the mechanisms of transcriptional regulation by NR have been very well studied, in particular for ER α , where the histone modification, chromatin remodelling and the transcriptional coregulator proteins have been well defined for NR regulated genes, at least for some receptors and model regulated genes (McKenna and O'Malley, 2010; Perissi et al., 2010a; Perissi and Rosenfeld, 2005; Rosenfeld et al., 2006; Wolf et al., 2008). Recent studies have, however, provided a number of new insights into the mechanisms by which NR regulate genes expression, for which the mechanisms remain poorly defined, including the demonstration that (a) several proteins involved in DNA damage recognition and repair acts as transcriptional regulators for ER α and other NR, (b) chromatin remodelling at promoters of estrogen regulated genes involves DNA strand break formation that facilitates nucleosome movement and hence chromatin remodelling and (c) there is rapid DNA methylation and demethylation at promoters of, at least some, estrogen regulated genes following ER α recruitment (Bretschneider et al., 2008; Kangaspeska et al., 2008; Metivier et al., 2008a). To study these aspects of transcription initiation by ER α , we have investigated the potential role of proteins capable of modifying DNA, in order to determine the mechanisms by which DNA modification, including DNA strand break might occur.

We previously showed that TDG acts as an ER α coactivator that is recruited to promoters of estrogen regulated genes in breast cancer cells (Chen et al., 2003). The enzymatic activity of TDG is required for its coactivator activity (Chiang et al., 2010), indicating that it is involved in ER α mediated genes transcription. As TDG is required for repair of T:G and U:G mismatches, its role as an ER α coactivator raised the

possibility that CpG base pairs deaminated to T:G mismatches might be involved in the process of transcriptional initiation at promoters of estrogen regulated genes.

The AID/APOBEC cytosine deaminase family of genes could be candidates for generation of T:G and U:G from methylated CpG (meCpG) or CpG base pairs. Prior to the start of this PhD project, Dr Dongsheng Chen and Dr Ross Thomas in the laboratory demonstrated that APOBEC3B uniquely in the AID/APOBEC family, stimulates ER α activity in reporter gene assays. Moreover, mutation of the cytosine deaminase domains in APOBEC3B blocked its coactivator activity. In light of these results, the focus of this project was to determine the importance of APOBEC3B for ER α function in breast cancer cell lines and investigate the molecular mechanisms by which it regulates ER α activity.

In agreement with the previous reporter gene data, overexpression of APOBEC3B, but not of APOBEC3G stimulated MCF-7 cell growth. Furthermore, cytosine deaminase domain mutants that do not stimulate ER α activity in reporter gene assays also did not stimulate breast cancer cell growth. Conversely, siRNA-mediated knockdown of APOBEC3B in MCF-7, T47D and ZR-75-1 ER α -positive and estrogen-responsive breast cancer cells inhibited their growth. Growth of the ER α -negative and estrogen non-responsive MDA-MB-231 breast cancer cell was not affected by APOBEC3B siRNA, indicating that APOBEC3B can regulate the growth of breast cancer cells that express ER α , in agreement with reporter gene assays which showed that APOBEC3B does not modulate the activity of NR other than ER α , although its importance for transcription factors other than NR has not to date been determined.

4.2 APOBEC3B interacts with ER α and is recruited to the promoters of estrogen regulated genes

As expected from reporter gene studies, APOBEC3B overexpression in MCF-7 cells stimulated expression of the estrogen responsive genes, pS2 and CTSD, whereas transfection of cytidine deaminase domain mutants did not stimulate pS2 or CTSD expression. Also, as expected, siRNA-mediated APOBEC3B knockdown reduced

expression of the estrogen-regulated genes CTSD, pS2 and PR at the mRNA and protein levels. Many transcriptional coregulators of ER α , including TDG, CBP, p300 and members of the p160 family interact with ER α in a ligand-dependent manner, (Anzick et al., 1997; Chen et al., 2003; Smith et al., 1996; Voegel et al., 1996). Unlike these ligand-dependent interactions, however, co-immunoprecipitation of HA-tagged APOBEC3B with ER α following transient transfection in COS-1 cells, showed that APOBEC3B interacts with ER α in a ligand-independent manner. Nuclear colocalization of APOBEC3B-HA and ER α was observed in U2OS transfected with HA-tagged APOBEC3B and ER α that was also ligand-independent. Ligand-independent interaction with ER α has been described for other coactivators, for example PRMT2 (Qi et al., 2002). Moreover, co-immunoprecipitation using MCF-7 cell lysates showed that endogenous APOBEC3B and ER α in MCF-7 also interact. Collectively, these results show that APOBEC3B can interact with ER α in a ligand-independent manner.

ChIP analysis showed that APOBEC3B is recruited to the promoters of estrogen responsive genes, as would be expected for an ER α coactivator. Previous studies have shown that ER α is rapidly recruited to promoters of estrogen responsive genes. Moreover, detailed analysis of ER α recruitment to the pS2 gene promoter in MCF-7 cells has shown that within three hours of estrogen treatment, ER α recruitment to the pS2 promoter is characterized by several cycles of association and dissociation, as confirmed by the findings in this thesis. Moreover, ChIP over the same period for 60 coactivator proteins and histone marks shows ordered cycling on and off of distinct coactivators, accompanied by cyclical histone acetylation, methylation, deacetylation and demethylation (Burakov et al., 2000; Metivier et al., 2003; Metivier et al., 2006; Shang et al., 2000). As with other coactivators, APOBEC3B also cycled on and off the pS2 promoter following estrogen treatment. Similar ChIP results were obtained for the GREB1 gene. The timing of APOBEC3B recruitment and dissociation will determine the timing of chromatin and/or DNA modification carried out by APOBEC3B. Hence, identification of the modification and performing detailed time course profile of the modification following estrogen additions would provide evidence to support a role for APOBEC3B in the process.

4.3 Active DNA demethylation at promoters of estrogen-regulated genes and the role of APOBEC3B in DNA demethylation

The cytosine deaminase activity of APOBEC3B raised the possibility that APOBEC3B might deaminate meCpG, followed by DNA repair, resulting effectively in DNA demethylation. Two recent reports showed cyclical and rapid DNA demethylation at the pS2 gene promoter (Kangaspeska et al., 2008; Metivier et al., 2008a). Whilst GST-MBD pulldown assays for enriching methylated DNA, followed by PCR for detection of the pS2 gene, provided evidence for cycles of rapid DNA demethylation, in agreement with the previous reports, bisulfite treatment, followed by pyrosequencing or by cloning and sequencing of PCR products encoding the pS2 gene promoter failed to show significant DNA methylation at the pS2 gene promoter; nor was there evidence for estrogen stimulation of DNA methylation. It is not clear why there should be a difference between the results of the GST-MBD pulldown assay and the direct sequencing methods. However, the GST pulldown assay was carried out following sonication to generate average DNA lengths of 300-500 bp, whilst the direct sequencing methods were based on amplification of specific sequences, raising the possibility of DNA methylation in the pS2 gene at regions outside those examined by sequencing. This is a possibility that should be further examined. However, my findings are in general agreement with those reported previously for the GST-MBD pulldown assay (Kangaspeska et al., 2008), but are in apparent contradiction with the sequencing results for the region of the pS2 gene promoter that I have sequenced (Metivier et al., 2008a). It should be noted, however, that although the reported GST-MBD pulldown results are for MCF-7 cells, the sequencing to establish methylation/demethylation profiles for specific CpG dinucleotides was carried out in the ER α -negative MDA-MB-231 cells, in which ER α has been stably expressed. As such, regulation of the pS2 gene, which is not normally expressed in MDA-MB-231 cells, may be different from that of the pS2 gene in the ER α -positive MCF-7 cells. By contrast with the results for the pS2 gene, there was considerable change in DNA methylation at the -392 site in the CTSD gene promoter, with no significant methylation being detected for other CpG dinucleotides. APOBEC3B siRNA did not influence the methylation/demethylation profile for this site.

Recent studies have implicated members of the AID/APOBEC family in active DNA demethylation. AID-mediated DNA deamination, coupled with DNA repair by the MBD4, T:G mismatch DNA glycosylase, following injection of methylation DNA in Zebrafish embryos has been reported (Guo et al., 2011; Rai et al., 2008). In HEK293 cells, overexpression of APOBEC1, APOBEC2, APOBEC3A, APOBEC3C and APOBEC3E increased DNA demethylation (Guo et al., 2011), although these authors did not find evidence for DNA demethylation by APOBEC3B in this system. Thus, there is mounting evidence implicating AID/APOBEC family in active DNA demethylation. Using the GST-MBD pulldown assay APOBEC3B siRNA transfection resulted in increased DNA methylation in MCF7 cells, suggesting that APOBEC3B might be involved in regulating DNA methylation in the pS2 gene. However, the increased in methylation did not reach statistically significant. Further study of DNA methylation changes in the pS2 gene and the possible involvement of APOBEC3B in this process is therefore warranted.

4.4 DNA double strand break formation at the pS2 gene promoter and the possible involvement of APOBEC3B in DNA strand breaks

New findings have reported a requirement for specific DNA strand breaks in the promoters of ER α and AR regulated genes that facilitate transcription by allowing nucleosome movement and facilitate chromatin remodeling (Haffner et al., 2010; Ju et al., 2006). This report shows that topoisomerase II β (TOP2B) is recruited with AR and ER α to regulatory sites on target genes to promote DNA strand break, which is repaired by the subsequent recruitment of DSB repair proteins including PARP1, DNA-PK and ATM (Haffner et al., 2010; Ju et al., 2006; Nitiss, 2009a, b). Another study has provided evidence for androgen stimulation of AR resulting in the recruitment of AID to promoters of androgen regulated genes and in the case of prostate cancer cells, the resultant translocation of the TMPRSS2 gene to the ERG and ETV1 genes, translocations that are commonly observed in prostate cancer (Lin et al., 2009). Using biotin DNA end labeling of MCF-7 cells and immunoprecipitation using a biotin antibody confirmed that estrogen induces DSB at the pS2 gene

promoter within 10 minutes of estrogen treatment that showed a maximum DSB of 9 fold, with DSB levels falling after this time, returning to baseline by 60 minutes, in agreement with previous findings (Ju et al., 2006). Furthermore, immunofluorescence staining for γ H2AX showed that estrogen induces γ H2AX phosphorylation in MCF-7 cells and the antioestrogens tamoxifen and ICI 182 780 inhibited the estrogen induced γ H2AX phosphorylation, indicating that the estrogen stimulation of γ H2AX phosphorylation is ER α mediated. APOBEC3B overexpression stimulated γ H2AX phosphorylation in MCF-7 cells, with cytosine deaminase domain mutants only weakly stimulating γ H2AX phosphorylation. The cytosine deaminase domain mutants also inhibited the estrogen stimulated DSB formation at the pS2 promoter. Similarly APOBEC3B knockdown inhibited DSB formation at the pS2 promoter. Together, these results indicate that APOBEC3B is important for this process. Clearly, a great deal of additional work is required to demonstrate the importance of APOBEC3B, as well as DSB formation for estrogen-regulated gene expression. Firstly, it would be important to determine if DSB formation is a general feature of ER α action at promoters of estrogen-regulated genes. In this context, we have carried out biotin immunoprecipitation following 10 minutes estrogen treatment of MCF-7 cells and carried out high-throughput DNA sequencing. Although this requires sequencing of further replicates, preliminary analysis maps biotin (and hence DSB) at many defined estrogen regulated gene promoters, in the vicinity of defined ER α binding sites, indicative of the general importance of DSB formation for estrogen-regulated gene expression. APOBEC3B ChIP-seq will also allow us to whether APOBEC3B is recruited to sites of DSB formation. Other experiments to perform will be to determine if the sites of γ H2AX phosphorylation induced by estrogen represent is occurring at estrogen responsive gene promoters and to confirm the importance of APOBEC3B in this process. In summary, these studies provide exciting new data linking the coactivator function of APOBEC3B with DSB formation required to initiate chromatin changes that facilitate gene expression.

4.5 APOBEC3B and NONO synergistically work to increase ER α transcriptional activity

Yeast 2-hybrid screening has further provided a link between APOBEC3B and proteins involved in RNA processing, through identification of an interaction with NONO. The results presented here provide confirmation of the interaction between APOBEC3B and NONO and show that APOBEC3B interacts with the NONO complex, which includes PSF and PSPC1. Moreover, NONO synergized with APOBEC3B to stimulate ER α activity in reporter gene assays. These findings are in agreement with previous reports demonstrating that NONO is a transcriptional coregulator for other NR, including AR, PR, GR, SF-1, the thyroid hormone receptor and retinoid X receptors (Dong et al., 2009; Ishiguro et al., 2003; Kuwahara et al., 2006; Mathur et al., 2001; Sewer et al., 2002). Although the mechanisms by which NONO and its dimerisation partners PSF and PSPC1 regulate transcription are not defined, NONO appears to promote recruitment of the Sin3A complex, with associated HDAC, to act as a corepressor. How it stimulates transcription factor activity remains unclear. However, NONO, PSPC1 and PSF proteins contain RNA recognition motifs, indicating that they bind RNA. NONO and PSF have also been implicated in pre-mRNA processing, RNA splicing, transcription termination and nuclear sequestration of adenine deaminase mediated A to I edited mRNA, which are released following activation of cellular stress responses, to allow rapid translation of some proteins (Bond and Fox, 2009; Kaneko et al., 2007; Zhang and Carmichael, 2001)

In terms of the significance of APOBEC3B interaction with NONO and regulation of estrogen-responsive genes, one possibility is the regulation of non-coding nuclear RNAs. It is becoming increasingly clear that much of the transcribed genome encodes noncoding RNAs (ncRNAs), many of which are likely to play a role in gene regulation. This includes small ncRNAs generated from longer transcripts and which act to repress gene expression using the RNA interference machinery, for example microRNAs. There are also large ncRNAs that directly regulate gene expression by influencing transcription and are likely to be involved in epigenetic regulation. Examples of long ncRNAs implicated in NR function are the steroid receptor activator (SRA), which was identified as an RNA that interacts with SRC1 (Lanz et

al., 1999). SRA has multiple stem-loops, associates with the ER α coactivator p68 RNA helicase, one of the first coactivators recruited to promoters of estrogen regulated genes following estrogen stimulation and appears to regulate the recruitment of other coactivator complexes, particularly those containing SRC1 (Caretto et al., 2007; Caretto et al., 2006; Watanabe et al., 2001). Another ncRNA that regulates glucocorticoid receptor (GR) activity is GAS5. GR is able to recognize and bind to a potential GR binding motif encoded in the stem-loops of the GAS5 RNA. As a result GAS5 RNA can competitively inhibit the binding of GR to genomically encoded GREs, hence inhibiting GR activity (Kino et al., 2010). It is possible that APOBEC3B is involved in RNA editing of ncRNAs and that this is important for the function of ncRNAs required for ER α activity. The nature of the ncRNA(s) that might be involved in regulating ER α activity are unclear, although it is unlikely given the fact that SRA can act as a coactivator for many NR, whereas APOBEC3B stimulates only ER α activity, that SRA would be a substrate for APOBEC3B. Moreover, APOBEC3B does not appear to edit single-stranded RNA in vitro, but can edit DNA, although its activity has not been determined using double-stranded RNA (Dr N Navaratnam, Imperial College London, personal communication). It would be interesting to determine if the coactivator function of APOBEC3B involves ncRNAs. This could be achieved in MCF-7 cells by preparing lysates following formaldehyde fixation as done for ChIP, but following immunoprecipitation, recovering bound RNA, followed by cDNA synthesis, cloning and sequencing, or directly sequencing the RNAs using next generation sequencing (RNA-seq) (Hafner et al., 2010).

5. Summary and importance of findings for breast cancer

In summary, I have shown that APOBEC3B is an ER α coactivator that is an important regulator of breast cancer cell growth and estrogen-responsive gene expression. Our first hypothesis was that APOBEC3B might be involved in active DNA demethylation in breast cancer cells. However, extensive analysis of DNA methylation at promoters of model estrogen responsive genes provides little evidence to support a role for APOBEC3B in DNA demethylation at promoters of genes whose expression it can regulate. Strong evidence for APOBEC3B's involvement in DSB formation was forthcoming and this is potentially an important mechanism underlying

APOBEC3B activity in breast cancer cells. Finally, the demonstration of APOBEC3B interaction with the NONO complex, as well as other interacting proteins also linked to RNA processing provides evidence for the involvement of RNAs in APOBEC3B function.

In terms of the potential clinical importance of these findings, analysis of the expression of APOBEC3B in gene expression microarray datasets indicates that APOBEC3B expression is significantly higher in breast cancer tissue compared to normal breast tissue and increases with tumour grade (Figure 4.1A, B) (Richardson et al., 2006). Furthermore, Kaplan-Meier analysis shows that high level APOBEC3B expression is associated with shorter relapse-free interval and overall survival (Figure 4.1C, D). Interestingly, a 29.5 kb deletion has been described that removes almost the entire APOBEC3B gene in some human populations, especially those of Chinese origin where the gene is deleted in 30-40% of the population, whereas the deletion is extremely rare in African or Caucasian populations (Kidd et al., 2007). It would be interesting to determine if the presence or absence of the APOBEC3B gene is associated with breast cancer risk. Certainly, another common deletion that removes exons 4-5 and hence would encode a truncated protein, has also been described in a Far-Eastern population (Komatsu et al., 2008). In this study, of 50 breast cancer patients and 50 healthy females, the exon 4-5 deletions were 7 times more prevalent in the breast cancer patients. My findings relating to APOBEC3B function offer a possible explanation for the gene expression profiling and polymorphism studies that are indicative of a significant link between APOBEC3B and breast cancer development and progression. Clearly, therefore elucidation of APOBEC3B mechanisms of action in breast cancer is deserving of further attention.

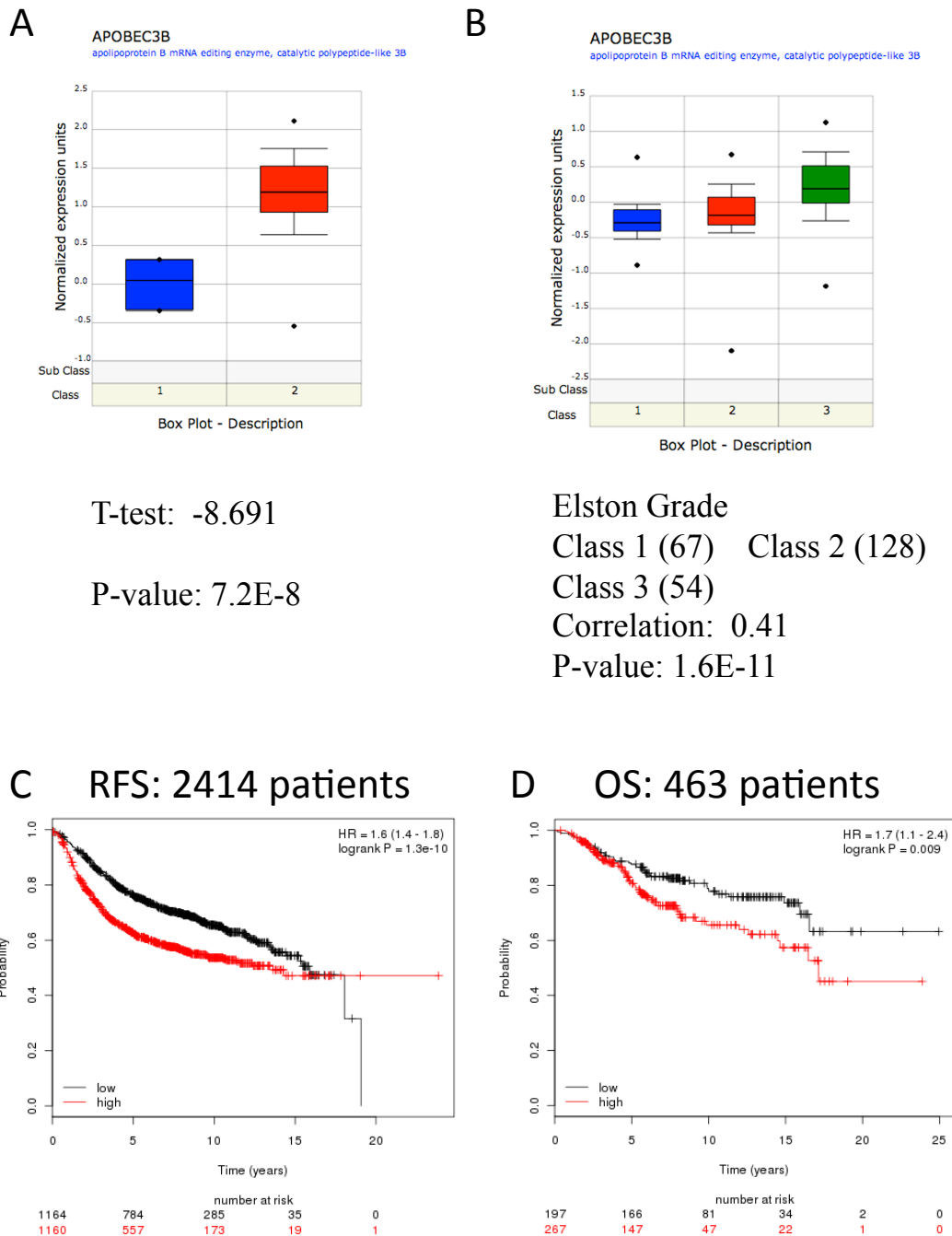


Figure 4.1. Association of APOBEC3B expression with clinical features in breast cancer. (A-B) Boxplots were generated using datasets present in Oncomine (<http://www.oncomine.org>). (A) Shown is a boxplot graph, comparing APOBEC3B expression in normal breast tissue (n = 7) and breast tumours (n = 40) (Richardson et al., Cancer cell 2006). (B) Boxplot showing increased APOBEC3B expression with increasing tumour grade (data from Miller et al PNAS 2005/09/21). (C-D) Kaplan-Meier survival curves were generated by analysing publicly available gene expression microarray data for breast tumours (<http://kmplot.com>) (Gyorffy et al., Breast Cancer Res Treatment, 2010 Oct;123 (3):725-31). These plots show that high level APOBEC3B expression is a poor prognostic marker in breast cancer.

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APPENDIX

Publications

ZNF366 is an estrogen receptor corepressor that acts through CtBP and histone deacetylases

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ABSTRACT

The regulation of gene expression by estrogen receptor- α (ER α) requires the coordinated and temporal recruitment of diverse sets of transcriptional co-regulator complexes, which mediate nucleosome remodelling and histone modification. Using ER α as bait in a yeast two-hybrid screen, we have identified a novel ER α -interacting protein, ZNF366, which is a potent corepressor of ER α activity. The interaction between ZNF366 and ER α has been confirmed *in vitro* and *in vivo*, and is mediated by the zinc finger domains of the two proteins. Further, we show that ZNF366 acts as a corepressor by interacting with other known ER α corepressors, namely RIP140 and CtBP, to inhibit expression of estrogen-responsive genes *in vivo*. Together, our results indicate that ZNF366 may play an important role in regulating the expression of genes in response to estrogen.

INTRODUCTION

Estrogens play diverse roles in the body, most notably in the development and maintenance of the female and the male, reproductive systems and secondary sexual characteristics (1). Estrogens also play a central role in promoting breast cancer growth (2), as well as being implicated in uterine and ovarian cancers (3,4), and are also implicated in the physiology of the brain, bone and the cardiovascular system, as evidenced by the increased risk of cardiovascular disease and osteoporosis engendered by the decline in estrogen levels during menopause (1).

Estrogen action is mediated by two highly related estrogen receptors (ER α and ER β), which are members of

the ligand-activated nuclear receptor (NR) superfamily of transcription factors (5,6). NRs are characterized by a DNA binding domain (DBD), comprised of two zinc fingers, which mediate receptor dimerization and binding to specific response elements in the promoters of target genes. Binding of the ligand to the ligand binding domain (LBD), located C-terminal to the DBD, results in a conformational change in the LBD and activation of the intrinsic transcription activation function AF2, which facilitates the recruitment of transcriptional coactivators (7,8). Transcription activation requires cooperation of AF2 with a region N-terminal to the DBD that encodes transcription activation function AF1, which is often, as in the case of ER α and ER β , regulated by phosphorylation at specific serine residues (9–11).

The liganded estrogen receptors regulate gene expression by direct binding to DNA at estrogen response elements in target genes, resulting in the recruitment of diverse transcriptional coregulators, including the SWI–SNF complexes that remodel chromatin to alter nucleosomal organization in an ATP-dependent manner (12), the p160 family of coactivators (SRC1/NC0-A1, TIF-2/GRIP1 and AIB1/pCIP/ACTR/RAC3/TRAM1) and TRAP/DRIP complexes. (13–15). The p160 coactivators facilitate the recruitment of other proteins, including CBP, its homologue p300 and p/CAF, which possess intrinsic histone acetyltransferase activity, as well as histone methyltransferases CARM1 and PRMT1 that methylate arginine residues in histone tails (16,17). The thyroid receptor-associated protein (TRAP) complex, similar to or identical with the vitamin D3 receptor-interacting protein (DRIP) complex, which is also similar in many respects to the Mediator complex acts to bridge RNA polymerase II with basal transcription factors and transcription activators. These and other coregulators are recruited to gene promoters in a sequential/ordered manner, resulting in cycles of chromatin remodelling and modification that facilitate transcription (13,15,18).

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The ER α LBD is composed of 12 α -helices packed in three layers, with a central hydrophobic pocket that accommodates the ligand (7). Helix 12, together with helices 3, 4 and 5, form a coactivator-binding groove. Most coactivators recruited by agonist-bound ER α contain LXXLL motifs, which form a two-turn amphipathic α -helix that fits into the coactivator-binding groove in the ER α LBD (8,19–22). In addition to the well-characterized recruitment of coactivators to the LBD/AF2, coactivator interaction with AF1 of estrogen receptors has also been described, the interaction being influenced by the phosphorylation status of the receptor in some cases (23–25).

When unliganded, some DNA-bound NR recruit the corepressors NCoR and SMRT and associated protein complexes implicated in transcriptional repression and histone deacetylation, these complexes being dissociated upon ligand binding (13–15,26). NCoR/SMRT bind to NR through CoRNR boxes, LXXI/HIXXXL/I motifs, that form a more extended α -helix than the LXXLL motifs, with helix 12 in the LBD being displaced from the conformation it occupies in the agonist-bound LBD (27,28). NCoR/SMRT is also recruited by antagonist-bound ER α to inhibit gene expression (29–31).

In addition to stimulating gene expression, estrogen-bound ER represses the expression of many genes. Indeed, gene profiling studies show that the down-regulation of gene expression is a significant feature of the response to estrogen in the ER α -positive, estrogen-responsive MCF7 breast cancer cell line (32). This is likely to involve transcriptional corepressors, such as LCoR and RIP140, which can be recruited to the agonist-bound ER α via LXXLL motifs (20,33,34). Repression by LCoR and RIP140 occurs through HDAC-dependent and -independent mechanisms and involves the recruitment of HDACs and the C-terminal binding protein (CtBP) corepressor (34–39). CtBP, originally identified based on its interaction with the C-terminal end of adenovirus E1A via the sequence PLDLS, is highly conserved in higher eukaryotes and plays a critical role in development (40,41). Other transcription factors also interact with CtBP1, and the highly related CtBP2, through PDXLS motifs. Although, the mechanisms by which CtBP acts as a corepressor have not been fully defined, a recent study has identified CtBP complexes that contain HDACs and histone lysine methyltransferases (42).

The ER α DBD participates in the recruitment of transcriptional co-regulator proteins. These include the coactivator XBP-1 (43), which modulates ER α signalling both in the absence and presence of estrogen, the signal transducer and activator of transcription-5 (STAT 5) (44) and the corepressor TAF-Ib that has been shown to decrease ER α acetylation (45). Here, we report that ZNF366, which encodes an evolutionarily conserved zinc finger protein, interacts with the ER α DBD. We also show that ZNF366 represses ER α activity through association with RIP140, CtBP and histone deacetylases.

MATERIALS AND METHODS

Plasmids

The mammalian expression plasmids and reporter genes have previously been described (37,46–49). Site-directed

mutagenesis was used to introduce an EcoRI site 5' to the GAL4 translation initiation site in the pBridge yeast expression plasmid (BD Biosciences, UK), enabling cDNA sequences encoding ER α and ER α - Δ LBD to be cloned at this position following removal of the GAL4 sequences encoded between the introduced EcoRI site and the multiple cloning site in pBridge. The pACTII-ZNF366 clone isolated from the yeast 2-hybrid screening encodes sequences corresponding to 439–2761 bp of the ZNF366 mRNA sequence with the accession number NM_152625 in the NCBI database (www.ncbi.nlm.nih.gov). The full-length ZNF366 open reading frame was reconstituted from the pACTII-ZNF366 clone and IMAGE EST clone 5204702 (accession no. B1770486), to generate pCMVSPORT6-ZNF366 in which ZNF366 is C-terminally FLAG-tagged. ZNF366 deletion and point mutants were generated by site-directed mutagenesis according to manufacturer's protocols (Stratagene, UK).

Yeast 2-hybrid screening

PL1 α (MAT α ura3- Δ 1 his3- Δ 200 leu2- Δ 1 trp1::ERE)1-URA3 yeast strain (50) was transformed with pBridge(Mod)-ER α - Δ LBD, together with a human placental cDNA expression library (BD Biosciences, UK), using the Alkali-Cation yeast transformation kit (BIO 101 systems, UK). Following transformation, the cells were plated on 15 cm trp⁻ leu⁻ ura⁻ plates. Positive clones arising from the screening of 2×10^4 transformants were re-screened and plasmid DNAs were isolated using the lyticase method from BD Biosciences, UK. Plasmids from positive clones were re-transformed, together with pBridge(Mod)-ER α - Δ LBD or pBridge(Mod)-ER α and interactions confirmed by growth on trp⁻ leu⁻ ura⁻ plates.

Northern blotting

Multiple tissue northern blots MTN I and MTN II (BD Biosciences, Europe) were probed following ³²P-labelling of the ZNF366 cDNA isolated from the pACTII-ZNF366 clone, as described (51).

Protein expression, purification and glutathione S-transferase (GST)-based interaction assay

In vitro transcription/translations were performed using TNT rabbit reticulocyte lysates (Promega, UK), in the presence of [³⁵S]-labelled methionine. GST proteins were induced and *Escherichia coli* lysates prepared as described previously (33). For pulldowns, GST fusion proteins were purified by affinity chromatography on glutathione-agarose beads and retained as 50% slurry in 20 mM HEPES (pH 7.6), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 20% glycerol, supplemented with protease inhibitors. A total of 100 μ l volumes of glutathione-agarose bead slurry loaded with 10 μ g of GST fusion proteins were then used directly in binding assays with 10 μ l of radiolabelled *in vitro* translation reactions and 890 μ l of low salt buffer [50 mM HEPES (pH 7.6), 250 mM NaCl, 0.5% NP-40, 5 mM EDTA, 0.1% BSA, 0.5 mM DTT, 0.005% SDS and protease inhibitors]. Following 1 h incubation at room temperature, the beads were washed twice with low salt buffer and twice with high-salt buffer (low salt buffer, but with 1 M NaCl). Samples were

boiled for 10 min in 80 µl of Laemmli buffer and fractionated by SDS-PAGE. Gels were dried and autoradiographed.

Reporter gene assays

COS-1 cells were maintained in DMEM, supplemented with 5% fetal calf serum (FCS). For transient transfection, cells were seeded in 24-well plates in DMEM lacking phenol red and supplemented with 5% dextran-coated charcoal-stripped FCS (DSS). Following seeding for 24 h, the cells were transfected using Fugene 6 (Roche Diagnostics, UK), with 100 ng of luciferase reporter gene and amounts of expression plasmids as indicated in the figure legends. E2 (10 nM), 4-hydroxytamoxifen (OHT; 100 nM) or ICI 182, 780 (ICI; 100 nM) were added as appropriate. Since the ligands were prepared in ethanol, an equal volume of ethanol was added to the no ligand controls. Luciferase activities were determined using the Dual-Glo Luciferase Assay kit (Promega, UK). For the other reporter gene assays, cells were maintained in DMEM, supplemented with 5% FCS and transfections carried out as above.

Immunoprecipitations and immunoblotting

COS-1 cells were plated in 9 cm dishes in DMEM supplemented with 5% FCS 16 to 24 h prior to transfection. The cells were transfected with 5 µg of the ZNF366-FLAG and ERα expression plasmids using Lipofectamine 2000 (Invitrogen, UK). Following transfection for 48 h, the cells were lysed in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS and 50 mM Tris-HCl (pH 7.5)] containing protease inhibitors. Lysates (2 mg) were immunoprecipitated (IP) using the M2 anti-FLAG mouse monoclonal antibody (Sigma-Aldrich, UK), or using an anti-ERα antibody (6F11; Novocastra, UK). Control IPs was carried out using mouse IgG (Sigma-Aldrich, UK). IPs were resolved by SDS-PAGE and immunoblotted using horseradish peroxidase (HRP)-labelled HA antibody (Sigma-Aldrich, UK) or using an anti-ERα rabbit polyclonal antibody HC20 (Santa Cruz, UK). Co-IP of ZNF366-FLAG with CtBP was carried out as above, except that a mouse monoclonal CtBP antibody (sc-17759; Santa Cruz) was used for the IPs and a rabbit polyclonal CtBP antibody (sc-11390; Santa Cruz) was used for immunoblotting.

MCF7 cells cultured for 3 days in DMEM lacking phenol red and supplemented with 5% DSS, were transfected with 1 µg of ZNF366-FLAG or vector control, using Fugene 6. The media were replaced with media containing E2 (10 nM) or vehicle, 24 h following transfection and the cells were harvested after a further 24 h. Immunoblotting was performed using antibodies for cathepsin D (ab6313; Abcam, UK), progesterone receptor (SC538; Santa Cruz Biotechnologies, UK), FLAG-M2 and β-actin (ab6276; Abcam, UK).

Immunofluorescence

COS-1 cells plated on glass coverslips placed in 24-well plates in DMEM lacking phenol red and containing 5% DSS, were transiently transfected with 50 ng of ZNF366-FLAG and/or [ERα-ΔNLS (HE257G; (48))] using Fugene 6. Five hours following transfection, culture media were replaced by fresh media containing E2 (100 nM), OHT

(1 µM) or ICI 182, 780 (100 nM), or an equal volume of vehicle (ethanol), as appropriate. 24 h later, cells were fixed by the addition of 4% formaldehyde for 10 min at room temperature, washed with phosphate-buffered saline (PBS) and 0.1 M glycine was added for 10 min to neutralize the formaldehyde. Following further washing with PBS, the cells were permeabilized in 1% Triton/PBS for 5 min. After washing with PBS, the cells were incubated at 37°C for 1 h with the 6F11 ERα antibody (1:50 dilution) and rabbit polyclonal FLAG antiserum (Santa Cruz Biotechnology, UK) (1:350 dilution). The cells were washed and incubated for 1 h at 37°C with Alexa Fluor 488 goat anti-mouse immunoglobulins (green) and Alexa Fluor 594 goat anti-rabbit immunoglobulins (red) (1:3000 dilution). The coverslips were mounted on microscope slides using mountant containing Dapi (Vector Laboratories, UK) and immunofluorescence observed using a Zeiss LSM510 confocal microscope.

Growth assays

MCF7 and MDA-MB-231 cells (2×10^5 cells per well) were seeded in 6-well plates in DMEM lacking phenol red and containing 5% DSS. After 48 h the cells were transfected with 1 µg of ZNF366 or empty vector using Fugene 6. E2 (10 nM) was added after 24 h and cell numbers determined using a haemocytometer after a further 48 h.

RNA interference and RT-PCR analysis of gene expression

PE04 cells (2.5×10^5 cells) seeded in 6-well plates in RPMI lacking phenol red and containing 5% DSS, were transfected with double-stranded RNA oligonucleotides for ZNF366, lamin A/C or a non-targeting siRNA (Ambion, UK), in serum-free DMEM lacking phenol red, using Oligofectamine (Invitrogen, UK), according to manufacturer's protocols. After 4 h the medium was changed to DMEM lacking phenol red, supplemented with 5% DSS and containing E2 (10 nM), as appropriate. RNA was prepared after a further 24 h and RT-PCR carried out using primers with the sequences: 5'-GGACCAGCTTCAGTCACCTTTCCAGTGGTGGCC-3' and 5'-GGGAGTAAAGCTGGTGCCTGGGACACAGGTCACG-3' (GREB1), 5'-CCCCATCCAGT-ACAACTGCT-3' and 5'-CTTCACGTCAGAGTGGACGA-3' (ZNF366), 5'-GCGTACGGCTCTCATCAACT-3' and 5'-GACACTGGAGGCAGAAGAGC-3' (Lamin A/C) and 5'-TCCCATCACCATCTTCCA-3' and 5'-CATCACGCCACAGTTTCC-3' (GAPDH).

RESULTS

Identification of ZNF366

We utilized yeast strain PL1α, encoding an integrated estrogen-responsive URA3 gene (50), for screening of a human placental cDNA expression library for proteins that interact with an ERα deletion mutant lacking the LBD (ERα-ΔLBD). Screening of 2×10^4 transformants yielded 24 positive clones. One of these encoded the C-terminal portion of ZNF366, described previously based on gene prediction of genomic DNA sequence of human chromosome

5q13.2. Human ESTs were identified using the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST/>) by carrying out a BLAST search against the database of human ESTs, using sequences derived from the yeast 2-hybrid clone. The exact intron/exon structure of ZNF366 was established by complete DNA sequencing of overlapping EST IMAGE clones with GenBank accession nos BI523869, 5201353, BI770486 and BE552137 to generate an mRNA sequence encoding a predicted polypeptide of 744 amino acids (Figure 1A), encoded within 5 exons. Based on the homology with the Fugu fZF1 gene, this was subsequently confirmed by Gilligan *et al.* (52).

Homology searches identified genes in mouse, rat, dog, chicken and *Xenopus tropicalis*, having very high amino acid sequence homology (93% or greater) in the zinc finger region and all of which encode 11 zinc fingers (Figure 1B). Regions N- and C-terminal to the zinc fingers were also well conserved, albeit less so than the zinc finger region. Together, the high degree of sequence conservation indicates that the identified vertebrate genes represent orthologues of human ZNF366. Support for this idea is provided by additional homology searches, which indicated that the human gene most closely related to ZNF366 is ZNF710, also encoding an 11 zinc finger protein, the zinc finger regions of human ZNF366 and ZNF710 showing amino acid sequence identity of 78%, considerably lower than the identity between human and the putative ZNF366 homologue in chicken or *Xenopus*. Moreover, no significant homology was detectable between human ZNF366 and ZNF710 outside the zinc finger region. All other human genes demonstrating homology to human ZNF366 displayed amino acid sequence identities no better than 39%, when the zinc finger region of ZNF366 was used in the BLAST searching. Together with the previous report demonstrating, based on very high (88%) amino acid sequence homology in the zinc finger region, as well as conservation of the chromosome location of human ZNF366 and the Fugu fZF1 gene (52), these analyses indicate that ZNF366 is highly conserved in vertebrate evolution.

Northern blotting of human tissue RNAs demonstrated that ZNF366 is broadly expressed at varying levels in human adult tissues, with highest expression in heart, placenta, muscle and spleen, with possible alternative splice forms in the liver and muscle (Figure 1C).

Interaction of ZNF366 with ER- α

To further confirm interaction between ER α and ZNF366, whole cell lysates were prepared from COS-1 cells transiently transfected with ER α and FLAG-tagged ZNF366, in the presence of E2. IP with FLAG antibody, followed by immunoblotting using the HC20 rabbit polyclonal ER α anti-serum, showed that ER α interacts with ZNF366 (Figure 2A), whilst IP of ER α co-immunoprecipitated ZNF366-FLAG (Figure 2B), indicating that ER α and ZNF366 interact *in vivo*.

As expected, PL1 α cells co-transformed with pBridge(Mod)-ER α - Δ LBD and pACTII-ZNF366 grew on minimal medium lacking lacking uracil (Figure 2C). However, for full-length ER α , productive interaction, as assayed by growth in the absence of uracil, was ligand-dependent, requiring the addition of estrogen (17 β -estradiol; E2) or an anti-estrogen 4-hydroxytamoxifen (OHT).

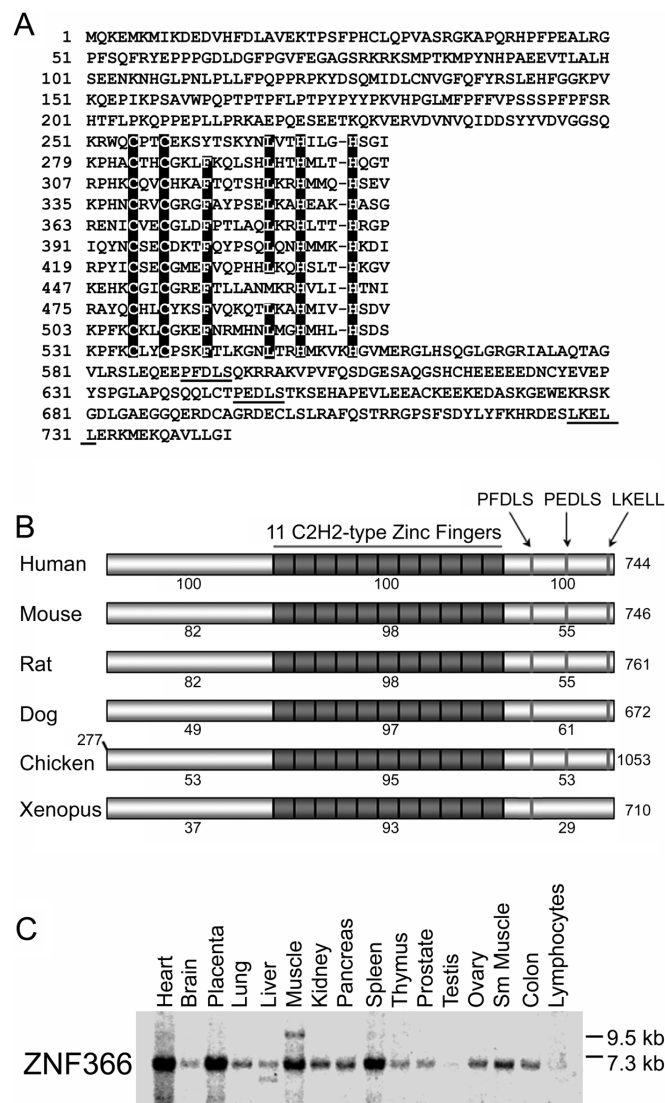


Figure 1. The amino acid sequence and tissue distribution of human ZNF366. (A) The deduced amino acid sequence of human ZNF366 is shown in the single letter amino acid code, with the cysteine (C) and histidine (H) residues of the 11 zinc fingers in ZNF366 highlighted, as are the conserved phenylalanine (F) and leucine (L) residues. Potential CtBP binding motifs and an LXXLL motif are underlined. (B) A schematic representation of ZNF366 from different species is shown, together with the position of the zinc fingers and the putative CtBP and LXXLL motifs, the sequences for these motifs in human ZNF366 being shown. The numbers below the representation of ZNF366 from each species refer to percentage amino acid sequence homology relative to human ZNF366. The numbers to the right refer to the predicted number of amino acids encoded by the ZNF366 genes from different species. The region encoding 277–1053 amino acids was used for alignment in the case of the chicken ZNF366. ZNF366 sequences used here have GenBank accession nos NM_152625 (human), NM_001004149 (mouse), XM_226715 (rat), XP_544370 (dog) and XP_429153 (chicken). The *Xenopus tropicalis* ZNF366 sequence was derived from the ENSEMBL database, from sequences having the Gene ID ENSGALG00000015008. (C) Northern blot of 2 µg of poly(A)+ RNA from the tissues indicated, probed with ZNF366 cDNA. Size markers are shown on the right.

In order to confirm the ligand requirement for the interaction between ER α and/or ZNF366 in mammalian cells, COS-1 cells were transfected with ER α - Δ NLS, which is excluded from the nucleus (48). ZNF366-FLAG was exclusively localized to the nuclei (Figure 2D), whilst

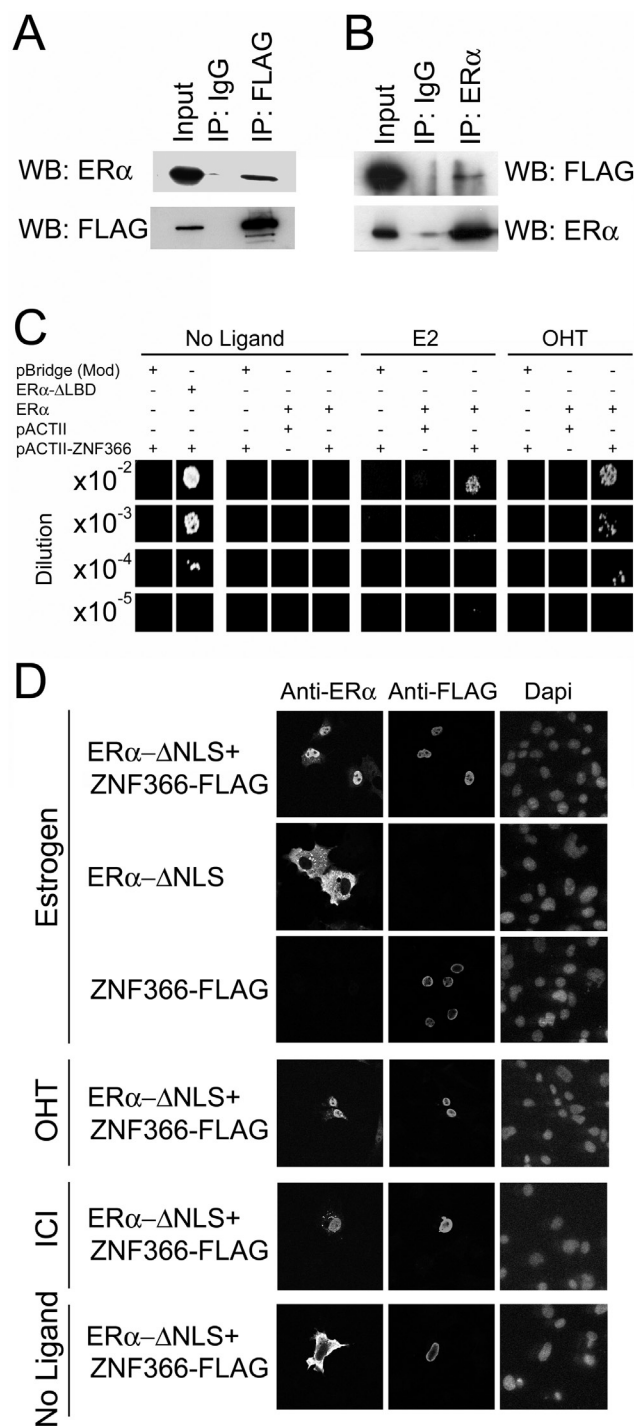


Figure 2. Interaction of ZNF366 with ERα. (A and B) Lysates prepared from COS-1 cells transiently transfected with ERα and ZNF366-FLAG were immunoprecipitated with mouse IgG, or with ERα or FLAG antibodies. Input represents 5% of the total volume of lysate used in the immunoprecipitations. (C) Cultures of PL1α yeast cells transformed with pBridge(Mod), pBridge(Mod)-ERα-ΔLBD or pBridge(Mod)-ERα, together with pACTII or ZNF366 were spotted at various dilutions, as indicated. Yeast growth was performed over 7 days at 30°C, in the presence of 17β-estradiol (E2; 1 nM), 4-hydroxytamoxifen (OHT; 100 nM) or in the absence of ligand (no ligand), on plates lacking tryptophan, leucine and uracil. (D) COS-1 cells transiently transfected with ERα-ΔNLS and/or FLAG-ZNF366 were visualized by immunofluorescent staining, as described in Materials and Methods. Dapi was used to visualize nuclei. Estrogen (100 nM), OHT or ICI (100 nM) were added as indicated.

ERα-ΔNLS was always cytoplasmic in the presence of E2, or in the presence of the anti-estrogens OHT or ICI 182, 780 (ICI), as well as in the absence of ligand, when expressed alone (data not shown). In cells co-transfected with ZNF366-FLAG and ERα-ΔNLS, ERα-ΔNLS re-localized to the nucleus when E2, OHT or ICI were present, but not in the absence of ligand, indicative of ligand-dependent interaction between the two proteins. These data further demonstrate that ERα-ΔNLS and ZNF366 interact *in vivo* and in agreement with the yeast 2-hybrid data, show that the *in vivo* interaction requires estrogen agonist or antagonist binding by ERα.

In contrast, *in vitro* binding assays showed that ERα bound to GST-ZNF366 in the absence of ligand, as well as in the presence of E2 or anti-estrogens, although the interaction appeared to be greater in the presence of E2 and ICI (Figure 3C), which may suggest that the interaction between ERα and ZNF366 *in vivo* is regulated by other factors (see Discussion). ERα can be phosphorylated at serine 118 within AF1, and substitution of this residue by alanine significantly reduces ERα activity (53). Substitution of leucine-539 and leucine-540 in the LBD also dramatically reduces ERα activity, by preventing coactivator recruitment (54). Substitution of serine 118 or of leucine-539/540 did not inhibit ZNF366 interaction with ERα, suggesting that AF1 and the LBD/AF2 may not be involved in the interaction of ZNF366 with ERα.

GST pulldowns were performed to delineate the regions of ERα and ZNF366 required for their interaction. ERα deletion mutants lacking AF1 (ERα-ΔAF1) or the LBD (ERα-ΔLBD) interacted with ZNF366, as did the isolated ERα DBD (Figure 3D), whereas the interaction with the LBD was weak, suggesting that the ERα DBD is required for interaction with ZNF366. GST fusion proteins encoding the N- and C-terminal regions of ZNF366, ZNF366(9–251) and ZNF366(558–744) respectively, did not interact with ERα, indicating that the zinc finger region is required for the interaction. Interestingly, this assay suggests that the interaction requires independent binding to several zinc fingers, since ZNF366(9–452) and ZNF366(455–744), which do not overlap, both interacted with ERα.

ZNF366 is a corepressor for ERα

In reporter gene assays, co-transfection with increasing amounts of ZNF366 showed a dose-dependent inhibition of ERα activity (Figure 4A). ZNF366 repressed AF1 (ERα-ΔLBD; Figure 4B) and AF2 (ERα-ΔAF1; Figure 4C), in agreement with the GST pulldowns that indicate interaction of ZNF366 with the ERα DBD. A trans-repression assay was employed to see whether ZNF366 is a repressor and encodes autonomous repression domain(s). For this, a luciferase reporter gene under the control of LexA and Gal4 binding sites upstream of an E1A TATA box was used. As expected, LexA-VP16 stimulated reporter gene expression (Figure 4D), with this activity being reduced in a dose-dependent manner by the Gal4 DBD fused to the NR corepressors RIP140 (Gal4-RIP140), as described previously (37). ZNF366 similarly repressed reporter gene activity in a dose-dependent manner, confirmative of its activity as a transcriptional repressor.

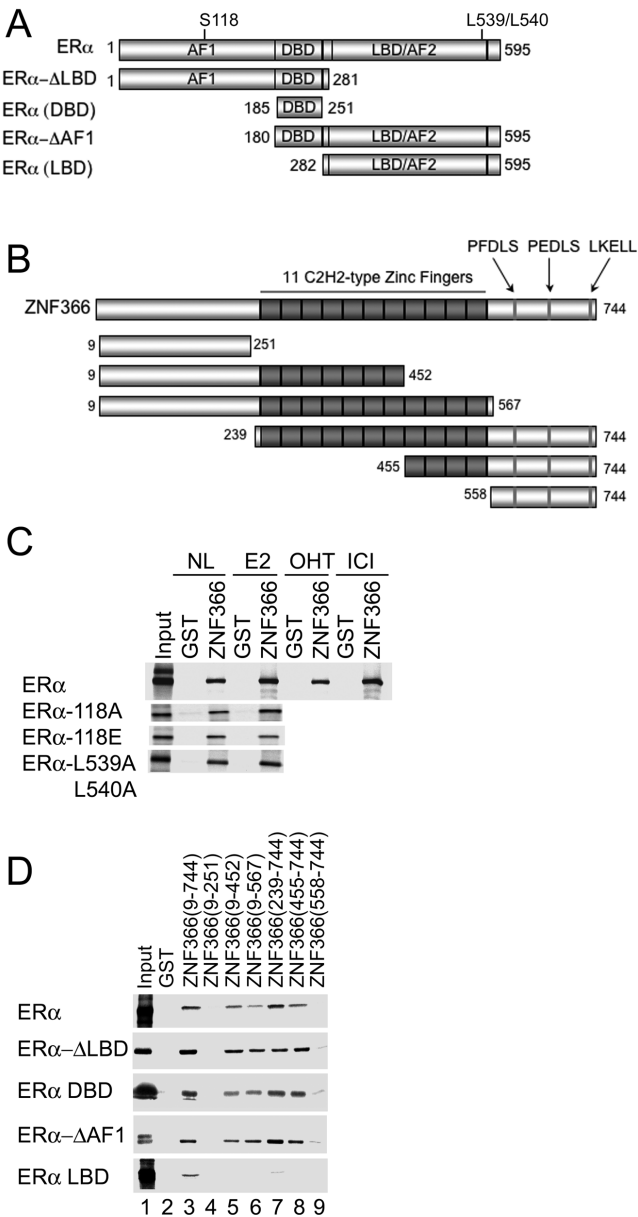


Figure 3. *In vitro* interaction between ER and ZNF366. (A and B) Schematic representations of ERα, ZNF366 and deletion mutants are shown. All N-terminal ZNF366 fusion proteins started at amino acid 9, thereby avoiding potential internal translation start sites. (C and D) GST binding assays were carried out by incubation of ³⁵S-labelled ERα or mutants with GST or GST-ZNF366 fusion proteins, in the absence of ligand (NL) or in the presence of 100 nM E2, OHT or ICI. E2 was present throughout in part (D). Input lanes represent 20% of the total volume of the *in vitro* translation reaction used in the binding assay.

In order to address the potential activity of ZNF366 on ERα-regulated gene expression, we looked for human cell lines in which the genes were co-expressed (data not shown). The PE04 ovarian cancer cell line was found to express both ERα (3) and ZNF366 (Figure 4E). Transfection with siRNA for ZNF366 resulted in down-regulation of ZNF366 expression and concomitant increase in the expression of the estrogen-responsive GREB1 and TERT genes, indicating that ZNF366 is involved in the regulation of estrogen-responsive gene expression *in vivo*.

ZNF366 interacts with CtBP *in vitro* and *in vivo*

Since ZNF366 can repress ligand-stimulated ERα activity, we wondered whether it interacts with other corepressors that are known to associate with ERα in a ligand-dependent manner. Amongst these is RIP140 (33), which acts by recruiting HDACs and C-terminal binding protein (CtBP) (37). In GST pulldown assays, ZNF366 interacted with RIP140 1–415 and 753–1158 amino acids (Figure 5A), which encode repression domains RD1 and RD4 (37). This interaction appeared to require the C-terminal-most zinc fingers 8–11 of ZNF366 (Figure 5B).

Interestingly, ZNF366 also interacted with CtBP1 (Figure 5A), the interaction apparently requiring sequences C-terminal to the zinc fingers (Figure 5B). Many proteins interact with CtBP through sequence motifs having the consensus sequence PxDLS, with a lysine residue two amino acids C-terminal to the serine also often being present (41). Two such motifs, 590-PFDLS(QK)-596 and 645-PEDLS(TK)-651 (Figure 1) are located within the region of ZNF366 required for interaction with CtBP. Mutation of the CtBP motifs by substituting the proline (P) and aspartic acid (D) residues by alanines, prevented interaction between ZNF366 and CtBP in GST pulldown assays (Figure 5C) and in a mammalian two-hybrid assay (Figure 5D). Mutation of M1 or the M2 motif reduced the interaction between ZNF366 and CtBP1. In these assays, the N-terminal CtBP motif (M1) appeared to be more important than the C-terminal motif (M2).

In the trans-repression assay, mutation of the N-terminal-most CtBP binding motif in ZNF366 (M1) partially relieved the repression of LexA-VP16 (Figure 5E), whereas mutation of the second motif (M2) did not significantly relieve the repression and mutation of both motifs almost completely abolished the repression by ZNF366. These findings show that the interaction between ZNF366 and CtBP is important for the repression activity of ZNF366, the interaction being mediated by two CtBP-interaction motifs, with both motifs being required for the interaction with CtBP, although motif M1 may be more important than M2 for the interaction.

Whole cell lysates prepared from COS-1 cells transiently transfected with CtBP1 and FLAG-tagged ZNF366 immunoprecipitated using a CtBP antibody resulted in co-IP of FLAG-ZNF366 (Figure 5F). In the reciprocal experiment, CtBP1 was co-immunoprecipitated with FLAG-ZNF366 (Figure 5G). CtBP1 was not co-immunoprecipitated with ZNF366 in which the CtBP binding motifs were mutated. Collectively these data demonstrate that ZNF366 interacts *in vitro* and *in vivo* with CtBP1, the interaction being mediated by two CtBP binding motifs. In agreement with these findings substitution of the CtBP motifs in ZNF366 significantly reduced the repression of ERα activity by ZNF366 (Figure 6A).

Gene repression by transcriptional corepressors, including RIP140 and CtBP, frequently requires HDAC recruitment and histone deacetylation. The HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) relieved the repression of ERα activity by ZNF366 (Figure 6B), whilst GST pull-downs showed that ZNF366 interacts with HDACs 1, 3 and 6 (Figure 6C), indicating that transcriptional repression by

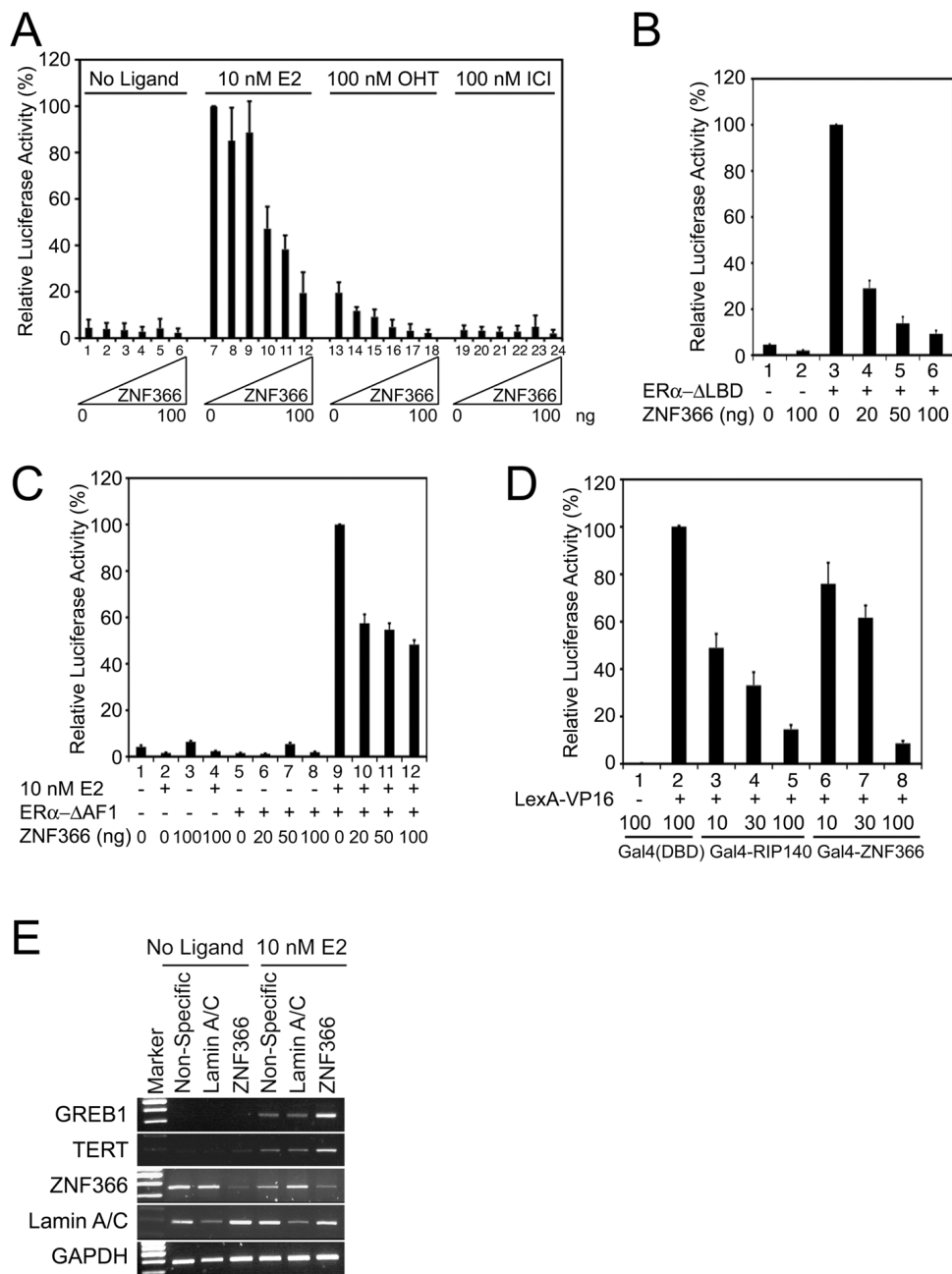


Figure 4. ZNF366 is a repressor of ERα activity. (A) COS-1 cells were transfected with ERE-3-TATA-luc (100 ng), ERα (100 ng), ZNF366 and the RLTK renilla luciferase reporter (100 ng). Results represent the mean of three independent experiments individually corrected for transfection efficiency against renilla luciferase activity. Error bars represent the standard error of the mean. The activity for ERα in the presence of E2 and in the absence of ZNF366 was taken as 100%. All other activities are shown relative to this. The amounts of ZNF366 transfected were 0 ng (lanes 1, 7, 13 and 19), 0.1 ng (lanes 2, 8, 14 and 20), 1 ng (lanes 3, 9, 15 and 21), 10 ng (lanes 4, 10, 16 and 22), 30 ng (lanes 5, 11, 17 and 23) or 100 ng (lanes 6, 12, 18 and 24). (B and C) Reporter gene assays were performed following transfection of 100 ng ERα-ΔLBD (B) or ERα-ΔAF1 (C), as for (A). (D) COS-1 cells were co-transfected with 100 ng Gal4 DBD, the Gal4 DBD fused to full-length RIP140 or ZNF366 together with LexA-VP16 and the Lex-Gal-luc reporter gene. Relative reporter gene activities from three independent experiments are shown. (E) Shown are RT-PCR carried out using PCR primers for GREB1, TERT, ZNF366, Lamin A/C and GAPDH, using total RNA prepared from PE04 cells transfected with non-targeting control, Lamin A/C, or ZNF366 siRNA.

ZNF366 is mediated, at least in part through histone deacetylation.

ZNF366 represses the expression of estrogen-responsive genes in breast cancer cells

The majority of breast cancers express ERα, and the growth of ERα-positive breast tumours is stimulated by estrogen,

as evidenced by the utility of anti-estrogens and inhibitors of estrogen biosynthesis in breast cancer treatment (2). The MCF7 breast cancer cell line expresses ERα and grows in response to estrogen, its growth being inhibited by anti-estrogens. Further, MCF7 cells demonstrate estrogen-stimulated expression of a number of well-characterized estrogen-responsive genes, including cathepsin D and pS2. In order to evaluate the effect of ZNF366 on ERα-regulation

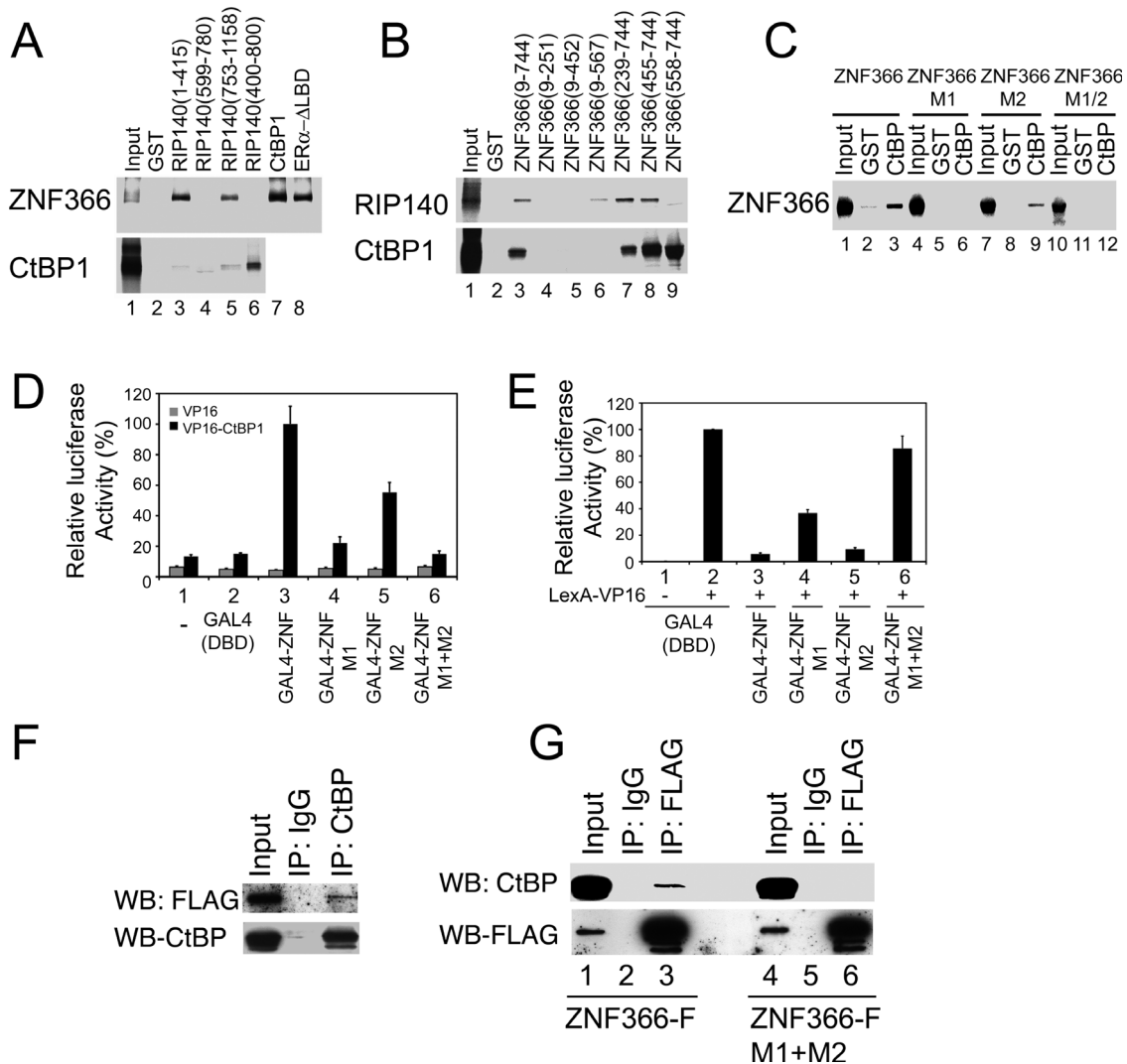


Figure 5. ZNF366 interacts with CtBP *in vitro* and *in vivo*. RIP140 fragments (A), ZNF366 fragments (B), CtBP1 (A and E) or ER α - Δ LBD (A) fused to GST, were immobilized on glutathione beads and incubated with 35 S-labelled ZNF366 (A and C), CtBP1 (A, B) or RIP140 (B). Bound proteins were eluted, resolved by 10% SDS-PAGE and exposed to autoradiography. (D) Mammalian 2-hybrid assay was performed by co-transfecting 100 ng Gal4 DBD or Gal4 fusions with ZNF366 558–744 amino acids, and 100 ng of VP16 or VP16 fused to CtBP. (E) COS-1 cells were co-transfected with LexA-VP16 and the Lex-Gal-luc reporter plasmid, together with the Gal4 DBD or Gal4 fused to 558–744 amino acids of ZNF366 in which the N-terminal (M1), the C-terminal (M2) or both (M1/M2) CtBP binding motifs have been mutated. Relative reporter gene activities from three independent experiments are shown. (F and G) Whole cell lysates prepared from COS-1 cells transiently transfected with CtBP1 and FLAG-tagged ZNF366 or FLAG-tagged ZNF366 mutated in the CtBP binding sites were immunoprecipitated using antibodies to CtBP1, followed by western blotting for CtBP1 and FLAG (F) or using the FLAG antibody followed by western blotting for FLAG and CtBP1 (G). In each case control immunoprecipitations were performed using mouse IgG.

of these genomically encoded estrogen-responsive genes, MCF7 cells were transfected with ZNF366. This resulted in a marked reduction in expression of both cathepsin D and pS2 (Figure 7A). MCF7 cell growth was also reduced following ZNF366 transfection (Figure 7B), whilst growth of an ER α -negative breast cancer cell line that is not estrogen-responsive, was not inhibited by ZNF366 (Figure 7C).

DISCUSSION

Zinc finger proteins constitute a very large family of transcriptional regulators and can be further subdivided into groupings based on the type of zinc finger present, as well

as by the presence of additional motifs elsewhere in the protein that mediate protein–protein interactions and transcriptional regulation. Sequence analysis of ZNF366 shows that it encodes a protein containing 11 Kruppel-type C2H2 zinc fingers, which is highly conserved in vertebrate evolution. However, ZNF366 does not belong to any of the major subfamilies of the Kruppel zinc finger family and shows most significant amino acid sequence similarity to one other Kruppel zinc finger protein, ZNF710, of unknown function, where the homology is restricted to the Kruppel zinc finger region. However, several other Kruppel-type zinc finger proteins act as transcriptional repressors, including ZNF217, a putative oncogene that is amplified and overexpressed in breast and other cancers (55,56), and

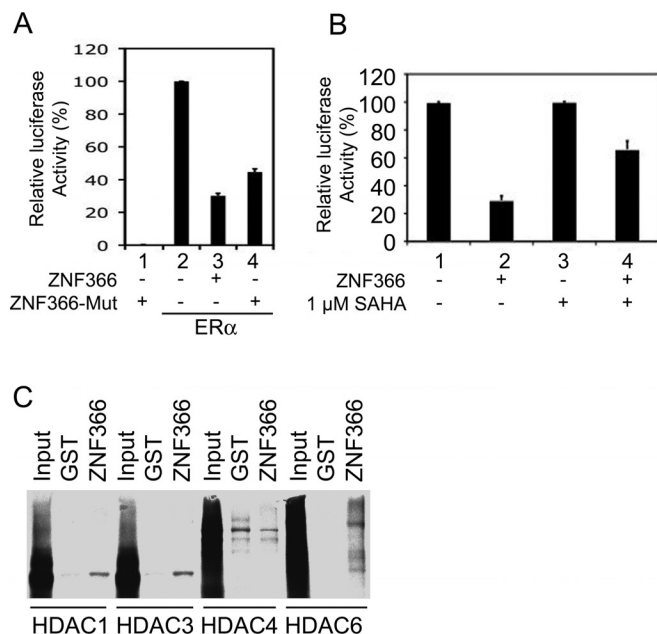


Figure 6. Repression of ER α activity by ZNF366 involves HDACs. (A) COS-1 cells were transfected with ERE-3-TATA-luc (100 ng), ER α (100 ng) and ZNF366 (10 ng) or ZNF366 in which the CtBP sites have been mutated (ZNF366-Mut). E2 (10 nM) was present throughout. Results represent the mean of three independent experiments. (B) SAHA and E2 were added 24 h following transfection with ER α and ZNF366 and cells processed as above. GST pull-downs were carried out by incubation of 35 S-labelled HDAC 1, 3, 4 or 6, with GST-ZNF366 fusion proteins. The input lanes represent 20% of the total volume of the *in vitro* translation reaction used in the binding assay.

copurifies with a CtBP corepressor complex (42). Further, the Bcl11 Kruppel zinc finger gene, which is translocated in B-cell chronic lymphocytic leukemias, acts as a corepressor for the COUP-TF NR (57).

Although we did not investigate the potential of ZNF366 to bind DNA, alter reporter gene activities in yeast or in mammalian cells in the absence of ER α (see Results and Supplementary Figure 1A). Further, ZNF366 did not bind to estrogen response elements in gelshift assays (Supplementary Figure 1B). Recruitment of ER α to the estrogen-responsive pS2 gene promoter was also not inhibited by ZNF366 expression in MCF7 cells (Supplementary Figure 1C). Collectively, these findings indicate that the repression of estrogen-responsive reporter genes by ZNF366 does not involve inhibition of DNA binding by ER α .

ZNF366 appears to be recruited to estrogen-responsive genes through interaction of the zinc finger region of ZNF366 with the zinc finger region (DBD) of ER α . Whilst the zinc finger region was required for ZNF366 interaction with ER α , the exact sequence requirements for the interaction with ER α were not established, although non-overlapping regions of ZNF366, encoding zinc fingers 1–7 or 8–11 were sufficient for the interaction. Whilst the interaction of ZNF366 and ER α did not require ligand for *in vitro* assays, the interaction was apparently better in the presence of estrogen. Further, *in vivo* assays demonstrated a requirement for estrogen or anti-estrogen binding for the interaction between ZNF366 and ER α . The *in vivo* requirement for ligand binding may be influenced by post-translational modifications. Additionally, steroid receptors, including ER α , are complexed in

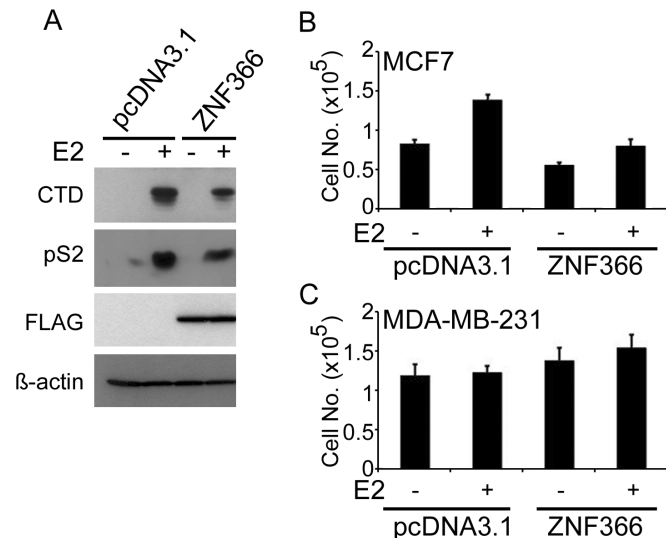


Figure 7. ZNF366 acts as a corepressor for endogenous estrogen-responsive genes. (A) MCF7 cells cultured in estrogen-free medium for 3 days were transfected with ZNF366 or control vector. E2 (10 nM) was added 24 h following transfection and lysates prepared after a further 24 h, were immunoblotted for cathepsin D (CTD), pS2, FLAG-ZNF366 and β -actin. (B and C) MCF7 and MDA-MB-231 cells were transfected as above and cell counts obtained 72 h after the addition of E2. The means of three experiments are shown, error bars representing the standard error of the mean.

the unliganded state, with the Hsp90 chaperone complex, required for appropriate folding of steroid receptors (58). The Hsp90-steroid receptor also likely interferes with steroid receptor interaction with some proteins. Ligand binding results in a conformational change in steroid receptors and Hsp90 dissociation. This could explain the estrogen and anti-estrogen regulation of ZNF366 recruitment by ER α .

In vitro, ZNF366 also interacted with other steroid receptors (ER β , androgen and glucocorticoid receptors), as well as the non-steroid retinoic acid, retinoid X and peroxisome proliferators-activated receptors (data not shown). In all cases the ZNF366 zinc finger region mediated the interaction, with no interaction being detected for the region C-terminal to the zinc finger region (558–744 amino acids), with the exception of retinoid X receptor- α (RXR α). In this case, ligand-stimulated interaction of RXR α with 558–744 amino acids was observed, indicating that the interaction between ZNF366 and RXR α is mechanistically distinct from the interaction of ZNF366 with other NRs, perhaps requiring the potential LXXLL motif located near the C-terminus of ZNF366.

ZNF366 inhibited ligand-dependent transactivation by ER α in a dose-dependent manner and functioned as a repressor when tethered to DNA by the GAL4 DBD. Its interaction with the ER α DBD suggests that the observed repression is not due simply to prevention of coactivator recruitment, nor does ZNF366 inhibit DNA binding by ER α (data not shown). Rather, ZNF366 appears to recruit multiple factors that act to repress transcription. Hence, the HDAC inhibitor SAHA partially relieved the repression of ER α by ZNF366 and *in vitro* binding assays showed that ZNF366 interacts with Class I HDACs 1 and 3, as well as the Class II HDAC6, indicating that the corepressor activity of ZNF366 is, at least in part, HDAC-dependent.

Several different corepressor complexes that are associated with NRs have been identified, most notably N-CoR–SMRT complexes that include HDACs (59). These are usually recruited to unliganded or antagonist-bound NRs, such as the tamoxifen-bound ER α (60). RIP140 is unusual in being a corepressor that is recruited by agonist-bound NRs. The repressive activity of RIP140 is achieved by the recruitment of class I HDACs and CtBP1 (35–39,61). CtBP1 and the related protein CtBP2 are potent corepressors that are present in protein complexes containing HDACs and histone lysine methyltransferases (42), and its corepressor activity is mediated through HDAC-dependent and—independent mechanisms, the HDAC-independent mechanisms likely involving PcG complexes (62). ZNF366 interacted with RIP140, the interaction requiring 455–558 amino acids of ZNF366, which encode zinc fingers 7–11. ZNF366 also interacted with CtBP1 *in vitro* and *in vivo*, the interaction being mediated by two PXDLS CtBP-interaction motifs located C-terminal to the zinc fingers in ZNF366. Mutation of the CtBP-interacting motifs prevented the interaction of ZNF366 and CtBP1 and relieved repression of ER α activity by ZNF366, confirming the importance of CtBP recruitment for the corepressor activity of ZNF366. However, the mutant ZNF366 still significantly repressed ER α activity, likely due to the fact that it directly interacts with HDACs and with RIP140.

These studies suggest that ZNF366 acts as a corepressor for ER α . In agreement with these findings, transfection of ZNF366 into the estrogen-responsive and ER α -positive MCF7 breast cancer cell line, which does not express ZNF366 (data not shown), reduced expression of genomically encoded ER α -regulated genes. Moreover, expression of ZNF366 inhibited MCF7 cell growth in response to estrogen, whereas ZNF366 expression did not inhibit growth of the ER α -negative, MDA-MB-231 breast cancer cell line that is not estrogen-responsive. Finally, RNAi-mediated down-regulation of ZNF366 in the ER α -positive PE04 ovarian cell line, stimulated expression of the estrogen-regulated GREB1 and TERT genes, further evidence for the *in vivo* role of ZNF366 as a corepressor for ER α .

In summary, we have identified a novel ER α -interacting protein ZNF366, which represses ligand-dependent ER α transactivation by recruitment of multiple factors, to regulate the expression of estrogen-responsive genes. ZNF366 is widely expressed in adult tissues and our preliminary findings of interaction between ZNF366 and many other NRs suggest that ZNF366 may have a widespread role as a NR corepressor, in addition to its action as an ER α corepressor, as defined in this study.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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ORIGINAL ARTICLE

The DEAD-box protein p72 regulates ER α -oestrogen-dependent transcription and cell growth, and is associated with improved survival in ER α -positive breast cancer

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The DEAD-box RNA helicases p68 (DDX5) and p72 (DDX17) have been shown to act as transcriptional co-activators for a diverse range of transcription factors, including oestrogen receptor- α (ER α). Here, we show that, although both proteins interact with and co-activate ER α in reporter gene assays, small interfering RNA-mediated knockdown of p72, but not p68, results in a significant inhibition of oestrogen-dependent transcription of endogenous ER α -responsive genes and oestrogen-dependent growth of MCF-7 and ZR75-1 breast cancer cells. Furthermore, immunohistochemical staining of ER α -positive primary breast cancers for p68 and p72 indicate that p72 expression is associated with an increased period of relapse-free and overall survival ($P=0.006$ and 0.016 , respectively), as well as being inversely associated with Her2 expression ($P=0.008$). Conversely, p68 shows no association with relapse-free period, or overall survival, but it is associated with an increased expression of Her2 ($P=0.001$), AIB-1 ($P<0.001$) and higher tumour grade ($P=0.044$). Our data thus highlight a crucial role for p72 in ER α co-activation and oestrogen-dependent cell growth and provide evidence in support of distinct but important roles for both p68 and p72 in regulating ER α activity in breast cancer.

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Keywords: p68 RNA helicase; p72 RNA helicase; oestrogen receptor- α ; gene regulation; breast cancer; tamoxifen

Introduction

The DEAD-box subfamily of RNA helicases, originally so named on the basis of the presence of a conserved motif having the sequence Asp-Glu-Ala-Asp, have been implicated in cellular processes involving the regulation of RNA structure, including pre-mRNA processing, RNA export, RNA degradation, ribosome assembly, translation and miRNA maturation (Linder *et al.*, 1989; Tanner and Linder, 2001; Fuller-Pace, 2006). The p68 RNA helicase (DDX5) and the closely related p72 RNA helicase (DDX17) are two key members of the DEAD-box RNA helicases that are essential for viability; p68 $-/-$ mice die *in utero* and p72 $-/-$ mice die shortly after birth, with mice for both knockouts exhibiting severe developmental defects (Fukuda *et al.*, 2007).

Recent findings have shown that p68 and p72 act as transcriptional co-regulators required for the action of diverse transcription factors. Evidence for this was first forthcoming through the demonstration that p68 associates with oestrogen receptor- α (ER α), with preferential interaction being observed for ER α phosphorylated at serine 118 (Endoh *et al.*, 1999). p72 has also been shown to be an ER α co-activator (Watanabe *et al.*, 2001). p68 also acts as a transcriptional co-activator of the ER α -related androgen receptor (Clark *et al.*, 2008), the tumour suppressor p53 (Bates *et al.*, 2005) and Runx2, a regulator of osteoblast differentiation (Jensen *et al.*, 2008). Both p68 and p72 have also been shown to co-activate MyoD, acting as regulators of muscle differentiation (Carette *et al.*, 2006). Although the mechanisms of p68 and p72 action as transcriptional co-activators remain to be defined, their interaction with other co-activators, including the histone acetyltransferases, CBP/p300 and P/CAF (Rossow and Janknecht, 2003; Shin and Janknecht, 2007), and the steroid receptor co-activator (SRC) family of nuclear receptor co-activators (Watanabe *et al.*, 2001), which themselves interact with CBP/p300 and P/CAF (Goodman and Smolik, 2000), is suggestive of roles for p68 and p72 in the regulation of histone modification by histone acetyltransferases. A potential role for the RNA

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binding/helicase activity of p68/p72 has been suggested by the finding that they co-immunoprecipitate with ER α , SRC and the RNA co-activator SRA (Watanabe *et al.*, 2001), although RNA helicase activity *per se* does not appear to be required for co-activation in reporter assays (Endoh *et al.*, 1999). Finally, p68 and p72 also have potential as co-repressors through association with histone deacetylase HDAC1 (Wilson *et al.*, 2004), the association being promoted by sumoylation of p68 (Jacobs *et al.*, 2007).

Expression of ER α is one of the key features of breast cancer, the most frequently observed cancer in women in industrialized nations, with ER α expression being found in approximately 70% of tumours. Its presence is both prognostic and predictive of response to endocrine therapies. Determination of ER status of tumours is therefore crucial in the management of ER-positive breast cancers by treatment with endocrine agents, such as tamoxifen, that inhibit ER α activity (Ali and Coombes, 2002), or aromatase inhibitors that reduce the levels of circulating estrogens. However, following an initial response to tamoxifen, a significant proportion of patients relapse. These tumours often remain ER α positive and may respond to an alternative endocrine agent, showing ER α -dependence for the continued growth of these tumours (Buzdar and Howell, 2001; Morris and Wakeling, 2002), and indicating altered ER α function as a possible mechanism underlying treatment failure.

Oestrogen receptor- α is a member of the nuclear receptor superfamily of ligand-activated transcription factors (Mangelsdorf *et al.*, 1995). Activation of gene expression by ER α is mediated by two transcription activation domains, AF1 and AF2, which act in a promoter- and cell-specific manner. AF1, located N-terminal to the DNA-binding domain (DBD), functions in a ligand-independent manner and its activity can be stimulated through growth factor-regulated signalling cascades in the absence of oestrogen (Bunone *et al.*, 1996). AF1 activity is enhanced by activating phosphorylation of serines 104, 106 and 118 through the mitogen-activated protein kinase (MAPK) pathway (Ali *et al.*, 1993; Le Goff *et al.*, 1994; Thomas *et al.*, 2008), and by cyclin-dependent protein kinases CDK2 (Ser-104/Ser106) (Trowbridge *et al.*, 1997) and CDK7 (Ser-118) (Chen *et al.*, 2000). In addition, these phosphorylation sites are substrates for GSK-3 β (Medunjanin *et al.*, 2005; Grisouard *et al.*, 2007). AF2 is integral to the ligand-binding domain and its activity requires oestrogen binding by the ligand-binding domain, which results in a conformational change facilitating co-regulator recruitment (Glass, 1994).

Transcriptional regulation by ER α requires the action of a plethora of transcriptional co-regulators at oestrogen-responsive gene promoters, which mediate chromatin remodelling (reviewed by Klinge, (2000)). Extensive chromatin immunoprecipitation analysis of model oestrogen-responsive genes, in particular the pS2 gene, has shown that these co-regulators are recruited and dissociated in an ordered, cyclical manner, where the promoter is initially activated to produce one round of transcribed mRNA, then is reset to an inactive state

before the next cycle of transcription begins (Shang *et al.*, 2000; Metivier *et al.*, 2003). In the cycles of co-regulator recruitment, p68 is one of the earliest ER α co-activators to be recruited, suggestive of a particularly important role for p68 in the regulation of oestrogen-responsive gene expression.

In this study, we have characterized further the importance of p68 and p72 in gene regulation by ER α , in particular using RNA interference-mediated down-regulation of p68 and p72 in the MCF-7 breast cancer cell line. Furthermore, we have investigated the expression of both proteins in a cohort of human breast cancers and correlated their expression with a number of prognostic markers. These studies have identified p72 as a key factor in the regulation of oestrogen-dependent cell growth and have identified associations with improved patient survival, indicating that it has an important role in breast cancer pathogenesis.

Results

p68 and p72 co-activate ER α in synergy with SRC-1

To examine p68/p72 co-activator function, we transfected COS-1 cells with an oestrogen-responsive firefly luciferase reporter plasmid and combinations of full-length ER α or mutants lacking either AF1 or AF2 (Figure 1a), together with p68, p72 and the well-characterized steroid receptor co-activator SRC-1. In agreement with previous reports (Endoh *et al.*, 1999; Watanabe *et al.*, 2001), ER α activity was stimulated by co-transfection with p68 and p72 (Figure 1b); this co-activation was synergistically enhanced by SRC-1. As has also previously been described (Endoh *et al.*, 1999), the RNA helicase activity of p68 is dispensable as co-transfection with a helicase-inactive p68 mutant did not prevent the stimulation of ER α activity by p68 (Figure 1c), nor was co-activation of ER α blocked by a similar mutation in p72.

p68 was originally identified as an ER α co-activator following *in vitro* purification of proteins that interacted preferentially with ER α phosphorylated at ser-118 by MAPK (Endoh *et al.*, 1999). In reporter assays using truncated ER α lacking the ligand-binding domain/AF2, p68 and p72 stimulated AF1 activity (Figure 1d). Although mutation of Ser-104 or Ser-106 did not prevent co-activation by p68 or by p72, mutation of Ser-118 gave little or no stimulation of ER α activity by p68 and p72, with mutation of all three residues completely preventing co-activation of AF1 by p68 and p72. Interestingly, however, both p68 and p72 stimulated the activity of an ER α deletion mutant lacking AF1 (Figure 1e), suggesting that co-activation of ER α by p68/p72 involves the ER α DBD and/or AF2, in addition to AF1. Again this co-activation was synergistically enhanced by SRC-1.

p68 and p72 interact with ER α in vitro and in vivo in an oestrogen-independent manner

Using glutathione *S*-transferase (GST)-pulldowns, an *in vitro* interaction between ER α and p68 was observed,

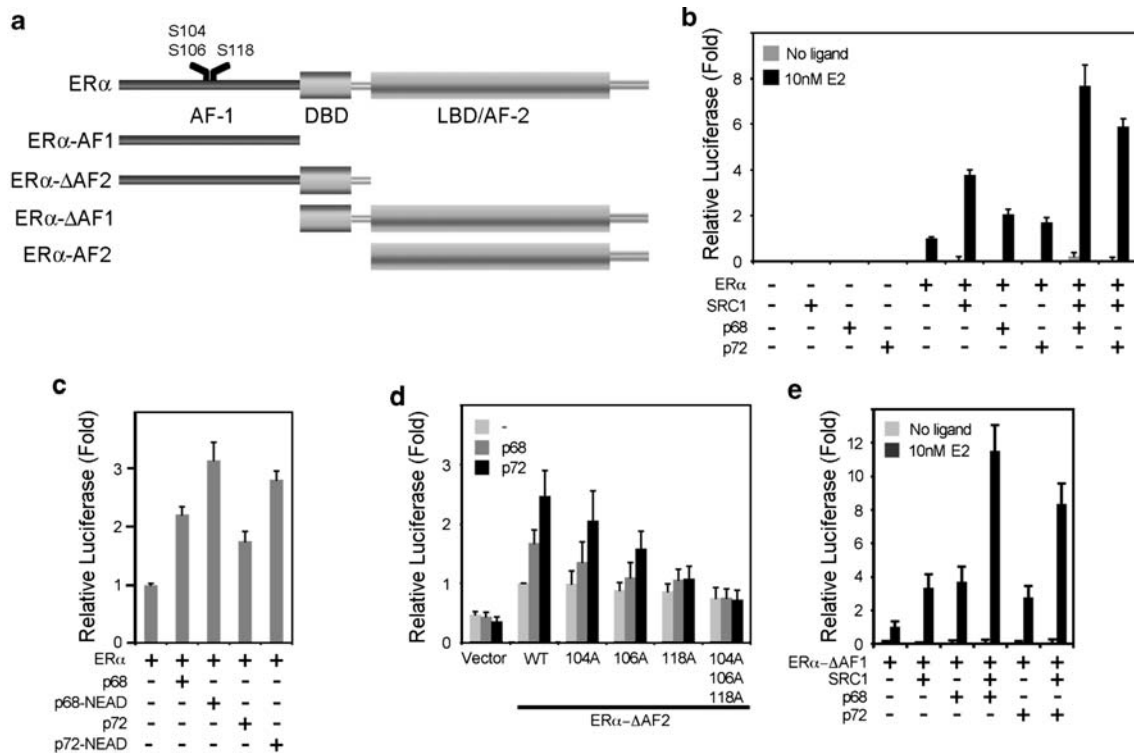


Figure 1 p68 and p72 co-activate oestrogen receptor- α (ER α) AF1 and AF2 in synergy with steroid receptor co-activator (SRC-1) but independently of RNA helicase activity. (a) A schematic representation of ER α , with positions of the functional domains, is shown. Positions of serines 104, 106 and 118 are highlighted. Also depicted are the ER α deletion constructs used in this study. (b) COS-1 cells were transfected with an oestrogen-responsive firefly luciferase reporter gene, the renilla luciferase control plasmid (RLTK), together with p68, p72 and SRC-1, as shown. Reporter gene activities were calculated relative to the renilla luciferase activity, to control for transfection efficiency, and are shown relative to the reporter gene activity for ER α in the presence of 10 nM 17 β -estradiol (E2). (c) COS-1 cells were transfected as above, with p68 and p72 mutants in which the DEAD-box motif had been mutated to a helicase-inactive 'NEAD' sequence. (d) Mutation of serines 104, 106 and 118 reduces co-activation of the ER α AF1 region by p68 and p72. (e) p68 and p72 co-activate the ER α LBD/AF2 in the presence of 10 nM E2. (b–d) The mean reporter activities of at least three independent experiments are shown, with the error bars representing the standard error of the mean (s.e.m.).

the interaction being oestrogen independent (Figure 2a). Interestingly, deletion mutants lacking AF1 or AF2 interacted with p68, although only weak interactions were observed for AF1 or AF2, indicative of interaction of p68 and p72 with ER α DBD. Similar results were obtained for p72, although interaction with the mutant lacking AF1 was weaker than that for p68, indicating that the interaction of p68 and p72 with ER α is similar, at least *in vitro*. The lack of interaction between ER α -AF1 and p68/p72 could be due to a requirement for AF1 phosphorylation. However, interaction of AF1-DBD (ER α - Δ AF2) and the AF1 region with p68 and p72 was unaffected by mutation of S118 or of S104/S106/S118 (Figure 2b). Nor was interaction between these ER α mutants and p68 or p72 stimulated by *in vitro* phosphorylation with ERK2 MAPK (Figure 2c). Taken together, these findings show that regions in addition to the AF1 are important for the interaction between ER α and p68/p72 and indicate that the ER α DBD is likely to be important for interaction of ER α with p68 and p72.

Co-immunoprecipitation of nuclear extracts prepared from MCF-7 cells treated with 10 nM oestrogen for 6 h showed that p68 and p72 co-immunoprecipitated with

ER α in an oestrogen-independent manner (Figure 2d). Furthermore, we found that although ER α co-immunoprecipitated both p68 and p72, p68 co-immunoprecipitated ER α efficiently, but only co-immunoprecipitated a very small amount of p72, implying that ER α -p68 and ER α -p72 complexes may be distinct within the cell.

RNAi-mediated knockdown of p68 and p72 indicates that p72 is required for the expression of oestrogen-regulated genes

To determine whether p68 and p72 are required for the expression of endogenous oestrogen-responsive genes, we knocked down the expression of these proteins by transfection of specific small interfering RNA (siRNA) molecules, which have previously been described (Bates *et al.*, 2005). Knockdown of p72 resulted in significantly reduced pS2 expression at both the mRNA and protein levels (Figures 3a and e). For cathepsin D, knockdown of p72 led to a significant decrease in protein expression, although there was only a small, but reproducible, decrease in mRNA levels (Figures 3b and e). Surprisingly, p68 knockdown did not affect pS2 or cathepsin D expression (Figures 3a,b and e). Knockdown of

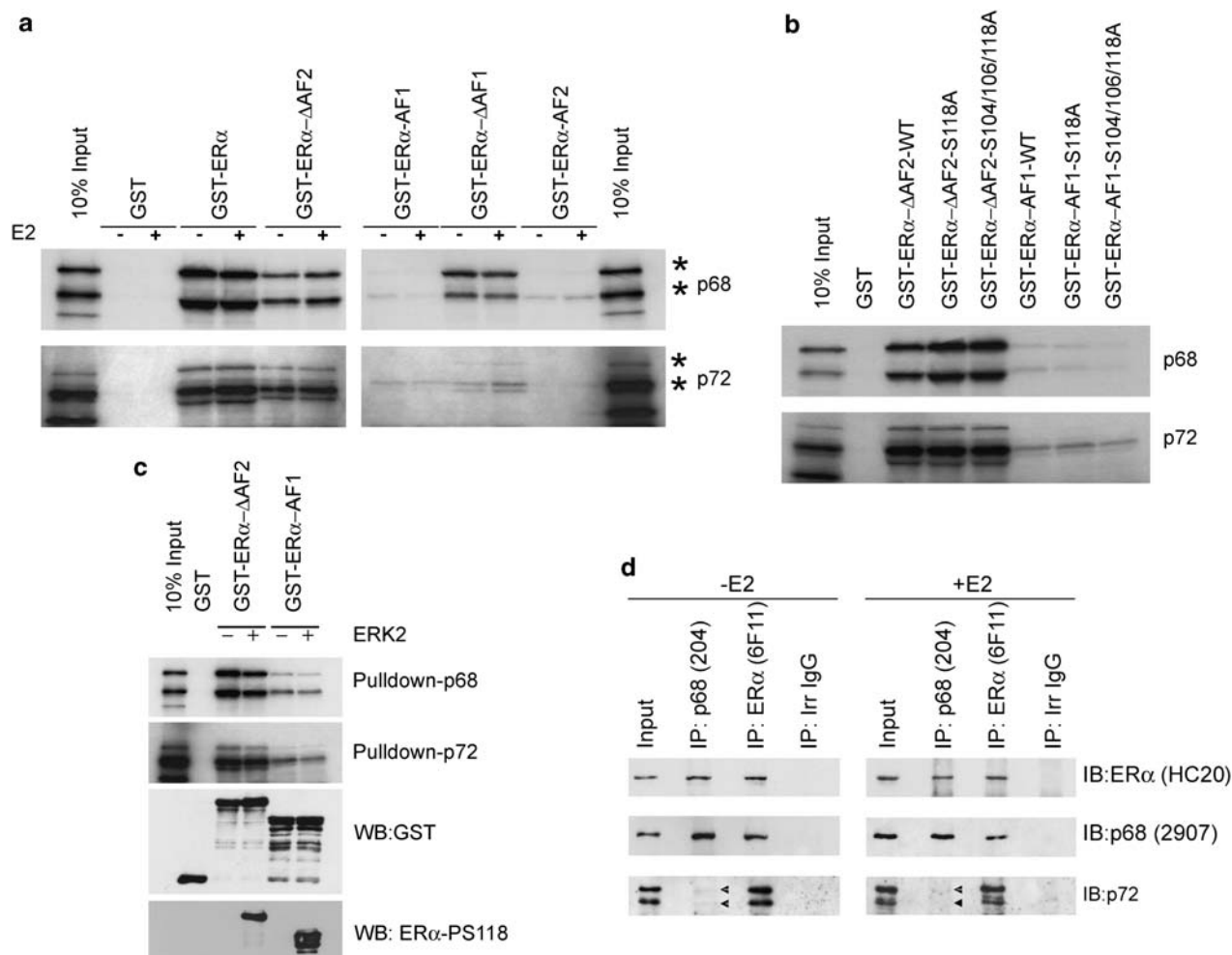


Figure 2 p68 and p72 interact with oestrogen receptor- α (ER α) in a ligand-independent manner *in vitro* and *in vivo*. (**a** and **b**) Glutathione *S*-transferases (GST)-pulldown assays were carried out using 35 S-labelled, *in vitro*-translated p68 and p72 and GST-fusions of full-length ER α or regions of ER α , as shown. Double bands are observed for p68 and p72 (marked *); these are the result of internal translation initiation. Full-length (upper band) and N-terminal truncated forms of p68 and p72 are indicated. (**c**) GST-ER α - Δ AF2 and GST-ER α -AF1 were phosphorylated *in vitro* with ERK2 following pulldown using glutathione beads. *In vitro*-transcribed/translated p68 or p72 were then added and pulldowns completed as above. Western blotting with an ER α -phospho-S118-specific antibody shows phosphorylation of the GST-tagged proteins. (**d**) Co-immunoprecipitation of p68 and p72 with ER α . Nuclear extracts were prepared from MCF-7 cells grown for 3 days in oestrogen-free medium, then treated with either 10 nM 17 β -estradiol (E2) or a vehicle control for 6 h. Lysates were immunoprecipitated with antibodies to either ER α (6F11) or p68 (PAb204) and analysed by western blotting for ER α (HC20), p68 (2907) and p72 (DRD K12). The doublet observed in the p72 western blot shows the p72 and p82 isoforms of p72, which have been reported previously (Uhlmann-Schiffler *et al.*, 2002; Ogilvie *et al.*, 2003).

p68 and p72 was confirmed by quantitative reverse transcription-PCR and western blotting (Figures 3c and d). Interestingly, we still observed oestrogen stimulation of pS2 and cathepsin D expression following p72 knockdown, although the overall level is markedly reduced, suggesting that p68 or other ER α co-regulators can compensate for p72, at least in part.

RNAi depletion of p72 inhibits the oestrogen-dependent growth of cells

As p72 appears to be important for stimulation of oestrogen-regulated gene expression, we determined the effect of p68 or p72 knockdown on oestrogen-stimulated growth of MCF-7 cells. Although knockdown of p68

had no significant effect on cell growth compared with a non-silencing siRNA (Figure 4a), knockdown of p72 (Figure 4b) significantly reduced cell growth. To test whether the effects of p72 were oestrogen-specific, we treated these cells with 4-hydroxytamoxifen alone or in combination with oestrogen. We found that tamoxifen treatment does not further slow the growth of the cells, suggesting that the inhibition of MCF-7 cell growth following p72 knockdown is due to the inhibition of ER α activity (Figure 4c). For all experiments, knockdown of p68 and p72 was confirmed by western blotting. Knockdown of p72 in ZR75-1 cells similarly inhibited oestrogen-stimulated growth, indicating that p72 is also required for ER function in these cells (Supplementary Figure 1).

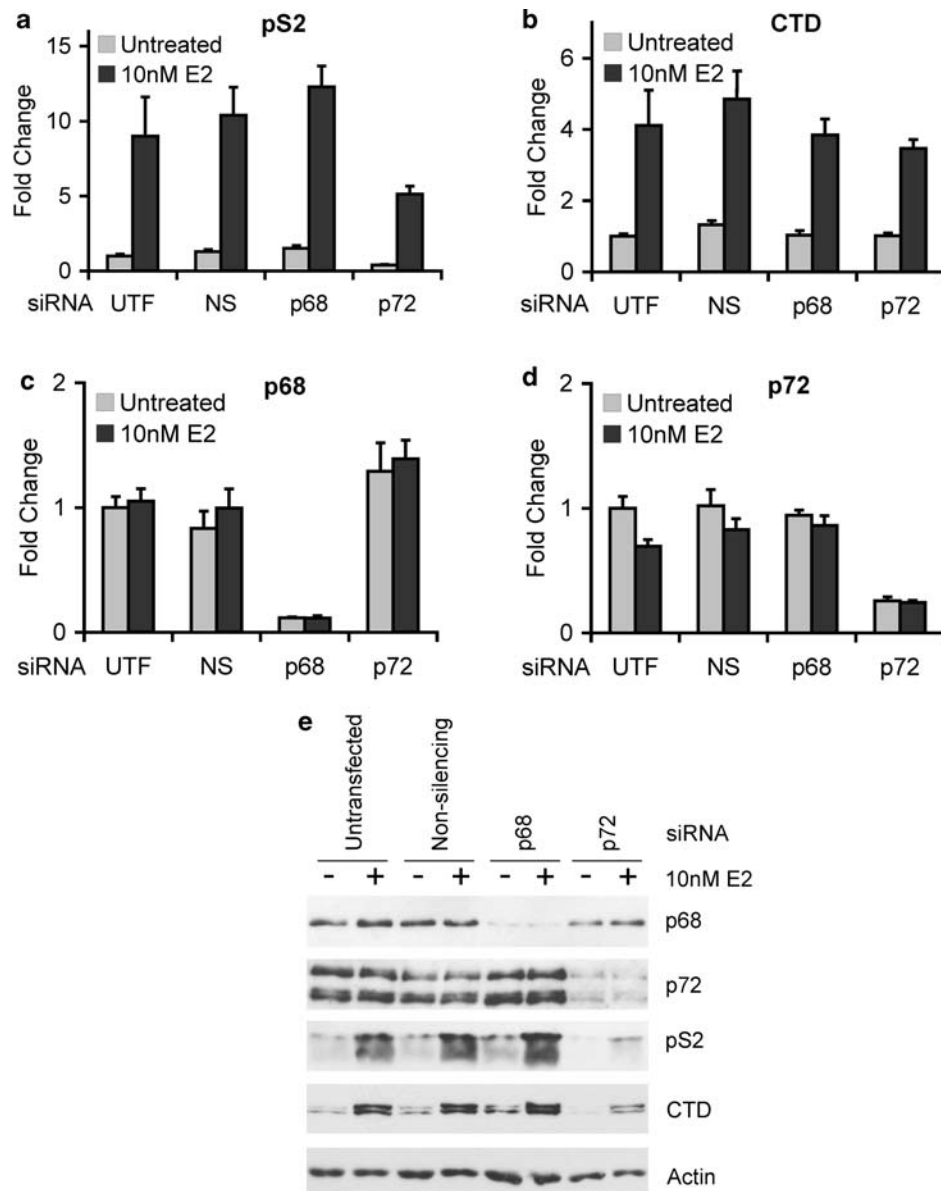


Figure 3 Short interfering RNA (siRNA)-mediated knockdown of p68 and p72 indicates that p72 is required for the expression of oestrogen-regulated genes. MCF-7 cells were grown in oestrogen-free medium for 72 h and then transfected using siRNAs against p68 or p72, or a non-silencing control (NS). After 56 h transfection, the cells were treated with 10 nM 17 β -estradiol (E2) or an equivalent volume of ethanol for 16 h and then harvested for RNA or protein. cDNA prepared from these cells was used to carry out Taqman quantitative reverse transcription-PCR to examine the expression of the endogenous oestrogen-responsive genes pS2 (**a**) and cathepsin D (**b**), and western blotting was used to examine the expression of these genes at the protein level, with actin as a loading control (**e**). All graphs (**a-d**) are the means of three independent experiments $\pm$ s.e.m.

p72 expression is associated with better prognosis in breast cancer

To assess their significance in breast cancer pathogenesis, immunohistochemical staining of 233 ER α -positive tumours was performed for p68 and p72 (Figure 5a). p68 and p72 staining data were obtained for 226 and 229 cases, respectively, of which 67 were p68 positive, and 173 were p72 positive. These tumours had previously been immunostained for ER α phosphorylated at S118, as well as a series of other markers including Her2, PR (Sarwar *et al.*, 2006) and AIB-1. We correlated the expression of p68, p72 and these markers with other

pathological and clinical data for each tumour, including histological grade, lymph node involvement, time to relapse and death (Table 1). There was no statistically significant association between p68 and p72 expression in this group of patients ($P=0.397$). p68 expression was associated with Her2 and AIB-1 positivity ($P=0.001$ and $P<0.001$, respectively), as well as with increasing tumour grade ($P=0.044$), all of which are markers of poor prognosis (Osborne *et al.*, 2003). However, no association was identified between p68 expression and either relapse-free or overall survival ($P=0.097$ and 0.200 respectively) in our patient cohort (Figures 5b

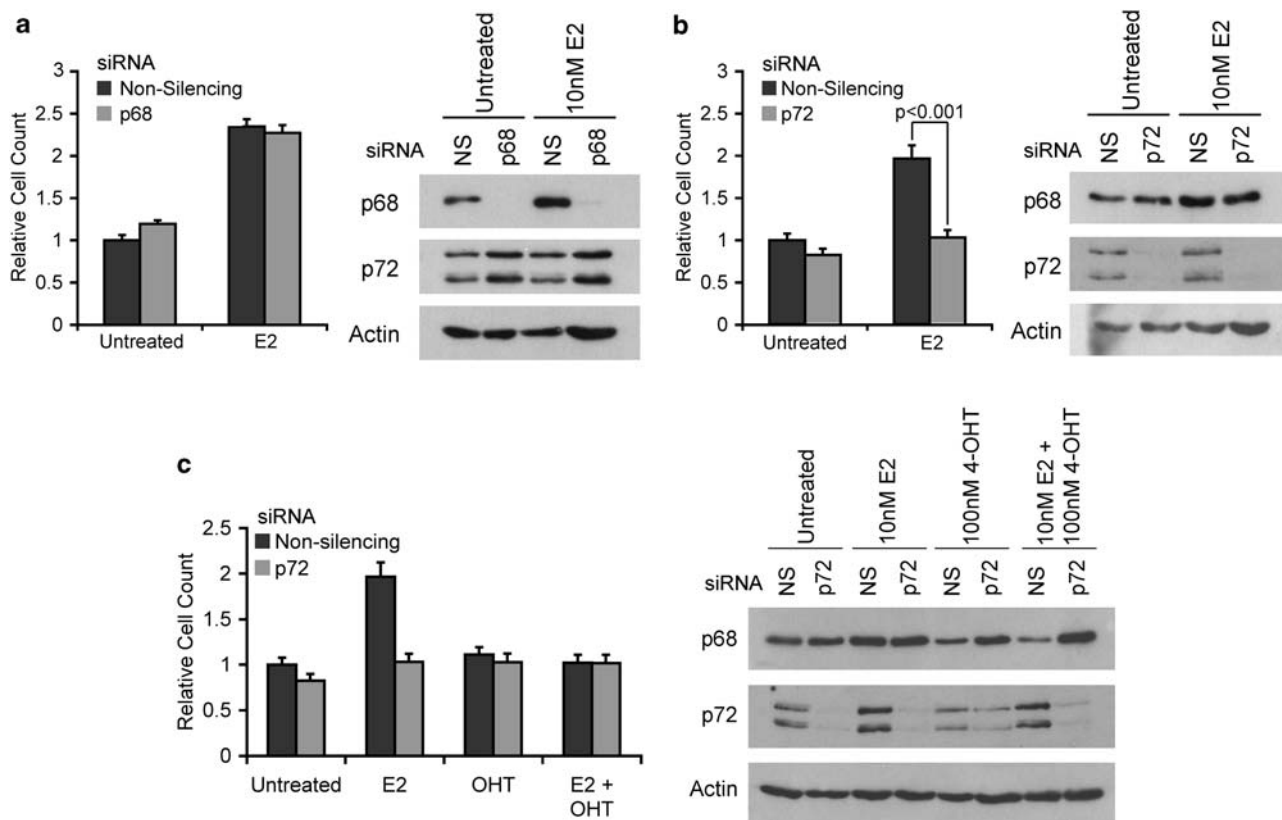


Figure 4 Small interfering RNA (siRNA)-mediated knockdown of p72, but not p68, results in a slowdown of oestrogen-dependent growth of breast cancer cells. MCF-7 cells were grown for 3 days in oestrogen-free medium, then transfected with siRNAs against p68 or p72, or a non-silencing control (NS), in the presence or absence of 10 nM 17 β -estradiol (E2) and/or 100 nM 4-hydroxytamoxifen (4-OHT). Cells were trypsinized and counted 4 days after transfection to assess cell growth. (a) Effect of p68 knockdown; (b) Effect of p72 knockdown; (c) Effect of p72 knockdown on growth in the presence of E2, 4-OHT or a combination of both ligands. Western blots showing knockdown of p68 and p72 at the time of counting accompany each graph. Cell counts are shown as relative to the untreated non-silencing control. Each count was carried out in triplicate within each experiment, and results are shown as the mean $\pm$ s.e.m. of at least three independent experiments. In all western blots, actin was used as a loading control.

and c). Conversely, p72 expression was associated with a favourable prognosis. Most strikingly, p72-positive tumours were associated with Her2 negativity ($P=0.008$), progesterone receptor positivity ($P=0.037$) and reduced likelihood of relapse ($P=0.025$) and cancer death ($P=0.014$). In agreement with this, Kaplan–Meier analysis showed that p72-positive tumours are associated with a longer relapse-free period and overall survival ($P=0.006$ and 0.016 respectively) (Figures 5d and e). However, multivariate analysis showed that p72 was not a significant predictor of relapse-free or overall survival (Table 2). Lymph node positivity, as well as Her2 and AIB1 expression were significant predictors of relapse-free survival ($P<0.001$, $P=0.003$ and 0.024 , respectively), although lymph node positivity, Her2 positivity and tumour grade predicted overall survival ($P=0.001$, 0.017 and 0.008 , respectively).

Dysregulation of ER α -mediated inhibition of Her2 expression is one mechanism through which breast tumours may become resistant to anti-oestrogen treatment. AIB-1 is thought to have a crucial role in this dysregulation by competing for ER α binding with Pax2 and reversing ER α -dependent repression of Her2 (Hurtado *et al.*, 2008). We examined the effects of expression of p72 in AIB-1-positive tumours and

its relation to Her2 expression. As has been previously described in a number of studies (Osborne *et al.*, 2003), AIB-1 positivity in the tumours was associated with poorer prognosis (Figures 6a and b). Although the expression of p72 had little effect on the prognosis of patients with AIB-1-negative tumours, p72 expression in AIB-1-positive tumours was associated with an increased period of relapse-free and overall survival (Figures 6c and d, $P=0.003$ and 0.043 , respectively). Furthermore, the proportion of Her2-positive tumours was significantly lower in AIB-1-positive tumours that were also positive for p72 ($P=0.005$, Figure 6e). Expression of p68 did not show any association with prognosis in AIB-1-positive tumours, although the proportion of Her2-positive tumours was significantly increased in AIB-1-positive tumours expressing p68 ($P=0.005$), nor did p72 expression influence the prognosis of Her2-positive tumours (Supplementary Figures 2 and 3).

Discussion

Recent studies have shown that the related p68 and p72 RNA helicases regulate gene expression by acting as

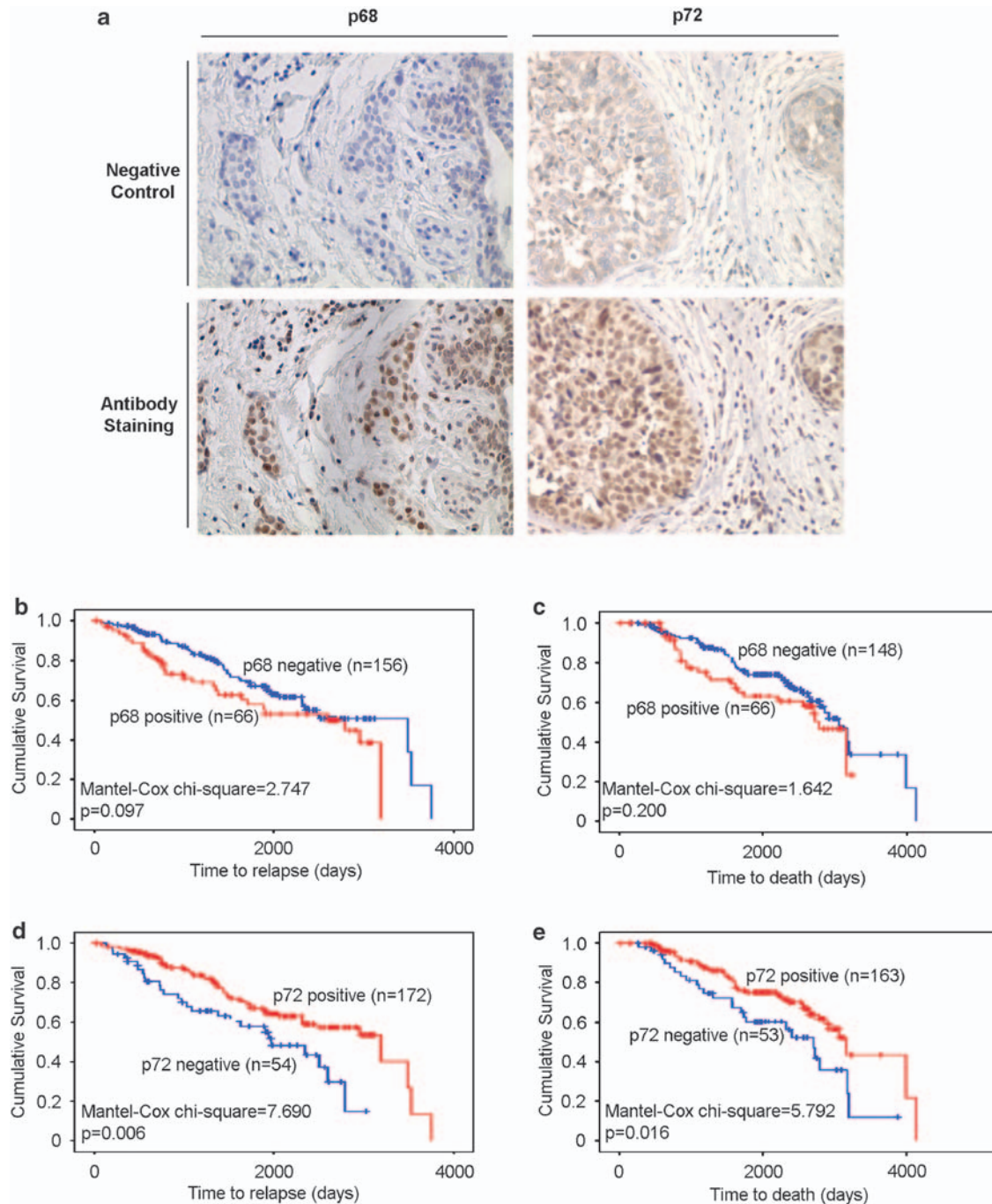


Figure 5 Immunohistochemical staining of breast cancer biopsies for p68 and p72 RNA helicases. (a) Representative examples of p68 and p72 negative and positive human breast cancer sections (magnification: $\times 20$) are shown. Kaplan-Meier survival curves showed no association of p68 with disease-free (b) or overall (c) survival, whereas p72 positivity was associated with increased disease-free (d) and overall (e) survival in breast cancer patients.

transcriptional co-regulators for diverse transcription factors (Fuller-Pace, 2006). The first evidence for a role in gene regulation for p68 and p72 was reported for ER α , where p68 was identified on the basis of a preferential association with ER α phosphorylated at S118 in the transcription activation function AF1 (Endoh *et al.*, 1999). Subsequently, p72 was also shown to co-activate ER α (Watanabe *et al.*, 2001). In this study, we have undertaken a study of the importance

of p68 and p72 in oestrogen-regulated gene expression in the ER α -positive and oestrogen-dependent MCF-7 cells. Our findings indicate that, although both proteins can act as ER α co-activators, p72 appears to have a more critical role in oestrogen signalling than p68.

In agreement with previous reports, both p68 and p72 act as ER α co-activators in a reporter gene assay, in a manner that is independent of p68 and p72 ATPase and

Table 1 Relationships between p68 and p72 expression and clinical features

	<i>p68 Negative</i> (%) ^{a,b}	<i>p68 Positive</i> (%) ^{b,c}	χ^2 -test ^d	P-value ^e	<i>p72 Negative</i> (%) ^{a,b}	<i>p72 Positive</i> (%) ^{b,c}	χ^2 -test ^d	P-value ^e
<i>p68 status</i>								
Negative	—	—	—	—	42 (75)	116 (69)	0.716	0.397
Positive	—	—			14 (25)	52 (31)		
ND	—	—			0	5		
<i>p72 status</i>								
Negative	42 (27)	14 (21)	0.716	0.397	—	—	—	—
Positive	116 (73)	52 (79)			—	—		
ND	1	1			—	—		
<i>Relapse</i>								
No	101 (65)	37 (56)	1.487	0.223	27 (50)	115 (67)	5.003	0.025
Yes	55 (35)	29 (44)			27 (50)	57 (33)		
Unknown	3	1			2	1		
<i>Death</i>								
No	101 (68)	40 (61)	1.185	0.276	28 (53)	116 (71)	6.051	0.014
Yes	47 (32)	26 (39)			25 (47)	47 (29)		
Unknown	11	1			3	10		
<i>AIB-1</i>								
Negative	95 (60)	20 (30)	17.259	<0.001	31 (55)	84 (50)	0.482	0.487
Positive	63 (40)	47 (70)			25 (45)	84 (50)		
ND	1	0			0	5		
<i>Her2</i>								
Negative	135 (86)	44 (66)	12.073	0.001	38 (68)	144 (84)	7.098	0.008
Positive	22 (14)	23 (34)			18 (32)	27 (16)		
ND	2	0			0	2		
<i>Lymph node</i>								
Negative	49 (37)	21 (36)	0.025	0.875	13 (28)	60 (41)	2.835	0.092
Positive	82 (63)	37 (64)			34 (72)	85 (59)		
Unknown	28	9			9	28		
<i>Grade</i>								
I	27 (18)	6 (9)			7 (13)	28 (17)		
II	99 (64)	39 (59)	6.251	0.044	31 (57)	109 (64)	2.574	0.276
III	28 (18)	21 (22)			16 (30)	33 (19)		
Unknown	5	1			2	3		
<i>PR</i>								
Negative	47 (30)	20 (30)	0.002	0.965	22 (39)	43 (25)	4.334	0.037
Positive	112 (70)	47 (70)			34 (61)	130 (75)		

Abbreviation: ND, not determined.

^aData for p68 were available from 226 to 233 tumours, data for p72 were available from 229 to 233 tumours.

^bp68 or p72-positive score, > 10% nuclei positive.

^cPercentage of cases in each category.

^dPearson's χ^2 -test. The analysis was carried out using known samples only.

^eA value of $P < 0.05$ denotes statistical significance.

Table 2 Odds ratios for significant predictive factors for (i) period of relapse-free survival and (ii) period of overall survival

<i>Factor</i>	<i>Odds ratio</i>	<i>95% Confidence intervals</i>	<i>P-value</i>
<i>(i) Period of disease-free survival</i>			
Lymph node metastasis	6.109	2.628–14.202	<0.001
Her2 expression	2.219	1.311–3.756	0.003
AIB-1 expression	1.805	1.079–3.019	0.024
<i>(ii) Period of overall survival</i>			
Lymph node metastasis	4.391	1.877–10.271	0.001
Tumour grade	1.957	1.188–3.226	0.008
Her2 expression	1.967	1.130–3.427	0.017

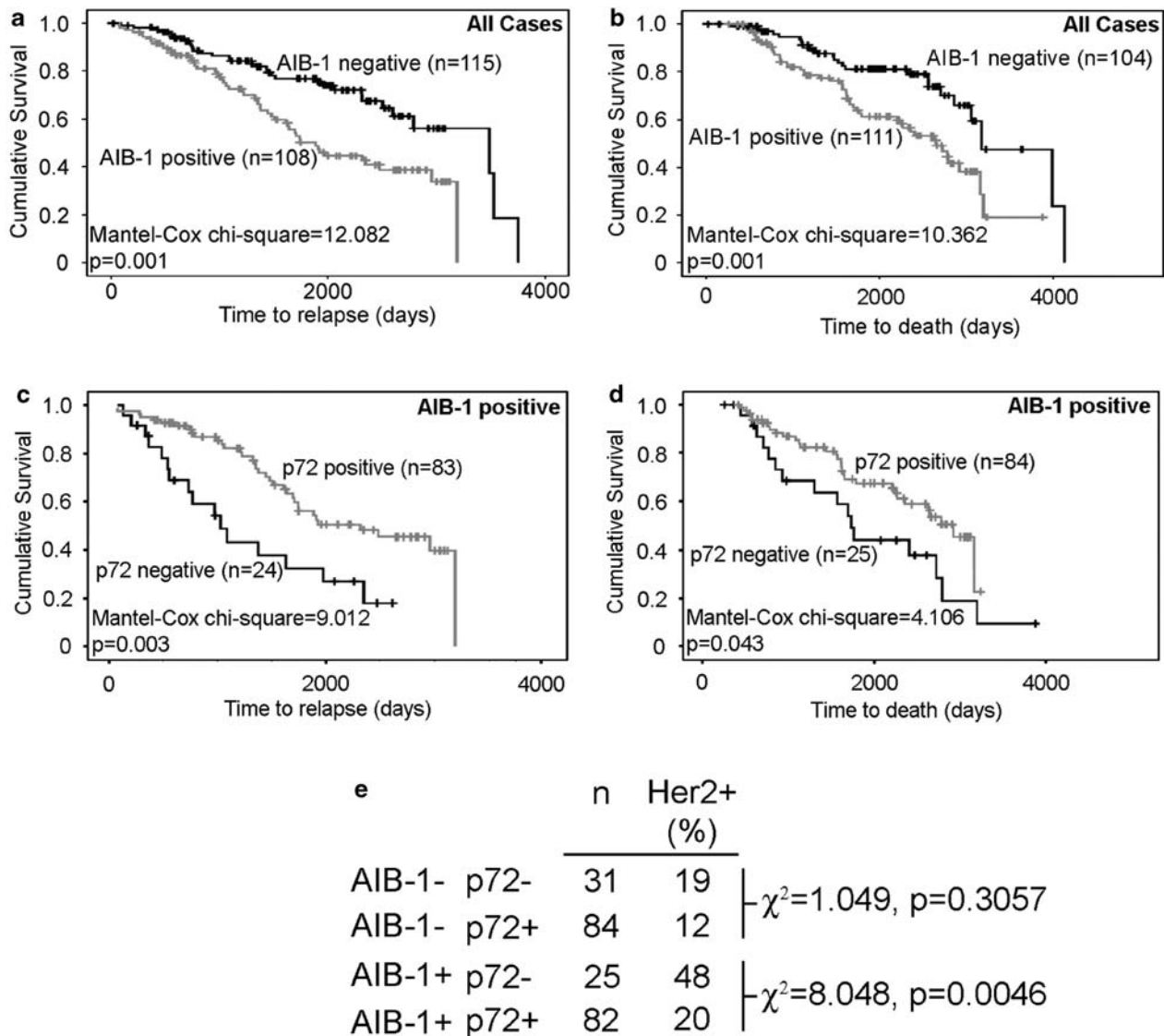


Figure 6 Expression of p72 in AIB-1-positive tumours is associated with Her2 negativity and improved patient prognosis. AIB-1 expression is associated with shorter relapse-free (a) and overall survival (b) in our patient cohort. However, in AIB-1-positive patients, p72 positivity is associated with a longer relapse-free period (c) and overall survival (d). The proportion of Her2-positive tumours is significantly less in AIB-1-positive tumours expressing p72 than in those that were p72 negative (e).

helicase activity. This implies that the N- and C-terminal regions of these proteins are important for co-activation and is consistent with our previous work, which also showed that these regions are critical for transcriptional regulation (Wilson *et al.*, 2004). Furthermore, although ser-118 is important for co-activation by p68 and p72 when assessing ER α lacking the ligand-binding domain/AF2, another region, most likely the ER α DBD, is also important for interaction with p68/p72.

Surprisingly, siRNA-mediated knockdown of p68 and p72 showed that although p72 is important for the regulation of oestrogen-responsive genes and for the oestrogen-stimulated growth of cells, p68 does not appear to be required. In contrast, immunohistochemical staining showed that p68 positivity is associated with markers of poor prognosis in ER α -positive breast cancer, suggesting that p68 and p72 may in fact have

distinct roles in the regulation of ER α activity in breast cancer cells.

Investigation of the expression of p68 and p72 in a panel of breast cancers revealed that increased p72 expression is associated with a favourable prognosis. Patients whose cancers were p72 positive had a significantly increased period of disease-free and overall survival. However, p72 was not itself an independent predictor of patient outcome. The implication from our data is that p72 is required for oestrogen-dependent cell growth. Therefore, tumours expressing p72 are likely to be oestrogen sensitive, and so treatment with endocrine agents may be more effective in these patients. As the tumours in our cohort were all ER α positive, we were unable to ascertain whether a relationship between ER α and p72 expression exists. However, meta-analysis of publicly available data sets in Oncomine (Rhodes *et al.*,

2007) showed a positive association between ER α and p72 mRNA in 10 independent studies (Supplementary Table 1), which supports the hypothesis that p72 is required for oestrogen signalling.

A significantly greater proportion of p72-negative tumours were positive for Her2, which is associated with poor response to endocrine treatment in ER α -positive tumours (Osborne *et al.*, 2003). Interestingly, AIB-1, another marker of endocrine resistance and poor prognosis (Osborne *et al.*, 2003), did not correlate with p72 expression. Overexpression of AIB-1, usually through amplification of 20q13 (Anzick *et al.*, 1997), is thought to override ER α -mediated repression of Her2 by competing out Pax2 (Hurtado *et al.*, 2008). As would be expected, AIB-1 and Her2 expression are strongly correlated in our data set ($P=0.002$, data not shown). However, we found that in AIB-1-positive tumours the presence of p72 is associated with lower Her2 positivity compared with the p72-negative AIB1-positive tumours, which is suggestive of a role for p72 in ER α -mediated repression of Her2 expression. The role of p68 in breast cancer is less clear. Although p68 positivity appears to have no overall effect on survival, there is a strong association between the presence of p68 and markers of poor prognosis, in particular Her2 and AIB-1.

In summary, our data provide evidence for distinct roles for p68 and p72 in regulating ER α activity. Furthermore, we have shown that p72 is important for transcriptional regulation by ER α and oestrogen-dependent cell growth in breast cancer cells. Finally, immunohistochemical staining indicates that p72 is a marker of good prognosis in breast cancer.

Materials and methods

Cell maintenance

MCF-7, ZR75-1 and COS-1 cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μ g/ml streptomycin and 100 U/ml penicillin (all supplied by Invitrogen, Paisley, UK) in 5% CO₂ at 37 °C. Before transfection, cells were placed for 72 h in Dulbecco's modified Eagle's medium without phenol red (Invitrogen) supplemented with 5% double dextran-coated charcoal-stripped fetal calf serum (First Link, Birmingham, UK), 2 mM glutamine, 100 μ g/ml streptomycin and 100 U/ml penicillin.

Plasmids

Plasmids expressing full-length and deletion mutants of ER α have previously been described (Tora *et al.*, 1989), as have the GST-ER α fusion proteins and the oestrogen-responsive luciferase reporter gene (Lopez-Garcia *et al.*, 2006). p68, p72 and the DEAD-box mutants have also previously been described (Bates *et al.*, 2005). The SRC-1 expression plasmid was a kind gift of Professor M Parker. pERE3-TATA-luc has also previously been described (Thomas *et al.* 2008). A thymidine kinase promoter Renilla luciferase reporter plasmid (RLTK; Promega, Madison, WI, USA) was used to control for transfection efficiency.

siRNA transfection

Small interfering RNA transfections were carried out using Lipofectamine RNAiMax (Invitrogen) by reverse transfection.

siRNA oligos against p68, p72 and a non-silencing control have been previously described (Bates *et al.*, 2005). 17 β -estradiol (Sigma-Aldrich, Poole, UK) was added to a final concentration of 10 nM, 56 h following transfection, and cells were harvested after a further 16 h for RNA or protein preparation. An equal volume of ethanol (vehicle) was added to the 'no ligand' controls.

Reporter gene assays

COS-1 cells were transiently transfected with ER α or ER α deletion constructs, together with p68, p72, SRC1, together with pERE3-TAT-luc and pRL-TK reporter genes, as previously described (Thomas *et al.*, 2008). Luciferase activities were determined using Dual-Glo reagents (Promega). Firefly luciferase levels were corrected for transfection efficiency using corresponding renilla luciferase levels. All experiments were independently repeated at least three times, and the data graphed as mean values, with error bars representing the s.e.s of the mean.

GST-pulldown assay

Glutathione *S*-transferases-tagged proteins were expressed in Rosetta BL21 *Escherichia coli* cells and purified on glutathione beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as previously described (Bates *et al.*, 2005). p68 and p72 were translated and ³⁵S-labelled using the TNT *in vitro* transcription/translation kit (Promega). The GST pull-down of *in vitro*-translated protein was carried out as described by Hsieh *et al.* (1999). Where GST-tagged proteins were phosphorylated, the following modifications were made to the protocol. Following binding of GST lysates, the beads were washed and resuspended in 2.5 bed volumes of 1 $\times$ MAPK buffer (New England Biolabs, Ipswich, MA, USA), containing 200 μ M adenosine triphosphate. A volume of 0.5 μ l per 30 μ l of buffer of activated ERK2 (New England Biolabs) was added to the beads, which were then incubated at 30 °C for 30 min. Following this, the beads were washed and the protocol proceeded with incubation with ³⁵S-labelled proteins as previously described (Hsieh *et al.*, 1999).

Nuclear extract preparation and co-immunoprecipitation

Nuclear extracts were prepared as described previously (Bates *et al.*, 2005) from MCF-7 cells grown in oestrogen-depleted media (Dulbecco's modified Eagle's medium containing 5% dextran-coated charcoal-stripped fetal calf serum) for 72 h with the addition of 10 nM 17 β -estradiol or an equivalent volume of ethanol for 6 h, before extract preparation. Immunoprecipitations were carried out using antibodies against ER α (6F11; Novocastra, Newcastle-upon-Tyne, UK) and p68 (PAb204; Millipore, Temecula, CA, USA) as described previously (Bates *et al.*, 2005).

Western blot analysis

Western blot analysis was carried out as described previously (Bates *et al.*, 2005) using antibodies against p68 (PAb204 and 2907 (rabbit polyclonal raised against the C-terminal 15 amino acids of p68)); p72 (DRD-K12 (rabbit polyclonal raised against amino acids 12–26 of p72)); ER α (HC20; Santa Cruz Biotechnologies, Santa Cruz, CA, USA); ER α -phospho-S118 (Cell Signalling Technology, Danvers, MA, USA); pS2 (FL-84, Santa Cruz Biotechnologies); cathepsin D (D-19; Abcam, Cambridge, UK); and actin (A2066; Sigma-Aldrich). Appropriate horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies for immunoblotting were purchased from DAKO (Ely, UK).

Cell growth assays

Cells were transfected in six-well plates with siRNA, as described above, except with the addition of 10 nM 17 β -estradiol (E2), 100 nM 4-hydroxytamoxifen (Sigma-Aldrich), a combination of both ligands, or an equivalent volume of ethanol at the time of transfection. Transfections were carried out in triplicate. After 96 h transfection, cells were trypsinized and counted using a haemocytometer. Following counting, cells were washed twice in phosphate-buffered saline and lysed in RIPA buffer. Lysates were sonicated and cleared by centrifugation at 14000 r.p.m. for 15 min. Protein concentrations were measured by Bradford assay (Sigma-Aldrich), equilibrated and analysed by western blotting.

Quantitative reverse transcription-PCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's protocol. A quantity of 1 μ g of RNA was treated with RQ1 RNase-free DNase (Promega) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Quantitative (Taqman) reverse transcription-PCR was carried out using the Stratagene MX3005-P instrument (Agilent, Santa Clara, CA, USA). Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) were purchased for each gene (Supplementary Table 2). β -Actin was used as a control and fold changes and differences between samples were calculated using the $\Delta\Delta C_t$ method in Microsoft Excel.

Immunohistochemistry

Two hundred and thirty three patients with primary invasive breast cancer, who had undergone surgery at Charing Cross Hospital between 1981 and 2003, were selected on the basis of the availability of clinical details at presentation and follow-up, including time to relapse, time to death, ER and PR status (Sarwar *et al.*, 2006). ER and PR status was confirmed using ER (VP-E613; Vector Laboratories, Peterborough, UK), PR

(MU328-UC; Biogenex, San Ramon, CA, USA) antibodies, and Her2 status was determined, as described (Jiang *et al.*, 2007). The clinico-pathological characteristics of the patient cohort are shown in Table 1. Immunohistochemical staining and scoring was carried out as previously described (Sarwar *et al.*, 2006). Immunostaining and scoring for AIB1 was carried out as described (Hurtado *et al.*, 2008). Staining for p68 and p72 was carried out using the antibodies PAb204 (Stevenson *et al.*, 1998) and DRD-K12, respectively. p68 and p72 were scored positive, if greater than 10% of tumour cell nuclei were stained. This study fulfilled the Institutional Ethics Review Board's guidelines for the use of stored tissue samples.

Statistical analyses

All statistical analyses were carried out using SPSS v14.0. Comparisons of cell counts were carried out by *t*-test. Immunohistochemical scores for p68 and p72 were compared with clinico-pathological features using the Pearson χ^2 -test. Survival analyses were carried out using the Kaplan-Meier survival function and the Mantel-Cox log-rank test. Multivariate analysis was carried out using the Cox Proportional Hazards model.

Conflict of interest

The authors declare no conflict of interest.

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The Development of a Selective Cyclin-Dependent Kinase Inhibitor That Shows Antitumor Activity

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Abstract

Normal progression through the cell cycle requires the sequential action of cyclin-dependent kinases CDK1, CDK2, CDK4, and CDK6. Direct or indirect deregulation of CDK activity is a feature of almost all cancers and has led to the development of CDK inhibitors as anticancer agents. The CDK-activating kinase (CAK) plays a critical role in regulating cell cycle by mediating the activating phosphorylation of CDK1, CDK2, CDK4, and CDK6. As such, CDK7, which also regulates transcription as part of the TFIIF basal transcription factor, is an attractive target for the development of anticancer drugs. Computer modeling of the CDK7 structure was used to design potential potent CDK7 inhibitors. Here, we show that a pyrazolo[1,5-*a*]pyrimidine-derived compound, BS-181, inhibited CAK activity with an IC_{50} of 21 nmol/L. Testing of other CDKs as well as another 69 kinases showed that BS-181 only inhibited CDK2 at concentrations lower than 1 μ mol/L, with CDK2 being inhibited 35-fold less potently (IC_{50} 880 nmol/L) than CDK7. In MCF-7 cells, BS-181 inhibited the phosphorylation of CDK7 substrates, promoted cell cycle arrest and apoptosis to inhibit the growth of cancer cell lines, and showed antitumor effects *in vivo*. The drug was stable *in vivo* with a plasma elimination half-life in mice of 405 minutes after i.p. administration of 10 mg/kg. The same dose of drug inhibited the growth of MCF-7 human xenografts in nude mice. BS-181 therefore provides the first example of a potent and selective CDK7 inhibitor with potential as an anticancer agent. [Cancer Res 2009;69(15):6208–15]

Introduction

Cyclin-dependent kinases (CDK) control cell proliferation by regulating entry into and passage through the cell cycle, initiation of DNA synthesis (S phase), and mitosis (M phase; ref. 1). CDKs are the catalytic subunits of a large family of serine/threonine protein kinases. Activation of specific CDKs is required for the appropriate progression through the cell cycle and into the next stage in the cell cycle. Hence, regulation of CDK activity is pivotal for the correct timing of cell cycle progression and CDK activity is tightly regulated at many levels, including complex formation with cyclins

and CDK inhibitors (CDKI) and by phosphorylation and dephosphorylation. Central to the activation of a given CDK is the requirement for association with specific cyclins and phosphorylation at a threonine residue in the activation loop (T-loop; ref. 2). In metazoans, phosphorylation of the CDKs that are required for cell cycle progression (CDK1, CDK2, CDK4, CDK6) is mediated by the CDK-activating kinase (CAK) having three subunits, CDK7, cyclin H (CycH), and MAT1 (3–5).

Deregulation of CDK activity forms an important part of many cancers, as well as other disease states, generally through elevated and/or inappropriate activation, as CDKs are infrequently mutated. Important mechanisms of CDK deregulation include cyclin overexpression, e.g., cyclin D1 (6), and loss of CDKI expression through mutational or epigenetic alterations (for review, see ref. 7). As such, CDKs are important targets for the design of anticancer drugs. Inhibitors of some CDKs, particular emphasis being placed on inhibitors of CDK2 as it controls S-phase entry, have been developed and a few have been tested in the clinical setting as anticancer agents (8–10). One of these is flavopiridol, which has modest selectivity for CDKs over other kinases and inhibits many members of the CDK family (11). The compound class that has yielded many CDK-selective ATP antagonists is 2,6,9-trisubstituted purines, exemplified by roscovitine, which shows good biological and pharmacologic properties (12, 13). CDK7 is an attractive target for drug development due to its critical role in the activation of the CDKs required for cell cycle progression (3, 5). This is especially significant as there is evidence that inhibition of some cell cycle CDKs may be compensated for by other CDKs. Hence, cells from mice that have been ablated for CDK2 are able to cycle, and CDK2^{-/-} mice are viable (14, 15). Similarly, CDK4^{-/-} and CDK6^{-/-} mice are viable, although the double-null mice show late embryonic lethality. However, mice lacking MAT1 die early in embryogenesis (16), indicative of a cellular requirement for CAK. Most described CDK inhibitors that potently inhibit CDK2 also inhibit CDK7, albeit at considerably higher concentrations than the concentrations required for CDK2 inhibition (13, 17). These compounds generally also inhibit other CDKs such as CDK5 and CDK9, which play important roles in neuronal development and transcription (15, 17–19). In addition to its role in cell cycle regulation, CDK7/CycH/MAT1 are components of the general transcription factor TFIIF (20, 21), required for initiation of transcription of RNA polymerase II (PolII)-directed genes. As part of the TFIIF complex, CDK7 phosphorylates the COOH-terminal domain of the largest subunit of RNA polymerase II (3). Further, CAK or TFIIF-associated CAK phosphorylates several transcription factors to regulate their activities (e.g., see refs. 22, 23). Inhibition of CDK7 activity would therefore be expected to inhibit transcription

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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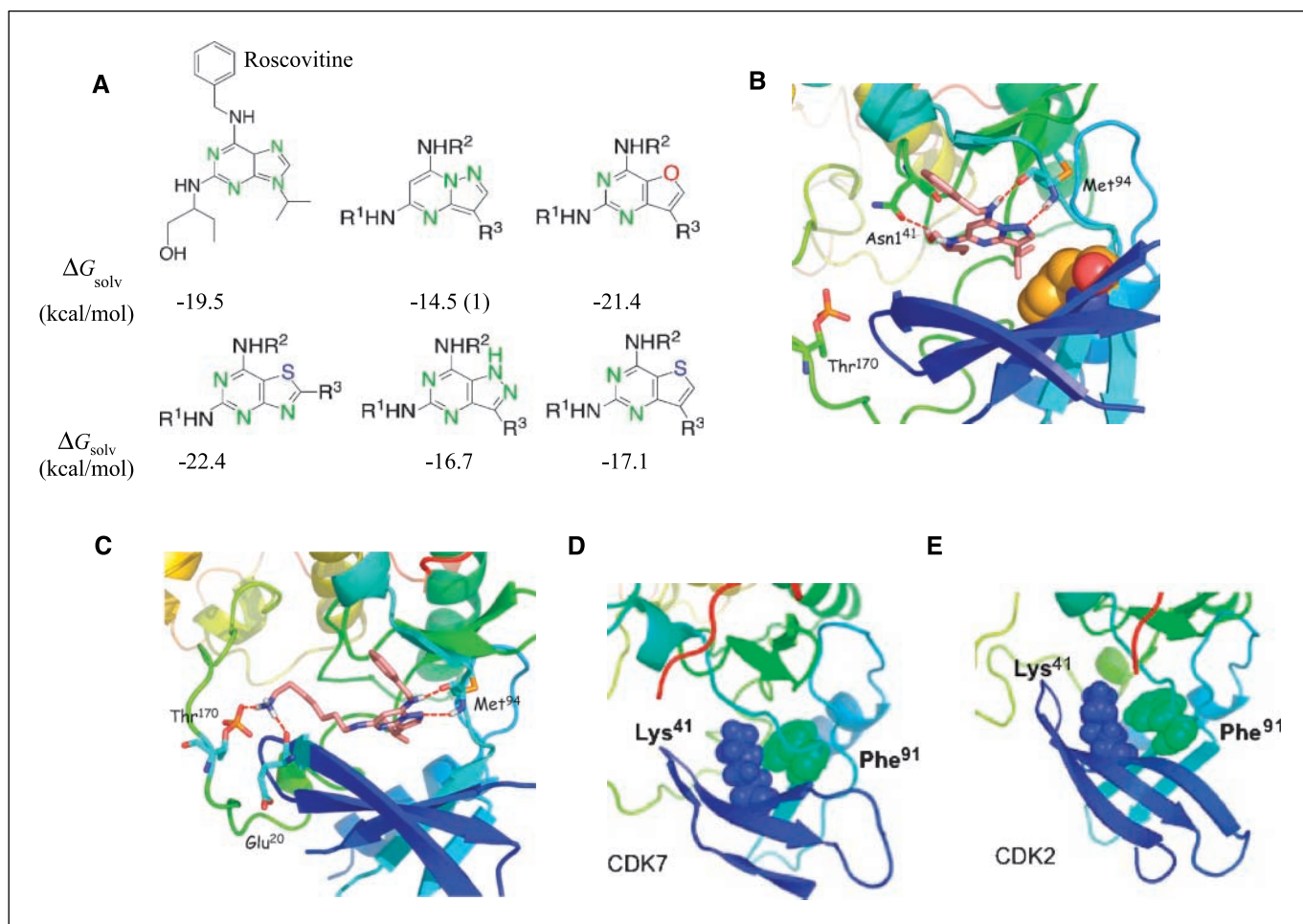


Figure 1. Computational analyses for design of CDK7 Inhibitors. **A**, AMSOL solvation energies of six core motifs based on roscovitrine (ΔG_{solv} , kcal/mol). R^1 , R^2 , and R^3 are the same as those in roscovitrine. **B**, lowest energy Glide XP pose for pyrazolopyrimidine **1**. The orange space-filling Phe⁹¹ is the gatekeeper residue. Dashed red lines, hydrogen bonds with Met⁹⁴ and Asn¹⁴¹. **C**, glide XP pose for BS-181 with hydrogen bonds to Glu²⁰, Met⁹⁴, and Thr¹⁷⁰ phosphate. Gatekeeper residue packing for CDK7 (**D**) and CDK2 (**E**). The packing is tighter for CDK7 relative to CDK2 as measured by surface exposure of the hydrophobic aggregation of Lys⁴¹ (blue) and Phe⁹¹ (green), 161 and 165 Å², respectively.

as well as cell cycle progression. Selective inhibitors should therefore provide potentially significant tools for dissecting further the multiple roles of CDK7 and could have utility as anticancer drugs. Here, we have performed computer modeling of the CDK7 structure to identify potential chemical structures that could act as selective CDK7 inhibitors. Based on these analyses, we have identified a novel selective inhibitor of CDK7, named BS-181, which inhibits phosphorylation of CDK7 substrates and inhibits cancer cell growth *in vitro* and *in vivo*.

Materials and Methods

Computer-aided drug design of BS-181. AMSOL v6.6⁴ was used to calculate solvation free energies of five preliminary core fragments based on roscovitrine. GLIDE (v 4.5) was used to dock the ligands into the CDK7 (PDB ID 1UA2) ATP-binding pocket. All manipulations using GLIDE were performed within the Maestro interface from Schrodinger (v 6.5). The Protein Preparation workflow in Maestro was first used to add protons and

assign ionization states to the protein. Following this protocol, a grid centered on the ligand was generated using the default Glide settings. All ligands were docked into this grid structure. Dockings were run using GLIDE SP mode to generate initial poses and then XP and MMGBSA modes to evaluate docking scores and rank ligands with roscovitrine as a reference. Ten poses were generated for each docking run and the top pose was picked for evaluation.

General synthetic methods. All manipulations of air- or moisture-sensitive materials were carried out in oven- or flame-dried glassware under an inert atmosphere of nitrogen or argon. Syringes, which were used to transfer reagents and solvents, were purged with nitrogen before use. Reaction solvents were distilled from CaH₂ (dichloromethane, toluene, triethylamine), Na/Ph₂CO (tetrahydrofuran, diethyl ether), or obtained as dry or anhydrous from Aldrich Chemical Company (*N,N*-dimethylformamide, acetonitrile) or BDH (ethanol). Other solvents and all reagents were obtained from commercial suppliers (Fluka, Aldrich Chemical Company, and Lancaster Chemicals) and were used as obtained if purity was >98%. All flash column chromatography experiments were carried out on BDH silica gel 60, particle size 0.040 to 0.063 mm, unless otherwise stated. TLC was performed on precoated aluminum-backed or glass-backed plates (Merck Kieselgel 60 F₂₅₄) and visualized with UV light (254 nm) or potassium permanganate (KMnO₄), vanillin or phosphomolybdic acid stains as deemed appropriate. Details

⁴ <http://comp.chem.umn.edu/amsol>

of the synthesis of BS-181 are shown in Supplementary Fig. S1 and in Supplementary Data.

In vitro kinase assays. The purified recombinant CDK2/cycE, CDK4/cycD1, CDK5/p35NCK, CDK7/CycH/MAT1, and CDK9/CycT were purchased from ProQinase GmbH. Kinase assays were performed according to manufacturer's protocols, using substrate peptides purchased from ProQinase GmbH, as described below. A luciferase assay (PKLight assay; Cambrex) was used to determine ATP remaining at the end of the kinase reaction, which provides a measure of kinase activity, according to the manufacturer's protocols.

Cell growth assays. All cells were purchased from the American Type Culture Collection and were routinely cultured in DMEM supplemented with 10% FCS (First Link). Cell growth was assessed using the sulforhodamine B assay, as described (24).

Flow cytometry. MCF-7 cells were seeded (4×10^5) in six-well plates in DMEM containing 10% FCS and allowed to adhere for 24 h, followed by addition of compounds or DMSO and incubation for 24 h. Cells were trypsinized, centrifuged at 1,100 rpm for 5 min, and resuspended in 5 mL of ice-cold PBS, centrifuged as above, gently resuspended in 1 mL ice-cold 70% ethanol, and incubated at 4°C for 1 h. Cells were washed twice with 5 mL of ice-cold PBS and resuspended in 100 μ L of PBS containing 100 μ g/mL RNase (Sigma-Aldrich) and 1 mL of 50 μ g/mL propidium iodide (Sigma-Aldrich) in PBS. Following incubation overnight in the dark at 4°C and filtering through 70- μ m muslin gauze into fluorescence-activated cell sorting tubes (Becton Dickinson) to remove cell clumps, stained cells were acquired using the RXP cytomics software on a Beckman Coulter Elite ESP (Beckman Coulter, High Wycombe) and data were analyzed using Flow Jo v7.2.5 (Tree Star, Inc.). For dual labeling with propidium iodide and Annexin V, the cells were trypsinized and collected with the culture medium, centrifuged at 1,100 rpm for 5 min, and washed twice with 5 mL of ice-cold PBS containing 2% (w/v) bovine serum albumin (Sigma-Aldrich). Cells were labeled with Annexin V-FITC using the Annexin V-FITC apoptosis detection kit I (BD Pharmingen), as per the manufacturer's instructions. Labeled cells were acquired within 1 h, by using the RXP cytomics software on a Beckman Coulter Elite ESP, and the data were analyzed using Flow Jo v7.2.5. Statistical analysis was performed for three independent experiments, carried out using the unpaired Student's *t* test to determine *P* values.

Immunoblotting. Cells (1×10^6) were plated in 10-cm plates and were treated with compounds after 24 h. Four hours later, cell lysates were prepared by the addition of 500 μ L of hot lysis buffer [4% SDS (w/v), 20% glycerol (v/v), 0.1% bromophenol blue (w/v), 0.1 mol/L Tris-HCl (pH 6.8), 0.2 mol/L DTT, in H_2O], preheated to 100°C. Immunoblotting was performed as described previously (25), using antibodies for PolII (N-20; Santa Cruz Biotechnologies), PolII phosphoserine-2, PolII phosphoserine-5, Rb phosphoserine-795, CDK7, cyclin D1, XIAP, Bcl2, and β -actin from Abcam plc. Antibodies for Rb, CDK2, CDK4, CDK5, cyclin A, cyclin B, cyclin E, and Bcl-xL were purchased from New England Biolabs.

Human tumor xenografts. Seven-week-old female nu/nu-BALB/c athymic nude mice were purchased from Harlan Olac Ltd. All procedures were approved by the CBS, Imperial College London Ethics Committee, and were covered by a Government Home Office project license. Before inoculation of animal with cells, a 0.72-mg 17 β -estradiol 60-d release pellet was implanted s.c. (Innovative Research of America). MCF-7 cells (5×10^6) were injected s.c. in not more than 0.1 mL volume into the flank of the animals. Tumor measurements were performed twice per week, and volumes were calculated using the formula $1/2 [\text{length (mm)}] \times [\text{width (mm)}]^2$. The animals were randomized and when tumors had reached a volume of 100 to 200 mm³, animals were entered into the different treatment groups, and treatment with test drug or vehicle control was initiated. Animals were treated with compound twice daily by i.p. injection for a total of 14 d. The compounds were prepared in the vehicle of 10% DMSO/50 mmol/L HCl/5% Tween 20/85% saline. Control mice were injected with the vehicle. Compounds were administered by exact body weight, with the injection volume being not more than 0.2 mL. At the end of the 14-d treatment period, the mice were sacrificed. Throughout the 14-d treatment period, animal weights were determined each day and tumor volumes were determined on alternate days.

Results

Computer-aided drug design approach for the development of potential CDK7 inhibitors. To design CDK7 inhibitors, computational analysis for selection of an alternative core heterocyclic ring structure able to preserve side chain functionality of roscovitine, offer synthetic access, and incorporate suitable solubility properties was carried out. Five motifs (Fig. 1A) were evaluated using Amsol 6.6, with the expectation that the structure with the least favorable aqueous solvation energy would be transferred most readily into the hydrophobic kinase active site pocket. The calculated free energies of solvation suggested that pyrazolopyrimidine **1** would be soluble, but the core structure most readily expelled from the water environment and into the protein. Accordingly, this motif was selected for synthetic modification. Docking studies performed using Glide (26, 27) also yielded the best Glide scores for pyrazolopyrimidine **1** compared with the other templates, including roscovitine (Supplementary Table S1).

A key observation in preliminary studies was that **1** with the same side chains as roscovitine docked into the CDK7 active site in the same orientation as the latter, but with slightly better scores and substantially more favorable solvation energies. The best pose for **1** is similar to that for other pyrazolopyrimidines (28). The 3-isopropyl group protrudes into the cavity formed by the gatekeeper Phe⁹¹. The N1 and N6 centers form hydrogen bonds with backbone atoms of Met⁹⁴ in the hinge region of the kinase, whereas the side chain hydroxyl makes a hydrogen bond with Asn¹⁴¹ (Fig. 1B).

The docked pose suggested that **1** is incapable of completely occupying the CDK7 active site pocket. Left unused was a sector in the back of the cleft occupied by two lysines (Lys⁴¹ and Lys¹³⁸) and a phosphorylated threonine (Thr¹⁷⁰). In an attempt to exploit the lipophilic nature of this subsite, the hydroxy ethyl moiety of **1** borrowed from roscovitine was excised and the resulting propyl side chain was extended. Nonpolar alkyl linkers of different chain length terminating in a variety of polar groups attached to the NH at the C2 position were conceived and docked. A six-carbon linker with a primary amine terminus (Fig. 1C) delivered the best docking score and suggested a considerable affinity improvement relative to roscovitine. At the same time, the corresponding Glide pose was similar to that for **1**. In addition to hydrogen bonds in the hinge region, the protonated distal amine was predicted to participate in

Table 1. Inhibition of cyclin-dependent protein kinase activity by BS-181

Kinase	Roscovitine IC ₅₀ , μ mol/L (SD)	BS-181 IC ₅₀ , μ mol/L (SD)
CDK7	0.51 (0.1)	0.021 (0.002)
CDK1	1.8 (0.3)	8.1 (0.6)
CDK2	0.1 (0.02)	0.88 (0.08)
CDK4	15.3 (6.6)	33 (1.5)
CDK5	0.24 (0.1)	3.0 (0.5)
CDK6	28 (4.9)	47 (4)
CDK9	1.2 (0.8)	4.2 (0.5)

NOTE: The mean IC₅₀ values (μ mol/L) for roscovitine and BS-181, obtained from three experiments, are shown together with the SD from the mean. The line graphs from which IC₅₀ values were obtained are shown in Supplementary Fig. S2.

Table 2. *In vitro* growth inhibitory activity of BS-181

Cell type	Cell line	Roscovitine IC ₅₀ , $\mu\text{mol/L}$ (SD)	BS-181 IC ₅₀ , $\mu\text{mol/L}$ (SD)
Breast	MCF-7	13 (1.0)	20 (0.5)
	MDA-MB-231	18 (1.0)	15 (0.8)
	T47D	25 (6.5)	16.5 (4.0)
	ZR-75-1	33.5 (2.0)	25.5 (1.3)
	BT474	30.5 (1.0)	30.5 (0.5)
	BT20	32.5 (1.25)	19 (1.25)
	MCF-10A	12.5 (2.3)	15 (1.8)
	HMEC	20.5 (1.3)	17.3 (2.3)
Colorectal	COLO-205	8.0 (3.2)	11.5 (1.5)
	HCT-116	17.5 (1.3)	15.3 (3.8)
	HCT-116 (p53 ^{-/-})	8 (2.8)	12.5 (1.5)
Lung	A549	17 (0.8)	37 (3.3)
	NCI-460	9.5 (1.2)	21 (2.0)
Osteosarcoma	U2OS	10.5 (1.0)	14.5 (1.2)
	SaOS2	13.9 (1.0)	20 (1.3)
Prostate	PC3	10.8 (0.3)	18.2 (2.0)
	LNCaP	8.4 (0.3)	24 (0.7)
Liver	HepG2	12.3 (1.0)	15.1 (0.1)

NOTE: The mean IC₅₀ values ($\mu\text{mol/L}$) were obtained using the sulforhodamine B assay. Shown are the mean values derived from at least three replicates, together with the SDs from the means.

strong electrostatic interactions with the phosphate group of Thr¹⁷⁰ and the backbone carbonyl of Glu²⁰. Simultaneously, the six-carbon linker exhibited productive van der Waals contacts with the floor of the kinase binding pocket (Fig. 1C). The same structure was estimated to possess a favorable solvation energy.

Additional docking studies also indicated the structure to favor CDK7 relative to CDK2 (PDB ID: 1B38), CDK5 (PDB ID: 1UNL), and CDK6 (PDB ID: 1XO2), suggesting possible selectivity (Supplementary Table S2). The relative docking scores were confirmed by induced-fit docking.

Synthesis of BS-181 and *in vitro* kinase inhibition. BS-181 was synthesized from dichloropyrazolo[1,5-*a*]pyrimidine **2** (29) by sequential selective substitution of the C-7 chloride using benzylamine, Boc protection, palladium-catalyzed displacement of the C-5 chloride using di-Boc-1,6-hexanediamine under Buchwald-Hartwig reaction conditions (30), and deprotection in acidic methanol (Supplementary Fig. S1). Inhibition of CDK7 activity was measured by incubation of increasing amounts of BS-181 with purified recombinant CDK7/CycH/MAT1 complex, followed by measurement of free ATP remaining in the reaction using a luciferase assay (PKLight, Cambrex), luciferase activity therefore providing a measure of inhibition of CDK7 activity. BS-181 inhibited CDK7 activity with an IC₅₀ of 21 nmol/L (Table 1; Supplementary Data Fig. S2), whereas the IC₅₀ achieved with roscovitine was 510 nmol/L, in agreement with previous reports for inhibition of CDK7 by roscovitine (13). The IC₅₀ values for inhibition of CDK1/cycB, CDK4/cycD1, CDK5/p35NCK, CDK6/cycD1, and CDK9/cycT by BS-181 were considerably higher than 1 $\mu\text{mol/L}$, with inhibition of CDK2/cycE having an IC₅₀ of 880 nmol/L, ~40-fold higher than the IC₅₀ for CDK7.

Seventy protein kinases from many different classes were tested for inhibition by BS-181. Some inhibition of the activities of several kinases was observed using high concentrations (10 $\mu\text{mol/L}$) of BS-

181 (Supplementary Table S5). Performing IC₅₀ measurements for those kinases that showed the greatest inhibition, CDK2/cycA, CK1, and DYRK1A, showed an IC₅₀ of 730 nmol/L, 7.36 $\mu\text{mol/L}$, and 2.3 $\mu\text{mol/L}$, respectively. These data confirm that BS-181 is a highly selective inhibitor of CDK7 activity.

BS-181 promotes cell cycle arrest and inhibits cancer cell growth. To assess the antiproliferative activity of BS-181, a panel of cell lines representing a range of tumor types, including breast, lung, prostate, and colorectal cancer, were treated with increasing concentrations of BS-181 for 72 hours. Determination of proliferation using the sulforhodamine B assay showed that growth was inhibited for all cell lines tested, with IC₅₀ values ranging from 11.5 to 37 $\mu\text{mol/L}$ (Table 2). The growth inhibition observed for BS-181 was similar to that observed for roscovitine, which gave IC₅₀ values in the range of 8 to 33.5 $\mu\text{mol/L}$.

Immunoblotting of whole cell lysates prepared from MCF-7 cells treated with BS-181 showed inhibition of phosphorylation of the RNA polymerase II COOH-terminal domain (CTD) at the well-established CDK7 phosphorylation site, namely serine 5 (P-Ser5; Fig. 2A). Densitometric analysis of the immunoblotting results of three independent experiments indicated that the IC₅₀ of inhibition of Ser⁵ phosphorylation is 9 $\mu\text{mol/L}$, whereas 50% inhibition of Ser⁵ phosphorylation by roscovitine was not reached at 50 $\mu\text{mol/L}$. Serine 2 in the CTD, which is not believed to be a substrate for CDK7, but is a substrate for CDK9 and can be phosphorylated by CDK2, was inhibited to a greater extent by roscovitine than by BS-181. BS-181 also inhibited RB phosphorylation at Ser⁷⁹⁵ and Ser⁸²¹ with an apparent IC₅₀ of 15 $\mu\text{mol/L}$ (Fig. 2B), similar to the IC₅₀ obtained for P-Ser2 inhibition.

Immunoblotting for CDKs and cyclins following BS-181 or roscovitine treatment for 4 hours showed down-regulation of CDK4 and cyclin D1 (Fig. 2C), with levels of the other CDKs and cyclins remaining unaffected. Additionally, levels of the antiapoptotic

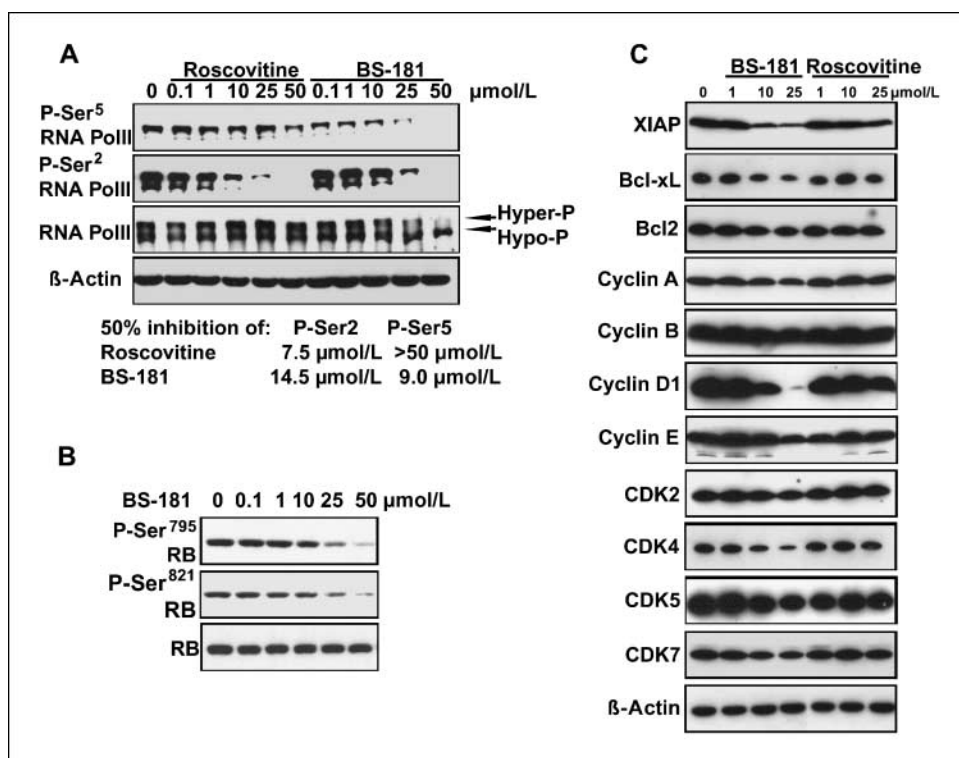


Figure 2. BS-181 inhibits phosphorylation of CDK7 substrates. Whole cell lysates were prepared from MCF-7 cells treated with BS-181 or roscovitine for 4 h, at the concentrations shown. A, immunoblotting was carried out using antibodies for RNA polymerase II or Pol II phosphorylated at Ser² or Ser⁵ in the COOH-terminal domain. The concentration at which apparent inhibition of PolII phosphorylation by 50% would be achieved was determined following densitometry of immunoblots from three experiments. B and C, immunoblotting was carried out as in A, using the antibodies as labeled.

proteins XIAP and Bcl-xL was reduced by BS-181, with Bcl-2 levels being unchanged.

Treatment with low concentrations of BS-181 for 24 hours showed an increase in cells in G₁, accompanied by a reduction in cell numbers in S and G₂-M (Fig. 3A; Supplementary Fig. S3). At higher concentrations, however, cells accumulated in the sub-G₁, indicative of apoptosis. This was confirmed by Annexin V staining of cells following BS-181 treatment for 24 hours, with 30% and 83% of cells staining positive for Annexin V with 25 and 50 μmol/L BS-181, respectively (Fig. 3B). No significant apoptosis was observed for roscovitine.

In vivo pharmacokinetic studies and tumor growth inhibition. The maximum tolerated single dose for BS-181 given i.p. was determined as 30 mg/kg, with 10 and 20 mg/kg being well tolerated (data not shown). For xenograft tumor growth inhibition studies, therefore, the animals were injected i.p. twice daily with 5 or 10 mg/kg, to give total daily doses of 10 or 20 mg/kg over a period of 14 days. Tumor growth was inhibited in a dose-dependent manner, with 25% and 50% reduction in tumor growth, compared with the control group, for 10 and 20 mg/kg/d doses of BS-181, respectively (Fig. 4A). At these doses, there was no apparent toxicity, as judged by lack of significant adverse effects on animal weights (Fig. 4B).

I.v. and i.p. administration of 10 mg/kg BS-181 showed rapid clearance (Supplementary Data Fig. S4). The terminal half-lives were 405 and 343 minutes for i.p. and i.v. administration, respectively, with the measured plasma concentration at 15 minutes of 1,950 (SE = 203) and 2,530 (SE = 269) ng/mL, respectively, and bioavailability being 37% for i.p. administration of BS-181 (Supplementary Tables S3 and S4).

Discussion

We show here that BS-181 selectively inhibits CDK7 *in vitro* and treatment of MCF-7 cells results in inhibition of phosphorylation

of the CDK7 substrate, Ser⁵, in the RNA polymerase II CTD. Further, BS-181 inhibits cell proliferation *in vitro* and *in vivo*. Together, these findings indicate that CDK7 is a potential target for cancer therapy.

The modeling predictions were substantiated when BS-181 proved to be a more potent and selective CDK7 inhibitor than roscovitine. For example, as shown here, roscovitine is an inhibitor of CDK2, CDK5, CDK7, and CDK9, with IC₅₀ values of 100, 240, 510, and 1,200 nmol/L, respectively. BS-181, on the other hand, exhibits a substantially higher preference for CDK7 with an IC₅₀ value of 21 nmol/L. Excellent selectivity against CDK2, CDK5, and CDK9 is shown by high IC₅₀ values of 880, 3,000, and 4,200 nmol/L, respectively. BS-181 also fails to block CDK1, CDK4, and CDK6, with IC₅₀ values being >5,000 nmol/L. As such, BS-181 is a highly selective CDK inhibitor and is the most potent CDK7 inhibitor described to date.

It is difficult to rationalize computationally the selectivity of BS-181 for CDK7 over CDK2 and CDK5 in terms of specific ligand-protein interactions. However, different packing interactions of the nonpolar isopropyl side chain at C-3 in BS-181 with the amino acids in the kinase pocket may aid in explaining the phenomenon. For example, the 3-isopropyl side chain protrudes into a cavity formed in part by the important gatekeeper residues Phe⁹¹ and the C4 carbon chain of Lys⁴¹ in both CDK2 and CDK7. However, the hydrophobic packing of the two residues is much tighter in the case of CDK7 (Fig. 1D) than it is in CDK2 (Fig. 1E). This volume-based realignment in the gatekeeper sector of the binding site may well exert a subtle effect that influences selectivity.

The first generation of general CDK inhibitors, such as olomoucine, showed activity against CDK1, CDK2, and CDK5. This was followed by the description of compounds such as UCN-01, which although showing antitumor activity showed side effects that limited their use. Other compounds include

flavopiridol, which is moderately selective against CDK4, CDK6, and CDK1, and CINK4, which is active against CDK4 and CDK6. Paullones have also been shown to have good selectivity against CDK1, CDK2, and CDK5. P276-00 is active against CDK9, with some activity against CDK4 and CDK1 (17, 31). It is only recently, however, that the concept of inhibition of transcriptional control by inhibiting CDK7 or CDK9 has gained some popularity. Inhibition of these kinases may be expected to be particularly important for transcripts that have a short half-life. Examples include transcripts for *bcl-2*, cyclin D, *Mcl-1*, and other genes involved in cell cycle progression and apoptosis. For example, flavopiridol, the most potent described inhibitor of CDK9, inhibits phosphorylation of the PolII CTD at Ser² and Ser⁵ (32, 33) and reduces expression of the antiapoptotic *Mcl-1* gene in primary CLL cells (34). Roscovitine has also been shown to inhibit PolII Ser² and Ser⁵ phosphorylation and roscovitine (Seliciclib) has been evaluated in a phase 1 study (10). This study

showed that the dose-limiting toxicity was fatigue, sickness, and hypokalemia and hyponatremia, with some patients showing evidence of renal failure. No responses were seen although disease stabilization was seen in some patients; the compound was insufficiently active and bioavailable to inhibit PolII phosphorylation. Clinical trials in chronic lymphocytic leukemia (CLL), lymphoma, and multiple myeloma are ongoing for flavopiridol; however, several studies have failed to show clinical responses, although more recent studies in CLL are encouraging and suggest that flavopiridol synergizes with other compounds such as imatinib and tumor necrosis factor-inducing compounds in leukemia (for review and references, see ref. 17).

BS-181 inhibits phosphorylation of the PolII CTD at Ser⁵, a known CDK7 substrate. Although CDK7 does not target Ser², BS-181 inhibited Ser² phosphorylation, likely through inhibition of CDK2 and CDK9. Indeed, Ser⁵ inhibition was observed at lower concentrations of BS-181 than the concentrations required for inhibition of Ser² phosphorylation. In this respect, roscovitine, which is a more potent inhibitor of CDK2 and CDK9 than BS-181, inhibited Ser² phosphorylation at lower concentrations than BS-181, but only poorly inhibited Ser⁵ phosphorylation. Together, these findings suggest that CDK7 is a key target of BS-181 in MCF-7 cells. Inhibition of Rb phosphorylation was also observed, but the inhibition was similar to that observed for Ser² of PolII, suggesting that reduction in Rb phosphorylation was indirect.

As outlined above, inhibition of CDK7 and CDK9 has been linked to down-regulation of cyclin D1, *bcl-2*, and *Mcl-1*. BS-181 treatment for as little as 4 hours reduced cyclin D1, XIAP, and Bcl-xL expression, although Bcl-2 levels were only slightly reduced. Interestingly, CDK4 levels were also reduced. Together, these data may explain the G₁ arrest and potent apoptosis brought about by BS-181 treatment of MCF-7 cells. Transcriptional inhibitors that block PolII activity, such as α -amanitin and actinomycin D, as well as compounds that inhibit PolII phosphorylation, such as 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), have been shown to induce apoptosis by activating p53 (35). Treatment of p53-null HCT116 cells (36) showed considerably reduced, albeit significant, induction of apoptosis by BS-181, compared with p53-positive HCT116 cells (Supplementary Fig. S5), suggesting that p53 is important for BS-181-mediated apoptosis, as described previously for DRB (37). Similarly, greater apoptosis was observed in the p53-positive U2OS osteosarcoma line, when compared with the p53-negative SaOS2 osteosarcoma line (Supplementary Fig. S6). Together, these findings indicate that p53 is important but not essential for BS-181-induced apoptosis.

I.p. injection of BS-181 inhibited MCF-7 tumor xenografts, lending further support to a potential utility of CDK7 inhibitors in cancer treatment. Pharmacokinetic studies showed rapid clearance of BS-181 administered i.p. or i.v. In the case of i.p. administration, the maximal blood concentration of BS-181 was 1,317 ng/mL. Further, bioavailability was only 37%, indicating a need for further refinement of the BS-181 structure to improve stability and bioavailability. As it stands, the studies described here indicate that continuous i.v. infusion or repeated administration is needed for further *in vivo* evaluation. The observed efficacy, despite the low plasma levels (lower than the IC₅₀ for growth inhibition *in vitro*), could therefore be due, at least in part, to more active metabolites generated following i.p. administration. Elucidation of the structures of possible metabolites and their activities will be the subject of future studies.

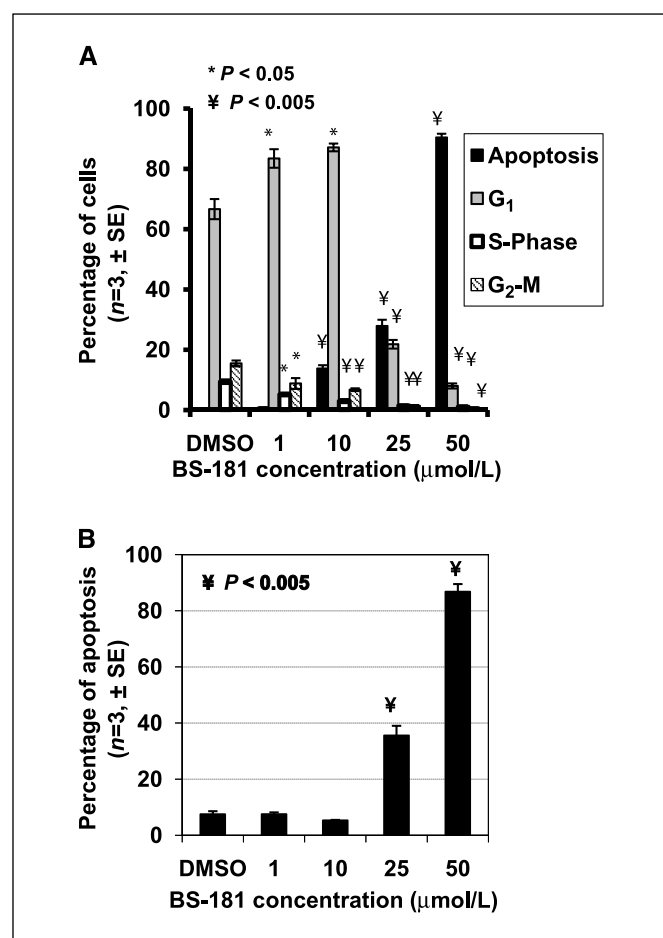


Figure 3. BS-181 treatment of MCF-7 cells leads to G₁ arrest and apoptosis. **A**, MCF-7 cells were treated with BS-181 at the concentrations shown, or with vehicle (DMSO) for 24 h, before fixation, staining with propidium iodide, and flow cytometric analysis. The percentage of cells in the sub-G₁ (apoptosis), G₁, S-phase, and G₂-M, as determined from three independent experiments, are shown. Error bars, SE. **B**, cells treated with DMSO or BS-181 were stained with an antibody for Annexin V and with propidium iodide. The percentage of cells that stained positive for Annexin V following the addition of BS-181 or roscovitine are shown for three independent experiments. Error bars, SE. *P* values were determined using Student's *t* test, in which comparison between the DMSO control and BS-181 treatment at each concentration was carried out. *, *P* < 0.05; ¥, *P* < 0.005.

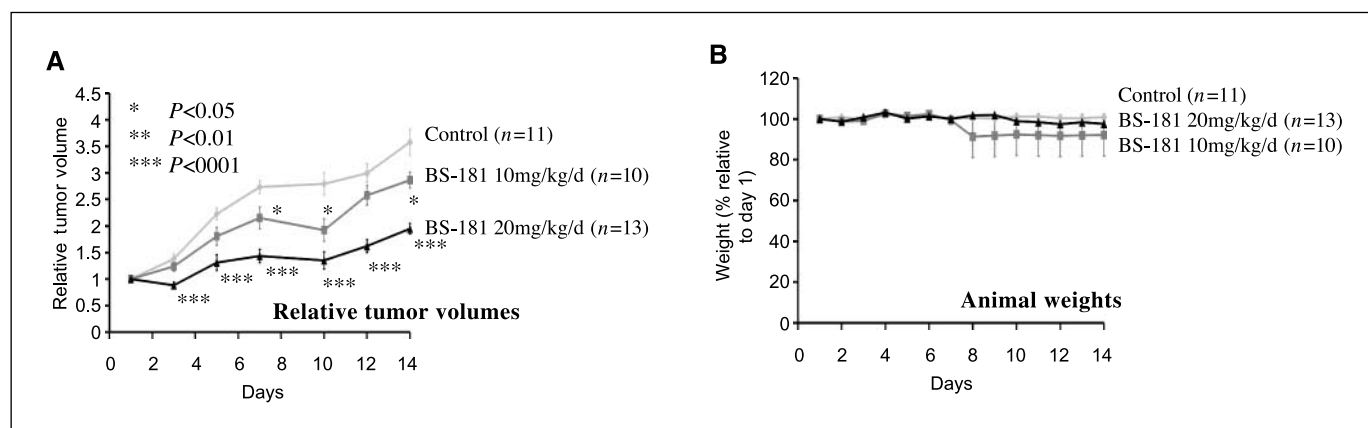


Figure 4. BS-181 inhibits the growth of MCF-7 tumors in nude mice. Randomized MCF-7 tumor-bearing mice were injected i.p. twice daily with 5 or 10 mg/kg BS-181, giving a total daily dose of 10 or 20 mg/kg/d, respectively, over a period of 14 d. Mouse weights were determined daily, tumor volumes being measured every 2 d. **A**, the change in tumor volume was determined for each animal, as tumor volume relative to the tumor volume of each animal at day 1. The line graphs show the mean tumor volumes for the animals in each treatment group. Error bars, SE. Asterisks depict the statistical significance of the differences between the control group and each of the BS-181 treatment groups, carried out using Student's *t* test. **B**, animal weights, provided as percentage change relative to the animal weights at day 1. Error bars, SE.

In summary, we have discovered the most potent CDK7-selective inhibitor to date by computer-aided drug design. BS-181 selectively exhibited nanomolar enzymatic potency and inhibited all cell lines tested at low micromolar concentrations. For the given route of administration (37% bioavailability), the drug showed *in vivo* activity in human tumor xenografts. BS-181 warrants further preclinical and clinical evaluation as a candidate cancer therapeutic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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A Novel Pyrazolo[1,5-*a*]pyrimidine Is a Potent Inhibitor of Cyclin-Dependent Protein Kinases 1, 2, and 9, Which Demonstrates Antitumor Effects in Human Tumor Xenografts Following Oral Administration

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Cyclin-dependent protein kinases (CDKs) are central to the appropriate regulation of cell proliferation, apoptosis, and gene expression. Abnormalities in CDK activity and regulation are common features of cancer, making CDK family members attractive targets for the development of anticancer drugs. Here, we report the identification of a pyrazolo[1,5-*a*]pyrimidine derived compound, **4k** (BS-194), as a selective and potent CDK inhibitor, which inhibits CDK2, CDK1, CDK5, CDK7, and CDK9 (IC₅₀ = 3, 30, 30, 250, and 90 nmol/L, respectively). Cell-based studies showed inhibition of the phosphorylation of CDK substrates, Rb and the RNA polymerase II C-terminal domain, down-regulation of cyclins A, E, and D1, and cell cycle block in the S and G₂/M phases. Consistent with these findings, **4k** demonstrated potent antiproliferative activity in 60 cancer cell lines tested (mean GI₅₀ = 280 nmol/L). Pharmacokinetic studies showed that **4k** is orally bioavailable, with an elimination half-life of 178 min following oral dosing in mice. When administered at a concentration of 25 mg/kg orally, **4k** inhibited human tumor xenografts and suppressed CDK substrate phosphorylation. These findings identify **4k** as a novel, potent CDK selective inhibitor with potential for oral delivery in cancer patients.

Introduction

Cyclin-dependent kinases (CDK^a) are serine/threonine kinases that regulate progression through the cell cycle. CDK4 and CDK6 mediate progression through the G₁ growth phase, CDK2 is required for entry into and transition through the DNA replication or synthesis (S) phase, and CDK1 controls progression through G₂ and mitosis. CDK7, together with its partners cyclin H and MAT1, form the CDK activating kinase (CAK), which activates the cell cycle CDKs by phosphorylating them at a threonine residue in the activation segment (T-loop).^{1–3}

Deregulation of cell cycle progression is a universal characteristic of cancer, and the majority of human cancers have abnormalities in some component of CDK activity, frequently through elevated and/or inappropriate CDK activation. Examples include cyclin overexpression, for example, cyclin D1 overexpression is commonly observed in breast cancer,⁴ and loss of expression of CDK inhibitory proteins (CKI) through

mutational or epigenetic alterations, for example, p16 loss has been observed in skin, lung, breast, and colorectal cancer (for review see ref 5). Synthetic inhibitors of CDK activity therefore present as a logical approach in the development of new cancer therapies. However, the development of inhibitors selective for a particular CDK has been questioned by the finding that inhibition of some CDKs may lead to compensation by other CDKs. For example, cells from mice that have been ablated for CDK2 are able to cycle, and CDK2^{−/−} mice are viable,^{6,7} while evidence for compensation of CDK2 by CDK1 has been forthcoming.^{8,9} Similarly, CDK4^{−/−} and CDK6^{−/−} mice are viable, although the double null mice show late embryonic lethality.^{10–12} Hence, a highly selective inhibitor of CDK2, 4, or 6 may be subject to clinical resistance due to the potential for compensation. However, acquisition of CDK dependence seems to feature in some tumor types. For example, despite the fact that CDK4 is not required for normal mammary gland development, it does appear to be important for induction of mammary tumors in mouse models.^{13–15} The CDK4/CDK6 partner cyclin D1 is similarly required for HER2- and Ras-induced mammary tumors, but not for Myc and Wnt1 induced mammary tumors,¹⁶ indicating that, at least in some tumor types, requirement for selective CDKs is acquired during the process of tumorigenesis in a process that is dependent on the pathway leading to tumor formation.^{3,17}

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^aAbbreviations: CDK, cyclin dependent kinase; Rb, retinoblastoma; PolII, RNA polymerase II; CKI, CDK inhibitory protein; HUVEC, human normal vascular endothelial cells; PBMC, peripheral blood mononuclear cells; SRB, sulforhodamine B; FACS, fluorescence-activated cell sorter; ADME-Tox, absorption, distribution, metabolism, excretion, and toxicity; PO, per oral, IV, intravenous; IP, intraperitoneal.

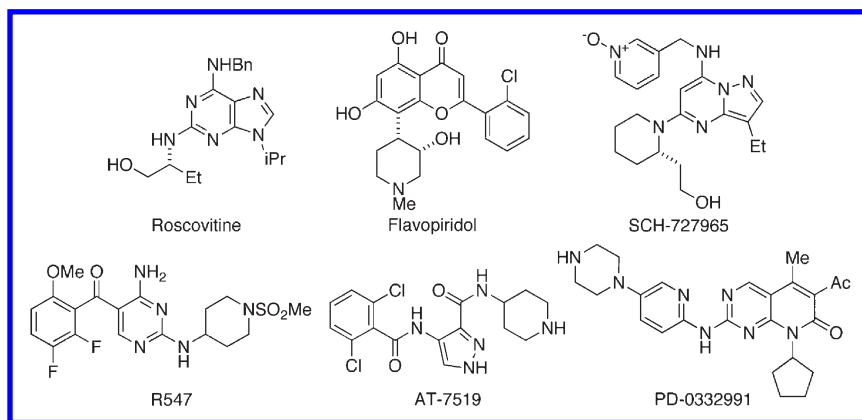
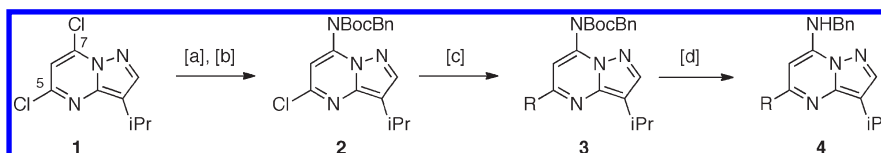


Figure 1. Structures of some CDK inhibitors currently in clinical evaluation. See text for references.

Scheme 1. Synthesis of Pyrazolopyrimidines^a



^a (a) BnNH_2 , EtOH, reflux, 16 h, 95%; (b) Boc_2O , THF, cat. DMAP, 25 °C, 20 h, 96%; (c) amine, $\text{Pd}_2(\text{dba})_3$, *rac*-BINAP, NaOtBu , 100 °C, 16 h, 40–99%; (d) MeOH, HCl, 25 °C, 3 h, 67–99%.

We reasoned that inhibitors of CDK7 could be potentially important in cancer therapy, as they would inhibit activation of many of the cyclin-dependent kinases involved in cell cycle progression. Moreover, as part of the TFIIF transcription factor complex, CDK7/CycH/MAT1 mediates the phosphorylation of the C-terminal domain of the largest subunit of RNA polymerase II, an event required for promoter clearance and transcription initiation.^{18–20} In carrying out a screen for CDK7 inhibitors, we identified a compound, **4k**, which inhibits CDK7 but is also a potent inhibitor of CDK1, CDK2, CDK5, and CDK9. In further evaluating the activity of **4k**, we show that **4k** potentially inhibits growth of the great majority of cancer cell lines examined and inhibits phosphorylation of CDK substrates at submicromolar concentrations. A number of inhibitors that target cell cycle CDKs have been identified and some have been evaluated clinically, including roscovitine (Seliciclib), SCH-727965, and AT7519 (Figure 1).^{21–23} **4k** is a pyrazolopyrimidine as is SCH-727965. Unlike many other CDK inhibitors, however, we show that **4k** is orally bioavailable and inhibits tumor growth in vivo. As such, **4k** is an important new CDK inhibitor with clinical potential.

Results

Identification of 4k. Of the CDK inhibitors currently undergoing clinical trials (Figure 1), a structural class that has yielded several CDK-selective ATP antagonists is the 2,6,9-trisubstituted purines, exemplified by roscovitine, which shows good biological and pharmacological properties. However, our computational analysis has shown that the pyrazolo[1,5-*a*]pyrimidine core has a less favorable aqueous solvation energy than the corresponding purine, suggesting that this compound class would be more readily transferred into the hydrophobic kinase active site.^{24,25} In this study, a range of pyrazolopyrimidines were synthesized and tested for CDK activity.

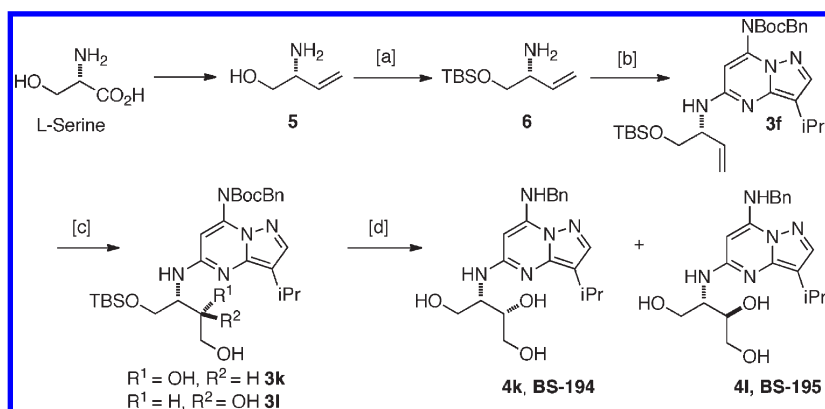
The compounds were readily accessible from dichloropyrazolo[1,5-*a*]pyrimidine **1** by sequential selective substitution of

the C-7 chloride using benzylamine followed by palladium-catalyzed displacement of the C-5 chloride under Buchwald–Hartwig reaction conditions, as described previously (Scheme 1).²⁴ Protection of the benzylic amine as a carbamate was essential for successful C-5 amination. Following deprotection, the analogues were assayed for CDK activity by measurement of free ATP remaining in the kinase reaction, as determined using a luciferase assay.

The high potency offered by the pyrazolopyrimidine core was highlighted by the effective inhibition of several CDKs by **4a**, which bears the roscovitine side chain (Supporting Information Table 1). Deletion of the free hydroxyl group in **4b** had a large detrimental effect on the potency. Interestingly, chain elongation and replacement of the hydroxyl by a terminal amine **4c** selectively increases potency toward CDK7. Compound **4c**, which we have previously reported (BS-181), inhibits CDK7 activity up to 83% while demonstrating only weak inhibition of the other CDKs. Compound **4c** inhibited CDK7 activity, with an IC_{50} of 21 nM/L, with greater than 40-fold selectivity over other CDKs and a wider range of 70 kinases and additionally showed antitumor activity in vivo.²⁴

Further long chain C-5 analogues were synthesized, and nitrile **4d** showed a similar inhibition of CDK7 activity (IC_{50} 20 nM/L) while also showing increased CDK2 inhibition. Similarly, amidine **4e** showed only a slight decrease in CDK7 inhibition (IC_{50} 40 nM/L), however, this also proved to be a highly potent CDK2 inhibitor.

Having identified **4c** as a potent and selective CDK7 inhibitor, it was decided to further investigate the SAR around the C-5 side chain against CDK potency and selectivity. Several analogues containing a terminal basic residue in combination with a free hydroxyl group were investigated. These were readily synthesized from alkene **6** (see Scheme 2) via cross metathesis, or oxidation/Wittig reactions followed by reduction of the resultant alkene prior to Buchwald–Hartwig coupling. However, the parent alkene **4f** showed decreased inhibition compared to the analogue with the roscovitine side chain **4a**,

Scheme 2. First Generation Synthesis of **4k** and **4l**^a

^a (a) *t*-BuMe₂SiCl, Et₃N, cat. DMAP, CH₂Cl₂, 25 °C, 16 h, 99%; (b) **2**, Pd₂(dba)₃, *rac*-BINAP, NaO^tBu, 100 °C, 16 h, 43%; (c) cat. OsO₄, NMO, MeCN, H₂O, 25 °C, 45 min, **3k** 42%, **3l** 33%; (d) MeOH, HCl, 25 °C, 3 h, **4k** 79%, **4l** 67%.

Table 1. Inhibition of Cyclin-Dependent Protein Kinase Activity by **4k**^a

kinase	roscovitine IC ₅₀ (μM) (SD)	4k IC ₅₀ (μM) (SD)
CDK1	2.1 (0.5)	0.033 (0.01)
CDK2	0.1 (0.05)	0.003 (0.001)
CDK4	13.5 (0.2)	20 (1.3)
CDK5	0.16 (0.2)	0.03 (0.006)
CDK6	23.5 (1.3)	35.5 (1.3)
CDK7	0.54 (0.09)	0.25 (0.04)
CDK9	0.95 (0.17)	0.09 (0.01)

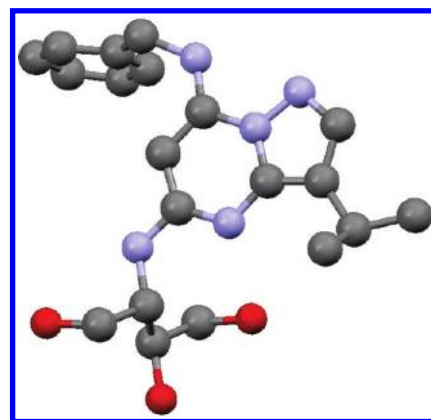
^a The mean IC₅₀ values (μM) for roscovitine and **4k**, obtained from three experiments, are shown, together with the standard deviation (SD) from the mean. The line graphs from which IC₅₀ values were obtained are shown in Supporting Information Figure 1.

and none of the analogues with the functionalized side chains **4g**, **4h**, or **4i** showed any significant inhibition of the CDKs.

The importance of free hydroxyl groups however, was reinforced by the high CDK7 inhibition and good selectivity demonstrated by the 2-(2-hydroxyethoxy)-ethylamino analogue **4j**. Accordingly, further hydroxylated compounds were synthesized by dihydroxylation of the vinyl residue present in **3f**, which after diastereoisomer separation and deprotection yielded the two amino triols **4k** and **4l** (BS-195). Both diastereoisomers proved to be highly active against a range of CDKs.

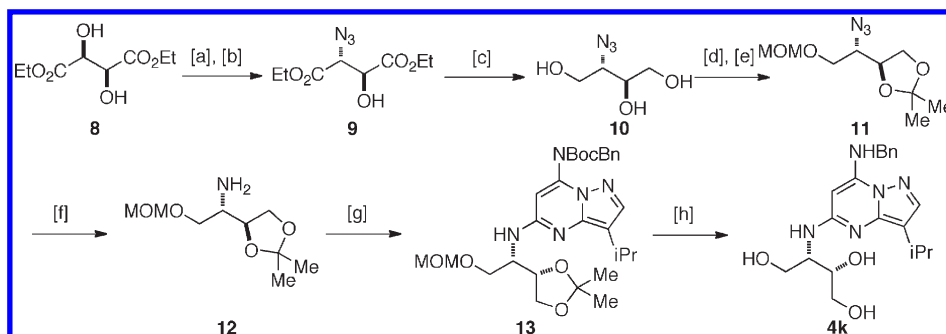
4k was chosen for further study because it inhibited CDK2 with an IC₅₀ = 3 nmol/L (Table 1; Supporting Information Figure 1), an IC₅₀ more than 30-fold lower than that showed by roscovitine (IC₅₀ = 100 nmol/L).²⁶ **4k** also inhibited CDK1 (IC₅₀ = 33 nmol/L), CDK5 (IC₅₀ = 30 nmol/L), and CDK9 (IC₅₀ = 90 nmol/L) and was a less potent inhibitor of CDK7 (IC₅₀ = 250 nmol/L), whereas CDK4 and CDK6 were not inhibited by **4k**. Compound **4l** gave a similar profile of CDK inhibition, however, **4k** had the advantage of being a crystalline solid. Compound **4k** was therefore selected for development due to both potency and ease of purification. In addition, the relative configuration of the molecule and by analogy its absolute stereochemistry was readily assigned by an X-ray crystal structure determination (Figure 2).

The initial synthesis of **4k** using a nonselective late stage alkene dihydroxylation reaction usefully gave access to both diastereoisomers, however, the diastereoisomer separation was laborious and unsuitable once **4k** had been selected for development (Scheme 2). Accordingly, an alternative route to

**Figure 2.** Crystal structure of **4k**.

enantiomerically and diastereomerically pure **4k** was investigated. We decided to introduce a fully functionalized C-5 side chain through the existing Buchwald–Hartwig coupling reaction, therefore a synthesis of the requisite fully protected amino-triol was required.

(–)-Diethyl tartrate (**8**) was converted to the corresponding cyclic sulfate using reaction conditions developed by Sharpless, by conversion to the cyclic sulfite with thionyl chloride, followed by ruthenium catalyzed oxidation to the cyclic sulfate.²⁷ Subsequent ring S_N2 opening using sodium azide gave the requisite azido-alcohol **9**. However, the oxidation reaction proved to be capricious, and as a consequence of difficulties encountered with handling of the cyclic sulfate, a more reliable method was investigated. Direct S_N2 ring-opening of the cyclic sulfite with sodium azide with careful control of temperature was highly effective, giving azide **9** in 70% yield.²⁸ The diester was readily reduced to triol **10** by lithium borohydride generated in situ in EtOH.^{28,29} It was necessary to protect the free hydroxyl groups to prevent competitive *O*-arylation in the Buchwald–Hartwig coupling, and it was hoped to achieve this in a single operation. However, attempts to protect all the hydroxyl groups as an orthoester by condensation with trimethyl orthoacetate or trimethyl orthobenzoate with acid catalysis failed. Attempts to prepare the triple methoxymethyl ether were complicated by formation of cyclic acetals and were only low yielding. A two-step procedure was therefore employed with the vicinal diol first being protected as an acetonide and the primary alcohol subsequently protected by methoxymethylation.

Scheme 3. Second Generation Synthesis of **4k**^a

^a (a) SOCl₂, NEt₃, CH₂Cl₂, 0 °C, 1 h; (b) NaN₃, DMF, 12 h, 70% (2 steps); (c) NaBH₄, LiCl, EtOH, 0 °C, 12 h; (d) (MeO)₂CMe₂, TsOH, Me₂CO, 50 °C, 3 h; (e) MOMCl, iPr₂NEt, CH₂Cl₂, 0 °C, 12 h, 70% (3 steps); (f) H₂, Pd/C, MeOH, 1 h, 97%; (g) 2, Pd₂(dba)₃, *rac*-BINAP, NaO^tBu, 100 °C, 16 h, 95%; (h) MeOH, HCl, 25 °C, 3 h, 90%.

Catalytic hydrogenation over palladium on charcoal gave the required fully protected amine **12**, which underwent high yielding Buchwald–Hartwig coupling to produce the protected **4k** precursor **13**. Global deprotection under acidic conditions gave **4k** as a single stereoisomer (Scheme 3). The ¹H and ¹³C NMR spectra of **4k** are shown in Supporting Information Figures 2 and 3.

4k Is a Potent Inhibitor of CDK1, CDK2, CDK7, and CDK9. Determination of the CDK selectivity of **4k**, performed by assaying inhibition of the activities of 76 proteins kinases representing members of different kinase classes (Table 2), showed significant inhibition of CAMKKβ, CK1, DYRK1A, ERK1, ERK2, and IRR at 10000 nmol/L but a less than 50% inhibition of these kinases when **4k** was used at a concentration of 1000 nmol/L (data not shown), suggestive of IC₅₀ values > 1000 nmol/L. In confirmation of this, the IC₅₀ values for CAMKKβ, CK1, DYRK1A, ERK1, ERK2, and IRR were determined as 2450, 1040, 2100, 3730, 3110, and 1800 nmol/L, respectively. The only exception was ERK8, whose activity was inhibited by 74% with 10 μmol/L **4k** and the IC₅₀ was determined as 330 nmol/L (data not shown), 100-fold higher than the IC₅₀ for CDK2, about 10-fold higher IC₅₀ than that obtained for CDK1 or CDK5.

A 1.8 Å crystal structure of **4k** bound to the inactive form of CDK2 was obtained by soaking the ligand into preformed CDK2 crystals. After molecular replacement with the published CDK2 structure, clear and unambiguous density for the ligand was found within the CDK2 ATP binding site (Figure 3A,D). Compound **4k** binds in a similar fashion to roscovitine, with the core heterocyclic ring occupying approximately the same position as the ATP purine ring.³⁰ The phenyl ring substituent occupies a hydrophobic pocket outside the ATP binding site, while N1 and N6 each form a hydrogen bond to the L83 main chain, as seen in the structure of roscovitine bound to CDK2 (Figure 3B,C). In addition, the three side chain hydroxyl groups of **4k** form several water-mediated interactions with CDK2 backbone and side chain atoms, involving residues E12, T14, D86, Q131, and D145 (Figure 3B). This is in contrast to roscovitine, where the number of stabilizing water-mediating interactions between the single ligand hydroxyl group and CDK2 is limited (Figure 3C). The structure suggests that the increased potency of **4k** in CDK2 inhibition assays compared to roscovitine could be due to tighter binding of the former to the inactive state of CDK2. The increased affinity of **4k** compared to roscovitine stems from its ability to form a network

Table 2. Inhibition of Protein Kinases by **4k**^a

4k (10 μM)			4k (10 μM)		
enzyme	% activity remaining	SD	enzyme	% activity remaining	SD
MKK1	65	15	AMPK	88	1
ERK1	42	15	MARK3	82	4
ERK2	26	4	BRSK2	108	14
JNK1	93	3	MELK	88	1
JNK2	94	12	CK1	19	1
p38a MAPK	98	12	CK2	89	7
P38b MAPK	94	3	DYRK1A	15	3
p38g MAPK	112	14	DYRK2	75	1
p38s MAPK	86	0	DYRK3	88	1
ERK8	26	4	NEK2a	102	3
RSK1	75	15	NEK6	87	15
RSK2	90	1	IKKb	80	3
PDK1	84	11	PIM1	103	6
PKBa	103	6	PIM2	94	1
PKBb	105	9	PIM3	101	5
SGK1	92	1	SRPK1	62	14
S6K1	77	0	MST2	86	8
PKA	93	2	EFK2	92	1
ROCK 2	102	14	HIPK2	73	6
PRK2	106	3	PAK4	55	7
PKCa	96	4	PAK5	65	3
PKC ζ	64	14	PAK6	85	6
PKD1	97	15	Src	95	13
MSK1	96	1	Lck	85	2
MNK1	98	3	CSK	77	1
MNK2	136	15	FGF-R1	86	2
MAPKAP-K2	111	14	IRR	32	11
PRAK	97	8	EPH A2	116	10
CAMKKb	23	1	MST4	92	14
CAMK1	93	5	SYK	120	11
SmMLCK	101	13	YES1	89	7
PHK	62	8	IKKe	94	7
CHK1	98	8	TBK1	87	8
CHK2	61	5	IGF1-R	67	15
GSK3b	63	15	VEG-FR	92	8
CDK2-cyclin A	3	0	BTk	91	8
PLK1	68	4	IR-HIS	70	2
PLK1 (okadaic acid)	97	14	EPH-B3	72	13

^a Inhibition of 76 protein kinases by **4k** at a concentration of 10 μmol/L. The assay was carried out in duplicate. Percentage of kinase activity remaining is shown, together with the standard deviations (SD).

of water mediated interactions within the CDK2 ATP binding pocket. Due to a major movement of the glycine loop (residues 12–20) upon cyclin binding to CDK2, it is expected that the **4k** hydroxyl substituents will form different interactions

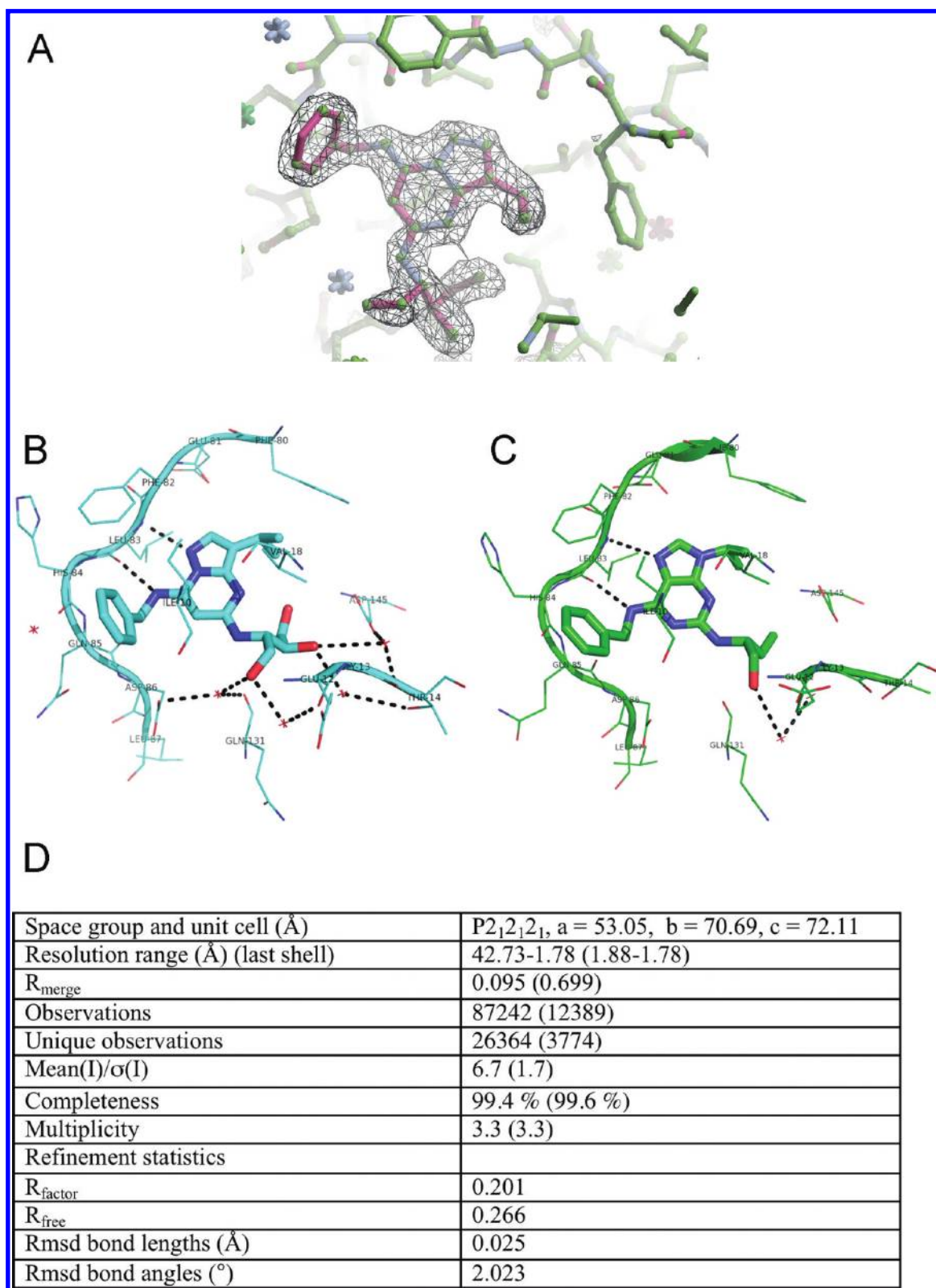


Figure 3. Crystal structure of **4k** bound to CDK2. (A) Shown is the **4k** difference electron density map after refinement, contoured at 2.9σ . (B) **4k** binding site in CDK2 is shown with hydrogen bonding interactions depicted as dashed lines. Water-mediated interactions are also shown. Carbon atoms are colored in cyan, nitrogen in blue, and oxygen in red. Water molecules are represented as red crosses. (C) Roscovitine binding site in CDK2 (PDB code 2A4L), as (B). (D) Summary of CDK2-**4k** data collection and refinement statistics.

in the active complex, and we are currently progressing studies to obtain a structure for **4k** bound to the active CDK2-cyclin A complex. Notwithstanding, the crystallography data confirm **4k** structure and binding to CDK2.

4k Promotes Cell Cycle Arrest and Inhibits Cancer Cell Growth. A panel of cell lines representing a range of tumor types, including breast, lung, prostate, and colorectal cancer, were treated with increasing concentrations of **4k** for 72 h.

Table 3. In Vitro Growth Inhibitory Activity of **4k**^a

cell type	cell line	roscovitine	4k
		GI ₅₀ (μM) (SD)	GI ₅₀ (μM) (SD)
breast	MCF-7	7.8 (0.2)	0.3 (0.1)
	MDA-MB-231	22.6 (0.1)	0.3 (0.1)
	MCF-10A	16.3 (0.1)	0.1 (0.1)
colorectal	COLO-205	24.9 (0.2)	0.12 (0.1)
	HCT-116	15.5 (0.1)	0.1 (0.1)
lung	A549	15 (0.2)	0.2 (0.1)
osteosarcoma	SaOS2	13.9 (0.1)	0.25 (0.1)
prostate	PC3	25.2 (0.1)	0.5 (0.1)
liver	HepG2	25.9 (0.2)	1.0 (0.1)
ovarian	Sk-Ov-3	35.7 (0.2)	0.3 (0.2)
human normal umbilical vein endothelial cells (HUVEC)		3.7 (0.1)	6.3 (0.1)

^a The mean IC₅₀ values (μM) were obtained using the sulforhodamine B (SRB) assay. Shown are the mean values derived from at least three replicates, together with the standard deviations (SD) from the means.

Determination of proliferation using the sulforhodamine B (SRB) assay showed that growth was inhibited for all cell lines tested, with 50% growth inhibition (GI₅₀) values of 100–500 nmol/L (Table 3). By contrast, GI₅₀ values for roscovitine ranged between 7.8–35.7 μmol/L for the same cell lines. Interestingly, human normal vascular endothelial cells (HUVEC) (GI₅₀ = 6.3 μmol/L) were somewhat less sensitive to **4k** than the cancer lines.

On the basis of our findings, **4k** was submitted to the Developmental Therapeutics Program at the NCI (<http://dtp.nci.nih.gov>) for testing using the NCI60 cancer cell line screen. In this screen, treatment with **4k** showed potent inhibition of all 60 cancer lines (Figure 4A, Supporting Information Figure 4), with GI₅₀ < 1 μmol/L (mean GI₅₀ = 2.81 × 10⁻⁷ mol/L). For the great majority of the lines, the 50% lethal concentration (LC₅₀) was > 100 μmol/L (mean LC₅₀ > 10 × 10⁻⁴ mol/L) (Supporting Information Table 2). For comparison, in the same cell panel, flavopiridol is a potent inhibitor of cancer cell growth (mean GI₅₀ = 7.47 × 10⁻⁸ mol/L) but lethal concentration for flavopiridol is reached at submicromolar concentrations (LC₅₀ = 9.04 × 10⁻⁷ mol/L) (<http://dtp.nci.nih.gov>).

Flow cytometric analysis of HCT116 cells treated with 1 and 10 μmol/L **4k** showed a significant reduction in cells in G1, with a concomitant increase in cells in S and G2/M phases of the cell cycle (Figure 4B; Supporting Information Figure 5), as would be expected from the potent inhibition of CDK1 and CDK2, but not of CDK4 and CDK6, by **4k**. Similar effects were observed in MCF-7 cells (data not shown). There was also an increase in the sub-G1 population, suggestive of a small, but significant amount of apoptosis at 1 and 10 μmol/L **4k**, which was confirmed by annexin V staining (data not shown).

4k Inhibits Phosphorylation of CDK Substrates and Promotes Loss of Cyclin Expression. Immunoblotting of whole cell lysates prepared from HCT116 colon cancer cells treated with **4k** for 4 and 24 h showed inhibition of the phosphorylation of the CDK2 substrate RB at Ser-780, Ser-795, Ser-801, Ser-807/Ser-811, and Thr-821 (Figure 4C,D), the CDK1 substrate protein phosphatase type 1α (PP1α) at Thr-320, and the CDK9 substrate Ser-2 in the C-terminal domain (CTD) of RNA polymerase II (PolII). Altered levels of CDKs or cyclins did not accompany inhibition of RB and PolII phosphorylation at 4 h following **4k** addition (Figure 4E). By contrast, at 24 h, levels of cyclin A, cyclin

B, and cyclin D1 were dramatically reduced in the case of **4k** at a concentration of 1 μmol/L. Reduction in the levels of these cyclins was also observed with higher concentrations (30 and 40 μmol/L) of roscovitine. CDK2 levels were not influenced by **4k** treatment, although its levels were reduced at high concentrations of roscovitine. Phosphorylation of Thr-170 of CDK2 was, however, reduced following 24 h treatment with **4k**. Together, these results indicate a rapid inhibition of CDK activity following **4k** addition, with slower acting effects on cyclin levels.

In Vivo Pharmacokinetic Studies and Tumor Growth Inhibition. To determine whether **4k** inhibits tumor growth in vivo, nude mice bearing MCF-7 tumors were injected intraperitoneally twice daily with 5 or 10 mg/kg, to give total daily doses of 10 mg/kg or 20 mg/kg, over a period of 14 days. Tumor growth was inhibited in a dose-dependent manner, with 30% and 40% reduction in tumor growth, compared with the control group, for 10 and 20 mg/kg/day doses of **4k**, respectively (Figure 5A). At these doses, there was no apparent toxicity, as judged by lack of significant adverse effects on animal weights (Figure 5B).

Compound **4k** was found to have favorable absorption, distribution, metabolism, elimination, and toxicity (ADME-Tox) properties. The drug had an aqueous solubility (PBS, pH 7.4) of 54.0 μmol/L (range, 47.16–60.89 μmol/L) and partition coefficient (Log *D*, *n*-octanol: PBS pH 7.4) of 3.0. In vitro cellular absorption (A–B permeability, TC7, pH 6.5/7.4) of **4k** was 9.7 × 10⁻⁶ cm·s⁻¹ (range, 9.32–10.06 × 10⁻⁶ cm·s⁻¹; recovery = 91±%) at a test concentration of 10 μmol/L. In the same assay, the absorption of propranolol used as control was 54.2 × 10⁻⁶ cm·s⁻¹ (range, 53.64–54.81 × 10⁻⁶ cm·s⁻¹; recovery = 85±%). Whereas plasma protein binding did not differ significantly between mouse and human, microsomal metabolism was found to be more favorable for human compared to mouse liver microsomes (Table 4). Injection of **4k** at 10 mg/kg into mice via the intraperitoneal (IP), intravenous (IV), and oral (PO) routes of administration of resulted in terminal elimination half-lives of 147, 210, and 178 min, respectively, with the measured plasma concentration at 15 min of 10474 (SEM = 5634), 25351 (SEM = 780), and 9347 (SEM = 551) ng/mL, respectively (Table 5; Figure 6). One of two metabolites detected after intravenous administration of **4k** was identified as an oxidative (hydroxylated) metabolite (data not shown).

Pharmacokinetic studies indicated good oral bioavailability (88%; *n* = 3 animals for 8 time points analyzed for each of PO and IV administration) for **4k**. To determine whether treatment with **4k** elicits responses in vivo, **4k** was administered by oral gavage to nu/nu-BALB/c athymic nude mice. Immunostaining and flow cytometric analysis of peripheral blood mononuclear cells (PBMC) isolated 6 h following administration showed that **4k** at concentrations of 25 mg/mL induced a 60–70% reduction in RB phosphorylation at Thr-821 (Figure 7). There were also 25–50% fall in PolII Ser-2 and Ser-5 phosphorylation with 25 mg/mL **4k**, with reductions in phosphorylation of PolII being maximal at 100 mg/mL **4k**, although at concentrations of 50 mg/mL or higher, PolII levels also fell. No difference in RB Thr-821 or PolII phosphorylation was evident 24 h following administration with 50 mg/mL **4k**, although RB Thr-821 phosphorylation was reduced by 50% (*p* < 0.01), 24 h after administration of 75 mg/mL **4k** (data not shown).

The above results indicated that oral administration of **4k** results in rapid down-regulation of RB and PolII phosphorylation

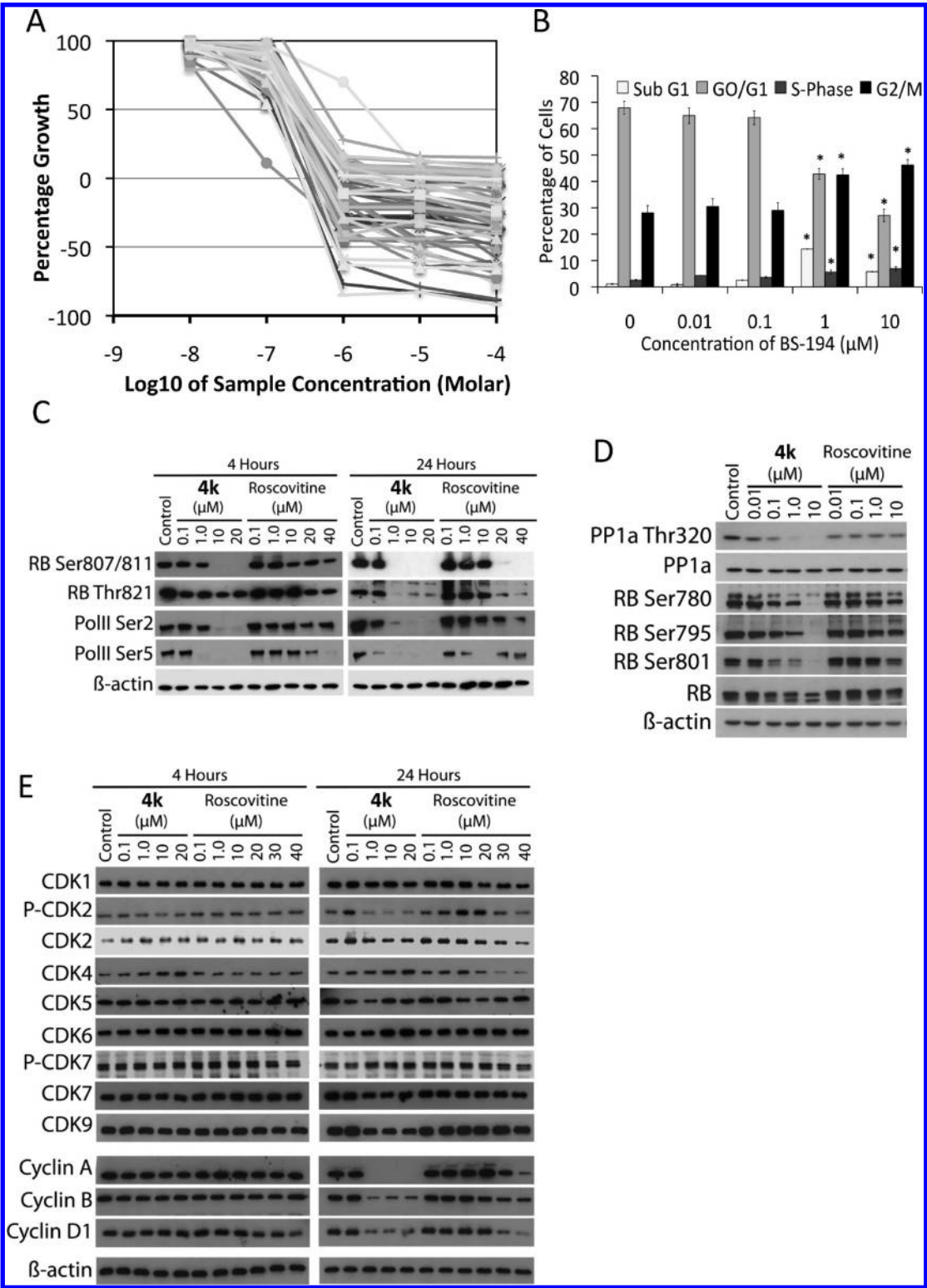


Figure 4. Compound **4k** treatment of HCT116 cells leads to cancer cell growth inhibition, cell cycle arrest, and inhibition of CDK substrate phosphorylation. (A) Growth inhibition profiles for the NCI 60 cancer cell panel. For details of the growth profiles of the individual cell lines see Supporting Information. (B) Effect of **4k** on cell cycle kinetics. **4k** at the concentrations shown was added to HCT116 cells for 24 h prior to fixation, staining with propidium iodide (PI), and flow cytometric analysis. The percentage of cells in the sub-G1 (apoptosis), G1, S-phase, and G2/M, as determined from three independent experiments, are shown. Error bars represent the standard errors of the mean (SEM). *P*-Values were determined using Student's *t* test, in which comparison between the DMSO control, and **4k** treatment at each concentration was carried out. Asterisks (*) denote *p* < 0.05. (C–E) Molecular pharmacology of **4k** on HCT116 cells. Cell lysates prepared from HCT116 cells treated with **4k** or roscovitine for 4 or 24 h were immunoblotted for various proteins or phosphorylated forms, as labeled.

but recovery within 24 h. To determine if oral administration of **4k** inhibits the growth of HCT116 tumor xenografts, animals

were treated once daily with 25 mg/kg **4k** by oral gavage. Tumor growth was inhibited in a dose-dependent manner, with

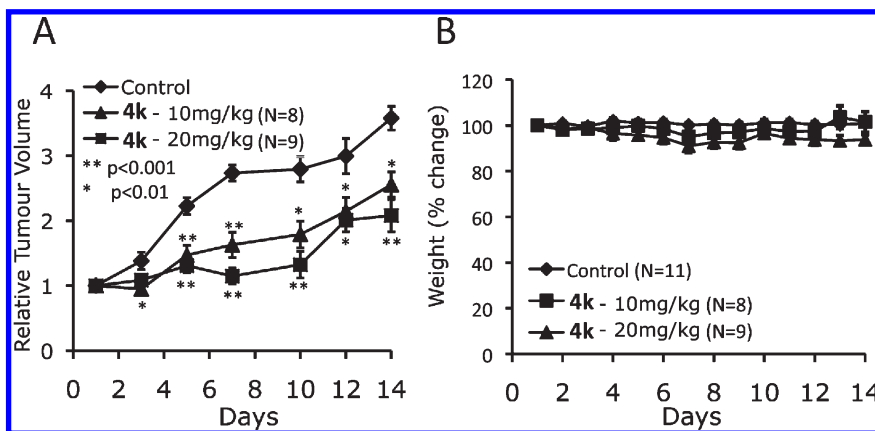


Figure 5. Compound **4k** inhibits growth in human breast tumor xenografts. (A) Randomized MCF-7 tumor bearing mice were injected intraperitoneally twice daily with vehicle (control, $n = 11$) or with 5 or 10 mg/kg of **4k**, giving a total daily dose of 10 mg/kg/day ($n = 8$) or 20 mg/kg/day ($n = 9$), respectively, over a period of 14 days. The change in tumor volume was determined for each animal, as tumor volume relative to the tumor volume of each animal at day 1. The line graphs show the mean tumor volumes for the animals in each treatment group. (B) Shown are the weights of the animals used over the time course of the study. Error bars represent the standard errors of the mean.

Table 4. In Vitro Plasma Protein Binding and Microsomal Metabolism of **4k**^a

plasma protein binding		
test concentration, μM	% protein bound (human)	% protein bound (mouse)
4k , 10	94.3 (94.0–94.5)	88.3 (86.0–90.6)
quinidine, 10	71.1 (65.3–76.9)	71.1 (70.0–72.2)
in vitro metabolism (metabolic stability) (liver microsomes)		
test concentration, μM	parent remaining (human) (%)	parent remaining (human) (%)
4k , 1.0	83 (80.6–84.8)	29 (28.8–29.1)
propranolol, 1.0	74 (73.5–75.2)	37 (34.7–39.6)

^a Data are mean values (range). Incubation: 0 and 60 min at 37 °C. Substrate/co-factor: test compound (1 μM), NADP (1 mM), G6P (5 mM), G6PDHase (1 U/mL) with 0.6% methanol, 0.6% acetonitrile ($n = 2$).

50% reduction in tumor growth in the 25 mg/kg/day group (Figure 8A), with no significant loss in animal weights in the vehicle or **4k** treatment groups. (Figure 8B).

Immunohistochemical staining of resected tumors showed no difference in Rb level in the vehicle or **4k** treatment groups (Figure 8C,D). By contrast, levels of Rb phosphorylation at Ser807/811 and Thr821 were decreased in the **4k** treated tumors when compared with the vehicle treated group ($p < 0.005$).

Discussion

Recent studies show that inhibition of individual CDKs required for cell cycle progression may be ineffective cancer treatments either because they are not essential or due to potential compensation by other CDKs.³ For example, in the case of CDK2, a popular target for drug discovery due to its role in progression through the S phase, inhibition of this kinase was insufficient to prevent cancer cell proliferation,³¹ whereas combined depletion of CDK2 and CDK1 in cancer cells resulted in greater cell cycle arrest than seen with depletion of CDK2 or CDK1 alone.⁹ In this context, the potent CDK1 and CDK2 inhibitory activity of **4k** provides an attractive therapeutic approach. Further, inhibition of CDK9 activity and consequent reduction in PolII activity has been shown to augment the growth inhibitory effects of CDK1 and CDK2 inhibition in cancer cell lines;⁹ here, **4k** inhibited CDK9, as well as CDK7 and

reduced PolII phosphorylation at Ser-2 and Ser-5 in vitro and in vivo. Phosphorylation of Rb regulates its interaction with E2F transcription factors, interaction with hypophosphorylated Rb repressing the expression of E2F-regulated genes. Many other interactions have been described, in particular, with proteins containing LXCXE motifs, including histone deacetylases HDAC1 and HDAC2 and chromatin remodelling proteins, with Rb phosphorylation also being critical for these interactions.^{32,33} Rb phosphorylation was inhibited at all sites studied, with the observed inhibition being considerably greater for **4k** than that observed for equivalent concentrations of roscovitine, in keeping with the much better growth inhibition seen for **4k** compared with roscovitine. Phosphorylation of all Rb sites was abolished with 10 $\mu\text{mol/L}$ of **4k**, with substantial inhibition being obtained at concentrations as low as 0.1 $\mu\text{mol/L}$ for Thr821, although complete inhibition of Thr821 phosphorylation was not achieved even at high concentrations of **4k**, which may be a reflection of that fact that in addition to being phosphorylated by CDK2, Thr821 can be phosphorylated by CDK6.^{34,35} Phosphorylation of PP1a at Thr-320 was inhibited in a dose-dependent manner, likely reflecting the inhibition of CDK1 activity by **4k**. These analyses were reflected in the accumulation of cells in the S and G2/M phases of the cell cycle, with a concomitant reduction in cells in the G1 phase.

As expected from the potent inhibition of CDK1, CDK2, and CDK9 by **4k** and the inhibition of Rb and PolII phosphorylation, the growth of diverse cancer cell lines was inhibited, with GI_{50} values ranging between 0.1 and 1.0 $\mu\text{mol/L}$. This was confirmed by growth studies performed with the NCI60 cancer cell line panel, GI_{50} values ranging between 0.1 and 0.5 $\mu\text{mol/L}$, with the exception of the multidrug resistant NCI/Adr-Res ovarian cancer cell line, which was inhibited somewhat less well ($\text{GI}_{50} = 2.0 \mu\text{mol/L}$). The growth of human normal vascular endothelial cells was also inhibited less well than the growth of the cancer lines ($\text{GI}_{50} = 6.3 \mu\text{mol/L}$), suggestive of some selectivity for cancer cells over normal cells.

The physicochemical and predictive ADME-Tox properties of **4k**, including aqueous solubility, cell permeability, and plasma protein binding were consistent with utility for in vivo studies. Pharmacokinetic studies demonstrated good oral bioavailability, which was confirmed by single oral dose administration, followed by collection of PBMC and FACS

Table 5. Pharmacokinetic Parameters for **4k**^a

route of administration	dose (mg/kg)	bioavailability (%)	$T_{\max}$ (min)	$C_{\max}$ (min)	$T_{1/2}$ (min)	AUC_{last} (min·ng/mL)	AUC_{INF} (min·mg/mL)	terminal points
PI	10	73	30	15604	147	1583520	158188	3
PO	10	88	15	9347	178	1922912	1926751	3
route of administration	dose (mg/kg)	$T_{1/2}$ (min)	Cl (mL/min/kg)	V_z (mL/kg)	V_{ss} (mL/kg)	AUC_{last} (min·ng/mL)	AUC_{INF} (min·mg/mL)	terminal points
IV	10	210	5	1391	754	2174209	2182968	3

^aThe studies were carried out in mice and the pharmacokinetic parameters were derived from noncompartmental model using WinNonlin 5.2. $T_{1/2}$ = elimination half-life. Cl = total body clearance. V_z = volume of distribution. V_{ss} = an estimate of the volume of distribution at steady state. AUC_{last} = area under the concentration–time curve from the time of dosing to the time of last observation that is greater than the limit of quantitation. AUC_{INF} = area under the concentration–time curve from the time of dosing, extrapolated to infinity. $T_{\max}$ = time of maximum observed concentration. $C_{\max}$ = concentration corresponding to $T_{\max}$. The bioavailability was calculated based on AUC_{INF} . Terminal points = the number of observations used to calculate the terminal slope.

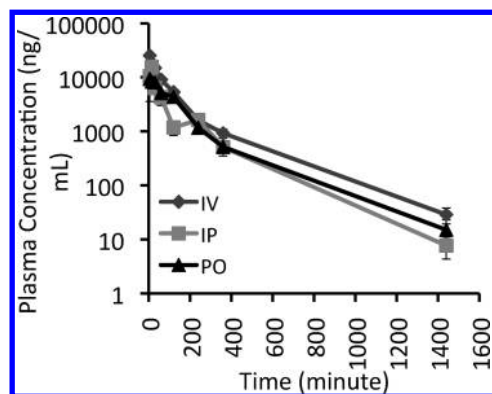


Figure 6. Pharmacokinetic determination of **4k** levels. The mean plasma concentration of **4k** over time was determined for four mice at each time point following **4k** administration. Error bars represent the standard errors of the mean.

analysis of Rb and PolII phosphorylation after 6 h. Inhibition of Rb Thr-821 phosphorylation was maximal with a dose of 25 mg/kg. Although PolII Ser-2 phosphorylation was decreased in a dose-dependent manner from 10 to 100 mg/kg, PolII levels were also seen to fall at doses of 50 and 100 mg/kg. Importantly, even at doses of 75 and 100 mg/kg, no significant reduction in PolII or Rb phosphorylation was noted 24 h following **4k** administration, which may be a reflection of the fact that the plasma concentration of **4k** are likely to be below the concentrations that demonstrate inhibition of Rb and PolII phosphorylation in cell lines beyond 6 h post administration. Nevertheless, single daily ip and oral administration of **4k** showed tumor growth inhibition in xenograft models at doses of 10 mg/kg ip in MCF-7 xenografts and 25 mg/kg po in HCT116 xenografts. Further, using Rb phosphorylation as an immunohistochemical marker of CDK activity, we have been able to observe that oral **4k** administration results in reduced Rb phosphorylation in the tumors. This is indicative of a relationship between pharmacodynamic factors and therapeutic outcome for **4k**.

Conclusions

Several selective and broad-specificity CDK inhibitors have now entered clinical trials. Phase I studies show that they can generally be safely administered. Of particular note is PD0332991, a selective CDK4/CDK6 inhibitor, as well as agents with broader selectivity, a few of which have been progressed to phase II studies either as single agents or in combination with chemotherapeutic agents such as cisplatin, gemcitabine, and capecitabine.²³ Here we have described the

identification and preclinical evaluation of a potent orally active CDK inhibitor, which demonstrates selectivity for CDK1, CDK2, CDK5, and CDK9, with moderate inhibition in vitro of CDK7 and which is a poor inhibitor of CDK4 and CDK6. Compound **4k** selectively exhibited nanomolar enzymatic potency, potently inhibited all cell lines tested, and demonstrated inhibition of phosphorylation of CDK substrates. A pyrazolopyrimidine compound (Dinaciclib; SCH 727965) which is similar to **4k** in selectively inhibiting CDK1, CDK2, CDK5, and CDK9 with IC_{50} values of 1–4 nmol/L, which shows tumor regression in mouse models²⁵ and is well tolerated in patients following IV administration in a phase I setting,²³ has recently been described. Our previous description of a CDK7 specific pyrazolopyrimidine-based compound,²⁴ together with the identification of **4k**, show that pyrazolopyrimidine-based compounds have considerable potential for development as potent CDK inhibitors. Finally, the oral bioavailability of **4k** highlights it as an important new CDK inhibitor, with potential for clinical development.

Materials and Methods

General Synthetic Methods. All manipulations of air or moisture sensitive materials were carried out in oven- or flame-dried glassware under an inert atmosphere of nitrogen or argon. Syringes, which were used to transfer reagents and solvents, were purged with nitrogen prior to use. Reaction solvents were distilled from CaH_2 (CH_2Cl_2 , PhMe, Et_3N), Na/Ph₂CO (THF, Et_2O), or obtained as dry or anhydrous from Aldrich Chemical Co. (DMF, MeCN) or BDH ($EtOH$). Other solvents and all reagents were obtained from commercial suppliers and were used as obtained if purity was > 98%. All flash column chromatography was carried out on silica gel 60 particle size 0.040–0.063 mm, unless otherwise stated. Thin layer chromatography (TLC) was performed on precoated aluminum backed or glass backed plates and visualized under ultraviolet light (254 nm) or following spraying with potassium permanganate, vanillin, or phosphomolybdic acid (PMA) stains as deemed appropriate. LC-MS analysis was performed using a Waters LCT Premier XE equipped with a Waters Atlantis C18-reverse phase column of length 30 mm, inner diameter 2.1 mm and particle size 3 μ m using a mobile phase of water (0.1% formic acid):acetonitrile, monitoring at 254 nm. Compound purity was determined by combustion analysis (elemental analysis) or LC-MS analysis and was confirmed to be > 95% for all compounds.

Dichloride **1** was prepared according to methods described by ref 51.

Benzyl-(5-chloro-3-iso-propylpyrazolo[1,5-*a*]pyrimidin-7-yl)-amine. 5,7-Dichloro-3-iso-propyl-pyrazolo[1,5-*a*]pyrimidine (**1**) (15.5 g, 67.0 mmol) and benzylamine (14.7 mL, 134 mmol) in $EtOH$ (500 mL) were heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature, concentrated in vacuo, and the residue recrystallized from $EtOAc$. The mother

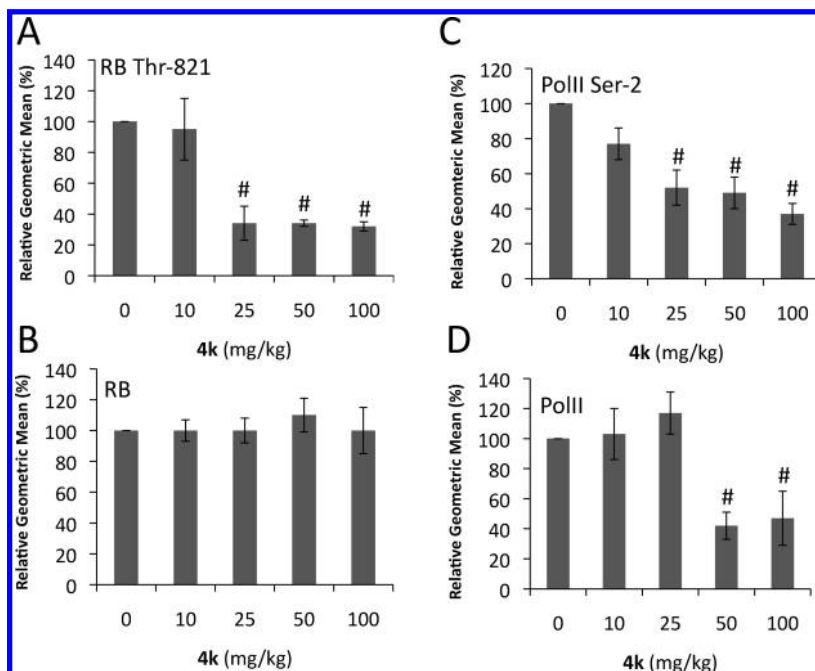


Figure 7. Oral administration of **4k** inhibits cyclin-dependent kinase substrate phosphorylation in mouse peripheral blood mononuclear cells. Mouse PBMCs were collected 6 h following dosing of female nude mice by oral gavage at the dose levels shown. PBMCs were incubated with the appropriate primary antibodies, followed by fluorescently labeled secondary antibodies and flow cytometric analysis ($n = 4$, except for the 100 mg/kg dose where $n = 3$) to determine the status of (A) Rb, (B) Rb phosphorylated at threonine-821 (Rb Thr821), (C) RNA polymerase II Ser-2 phosphorylation (PolII Ser-2), and (D) total PolII. Error bars represent the standard errors of the mean. P -Values were determined using Student's t test; # represents $p < 0.05$.

liquor was chromatographed on silica (EtOAc:hexanes 1:8) to yield the title pyrazolo[1,5-*a*]pyrimidine as a white solid. The combined yield of the crystallized and chromatographed material was 19.3 g, 96%; mp 78–79 °C (EtOAc); TLC Rf 0.16 (EtOAc:hexanes 1:16). IR (neat) 3380, 3239, 1616, 1585 cm^{-1} . ^1H NMR (CDCl_3 , 400 MHz) δ 7.84 (s, 1H), 7.40–7.32 (m, 5H), 6.88 (m, 1H), 5.91 (s, 1H), 4.55 (m, 2H), 3.28 (sept, $J = 6.9$ Hz, 1H), 1.33 (d, $J = 6.9$ Hz, 6H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 150.2, 146.9, 144.2, 141.6, 135.8, 129.1, 128.3, 127.3, 117.0, 84.7, 46.2, 23.5, 23.4. MS m/z (ESI) 301 ($M + \text{H}^+$). HRMS (ESI) calcd for $\text{C}_{16}\text{H}_{18}\text{ClN}_4^+$, 301.1215; found, 301.1227. Anal. Calcd for $\text{C}_{16}\text{H}_{17}\text{ClN}_4$: C, 63.89; H, 5.70; N, 18.63. Found: C, 63.95; H, 5.78; N, 18.59.

***tert*-Butyl-benzyl-(5-chloro-3-*iso*-propylpyrazolo[1,5-*a*]pyrimidin-7-yl)-carbamate Monohydrate (2).** Benzyl-(5-chloro-3-*iso*-propylpyrazolo[1,5-*a*]pyrimidin-7-yl)-amine (17.0 g, 56.7 mmol), Boc_2O (16.1 g, 73.7 mmol), and DMAP (100 mg) in THF (400 mL) were stirred for 20 h at ambient temperature. EtOAc (300 mL) was added, and the organic layer was washed with water (2×400 mL) and saturated aqueous NaHCO_3 (400 mL) and dried (Na_2SO_4). Concentration in vacuo, chromatography (EtOAc:hexanes 1:8), and recrystallization from EtOAc and hexanes (1: 8) gave carbamate **2** (22.6 g, 100%) as a pale-yellow solid; mp 100–103 °C (EtOAc); TLC Rf 0.40 (EtOAc:hexanes 1:8). IR (neat) 1612 cm^{-1} . ^1H NMR (CDCl_3 , 400 MHz) δ 8.02 (s, 1H), 7.34–7.21 (m, 5H), 6.48 (s, 1H), 5.03 (s, 2H), 3.31 (sept, $J = 6.9$ Hz, 1H), 1.52 (s, 2H), 1.39 (s, 9H), 1.37 (d, $J = 6.9$ Hz, 6H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 152.8, 148.0, 145.1, 144.3, 142.6, 136.8, 128.7, 127.8, 127.8, 118.3, 106.3, 83.0, 51.4, 28.0, 23.6, 23.4. MS m/z (ESI) 401 ($M + \text{H}^+$). HRMS (ESI) calcd for $\text{C}_{21}\text{H}_{26}\text{ClN}_4\text{O}_2^+$, 401.1739; found, 401.1735. Anal. Calcd for $\text{C}_{21}\text{H}_{27}\text{ClN}_4\text{O}_3$: C, 60.21; H, 6.50; N, 13.37. Found: C, 60.28; H, 6.58; N, 13.43.

General Procedure for Buchwald–Hartwig Coupling Reaction. *tert*-Butyl-benzyl-(5-chloro-3-*iso*-propylpyrazolo[1,5-*a*]pyrimidin-7-yl)-carbamate (1.0 equiv), tris(dibenzylideneacetone)dipalladium (0.05 equiv), 2,2'-bis(diphenylphosphino)-1,1'-binaphthalene (0.15 equiv), and sodium *tert*-butoxide (1.1 equiv) were suspended

in PhMe (0.30 M) and stirred for 15 min. The amine (1.1 equiv) was added and the mixture heated to 95 °C for 16 h and cooled and diluted with EtOAc. The organic phase was washed with water ($3 \times$), brine, dried (MgSO_4), and concentrated in vacuo. Chromatography on silica (EtOAc:hexanes) gave the pure coupled products.

General Procedure for the Final Acid Deprotection. AcCl was dissolved in MeOH (5.0 M) with stirring at 0 °C. After 15 min, the solution was warmed to room temperature and stirred for a further 15 min. The ambient was added and the mixture stirred at ambient temperature for 3 h and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 , washed with saturated aqueous sodium bicarbonate, dried (Na_2SO_4), and concentrated in vacuo. Crystallization (MeCN or MeOH) or chromatography (CH_2Cl_2 : MeOH) gave the pure analogues.

Compound **5** was prepared according to Campbell et al.³⁶

(*R*)-1-(*tert*-Butyldimethylsilyloxy)-3-buten-2-amine (6). Et_3N (5.00 mL, 35.6 mmol), DMAP (20.0 mg), and $t\text{-BuMe}_2\text{SiCl}$ (2.70 g, 17.8 mmol) were added to amino-alcohol **5** (2.00 g, 16.2 mmol) in CH_2Cl_2 (80 mL). The mixture was stirred overnight at ambient temperature. Water (80 mL) was added, and the mixture was vigorously stirred for 10 min. The organic phase was separated, washed with water (60 mL) and brine (60 mL), and dried (Na_2SO_4). Concentration in vacuo gave amine **6** (2.83 g, 43%) as a yellow oil; TLC Rf 0.55 (EtOAc:hexanes 1:1); $[\alpha]_D^{25}$ (c 1.09, CH_2Cl_2) + 22.8. IR (neat) 1471, 1254, 1095 cm^{-1} . ^1H NMR (CDCl_3 , 300 MHz) δ 5.80 (m, 1H), 5.14 (m, 2H), 3.60 (m, 1H), 3.42 (m, 2H), 1.61 (m, 2H), 0.89 (s, 9H), 0.05 (s, 6H). ^{13}C NMR (CDCl_3 , 75 MHz) δ 139.2, 115.1, 67.8, 55.8, 25.9, 18.3, -5.4. MS m/z (CI) 202 ($M + \text{H}^+$). HRMS (CI) calcd for $\text{C}_{10}\text{H}_{24}\text{NOSi}^+$, 202.1622; found, 202.1622.

(*R*)-*tert*-Butyl Benzyl(5-((1-(*tert*-butyldimethylsilyl)oxy)but-3-en-2-yl)amino)-3-*iso*-propylpyrazolo[1,5-*a*]pyrimidin-7-yl)-carbamate (3f). Synthesized according to the general procedure for Buchwald–Hartwig coupling, **2** (100 mg, 0.25 mmol), Pd_2dba_3 (12.0 mg, 0.013 mmol), BINAP (24.0 mg, 0.039 mmol), sodium *tert*-butoxide (36.0 mg, 0.38 mmol), amine **6** (58.0 mg, 0.27 mmol), and PhMe (0.80 mL) yielded carbamate **3f** (72.0 mg,

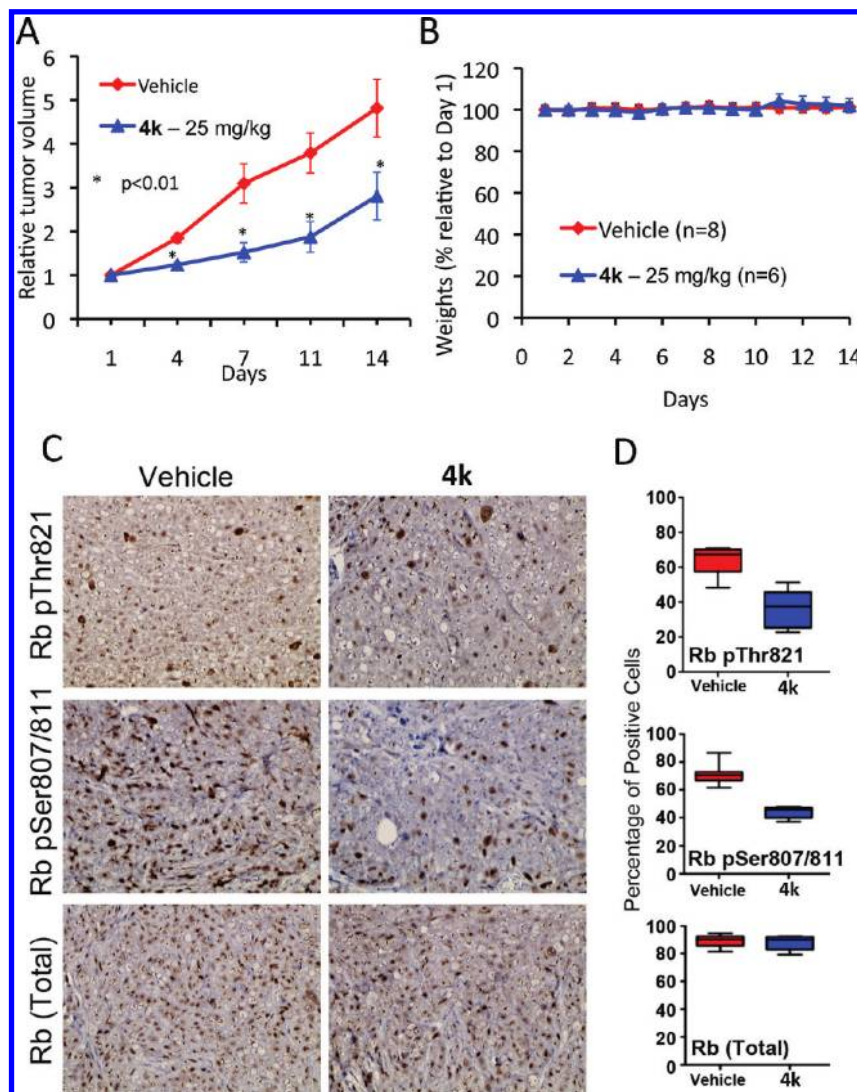


Figure 8. Oral administration of **4k** inhibits human tumor xenografts. (A) Female nude mice bearing HCT116 tumor xenografts were dosed by oral gavage with vehicle ($n = 8$) or **4k** at a concentration of 25 mg/kg ($n = 6$). (B) The weights of the animals used over the time course of the study are shown. Error bars represent the standard errors of the mean. P -Values were determined using Student's t test; # represents $p < 0.05$, * represents $p < 0.01$, and ** represents $p < 0.001$. (C) Shown are representative images for tumor xenografts immunostained using antibodies for Rb, Rb pSer807/811, and Rb pThr821. (D) One thousand nuclei were scored for determining percentage of stained cells in sections from each tumor. The results of the scoring are shown as box plots. Student's t test analysis showed that pSer807/811 and pThr821 staining was significantly different ($p < 0.005$) between the vehicle and **4k** treatment groups, whereas there was no significant difference in levels of total Rb.

0.13 mmol, 51%). Purification EtOAc:hexanes (1:10): [α]_D (c 0.59, CH₂Cl₂) + 16.3. IR (neat) 3370, 1722, 1642, 1581, 1516, 1368, 1158, 1107, 837, 777, 699 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz) δ 7.75 (s, 1H), 7.27 (m, 5H), 5.85 (m, 1H), 5.71 (s, 1H), 5.19 (m, 2H), 4.97 (br s, 1H), 4.95 (br s, 2H), 4.54 (m, 1H), 3.75 (m, 2H), 3.08 (m, 1H), 1.40–1.31 (m, 15H), 0.88 (s, 9H), 0.05 (s, 6H). ¹³C NMR (CDCl₃, 75 MHz) δ 154.0, 153.5, 146.1, 142.8, 141.5, 137.7, 136.4, 128.4, 127.9, 127.4, 116.2, 113.3, 97.2, 82.0, 65.0, 54.6, 51.3, 28.0, 25.9, 23.8, 23.1, 18.3, -5.4. MS m/z (CI) 566 (M + H)⁺. HRMS (CI) calcd for C₃₁H₄₈N₅O₃Si⁺, 566.3526; found, 566.3538.

tert-Butyl Benzyl(5-(((2S,3S)-1-((tert-butyldimethylsilyl)oxy)-3,4-dihydroxybutan-2-yl)amino)-3-iso-propylpyrazolo[1,5-*a*]pyrimidin-7-yl)carbamate (3k). *tert*-Butyl Benzyl(5-(((2S,3R)-1-((tert-butyldimethylsilyl)oxy)-3,4-dihydroxybutan-2-yl)amino)-3-iso-propylpyrazolo[1,5-*a*]pyrimidin-7-yl)carbamate (**3l**). OsO₄ (5.40 mL, 15 mol %, 2.5 wt % in ¹BuOH) was added to alkene **3f** (2.27 g, 4.00 mmol) and NMO H₂O (0.97 g, 8.3 mmol) in MeCN and H₂O (4:1, 80 mL). After 16 h, reaction was quenched by the addition of saturated aqueous Na₂SO₃ (20 mL). The mixture was stirred for

45 min at ambient temperature, and the aqueous phase was extracted with EtOAc (3 × 200 mL). The combined organic phases were dried (Na₂SO₄), concentrated in vacuo, and chromatographed (EtOAc:hexanes 1:20) to yield carbamate **3k** (1.00 g, 1.67 mmol, 42%) and carbamate **3l** (800 mg, 1.34 mmol, 33%). **3k**: IR (neat) 3363, 1721, 1644, 1518 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz) δ 7.80 (s, 1H), 7.36–7.27 (m, 5H), 5.72 (s, 1H), 5.24–5.22 (m, 1H), 4.98–4.94 (m, 3H), 4.18–4.11 (m, 1H), 3.95–3.90 (m, 1H), 3.79–3.76 (m, 1H), 3.64–3.57 (m, 3H), 3.10 (hept, $J = 6.9$ Hz, 1H), 2.84–2.81 (m, 1H), 1.43 (s, 9H), 1.33 (d, $J = 6.9$ Hz, 6H), 0.93 (s, 9H), 0.13–0.12 (m, 6H). ¹³C NMR (CDCl₃, 100 MHz) δ 154.8, 153.4, 141.7, 137.6, 128.5, 127.9, 127.6, 113.8, 97.4, 83.8, 82.5, 70.4, 62.5, 61.8, 53.5, 51.7, 28.0, 25.9, 23.6, 23.5, 23.2, -5.4. MS m/z (CI) 600 (M + H)⁺. HRMS (CI) calcd for C₃₁H₅₀N₅O₅Si⁺, 600.3576; found, 600.3578.

3l: IR (neat) 3361, 1719, 1644, 1518 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz) δ 7.77 (s, 1H), 7.32–7.23 (m, 5H), 5.78 (s, 1H), 5.27–5.25 (m, 1H), 4.98–4.89 (m, 2H), 4.41–4.31 (m, 2H), 4.14–4.09 (m, 1H), 4.00–3.90 (m, 2H), 3.79–3.75 (m, 1H), 3.61–3.57 (m, 1H), 3.40–3.36 (m, 1H), 3.11–3.04 (m, 1H), 1.40

(s, 9H), 1.32–1.28 (m, 6H), 0.89 (s, 9H), 0.08–0.06 (m, 6H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 141.7, 137.6, 128.5, 127.9, 127.6, 113.8, 97.4, 82.5, 70.4, 62.4, 61.8, 60.4, 53.5, 51.6, 28.0, 25.9, 23.6, –5.4. MS m/z (CI) 600 ($\text{M} + \text{H}$) $^+$. HRMS (CI) calcd for $\text{C}_{31}\text{H}_{50}\text{N}_5\text{O}_5\text{Si}^+$, 600.3576; found, 600.3574.

(2*R*,3*S*)-3-(7-(Benzylamino)-3-iso-propylpyrazolo[1,5-*a*]pyrimidin-5-ylamino)-1,2,4-butanetriol (4k). Synthesized according to the general procedure for acidic deprotection. **3k** (605 mg, 1.01 mmol), HCl/MeOH (50.0 mL). Yield triol **4k** (310 mg, 0.81 mmol, 79%). Purification crystallization from MeCN : mp 184–186 °C; $[\alpha]_{\text{D}}^{25}$ (c 0.20, CH_3OH) –25.0. IR (neat) 3229, 1639, 1581 cm^{-1} . ^1H NMR (CD_3OD , 400 MHz) δ 7.69 (s, 1H), 7.42–7.27 (m, 5H), 5.32 (s, 1H), 4.56 (s, 2H), 4.10–4.04 (m, 1H), 3.87 (dd, J = 11.4, 4.1 Hz, 1H), 3.84 (dd, J = 11.1, 5.5 Hz), 3.63–3.56 (m, 3H), 3.05 (hept, J = 6.9 Hz, 1H), 1.31 (d, J = 6.9 Hz, 3H), 1.29 (d, J = 6.9 Hz, 3H). ^{13}C NMR (CD_3OD , 100 MHz) δ 157.6, 146.9, 144.9, 140.6, 137.7, 128.4, 127.1, 126.6, 112.1, 72.8, 72.0, 63.0, 61.7, 54.3, 45.1, 23.3, 22.6, 22.2. MS m/z (ESI) 386 ($\text{M} + \text{H}$) $^+$. HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{28}\text{N}_5\text{O}_3^+$, 386.2187; found, 386.2181.

(2*R*,3*S*)-3-(7-(Benzylamino)-3-iso-propylpyrazolo[1,5-*a*]pyrimidin-5-ylamino)-1,2,4-butanetriol (4l). Synthesized according to the general procedure for acidic deprotection. **3l** (765 mg, 1.28 mmol), HCl/MeOH (50.0 mL). Yield **4l** (338 mg, 0.86 mmol, 67%). Purification chromatography (EtOAc): $[\alpha]_{\text{D}}^{25}$ (c 0.20, CH_3OH) –60.0. IR (neat) 3307, 1637, 1579 cm^{-1} . ^1H NMR (CD_3OD , 400 MHz) δ 7.68 (s, 1H), 7.40–7.25 (m, 5H), 5.34 (s, 1H), 4.54 (s, 2H), 4.23–4.20 (m, 1H), 3.93–3.89 (ddd, J = 8.3, 5.9, 2.2 Hz, 1H), 3.80 (dd, J = 11.0, 6.2 Hz, 1H), 3.77 (dd, J = 11.0, 7.0 Hz, 1H), 3.52 (dd, J = 11.5, 6.1 Hz, 1H), 3.39 (dd, J = 11.0, 8.7 Hz, 1H), 3.03 (hept, J = 6.9 Hz, 1H), 1.31 (d, J = 6.9 Hz, 3H), 1.29 (d, J = 6.9 Hz, 3H). ^{13}C NMR (CD_3OD , 100 MHz) δ 158.0, 146.9, 144.5, 140.1, 137.7, 128.3, 127.1, 126.7, 112.0, 72.8, 70.8, 62.2, 62.0, 53.4, 45.1, 23.2, 22.6, 22.2. MS m/z (ESI) 386 ($\text{M} + \text{H}$) $^+$. HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{28}\text{N}_5\text{O}_3^+$, 386.2187; found, 386.2189.

Second Generation Synthesis of 4k. (2*R*,3*S*)-Diethyl 2-azido-3-hydroxysuccinate (9). (–)-Diethyl tartrate (10.0 g, 48.5 mmol) in CH_2Cl_2 (250 mL) was cooled to –5 °C. Et_3N (13.5 mL, 97.0 mmol) was added, followed by the dropwise addition of SOCl_2 (5.28 mL, 72.7 mmol). After 30 min, the mixture was poured into water and the aqueous layer extracted into CH_2Cl_2 (3 $\times$). The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated in vacuo to yield the cyclic sulfite which was directly dissolved in DMF (400 mL), and sodium azide (6.30 g, 97.0 mmol) was added. The mixture was stirred for 12 h and then poured into water and extracted into Et_2O (3 $\times$). The combined organic layers were washed with brine, dried (MgSO_4), and concentrated in vacuo. Chromatography (hexanes: EtOAc 4:1) gave azide **9** (7.79 g, 33.7 mmol, 70%); $[\alpha]_{\text{D}}^{25}$ (c 1.0, EtOH) –39.4. ^1H NMR (CDCl_3 , 400 MHz) δ 4.66 (dd, J = 5.5, 2.7 Hz, 1H), 4.32 (m, 4H), 3.31 (d, J = 5.5 Hz, 1H), 1.35 (t, J = 7.1 Hz, 3H), 1.34 (t, J = 7.1 Hz, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 170.6, 166.9, 71.5, 64.2, 62.6, 62.0, 14.0.

(*S*)-4-((*S*)-1-Azido-2-(methoxymethoxy)ethyl)-2,2-dimethyl-1,3-dioxolane (11). Diester **9** (5.00 g, 21.6 mmol) in EtOH (80.0 mL) was cooled to –10 °C and lithium chloride (909 mg, 21.6 mmol) was added, followed by portionwise addition of sodium borohydride (4.80 g, 130 mmol). The suspension was warmed overnight, recooled to –10 °C, and acidified to pH4 by the dropwise addition of 1 M HCl in H_2O . The resultant clear solution was concentrated in vacuo to yield crude triol **10**, which was directly suspended in Me_2CO (80.0 mL) and 2,2-dimethoxypropane (25.0 mL). p -TsOH. H_2O (400 mg, 2.1 mmol) was added and the mixture heated to 50 °C for 2.5 h, cooled, and saturated ammonium chloride solution (60.0 mL) was added. The suspension was stirred for 1 h, poured into water, and extracted into Et_2O (3 $\times$). The combined organic layers were washed with brine, dried (MgSO_4), and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (25.0 mL), cooled to 0 °C, and N,N -di-*iso*-propylethylamine (7.51 mL, 43.2 mmol) was added followed by the slow

addition of MeOCH_2Cl (3.26 mL, 43.2 mmol) and the mixture was stirred overnight. The solution was poured into water and extracted into CH_2Cl_2 (3 $\times$). The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated in vacuo. Chromatography (hexanes: EtOAc 10:1) gave azide **11** (3.49 g, 15.1 mmol, 70%); $[\alpha]_{\text{D}}^{25}$ (c 1.2, CHCl_3) +18.6. IR (neat) 3229, 2100, 1373, 1259 cm^{-1} . ^1H NMR (CDCl_3 , 400 MHz) δ 4.68 (s, 2H), 4.14–4.06 (m, 2H), 3.97–3.94 (m, 1H), 3.84–3.78 (m, 1H), 3.71–3.64 (m, 2H), 3.42 (s, 3H), 1.47 (s, 3H), 1.37 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 109.8, 96.7, 74.8, 67.3, 66.6, 63.0, 55.5, 26.5, 25.2. MS m/z (ESI) 249 ($\text{M} + \text{NH}_4$) $^+$. HRMS (ESI) calcd for $\text{C}_9\text{H}_{18}\text{N}_3\text{O}_4^+$, 232.1297; found, 232.1303.

(*S*)-1-((*S*)-2,2-Dimethyl-1,3-dioxolan-4-yl)-2-(methoxymethoxy)-ethanamine (12). Azide **11** (3.49 g, 15.1 mmol) in MeOH (100 mL) and added to Pd/C (10 wt %, 350 mg). The atmosphere was replaced by hydrogen and hydrogen bubbled through the suspension for 1 h. The mixture was filtered through Celite and concentrated in vacuo to yield amine **12** (3.02 g, 14.7 mmol, 97%). IR (neat) 3330, 1600, 1461 cm^{-1} . ^1H NMR (CDCl_3 , 400 MHz) δ 4.65 (s, 2H), 4.07–4.01 (m, 2H), 3.93–3.87 (m, 1H), 3.66 (dd, J = 9.7, 3.5 Hz, 1H), 3.51 (dd, J = 9.7, 6.2 Hz, 1H), 3.38 (s, 3H), 3.10–3.06 (m, 1H), 1.45 (br. s, 2H), 1.43 (s, 3H), 1.36 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 108.9, 96.7, 77.1, 69.8, 66.3, 55.3, 53.2, 26.6, 25.3. HRMS (ESI) calcd for $\text{C}_9\text{H}_{20}\text{NO}_4$, 206.1392; found, 206.1395.

(2*R*,3*S*)-3-(7-(Benzylamino)-3-iso-propylpyrazolo[1,5-*a*]pyrimidin-5-ylamino)-1,2,4-butanetriol (4k). Synthesized according to the general procedure for Buchwald–Hartwig coupling, **2** (500 mg, 1.25 mmol), Pd_2dba_3 (57.0 mg, 0.062 mmol), BINAP (117.0 mg, 0.19 mmol), sodium *tert*-butoxide (132 mg, 1.38 mmol), **12** (283 mg, 1.38 mmol), and PhMe (5.0 mL). Purification EtOAc :hexanes (1:4). Analytically pure material could not be obtained at this stage. General procedure for acidic deprotection followed. HCl/MeOH (50.0 mL) yield **4k** (412 mg, 1.07 mmol, 86%). Purification: crystallization from MeCN .

Expression, Purification, and Crystallography of CDK2 Complexed with 4k. A cDNA encoding human CDK2 37 was cloned into a pProEx-HTb vector (Invitrogen) containing a 6 $\times$ His-SUMO-1 N-terminal tag and expressed in *E. coli* BL21 (DE3). To cleave the SUMO tag, SuPr-1/Senp2 (Imagenes, Berlin, Germany) was cloned into pET28b (Novagen) with both N- and C-terminal 6 $\times$ His tags and expressed in *E. coli* BL21(DE3). After a first nickel affinity purification, 6 $\times$ His-SUMO-CDK2 was incubated with partially purified SUMO protease at 4 °C overnight. Cleaved CDK2 was separated from the 6 $\times$ His-SUMO tag and the SUMO protease using a second nickel affinity column and further purified on a Superdex 75 gel filtration column (GE Healthcare). This purification method takes advantage of the fact that the SUMO protease cleaves the SUMO tag without leaving any extra N-terminal residues, which would hinder crystallization. Purified CDK2 was stored at –80 °C in the crystallization buffer (10 mM Hepes pH 7.4, 0.5 mM DTT). Crystallization and ligand soaking conditions were as previously described. 38 A 1.8 Å diffraction data set was collected at beamline I02 (Diamond Light Source, Didcot, England) and the structure solved by molecular replacement with Molrep 39 using the 1HCK PDB structure as the search model. **4k** was fitted automatically using ARP/wARP ligand 40 and the structure refined using Refmac5. 41 Coordinates and structure factors have been deposited to the Protein Data Bank, PDB code 3NS9.

In Vitro Kinase Assays. Purified recombinant CDK1/cycB1, CDK2/cycE, CDK4/cycD1, CDK5/p35NCK, CDK6/cycD1, CDK7/CycH/MAT1, and CDK9/CycT were purchased from ProQinase GmbH (Freiburg, Germany). Kinase assays were performed according to manufacturer's protocols, using substrate peptides purchased from ProQinase GmbH. Measurement of the amount of ATP remaining at the end of the reaction, carried out with a luciferase assay (PKLight assay; Cambrex, England), was used to determine inhibition of kinase activity by compounds.

Cell Growth Assays. Cell lines were obtained from the CRUK cell bank facility and were routinely cultured in DMEM supplemented with 10% fetal calf serum (FCS) (First Link, England). Cell growth was assessed using the Sulforhodamine B (SRB) assay, as described.⁴² Human normal umbilical vein endothelial cells (HUEVC) and the appropriate culture media were purchased from Lonza Wokingham Ltd., England, and the cells were cultured as per supplier's protocols.

Flow Cytometry. HCT116 colon cancer cells were seeded (4×10^5) in 6-well plates in DMEM containing 10% FCS and allowed to adhere for 24 h. Compounds prepared in DMSO were added, and after 24 h incubation, cells were trypsinized, centrifuged at 1100 rpm for 5 min, and resuspended in 5 mL of ice-cold PBS, centrifuged as above, gently resuspended in 2 mL ice-cold 70% ethanol, and incubated at 4 °C for 1 h. Cells were washed twice with 5 mL of ice-cold PBS and resuspended in 100 μ L of PBS containing 100 μ g/mL RNase (Sigma-Aldrich, England) and 1 mL of 50 μ g/mL propidium iodide (Sigma-Aldrich, England) in PBS. Following incubation overnight in the dark at 4 °C and filtering through 70 μ m muslin gauze into FACS tubes (Becton-Dickinson, England) to remove cell clumps, stained cells were processed using the RXP cytomics software on a Beckman Coulter Elite ESP (Beckman Coulter, High Wycombe, United Kingdom). The data were analyzed using Flow Jo v7.2.5 (Tree Star Inc., San Carlos, CA), and statistical analysis was performed using the unpaired Student's *t* test to determine *p*-values.

Immunoblotting. Cells (8×10^5), plated in 10 cm plates, were treated with compounds after 24 h. Four hours later, cell lysates were prepared by the addition of 500 μ L of RIPA buffer (Sigma-Aldrich, England), containing phosphatase inhibitor cocktail and complete protease inhibitor cocktail, both from Roche Diagnostics, England. Protein concentrations were determined using the BCA protein assay (Thermo-Scientific, England). Immunoblotting was performed as described previously.⁴³ Antibody details are available upon request.

In Vivo Assessment of Molecular Pharmacology. All procedures were approved by the Imperial College London Animal Ethics Committee. The experiments were done by licensed investigators in accordance with the United Kingdom Home Office's regulations under the Animal (Scientific Procedures) Act 1986 and were within guidelines set out by the United Kingdom Coordinating Committee for Cancer Research's Ad hoc Committee on the Welfare of Animals in Experimental Neoplasia.⁴⁴ Seven-week-old female nu/nu-BALB/c athymic nude mice were purchased from Harlan Olac Ltd. (England). **4k**, prepared in 28% (w/v) PEG300/5% (v/v) dimethylacetamide/10% (w/v) HPB cyclodextrin in H₂O, was administered by oral gavage. Drug was administered by exact body weight, with the injection volume being not more than 0.2 mL. After 6 h, the mice were anaesthetized and blood collected by cardiac puncture into tubes containing 200 μ L of PBS, 100 U of Heparin, and 10 mM EDTA. Blood was mixed with 1 mL of red blood cell lysis solution (Flowgen Bioscience, Nottingham, England) for 15 min at room temperature, followed by centrifugation for 5 min at 1,000 rpm to pellet the peripheral blood mononuclear cells (PBMC). PBMC were washed twice in PBS, and the cells were stained using the Fix and Perm kit (Caltag Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. Briefly, after washing the PBMCs in PBS, 2×10^4 – 10^5 cells were fixed using 50 μ L of reagent A (fixation medium) for 15 min at room temperature. At the end of the incubation, the cells were washed in PBS, and then 50 μ L of reagent B (permeabilization medium) was added together with antibodies for RNA polymerase II (PolII), PolII P-Ser2, RB, or RB P-Thr821 (purchased from Abcam, England) at a dilution of 1:100 for 1 h at room temperature. Cells were washed three times with PBS and were incubated for 1 h with goat antirabbit Alexafluor 488 (Invitrogen, Paisley, England) diluted at 1:200 in PBS in the dark at room temperature. Finally cells were washed in PBS three times and resuspended in 200 μ L of PBS and stored at 4 °C for flow cytometric analysis. Labeled cells

were processed using the RXP cytomics software on a Beckman Coulter Elite ESP, and the data were analyzed using Flow Jo v7.2.5.

Absorption, Distribution, Metabolism, Elimination and Toxicity (ADME-Tox) and Pharmacokinetic Studies. Studies were performed under contract by Cerep Inc. (USA), using described methods.^{45–48}

Tumor Xenografts. HCT116 cells (5×10^6) were injected subcutaneously in not more than 0.1 mL volume into the flank of the animals (female nu/nu-BALB/c athymic nude mice). Tumor measurements were performed twice per week, and volumes were calculated using the formula $\frac{1}{2}[\text{length (mm)}] \times [\text{width (mm)}]^2$. The animals were randomized, and when tumors had reached a volume of 100–200 mm³, animals were entered into the different treatment groups and treatment with **4k** or vehicle control was initiated. Animals were treated with **4k** prepared as above daily by oral gavage for a total of 14 days. At the end of the 14-day treatment period, the mice were sacrificed, and the tumors were fixed by formalin fixation and paraffin embedding. Throughout the 14-day treatment period, animal weights were determined each day and tumor volumes on alternate days. The MCF-7 xenograft study was performed exactly as described previously.²⁴

Immunohistochemistry. Tumors were dissected from mice and fixed in 10% formalin and embedded in paraffin blocks. Immunohistochemical staining was performed as described previously,⁴⁹ except that antigen retrieval was performed by microwaving for 10 min using 10 mmol/L citrate buffer (pH 6.0). Antibodies used were Rb pThr821 (ab4787) from Abcam (England) or Rb (9309) and Rb pSer807/811 (9308) from New England Biolabs, England. Images were acquired using the Automated Cellular Imaging System (ACIS) (Carl Zeiss Ltd., Welwyn Garden City, England). Cell positivity was determined by counting >1000 cells per section, and the results were tabulated using GraphPad Prism software, with statistical significance being determined using the Student's *t* test (GraphPad Prism).

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Supporting Information Available: In vitro kinase and cell growth inhibition data, cell cycle profiles, and spectral data for **4k**, as well as additional synthetic methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The liver receptor homolog-1 regulates estrogen receptor expression in breast cancer cells

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Abstract Estrogen receptor- α (ER) is expressed in the great majority of breast cancers, and the inhibition of ER action is a key part of breast cancer treatment. The inhibition of ER action is achieved using anti-estrogens, primarily tamoxifen, and with aromatase inhibitors that inhibit estrogen biosynthesis, thereby preventing ER activation. However, resistance to these therapies is common. With the aim of identifying new molecular targets for breast cancer therapy, we have identified the liver receptor homolog-1 (LRH-1) as an estrogen-regulated gene. RNA interference

and over-expression studies were used to investigate the role of the LRH-1 in regulating breast cancer growth and to identify the targets of an LRH-1 action. Promoter recruitment was determined using reporter gene and chromatin immunoprecipitation (ChIP) assays. We show that LRH-1 regulates breast cancer cell growth by regulating the ER expression. Reporter gene and in vitro DNA-binding assays identified an LRH-1-binding site in the ER gene promoter, and ChIP assays have demonstrated in vivo binding at this site. We also provide evidence for new LRH-1 variants in breast cancer cells arising from the use of alternative promoters. Previous studies have shown that LRH-1 functions in estrogen biosynthesis by regulating aromatase expression. Our findings extend this by highlighting LRH-1 as a key regulator of the estrogen response in breast cancer cells through the regulation of ER expression. Hence, inhibition of LRH-1 could provide a powerful new approach for the treatment of endocrine-resistant breast cancer.

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Introduction

Estrogen plays a critical role in the development and progression of breast cancer. Its actions are mediated by estrogen receptors, with estrogen receptor- α (ER) being expressed in the majority of breast cancers. ER is a member of the nuclear receptor (NR) superfamily of transcription factors, which acts by regulating specific gene expression upon binding estrogen. Inhibition of ER activity is achieved clinically through the use of selective estrogen receptor modulators (SERMs), such as tamoxifen, that compete with estrogen for binding to ER, to inhibit its activity [1, 2].

Aromatase inhibitors act by inhibiting the conversion of androgens into estrogen, and provide an alternative and effective approach for inhibiting ER activity, with newly introduced aromatase inhibitors, such as Anastrozole and Letrozole, already proving to have greater efficacy than tamoxifen [3, 4].

The regulation of ER gene expression has been subjected to intense study due to its important role in the regulation of breast cancer and in other important physiological processes, such as cardiovascular protection, bone homeostasis, and osteoporosis, and sexual development in males and females [5]. The coding region of the ER gene is located within eight exons spanning 140 kb on chromosome 6q25 [6, 7]. Regulation of an ER gene expression is complex, with transcription being initiated within multiple promoters spanning 150 kb [8]. Several of these promoters show tissue specificity [9], which has further complicated studies to define the transcriptional regulators of ER gene expression.

The liver receptor homolog (LRH-1), like ER, is a member of the NR superfamily. It belongs to the Ftz-f1 or NR5A subfamily that includes steroidogenic factor-1 (SF-1), members of which are characterised by the presence of an extended DNA-binding domain (DBD), the so-called Ftz-F1 box located at the C-terminus of the DBD [10]. Most NRs bind to DNA sequences conforming to the consensus NR-binding sequence, AGGTCA, either as homodimers (e.g., ER) to palindromes of the AGGTCA motif or to direct repeats of the AGGTCA motif as heterodimers with retinoid X receptor- α (RXR α) [11]. By contrast, members of the Ftz-F1 subfamily bind to sequences having a 5' extension to the NR DNA-binding motif as monomers, with the Ftz-F1 box targeting the 5'-YCA extension to the NR DNA-binding motif [12], where Y is C or T. Until recently classified as orphan receptors, structural studies have shown that LRH-1 and SF-1 bind phosphatidyl inositols, with their binding being required for maximal activity [13–15]. LRH-1 plays important roles in metabolism, being involved in the regulation of reverse cholesterol transport, lipid and cholesterol absorption, bile acid homeostasis, and steroidogenesis [10]. In particular, the LRH-1 has been implicated in the regulation of aromatase (CYP19) expression in the ovary [16]. Interestingly, in adipose tissue from normal women, CYP19 expression is low and mainly originates from the use of promoter I.4. By contrast, in breast cancer adipose tissue, activation of additional CYP19 gene promoters is seen, including importantly, the gonadal PII promoter. In the latter context, LRH-1 regulates CYP19 expression through binding to a response element in the PII promoter [17–20].

In this study, we show that in breast cancer cells, LRH-1 is co-expressed with ER in breast cancer cell lines and that RNAi-mediated LRH-1 knock-down inhibits breast cancer

cell growth. We demonstrate that this is due, at least in part, to the regulation of ER expression by LRH-1 through direct binding to the ER gene promoter. These studies show, for the first time, that ER is an LRH-1 target gene, a finding that is potentially relevant for the development of new therapies for breast cancer.

Materials and methods

Cell lines

COS-1, MCF-7, T47D, ZR75-1, BT474, and MDA-MB-231 cells were routinely cultured in DMEM containing 10% FCS. For estrogen-depletion experiments, the cells were transferred to DMEM lacking phenol red and containing 5% dextran-coated charcoal-stripped FCS for 72 h, as described previously [21]. 17β -Estradiol (estrogen), 4-hydroxytamoxifen, and ICI 182, 780 were prepared in ethanol and added to the medium at a final concentration of 10 nM (estrogen) or 100 nM (4-hydroxytamoxifen, ICI 182, 780). Compounds 5A, 5B, and 5L, which have previously been described [22], were prepared in DMSO and added to a final concentration of 10 μ M.

Plasmids

The renilla luciferase reporter gene was RLTK (Promega, UK). LRH-1 and SHP expression plasmids were pCI-LRH-1, pCDM8-hSHP, and the LRH-1; the firefly luciferase reporter gene was SF-1-luc (gifts from Dr. Donald McDonnell and Dr. David Moore) [14]. The F342W/I416W LRH-1 mutant was generated by site-directed mutagenesis of pCI-LRH-1, using primers having the sequences 5'-CA AGCACGAAAAGCTGAGCACCTGGGGGCTTATGTG-CAAAA-3' and 5'-ACTGGGCAACAAGTGGACTATTC CATATGGGCATCACAAGCCGG-3'. The PII-516 aromatase reporter gene was kindly provided by Drs. Colin Clyne and Evan Simpson. ERP-Luc was generated by amplifying the genomic region covering the ER gene promoter A to LRHRE3 (−5993/−117) using high fidelity *pfu* DNA polymerase (Fermentas, UK) with DNA prepared from the human ER gene Bacterial Artificial Chromosome clone RP3-443C4 (Geneservice Ltd, UK). The PCR product was cloned into pJET (Fermentas, UK) (pJET-ERP) and re-cloned into the pGL3-Basic luciferase reporter vector (Invitrogen), following digestion with *Bgl*/II restriction enzyme. The three putative LRH-1 response elements in this region, LRHRE1-3, were mutated by site-directed mutagenesis (Quickchange; Stratagene), changing the LRH-1-binding site to a *Bam*HI restriction site in each case (details available on request).

Transfections

MCF-7 and BT474 cells were transiently transfected with 100 ng of SHP or 50 ng of LRH-1, and the total RNA and protein lysates were prepared 48 h later, as previously described [21]. For reporter gene assays, COS-1 cells were transiently transfected in 96-well plates, using FuGene HD (Roche), according to manufacturer's protocols. The cells were transfected with 25 ng of the pGL3-Promoter-based reporter plasmids or 100 ng of the pGL3-Basic, SF-1 Luc, and RLTK, together with 50 ng of pCI-LRH-1. Cells were lysed 24 h following transfection and firefly and renilla luciferase activities were determined using the Dual Glo system (Promega, UK). A similar method was used for assaying ERP-luc and PII-516 aromatase reporter genes in COS-1 or MCF-7 cells.

siRNA

Cells were transfected using Lipofectamine RNAiMAX, according to manufacturer's methods (Invitrogen). RNA and protein were prepared 48 h following transfection. Cell number was estimated using the sulphorhodamine B (SRB) growth assay, as described previously [23]. siRNA LRH-1 On-TargetPlus Smartpool (Dharmacon) comprised siRNAs having the sequences: 5'-CAUAAUGGGCUAUUCAUAU-3' (#1), 5'-AGAGAAUUUGGACAGCUA-3' (#2), 5'-GGAGUGAGCUCUUAUCCU-3' (#3) and 5'-GAAGC CAUGUCUCAGGUGA-3' (#4). siGenome non-targeting siRNA (Dharmacon) had sequences 5'-UAAGGCUAUG AAGAGAUAC-3', 5'-AUGUAUUGGCCUGUAUUAG-3', 5'-AUGAACGUGAAUUGCUCAA-3', 5'-UGGUUUACA UGUCGACUAA-3'.

RT-PCR

Total RNA was collected, and real-time RT-PCR was performed as previously described [23]. Real-time RT-PCR was carried out using Taqman Gene Expression Assays (Applied Biosystems, UK) for ER (ESR1; Hs00174860_m1), CTD (Hs00157201_m1), LRH-1 (Hs00187067_m1), pS2 (TFF1; Hs00170216_m1), SHP (Hs00222677_m1), Dax1 (Hs00230864_m1), SF-1 (Hs00610436_m1) and GAPDH (Hs99999905_m1) on an ABI 7900HT machine.

Western blotting

Cells were cultured and protein lysates prepared as described previously [21]. Antibodies used were anti-ER α (Novacastra Laboratories), and anti-LRH-1 (Perseus Proteomics). SHP, cathepsin D, and β -actin antibodies were purchased from Abcam.

ChIP

Chromatin immunoprecipitation (ChIP) was performed as previously described [23]. Antibody used was LRH-1, and primers for real-time PCR were: LRHRE1 Fwd 5'-CTAGCCCAAGTGAACCGAGA-3', LRHRE1 Rev 5'-ACCTCAGGTCACGAACCAAA-3'. For normalization, qPCR was performed for the previously described control region for the c-myc gene [24], using oligonucleotides having the sequences: c-myc Fwd 5'-GCCAGTCCAAC CGGCTTATG-3', c-myc Rev 5'-GGTTCTCCCAAGCAG GAGCA-3'.

In vitro DNA-binding assay

Binding affinities for LRH-1 to LRHRE1 sequences were obtained using a fluorescence polarization assay, following expression and purification of the LRH-1 amino acid residues 79–184 as a MBP fusion protein in *E. coli*, as described previously [12]. Carboxyfluorescein (FAM)-labeled duplex oligonucleotides based on the LRHRE1 sequence 5'-AATTGCCAAAGCTTTGGT-3' and the CYP7A1 LRH-1-binding site described previously [12], (Integrated DNA Technologies, USA), were used. 10 nM of FAM-labeled oligonucleotide was mixed with MBP–LRH-1 DBD at varying concentrations, and polarization was measured in milli-polarization units (mP). The experiments were performed on a Panvera Beacon 2000, with an excitation wavelength of 495 nm, and an emission wavelength of 520 nm. The results were analyzed by SigmaPlot to generate binding data and dissociation constants.

Results

LRH-1 regulates the growth of breast cancer cells

As a strategy to identify key estrogen-regulated genes in breast cancer cells, we modified the estrogen-responsive and ER-positive MCF-7 breast cancer cell line, to conditionally express a transcriptionally repressive ER, PLZF-ER, composed of the PLZF transcriptional repressor fused to the ER DNA and ligand-binding domains [21]. The resulting line grows in an estrogen-dependent manner in the absence of PLZF-ER expression, but induction of PLZF-ER expression blocks its growth. Gene-expression microarray analysis carried out using this line, identified 1,627 genes which showed >1.5-fold regulation by estrogen within 16 h. Of these genes, 149 were repressed by PLZF-ER, suggesting that these genes are important for estrogen-regulated growth of MCF-7 cells (Buluwela et al., in preparation). One of the 149 genes whose expression was repressed by PLZF-ER in MCF-7 cells was LRH-1.

Estrogen treatment of MCF-7 cells showed a four-fold increase in the LRH-1 expression (Fig. 1a), confirming the microarray findings; no stimulation of LRH-1 expression was observed when the cells were treated with the anti-estrogens 4-hydroxytamoxifen or ICI182, 780. Further, LRH-1 expression was considerably higher in ER-positive, compared to ER-negative lines (Fig. 1b), suggestive of an association between the ER expression and the LRH-1 expression in breast cancer cells.

Analysis of the ER-binding and PolII occupancy data, generated from ChIP-chip and ChIP-seq profiling of global ER and PolII binding following treatment of estrogen-depleted MCF-7 cells with estrogen for 1 h [24, 25], showed ER binding to a region 10.5 kb upstream from the LRH-1 exon 1. Real-time PCR of ChIP following estrogen treatment of MCF-7 cells confirmed that estrogen

stimulates ER binding to this region of the LRH-1 promoter (Fig. 1d).

Several LRH-1 splice variants have been described [10, 26], including hLRH-1 and LRH-1v2 both of which lack exon 2 encoded amino acids 22–67 (Fig. 2b). In performing immunoblotting for the LRH-1, we noted that many of the commercially available antibodies, whilst detecting the transfected LRH-1 did not detect the LRH-1 in breast cancer lines. As these antibodies were directed to the LRH-1 N-terminus, we wondered whether this was due to the predominance of exon 2-deleted LRH-1 variants in these lines. 5'-Rapid amplification of complementary DNA ends (5'-RACE) for defining 5' ends of the LRH-1 transcripts in MCF-7 cells identified three main forms (Fig. 2a), the well-described LRH-1 variant 1 (v1) encoding a polypeptide of 541 amino acids, and two new variants, which we named variant 4 (v4) and variant 5 (v5) to distinguish them

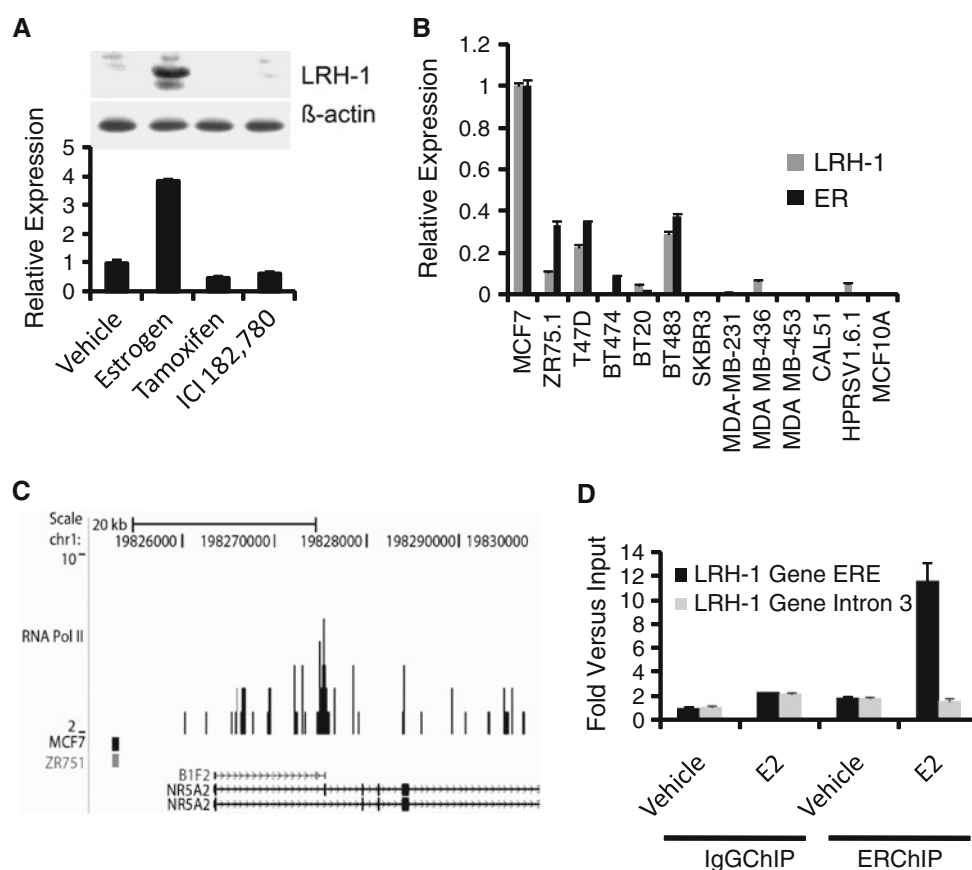


Fig. 1 LRH-1 is an estrogen-regulated gene that functions in breast cancer cell growth. **a** Hormone-depleted MCF-7 cells were stimulated with vehicle, estrogen, tamoxifen or ICI182780 and LRH-1 mRNA levels were assessed by real-time RT-PCR (lower panel). Protein lysates prepared following stimulation of hormone-depleted MCF-7 cells were immunoblotted (upper panel). **b** The LRH-1 and ER mRNA levels were determined by real-time RT-PCR analysis of total RNA. **c** Shown is the LRH-1 locus with the positions of ER-binding regions (black box) and RNA PolII occupancy for MCF-7 cells treated

with estrogen for 1 h. Also shown is the region of ER binding for ZR75-1. Shown are the ER and PolII occupancy representations, generated by uploading the ChIP-Seq datasets to the UCSC genome browser gateway (<http://genome.ucsc.edu>). **d** ChIP was performed using mouse immunoglobulins (IgG) or an ER antibody, and quantitative PCR was carried out for the ER-binding site identified in (c). PCR for a region in intron 3 served as a negative control for ER binding. The results of three independent replicates are shown

from the previously described LRH-1 variants. v4 and v5 would be expected to encode polypeptides of 501 and 482 amino acids, respectively. Interestingly, LRH-1-v4 transcription is initiated in intron 1 of the LRH-1 gene, extending the previously identified exon 2 further 5' into intron 1 (named exon 2a) (Fig. 2c; Supplementary Fig. 1). The mapped 5' end of LRH-1-v5 defined an exon in intron 2 (here, named exon 2b). Hence, v4 and v5 define transcripts from the previously undescribed, alternative LRH-1 gene promoters. Blast searches of the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) identified one EST clone, TRACH3028697 (GenBank number: DC418114.1; the 5' end of which precisely matched the 5' end determined by 5'-RACE. Also identified was an EST clone that contained the sequences present in v4 and extended the 5' untranslated region by a further 87 bp (Fig. 2b). No corroborating evidence for v5 was found in EST databases.

Expression analysis using isoform-specific real-time RT-PCR showed that v4 and v5 are highly estrogen-regulated, whereas variants hLRH-1, v1, and v2 expression were weakly stimulated by estrogen in MCF-7 cells (Fig. 3a). Note that using these primer sets, we did not distinguish between hLRH-1, v1, and v2. Furthermore, RT-PCR and immunoblotting showed that v4 is the predominant form of LRH-1 expressed in breast cancer cell lines (Fig. 3b, c). However, extensive reporter gene analysis did not reveal a significant difference between LRH-1 v1, and v4 (data not shown; Fig. 3d), despite the fact that v4 lacks the N-terminal 40 amino acids present in v1.

RNA interference (RNAi) used to determine whether LRH-1 is an important mediator of estrogen-stimulated growth of MCF-7 cells, showed that LRH-1 down-regulation using a pool of four small interfering RNAs (siRNA) potently inhibited MCF-7 growth (Fig. 4a). The individual siRNAs all inhibited MCF-7 growth, with the least growth inhibition being observed for siRNA #1, the siRNA that gave the smallest reduction in LRH-1 expression (Supplementary Figs. 2, 4b, f). Transfection with the LRH-1 siRNAs also inhibited the growth of the LRH-1 positive ZR-75-1 and T47D cells, but growth of the LRH-1-negative cell lines, BT474 and MDA-MB-231, was unaffected by the LRH-1 siRNA (Supplementary Fig. 3a).

LRH-1 regulates ER expression in breast cancer cells

Determination of the expression of estrogen-regulated genes showed that LRH-1 knockdown reduced expression of the pS2 and cathepsin D (CTD) genes (Fig. 4c, d), prompting us to determine whether the growth inhibitory effects of the LRH-1 knockdown could be due to the LRH-1 regulation of ER expression. Indeed, ER mRNA and protein were reduced following siRNA for LRH-1 (Fig. 4e, f). Inhibition of ER expression following LRH-1

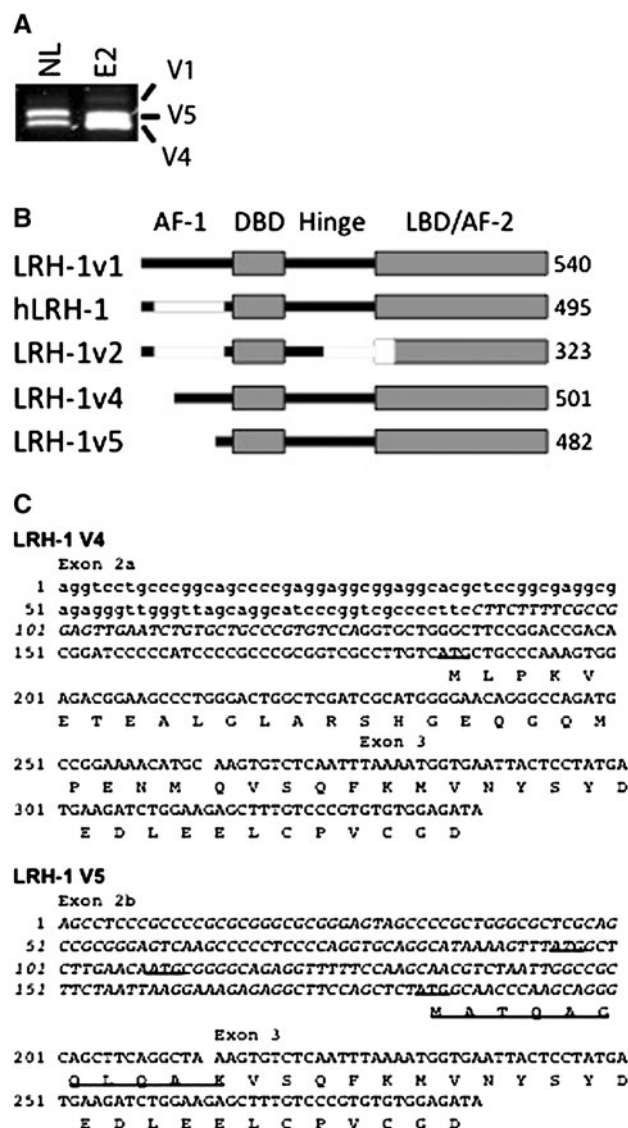


Fig. 2 Identification of new LRH-1 variants in breast cancer cells. **a** Products of 5'-RACE are shown for RNA prepared from MCF-7 cells treated with estrogen (E2) or vehicle (NL) for 8 h. The products were cloned and sequenced to assign their identities, as marked. **b** Shown is a schematic representation of the LRH-1 variants, including variants v4 and v5 identified here. **c** Sequences of LRH-1 variants v4 and v5. The DNA sequences in italics represent the 5' untranslated regions present in the 5'-RACE cloning products. The sequences in lower case shown for v4 are also present in an EST (UTERU3011183) (<http://www.ncbi.nlm.nih.gov/>). The underlined amino acids in v5 are the residues arising from the newly described exon (exon 2b)

knockdown was also observed in T47D and ZR75-1 cells (Supplementary Fig. 3b, c).

In order to confirm these findings, we investigated whether synthetic LRH-1 activating compounds could stimulate MCF-7 cell growth. A number of substituted *cis*-bicyclo[3.3.0]oct-2-enes have been identified as small molecule agonists of LRH-1 and SF-1 [22]. As there was no detectable expression of SF-1 in MCF-7 and BT-474

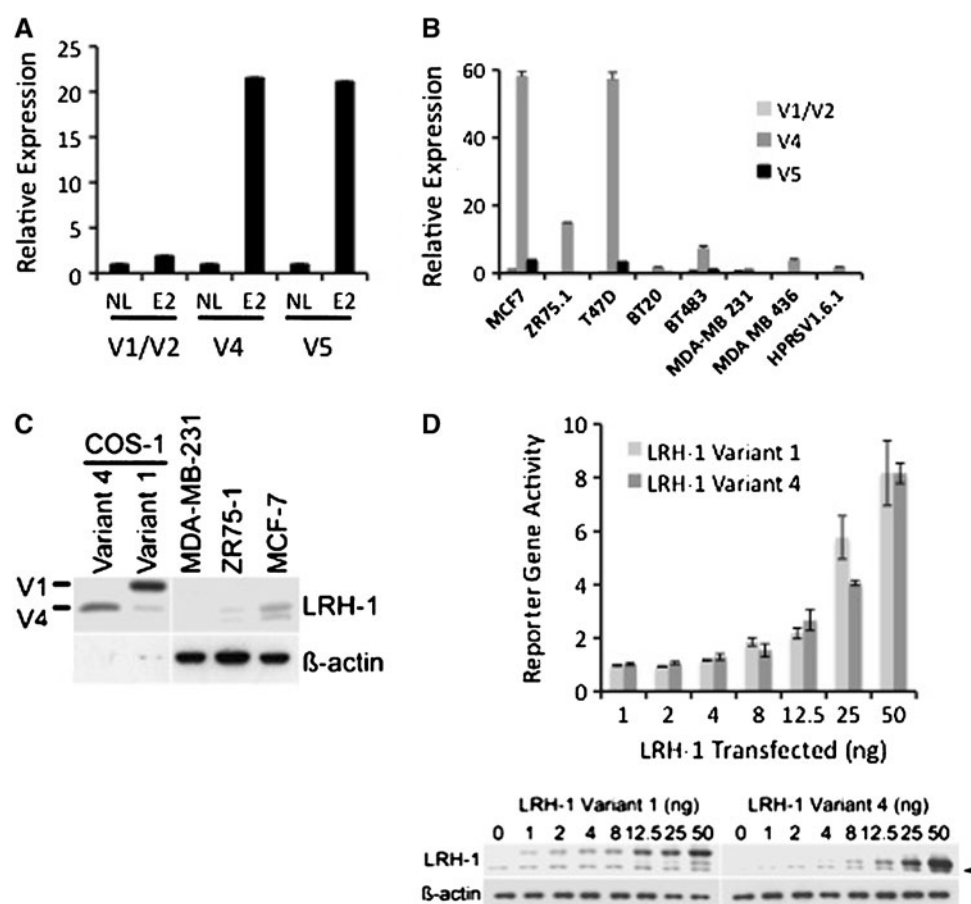


Fig. 3 LRH-1 variant 4 is the major form of the LRH-1 in breast cancer cells. **a** LRH-1 variant mRNA levels in MCF-7 cells were determined by real-time RT-PCR using primers that specifically amplify v4 or v5 sequences. Primers for v1/v2 amplify the LRH-1 variants 1 and 2, as well as hLRH-1. The results for three independent RNA samples are shown, with in each case expression level in the absence of ligand (NL) being taken as 1 and expression level in the presence of E2 shown relative to that. **b** Expression of the LRH-1 variants in breast cancer cell lines is shown. Expression of v1/v2 in

MCF-7 was taken as 1 and all other values are shown relative to this. **c** Protein lysates prepared from breast cancer lines and COS-1 cells transfected with LRH-1 v1 or v4 were immunoblotted for LRH-1 and β -actin. **d** COS-1 cells were co-transfected with an LRH-1-responsive luciferase reporter gene (SF-1-luc) and increasing amounts of LRH-1 v1 or v4. Reporter gene activities are shown relative to the vehicle control. Protein lysates prepared from these lysates were immunoblotted for LRH-1 to determine relative expression of the variants (lower panel)

cells (Supplementary Fig. 4), we determined the effect of three of these compounds (5A, 5B, and 5L) on cell growth. MCF-7 cell growth was stimulated, whereas growth of the ER-positive, but LRH-1-negative BT-474 cells, was unaffected (Fig. 5a). As seen with a reporter gene assay, these compounds stimulated the activity of an LRH-1 responsive luciferase reporter gene (Fig. 5b), and treatment with the compounds increased the ER levels in MCF-7 cells (Fig. 5c). Moreover, LRH-1 transfection stimulated ER expression in MCF-7 and BT474 cells (Fig. 5d, e), whereas transfection of the small heterodimer partner (SHP), a NR that acts as a co-repressor for LRH-1 [27], reduced ER expression in MCF-7 cells (Fig. 5f, g). Further, LRH-1 transfection stimulated the growth of MCF-7 cells, while SHP inhibited MCF-7 growth (Fig. 5h).

LRH-1 regulates ER expression by direct recruitment to the ER gene promoter

Expression of the human ER gene is initiated at multiple promoters spanning 150 kb [8]. RT-PCR showed that in the lines examined, the majority of ER expression is initiated at promoters A, B, and C, with some expression from promoter F (Fig. 5a; Supplemental Fig. 5). Analysis of the region encoding ER gene promoters A through F revealed the presence of 11 sequences conforming to the LRH-1 consensus binding site (YCAAGGYCR [28]), with the closest of these sites being located less than 6 kb 5' to promoters A/B (Fig. 6a). In addition, our analysis highlighted an extended palindrome centered around a *Hin*DIII restriction enzyme site (5'-CCAAAGCCTTTGG-3') which

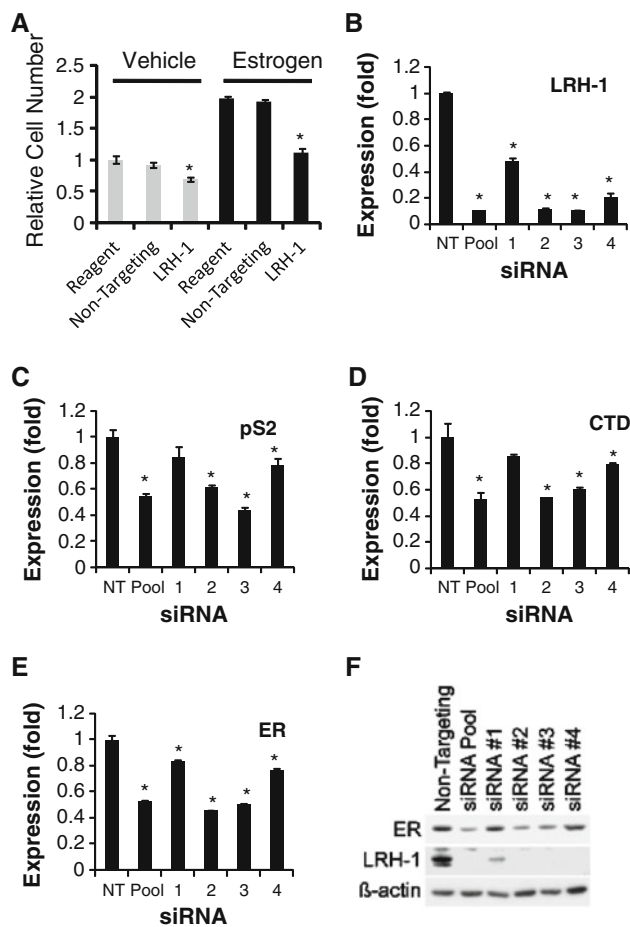


Fig. 4 Targeted knockdown of LRH-1 inhibits Estrogen Receptor (ER) expression. **a** MCF-7 cells grown in estrogen-depleted medium were transfected with the LRH-1 siRNA, treated with vehicle or estrogen and cell number estimated after 4 days using the sulphorhodamine B (SRB) assay. Growth is shown relative to the reagent alone control for three experiments. **b–e** siRNA pool for LRH-1 or individual siRNAs were transfected into MCF-7 cells, RNA and protein was prepared after 48 h. LRH-1 (**b**), pS2 (**c**) cathepsin D (CTD) (**d**) and ER (**e**) mRNA levels were determined by real-time RT-PCR analysis. Shown are the means and s.e.m. for three experiments. Asterisks show statistically significant difference (unpaired *t*-test, $P < 0.05$) from the reagent or non-targeting controls. **f** Immunoblotting was performed following preparation of protein lysates from MCF-7 cells transfected with siRNAs for LRH-1

encoded a sequence (underlined) similar to that identified in the mouse SEBP gene as an LRH-1-binding site [29], 912 bp 5' to the promoter A start site (named LRHRE1).

As promoters A and B constitute the major promoters utilized in MCF-7 cells, we generated an ER promoter luciferase reporter gene (ERP-luc) encoding a 6.0 kb region comprising promoters A through D and containing two putative LRH-1-binding sites (LRHRE2, and LRHRE3) that conform to the LRH-1 consensus binding site sequence, as well as LRHRE1. The ERP-luc reporter was activated by LRH-1 in MCF-7 cells (Fig. 6b). Structural studies have shown that the LRH-1 ligand-binding

domain (LBD) has phospholipids bound [13–15]. An LRH-1 mutant (LRH-1-F342W/I416W), which is impaired for phospholipid binding and shows reduced transcriptional activity [14] also showed reduced activation of ERP-luc. Mutation of LRHRE2 and LRHRE3 had modest effects on LRH-1 regulation of the reporter gene, with a considerably larger reduction in reporter gene activity being observed if LRHRE1 was mutated (Fig. 6c), suggesting that LRH-1 binds to the LRHRE1 sequence.

As the LRHRE1 sequence does not conform to the LRH-1 consensus binding site, LRH-1 binding to this sequence was determined using a fluorescence polarization assay using *E. coli*-expressed LRH-1 DBD and FAM-labeled oligonucleotides. The K_d of LRH-1 binding to LRHRE1 (126 nM) was similar to that obtained for the LRH-1 site in the Cyp7A1 gene promoter (200 nM) (Fig. 6d; Supplemental Fig. 5).

ChIP analysis of MCF-7 cell lysates with an LRH-1 antibody demonstrated LRH-1 recruitment to the region of the ER promoter containing the LRHRE1 sequence (Fig. 6e). Taken together, these findings indicate that LRH-1 is recruited to the LRHRE1 sequence in the ER gene promoter, and that this sequence is important for LRH-1 regulation of ER expression.

Discussion

LRH-1 is an estrogen-regulated gene in breast cancer cells

Estrogens play critical roles in the initiation and progression of human breast cancer, as well as other gynaecological cancers. Estrogen actions are mediated by ER which acts primarily as a transcription factor that, upon binding estrogen, regulates the expression of a large number of estrogen-responsive genes [30], causing, in the case of breast cancer cells, inhibition of apoptosis and promotion of proliferation. Defining the key estrogen-regulated genes in cancer cells would provide important insights into the mechanisms by which estrogen/ER promotes breast cancer growth. Using an engineered MCF-7 cell line that conditionally expresses a dominant-negative form of ER in which the PLZF transcriptional repressor was fused to ER [21], we identified LRH-1 as a gene whose expression was repressed upon PLZF-ER expression. RT-PCR analysis and immunoblotting confirmed estrogen-regulation of LRH-1, as has previously been described [31]. As also described previously, siRNA-mediated LRH-1 silencing potently inhibited the estrogen-stimulated growth of MCF-7 cells. Moreover, we observed inhibition of the growth of other ER/LRH-1-positive breast cancer cell lines (ZR75-1 and T47D).

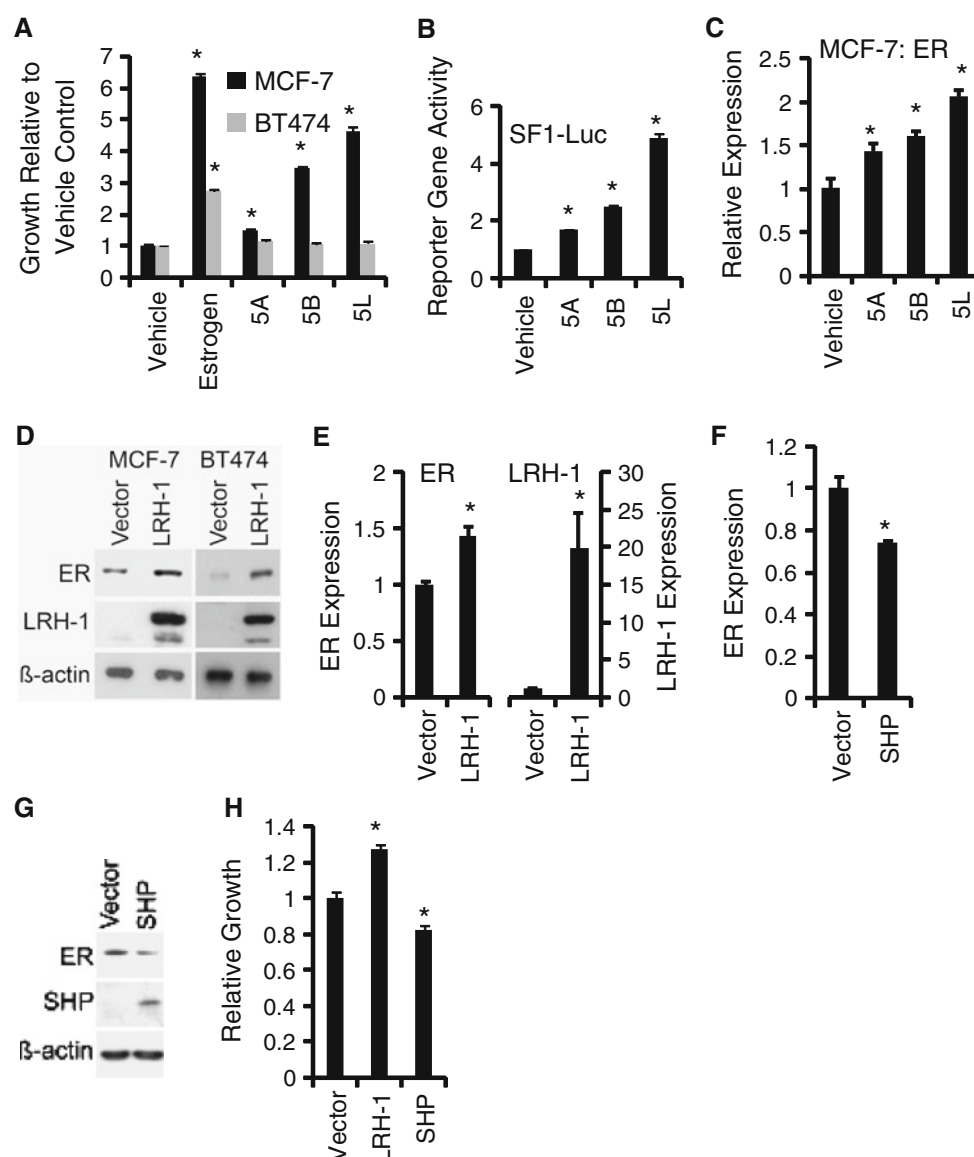


Fig. 5 LRH-1 stimulates Estrogen Receptor (ER) expression in breast cancer cells. **a** Hormone-depleted MCF-7 and BT-474 cells were stimulated with a vehicle, estrogen or LRH-1 activator, over a 12-day period. Shown is growth at day 12, relative to the vehicle control, as measured using the SRB assay. **b** COS-1 cells transfected with an LRH-1 responsive luciferase reporter gene (SF1-luc) were stimulated with vehicle (DMSO) or the LRH-1 activators 5A, 5B and 5L. Reporter gene activities are shown relative to the vehicle control. **c** RNA was prepared from MCF-7 cells treated with compounds 5A, 5B and 5L for 8 h. ER mRNA levels were determined by real-time RT-PCR. **d, g** Protein lysates prepared from MCF-7 cells following

transfection with LRH-1 (**d**) or SHP (**g**) were immunoblotted. **e, f** Real-time RT-PCR was performed using RNA prepared from MCF-7 cells transfected with LRH-1 (**e**) or SHP (**f**). **h** MCF-7 cells were transiently transfected with LRH-1 or SHP and cell numbers estimated after 4 days using the SRB assay. Growth is shown relative to the vector control. All graphical results are shown as the means and s.e.m. relative to the vehicle or vector controls, of at least three replicates. Asterisks denote statistically significant difference ($P < 0.05$) relative to the appropriate vehicle or vector control, determined using the unpaired *t*-test

The study by Annicotte et al. [31] previously identified an estrogen response element 2.3 kb upstream of the LRH-1 exon 1. ChIP based global analysis of ER-binding sites did not, however, detect significant binding to this region in MCF-7 and ZR75-1 cells. Instead, binding to a region 10.5 kb upstream of exon 1 was seen in both cell lines. This region contains the sequence 5'-AGGAcAcacTG ACCT-3'

(starting at chromosome 1 bp 198,252,853 in the NCBI hg18 human genome release, in which exon 1 of the LRH-1 gene is at bp 198,263,393 (<http://genome.ucsc.edu>)), which conforms well to the consensus ERE sequence (AGGTC AnnnTGACCT) [32]. ChIP analysis confirmed estrogen-stimulated ER recruitment to this site, indicating that this site is involved in ER regulation of LRH-1 expression.

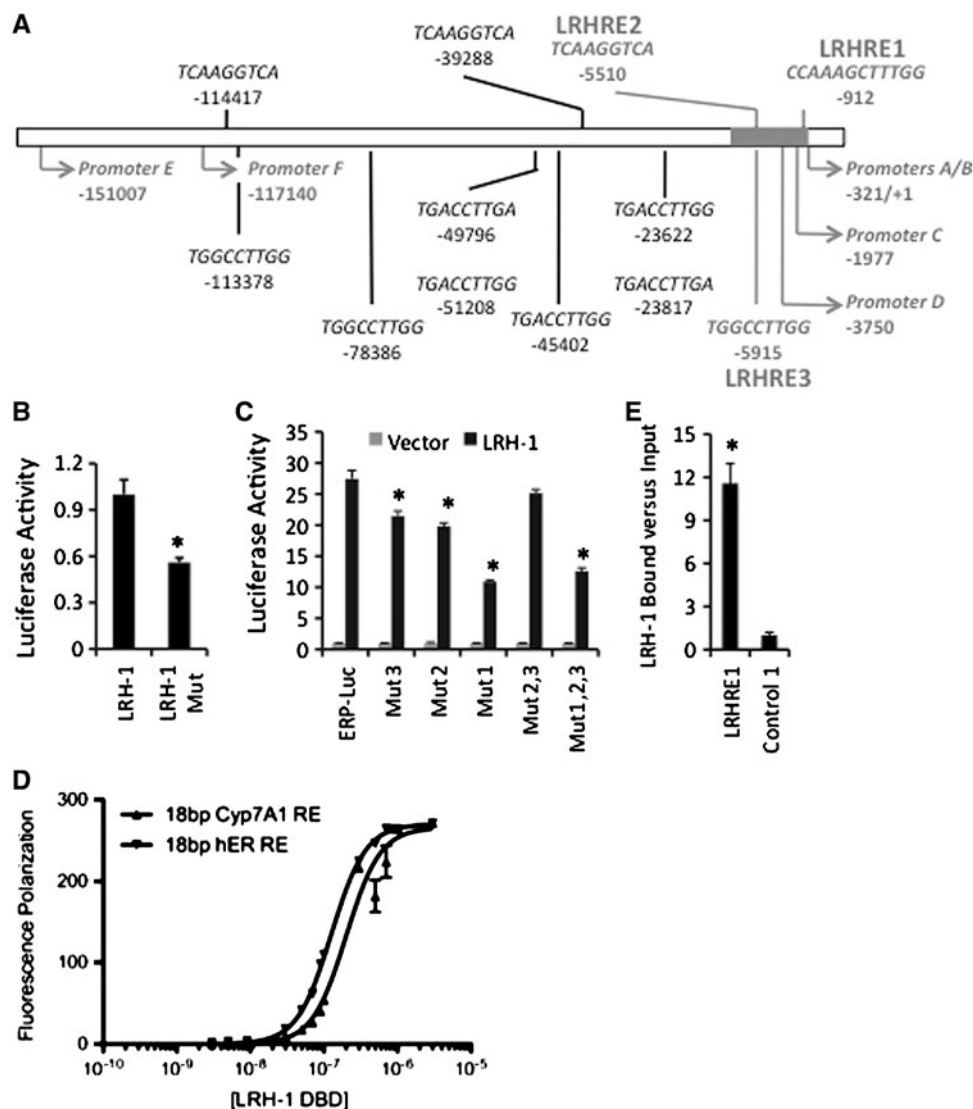


Fig. 6 LRH-1 binds to a specific site within the ER gene promoter. **a** Shown is a schematic representation of the ER gene, with the positions of ER promoters A through F highlighted. Transcription start site positions shown are as previously described [9]. Sequences that are similar to the consensus LRH-1-binding site are shown, either above or below the rectangle with the sequences shown below the rectangle denote sites on the lower DNA strand, whilst those above the rectangle represent sites on the upper DNA strand. The region of the ER gene used to generate the ER promoter luciferase reporter gene (ERP-luc) is shaded. **b** MCF-7 cells were transfected with ERP-luc, together with the LRH-1 or the LRH-1-F342W/I416W mutant. **c** Reporter gene activities were determined for MCF-7 cells

transfected with ERP-luc, or mutants in which LRHRE1-3 were mutated. All reporter gene activities are shown as the means and s.e.m., for three replicates, relative to the vector control. **d** DNA binding to LRHRE1 and the Cyp7A1 LRH-1 response element was measured by changes in fluorescence polarization for the LRH-1 DBD. The results of at least three replicates are shown. **e** ChIP was performed using lysates prepared from MCF-7 cells, and real-time PCR for three replicates using primers for a control region mapping to the c-myc locus and the ER promoter encompassing LRHRE1, are shown. In all cases asterisks show statistically significant differences ($P < 0.05$), determined using the unpaired *t*-test, between sample 1 and the other samples, or relative to the control samples

A new LRH-1 variant is the predominant form of LRH-1 in breast cancer cells

LRH-1 transcript mapping identified two new LRH-1 variants, both of which are expressed from distinct new promoters, one initiating within intron 1 and extending exon 2 127 bp 5' (v4), and the second initiating in intron 2 and lacking exon 1 and 2 sequences (v5). Both of these

variants are estrogen regulated, but v5 expression is low in breast cancer cells. LRH-1-v1 was the only other variant whose expression was observed by 5'-RACE, but it was also expressed at low levels, with v4 being the major form of LRH-1 in breast cancer cells, which was confirmed by immunoblotting. Forms of LRH-1 corresponding to v4, as well as v1 are seen in the mouse, chicken (Supplementary Fig. 1b, c), and rat, while v4 corresponds to LRH-1

transcripts predicted to come from the xenopus and zebrafish LRH-1 genes. Furthermore, analysis of the ChIP-chip for RNA polymerase II (PolII) [25] showed that at the LRH-1 locus, the strongest binding of PolII is centered on exon 2 sequences (see Fig. 2c), which indicates that in MCF-7 cells the major transcription initiation sites for the LRH-1 gene are centered around exon 2. Finally, real-time RT-PCR using RNA from a panel of human tissues showed that v4 is expressed more widely than the other variants. Indeed, we did not detect expression of the other variants in the absence of v4 expression. Taken together, these findings provide strong evidence for the *in vivo* importance of LRH-1-v4. A previous study examining LRH-1 expression immunohistochemically in invasive ductal carcinoma showed that 43% of breast tumours are LRH-1 positive [33], expression being negatively associated with clinical stage and histological status and positively associated with steroid receptor status. As this study was carried out using an antibody directed to amino acids 2–33 of LRH-1-v1 (absent in v4), it is possible that the LRH-1 positivity was underestimated, highlighting the need for re-evaluation of LRH-1 in breast cancer, the lack of currently ascribed differences in the activities of LRH-1 variants 1 and 4 notwithstanding. As the antibodies available to us have proved to be unsuitable for immunohistochemistry, we are currently developing new antibodies to address this issue.

LRH-1 regulates estrogen receptor expression in breast cancer cells

LRH-1 knockdown inhibits breast cancer cell growth, as previously described [31] and confirmed here. Importantly, LRH-1 knockdown reduced ER expression, LRH-1 overexpression or stimulation of its activity by synthetic agonists increased ER levels, whilst the LRH-1 repressor SHP reduced ER expression. Reporter gene studies and mutational analysis showed that three sites contribute to the LRH-1 regulation of the ER reporter gene, with an LRH-1-binding site, named LRHRE1 (5'-AATTGCCAAAGCTTGGT-3') having a sequence similar to LRH-1-binding site in the mouse SEBP gene (5'-CCCAAAGGCTT-3') [29], being the most important site for the LRH-1 regulation of ER expression. Finally, ChIP confirmed binding of LRH-1 to the ER gene in a region encompassing this site. Taken together, these results show that LRH-1 regulates ER-positive breast cancer cell growth through a mechanism involving regulation of ER gene expression through direct recruitment to the ER promoter.

Previous study has highlighted a positive regulatory loop in which ER and GATA-3 reciprocally regulate each other in breast cancer cells [34], and provides a possible explanation for the co-expression of ER and GATA-3 in breast cancers (see [34, 35]). Other estrogen-regulated

genes have been shown to be important for ER action in breast cancer cells, with FoxA1 being required for the recruitment of ER to the promoters of many estrogen-responsive genes [36, 37]. Our findings show that ER and LRH-1 form a positive cross-regulatory loop in which each transcription factor is required for the expression of the other gene.

The aromatase (CYP19) gene is transcribed from a number of different promoters, with expression from the different promoters being highly tissue selective. The aromatase promoter II (PII) is used in gonadal tissues, and its regulation by SF-1 has been demonstrated [38–40]. Whilst in normal breast tissue, aromatase is expressed at low levels, with promoter 1.4 being utilized, in tumor-bearing breast tissue, aromatase expression is elevated, with transcription being driven largely through PII promoter [19]. These studies have also indicated that SF-1 is not expressed in breast tumours, whilst LRH-1 is expressed in adipose tissue, with LRH-1 and aromatase co-expression being evident particularly in pre-adipocytes [17]. In this context, LRH-1 regulates aromatase expression through recruitment to the PII Promoter [17, 18]. Furthermore, regulation of the PII promoter by LRH-1 occurs synergistically with GATA3 [18]. Interestingly, regulation of ER expression by GATA3 has also previously been described [34]. Our preliminary analysis shows that LRH-1 and GATA3 also act synergistically at the ER promoter (Supplementary Fig. 7). Aromatase expression is additionally dependent on tumor-derived growth factors, particularly prostaglandin E₂, acting through protein kinase A (PKA) to stimulate aromatase PII activity [17, 18]. In agreement with these findings, activity of the aromatase PII reporter gene was strongly increased by PKA, whereas PKA did not similarly potentiate the synergism between LRH-1 and GATA3 at the ER gene promoter (Supplementary Fig. 7). Together, these results indicate that aromatase and ER expression are similarly regulated, albeit in different cell types, indicating that the cell-type specificity may be due to differences in cell signaling pathways, such as those involving PKA (for potential model of aromatase and ER regulation by LRH-1 see Supplementary Fig. 7c).

Of course, differential expression of the LRH-1 variants may also be important in specifying cell-type selectivity for the differential regulation of aromatase and ER genes in different cell types. Furthermore, we did not observe expression of the LRH-1 regulated SHP gene in the breast cancer cell lines; nor was Dax1 expression evident in LRH-1 positive breast cancer lines. As SHP and Dax1 are important regulators of LRH-1 activity [27], their presence or absence may also be important in defining promoter and cell-dependent activities of the LRH-1, as well as responses to other signaling pathways, such as PKA.

In conclusion, our studies show that LRH-1 is a key regulator of estrogen responses in breast cancer, which acts by regulating estrogen synthesis in breast tumour tissue and by regulating ER expression in breast cancer cells. As such, LRH-1 presents as an important target for the development of new therapeutic agents for use in breast cancer treatment.

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Conflict of interest statement The authors declare no conflict of interest.

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Transient over-expression of estrogen receptor- α in breast cancer cells promotes cell survival and estrogen-independent growth

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Abstract Estrogen receptor- α (ER α) positive breast cancer frequently responds to inhibitors of ER α activity, such as tamoxifen, and/or to aromatase inhibitors that block estrogen biosynthesis. However, many patients become resistant to these agents through mechanisms that remain unclear. Previous studies have shown that expression of ER α in ER α -negative breast cancer cell lines frequently inhibits their growth. In order to determine the consequence

of ER α over-expression in ER α -positive breast cancer cells, we over-expressed ER α in the MCF-7 breast cancer cell line using adenovirus gene transduction. ER α over-expression led to ligand-independent expression of the estrogen-regulated genes pS2 and PR and growth in the absence of estrogen. Interestingly, prolonged culturing of these cells in estrogen-free conditions led to the outgrowth of cells capable of growth in cultures from ER α transduced, but not in control cultures. From these cultures a line, MLET5, was established which remained ER α -positive, but grew in an estrogen-independent manner. Moreover, MLET5 cells were inhibited by anti-estrogens showing that ER α remains important for their growth. Gene expression microarray analysis comparing MCF-7 cells with MLET5 highlighted apoptosis as a major functional grouping that is altered in MLET5 cells, such that cell survival would be favoured. This conclusion was further substantiated by the demonstration that MLET5 show resistance to etoposide-induced apoptosis. As the gene expression microarray analysis also shows that the apoptosis gene set differentially expressed in MLET5 is enriched for estrogen-regulated genes, our findings suggest that transient over-expression of ER α could lead to increased cell survival and the development of estrogen-independent growth, thereby contributing to resistance to endocrine therapies in breast cancer patients.

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Introduction

Two-thirds of breast cancers express estrogen receptor- α (ER α), and estrogen plays a critical role in the development

and progression of these tumours. This understanding has led to the development of anti-estrogens, primarily tamoxifen, which compete with estrogen for binding to the ER α . Treatment with tamoxifen for 5 years following surgery leads to 50% lower annual recurrence rate and a 28% decrease in annual rates of mortality in patients with early stage ER-positive breast cancer [1, 2]. However, many patients who respond to tamoxifen, eventually relapse. Aromatase inhibitors act by preventing the conversion of androgens into estrogens by the aromatase enzyme, with new aromatase inhibitors displaying greater efficacy than tamoxifen. However, resistance to aromatase inhibitors also develops in many cases [3, 4].

In a proportion of cases, patients who initially present with ER α -positive breast cancer, become ER α -negative [5]. The mechanisms by which ER α expression is lost are unclear, although epigenetic silencing of the ER α gene may be involved [6]. In most cases, however, resistant tumours remain ER α -positive and show a response to a change of endocrine agent [2], indicating that ER α continues to be important in regulating tumour growth in these cases. For the latter, recent studies suggest that endocrine resistance could result from modulation of ER α activity by altered co-activator and co-repressor balance and/or crosstalk with growth factor signalling cascades, including phosphorylation of ER α at specific residues. In this context, elevated HER2 and EGFR expression have been observed in cell line models of tamoxifen resistance, whilst elevated ERK1/2 MAPK [7] and high levels of phosphorylated AKT have been associated with poor response to tamoxifen and a worse patient prognosis [8]. Further, phosphorylation of ER α at serine 118 (S118) is elevated in recurrence following tamoxifen treatment [9]. Finally, high-level expression of the coactivator AIB1 is associated with poor response to tamoxifen in ER α -positive breast cancer, with AIB1- and HER2-positive patients having the worst outcome following tamoxifen treatment [10].

Ectopic expression of ER α in ER α -negative breast cancer cell lines and in immortalised non-tumorigenic breast cells inhibits their growth, despite showing estrogen-dependent stimulation of expression of estrogen-responsive genes [11–13]. In contrast, over-expression of ER α did not inhibit the growth of ER α -positive breast cancer cell lines [12], although conditional over-expression of ER α did lead to increased growth of MCF-7 cells in the absence of ligand [14], suggesting that ER α over-expression in ER α -positive breast cancer cells may facilitate adaptation to estrogen deprivation.

To further investigate the consequences of ER α over-expression on the estrogen responses in ER α -positive breast cancer cells, we have transduced MCF-7 cells with an adenovirus encoding ER α . Prolonged culturing of the ER α -transduced cells in estrogen-free medium allowed the establishment of an estrogen-independent line,

MLET5, in which ER α expression was maintained. MLET5 cells did not retain adenoviral sequences and microsatellite genotyping confirmed their lineage as MCF-7-derived. In further characterising MLET5 cells by gene expression microarray analysis, we found changes in apoptosis associated gene expression when compared to MCF-7 cells, so as to favour cell survival. As a result of these differences, we have gone on to show that the MLET5 line has altered cell survival characteristics, as indicated by a greatly reduced sensitivity to etoposide-induced apoptosis. Together, these findings indicate that transient ER α over-expression in breast cancer cells could be sufficient to promote the development of endocrine resistance in breast cancer through altered expression of estrogen-regulated genes involved in cell survival.

Materials and methods

Cell culture and cell lines

MCF-7 cells were obtained from the ATCC (LGC Prochem, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Ltd., UK) supplemented with 10% fetal calf serum (FCS) (First Link Ltd., UK). For culturing in estrogen-free conditions, MCF-7 cells were cultured in DMEM lacking phenol red (DMEM-PR) (Gibco-BRL, UK), supplemented with 10% dextran-coated charcoal-stripped FCS (DSS) (First Link Ltd., UK). MLET5 cells were routinely cultured in DMEM-PR, containing 10% DSS.

Recombinant adenovirus construction and infection of MCF-7 cells

The human ER α open reading frame was cloned into the adenoviral shuttle vector pAdTrack-CMV [15], which encodes GFP, to generate pAdTrack-CMV-ER α . Recombinant adenoviral genome AdER α and control virus (AdGFP) were generated following recombination by co-transformation of *E. coli* BJ5183 cells with pAdEasy-1, as described and packaged in HEK293 cells, also as described [15]. The viruses were purified by caesium chloride banding and viral particle concentration was determined by spectrophotometric analysis. MCF-7 cells (6×10^6) were seeded in 10-cm plates in DMEM-PR, containing 10% DSS and allowed to settle for 24 h prior to infection. FACS analysis of single cell suspensions prepared 2 days following adenoviral transduction, was used to determine the percentage of cells transduced. Cell counts were performed using a haemocytometer with trypan blue exclusion for counting of viable cells.

Sulphorhodamine B (SRB) Growth assay

Sixteen hours following seeding of 3×10^3 cells in 96-well plates in DMEM-PR containing 10% DSS, the medium was replaced with fresh medium supplemented with 17 β -estradiol (E2), anti-estrogens, or an equivalent volume of the vehicle (ethanol). Medium was changed every 3 days. Cells were fixed using 40% (w/v) TCA, for 1 h at 4°C, washed five times with distilled, deionised H₂O, followed by incubation with 0.4% SRB in 1% acetic acid for 1 h at room temperature. Excess dye was removed with five washes with 1% acetic acid and drying at room temperature. Absorbance at 480 nm was determined following solubilisation of the dye by the addition of 100 μ l of 10 mM Tris base to each well.

For measuring growth following addition of Etoposide (Sigma–Aldrich), 2×10^3 cells were seeded in each well. Medium supplemented with the inhibitors in a titration of 2-fold dilutions, starting from 100 μ M, was added after 48 h. Cell growth was measured 48 and 72 h after treatment, using the SRB assay, with the GI₅₀ being defined as the concentration of drug required to obtain 50% of the growth exhibited by untreated, control cells.

Cell cycle analysis

Cells were seeded in six-well plates (10^5 /well) in DMEM containing 10% FCS and allowed to adhere for 48 h, followed by the addition of 1 nM–100 μ M etoposide (Sigma–Aldrich) or DMSO and incubation for 48 h. Cells were harvested, cell cycle Annexin V/propidium iodide staining and analysis was carried out as previously described [16].

RT-PCR analysis

RNA was prepared and RT-PCR performed as described previously [17]. For quantitative RT-PCR (Q-RT-PCR) measurements, Taqman Gene Expression Assays were used with a 7900HT Fast Real-time PCR machine (Applied Biosystems). Primer details are given in supplementary information.

Immunoblotting

MCF-7 and MLET5 cells (1×10^6) were seeded in 10-cm plates in DMEM containing 10% FCS and lysed after 48 h, as described [17]. For experiments where ligands were to be added, the cells were incubated in DMEM-PR containing 10% DSS for 3 days prior to seeding. Ligands were added, as appropriate, with an equal volume of ethanol being added to the controls. Cell lysates were prepared 24 h later. Immunoblotting was carried out as described

previously [17], using antibodies detailed in Supplementary material.

Gene expression microarray analysis

For gene expression microarray analysis, MCF-7 and MLET5 cultures were seeded in estrogen-depleted medium as described above for 3 days, with three bioreplicate cultures used for each cell line and treatment. Following 16 h treatment with 10 nM E2, RNA was purified from the cultures (RNeasy, Qiagen) and used to probe bead arrays (Illumina, Human WG-6) through Cambridge Genomic Services (<http://www.path.cam.ac.uk/cgs/>). Hybridisation data were obtained using BeadStudio software (Illumina) and raw gene expression data analysed using GeneSpring GX 10 software (Agilent, Santa Clara USA). All samples were normalised by quantile normalisation to minimise variation between microarray chips. Data were filtered to include only those probes expressed in at least one sample (present and marginal flags). The three replicates for the no ligand and E2 treatments were compared by unpaired *t* tests, and differentially expressed genes were considered significant at a multiple testing corrected *P* value (Benjamini Hochberg FDR) of <0.05.

Results

Adenoviral transduction of ER α stimulates MCF-7 cell growth and expression of estrogen-regulated genes

MCF-7 cells were infected with an adenovirus encoding GFP for monitoring infection (AdGFP), into which the human ER α coding region was inserted (AdER α) (Supplementary Fig. 1). FACS analysis showed that 47, 76 and 90% of the cells were GFP-positive using multiplicity of infection (MOI) of 400, 2,000 and 10,000 particles per cell, respectively. However, infection with AdGFP at MOI = 10,000 resulted in cell death, so all subsequent infections were performed using MOI = 2,000. Under these conditions, stimulation of growth was observed in the absence as well as in the presence of estrogen. ER α levels were 5–6-fold higher in the AdER α -infected cells, compared to the AdGFP-infected cells at the protein and RNA levels (Fig. 1a, b). However, in the presence of estrogen, expression of the estrogen-responsive PR and pS2 genes was similar in both cases (Fig. 1b–e), with inhibition of expression with the anti-estrogen ICI182,780 (ICI), but their expression was markedly elevated in the absence of ligand, suggesting that adenoviral over-expression of ER α leads to ligand-independent activation of E2-regulated genes, as well as growth in the absence of estrogen.

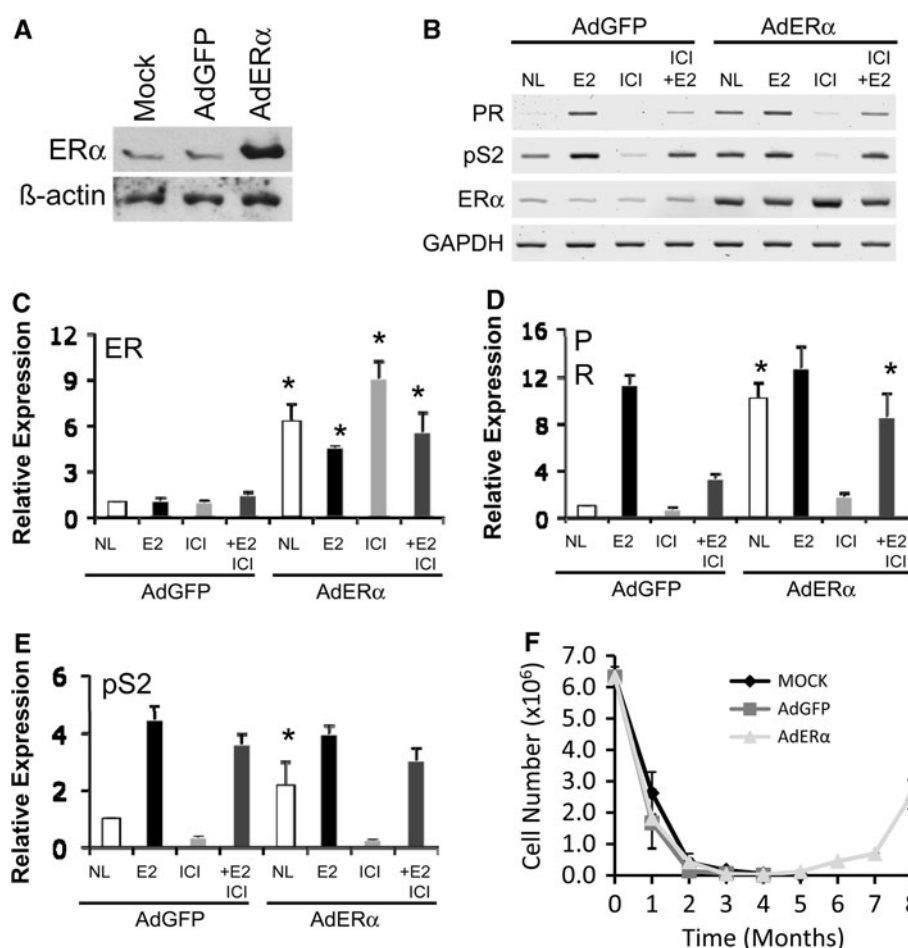


Fig. 1 Transduction of MCF-7 cells with an adenovirus encoding human ERα. **a** Immunoblots of total cell lysates prepared 48 h following infection of MCF-7 cells with AdGFP or AdERα at MOI 400. **b** For preparing RNA, estrogen (E2; 10 nM) and/or ICI182, 780 (ICI; 100 nM) were added 24 h prior to harvesting. RT-PCR was performed to analyse expression of the PR, pS2, ERα, and GAPDH genes. The expression of each gene was normalised to GAPDH following densitometric analysis and relative expression calculated for each gene, relative to the no ligand control for AdGFP-infected cells. The means for the relative expression levels of ERα (**c**), PR (**d**) and pS2 (**e**), as determined from densitometric analysis of

RT-PCR carried out on three replicate RNA preparations are shown, the error bars represent the SEM. Significance for gene expression differences was calculated using the *t* test. Statistically significant differences ($P < 0.05$) are shown for comparisons for any given treatment between the *lines* (*). **f** MCF-7 cells mock infected or infected with AdGFP or AdERα (MOI = 2,000), were grown over a period of 8 months, in estrogen-depleted medium. The cell number was determined at monthly intervals. The *graphs* show the average cell numbers from five independent experiments, the *error bars* representing the SEM

Survival of MCF-7 cells transduced with ERα is enhanced during long-term culturing in estrogen-depleted medium

Since transduction of ERα in MCF-7 cells conferred a growth advantage in estrogen-free culture conditions, we determined the effect of long-term culturing of AdERα-infected MCF-7 cells in E2-depleted growth medium (Fig. 1f). Under these conditions, cell numbers fell in all cultures over a period of 2–3 months, resulting in a complete loss in the mock and AdGFP-infected cultures by month 5. In contrast, cell numbers started to recover in the AdERα cultures. The loss of control AdGFP-infected and mock-infected MCF-7 cells, with the emergence of a small

number of surviving cells following infection with AdERα over the course of 5–6 months, was observed in four additional, independent transduction experiments. In order to confirm these findings, the infections were repeated. From 3 out of the 5 separate infections and selection over 8 months, the recovered cells were cultured, one of these giving rise to the MLET5 cell line. Monitoring of the cultures by fluorescence microscopy showed no evidence of GFP expression 2–3 months following infection; nor was GFP expression evident in the MLET5 line (Supplementary Fig. 1E). Furthermore, there was no detectable expression of GFP by RT-PCR, nor was there any evidence for the presence of adenoviral sequences by PCR (data not shown). These observations are consistent with adenoviruses being

non-integrative and, as a result of cell division, having transduced genomes that become diluted, resulting in loss of transgene expression. Finally, microsatellite genotyping analysis showed that MLET5 cells are indeed identical to MCF-7 cells for 10 markers examined (Supplementary Table 1). All of the markers used are highly polymorphic with multiple alleles. Assuming a conservative figure of 0.5 for the frequency of each allele at the 10 loci examined, the probability that the cell lines are not derived from MCF-7 is 0.5^{16} ($P < 0.0001$) (Supplementary Table 1).

MLET5 cells grow in an estrogen-independent manner, but show differential sensitivity to anti-estrogens

Estrogen is required for MCF-7 cell growth (Fig. 2a). In contrast, growth of the MLET5 cells was independent of E2, and was indistinguishable from growth in the absence of ligand (Fig. 2b). Moreover, in contrast to MCF-7 cells, the growth of MLET5 cells was only partially inhibited by anti-estrogens (Fig. 2c, d). At the RNA level ER α remained unchanged in MLET5 cells, although there was an apparent decrease in ER α expression in the absence of E2 (Fig. 3a). Overall however, ER α protein levels were elevated in MLET5 cells compared with MCF-7 cells (Fig. 3h), which may be indicative of a post-transcriptional mechanism of ER α protein stabilisation in MLET5 cells. Despite the elevated level of ER α protein, expression of several well-characterised estrogen-regulated genes was reduced (PR, CTD, TFF1/pS2), at the mRNA and protein level

(Fig. 3b–d and Fig. 3h), suggesting that the responsiveness of some, but not all E2-regulated genes (GREB1, MYC, CCND1; Fig. 3f–h) is attenuated in MLET5 cells.

This reduced stimulation in expression of some estrogen-regulated genes could be due to a reduction in ER α phosphorylation in MLET5 cells. However, levels of Ser118 and Ser167 phosphorylation (Supplementary Fig. 2), previously associated with increased ER activity were found to be higher in MLET5 cells than in MCF-7 cells, with the higher levels of phosphorylation reflecting the elevated ER α expression in MLET5 cells. Alternatively, altered ER α transcriptional co-regulator levels could lead to attenuation of expression of regulated genes [18–20]. Although expression of the p160 co-activators of ER SRC1 and AIB1 were not altered in MLET5 cells (Supplementary Fig. 3), there was a marked up-regulation of the co-repressor SMRT in MLET5 cells, suggesting the possibility of its involvement in reduced expression of E2-responsive genes in MLET5 cells.

Gene expression microarray analysis of MLET5

In order to determine if reduced estrogen regulation was a general feature of ER α activity in MLET5 cells, and to determine the gene expression changes that may have led to the development of the estrogen-independent outgrowth of MLET5 cells, gene expression microarray analysis was carried out. For this, RNA prepared from MCF-7 and MLET5 cells cultured in estrogen-depleted medium,

Fig. 2 MLET5 cells grow in an estrogen-independent manner MCF-7 (a, c) and MLET5 (b, d) cells were grown in the absence of ligand (NL) or in the presence of increasing concentrations of 17 β -estradiol (E2) (a, b). Growth in the presence of anti-estrogens was carried out following the addition of E2 (10 nM), 4-hydroxytamoxifen (OHT; 100 nM), ICI 182, 780 (ICI; 100 nM) or Raloxifene (RAL; 100 nM) (c, d). Growth was determined using the SRB assay for three experiments. Error bars depict the standard errors of the mean

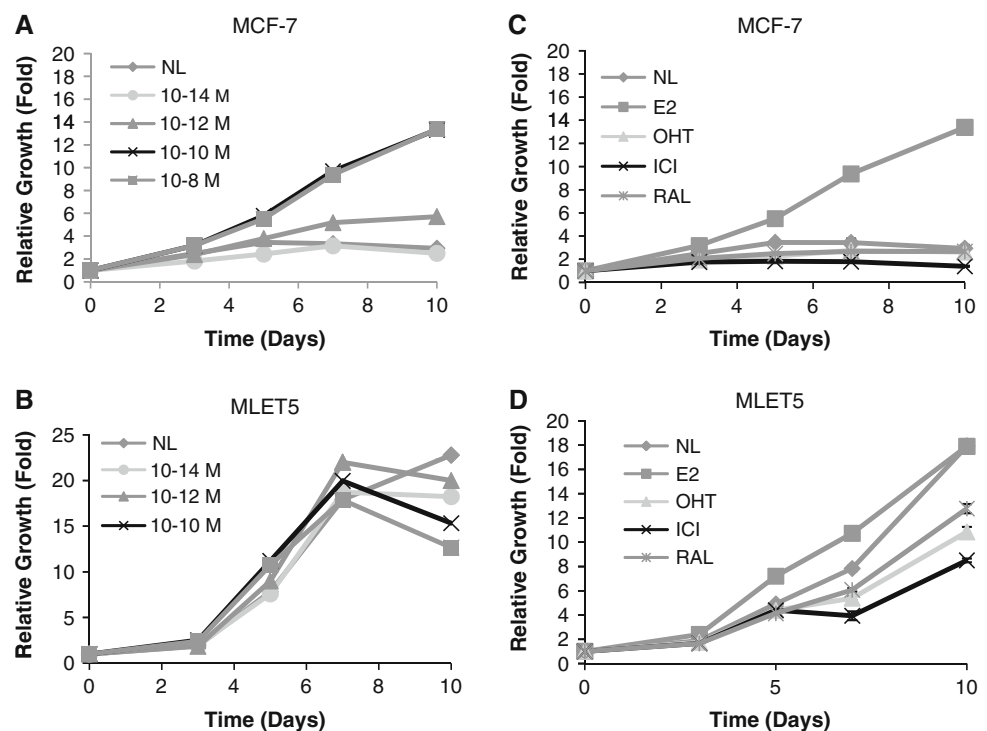
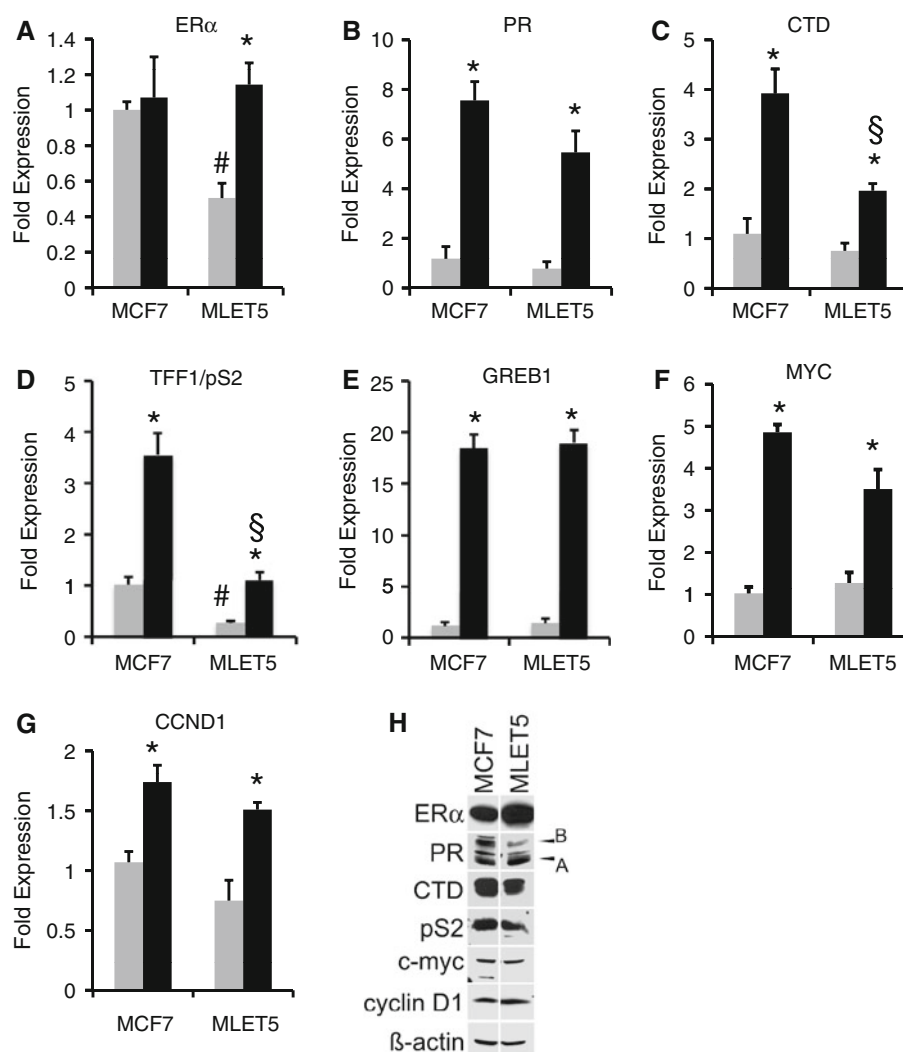


Fig. 3 The expression of estrogen-regulated genes in MLET5 cells. **a–g** Total RNA prepared from MCF-7 and MLET5 cells cultured in estrogen-depleted medium, to which 10 nM E2 was added 16 h prior to harvesting, was subjected to Q-RT-PCR. Expression level for MCF-7 cells in the absence of ligand was taken as 1, and expression for the other samples are depicted relative to this for three RNA preparations. The grey bars show expression in the absence of estrogen, with the black bars representing expression in the presence of E2. Error bars show the standard errors of the mean. Significance for gene expression differences was calculated using the *t* test. Statistically significant differences ($P < 0.05$) are shown for comparisons between the no ligand and estrogen-treated samples within each line (*), for the no ligand samples between lines (#) and for the estrogen-treated samples (§). **h** Whole cell lysates were immunoblotted, as shown



followed by addition of E2 for 16 h, was used to probe Illumina Human WG-6 bead chips. Of the 42,620 genes on the array, 4,353 genes showed significant differential expression in MCF-7 in the presence of E2, compared with the vehicle control, with similar numbers of genes showing up- and down-regulation. About half as many (2,134) genes showed significant differential expression in MLET5 cells, consistent with the reduced expression of estrogen-regulated genes observed using Q-RT-PCR (Fig. 3). Applying a fold change (FC) of 1.5 showed that 414 and 971 genes were up-, or down-regulated with estrogen in MLET5 and MCF-7 cells, respectively (Fig. 4a, b). Of the genes showing 1.5-fold up-regulation in expression in E2-treated MCF-7 cells, 34% (171 of 501) genes were also up-regulated ≥ 1.5 -fold in MLET5 cells (representing 79% of the genes up-regulated by estrogen) and included known estrogen-regulated targets such as GREB1, PDZK1, PGR, MYB, RET and IGFBP4 (Supplementary Table 2). It should also be noted that a further 140 genes with ≥ 1.5 -fold induction

by estrogen in MCF-7 cells showed a significant estrogen regulation in MLET5 cells per se.

Similarly, 115 (24%) of the estrogen down-regulated genes in MCF-7 cells were also down-regulated >1.5 -fold in MLET5 and a further 140 genes showed significant down-regulation in MLET5 cells. These data are shown in a scatterplot analysis (Fig. 4c) and highlights the fact that most of the genes that show estrogen regulation in MCF-7 cells are also estrogen-regulated in MLET5 cells, indicating that despite their adaptation to growth in the absence of estrogen, MLET5 cells still feature a marked, but attenuated estrogen-regulated gene response.

In examining the MCF-7 and MLET5 gene expression profiles we also noted that a number of previously described estrogen up-regulated genes which showed moderate, but significant estrogen regulation in MCF-7 cells, were up-regulated in MLET5 cells in the absence of ligand. For example, the expression of CXCL12 was 4.4-fold higher in MLET5 than in MCF-7. The same was also true for some

estrogen down-regulated genes, such as INHBB, whose expression was 0.25-fold in vehicle treated MLET5 cells compared to MCF-7 cells. This suggests the possibility that in MLET5 cells ER regulates the expression of estrogen-responsive genes in the absence of estrogen and that estrogen is able to further modulate the expression of these genes.

Estrogen regulation and apoptosis in MCF-7 and MLET5 cells

A total of 717 genes were differentially expressed ($P < 0.05$, $FC \geq 2.0$) in MLET5 compared with MCF-7, whilst 886 genes showed differential expression in the presence of estrogen, with 563 of these genes being differentially regulated in both the vehicle and estrogen-treated samples (Fig. 4d). Pathway analysis of the genes showing differential expression in MLET5 cells compared with MCF-7 cells ($P < 0.05$, $FC \geq 2.0$) using gene set enrichment analysis (GSEA) identified cellular and molecular functional groupings (Supplementary Table 3), the most

significant of which were functional groups encompassing apoptosis and included molecules involved in cell survival and the regulation of cell death. David (<http://david.abcc.ncifcrf.gov/>) also showed significant enrichment of cell death groups in MLET5 cells (Supplementary table 3).

With GSEA 5 of the top 6 annotated groups are associated with apoptosis and accounted for 22 genes showing differential expression in MLET5 vs. MCF-7 cells, including the key apoptotic regulators BCL2, BAD and BIK (Table 1; Supplementary Fig. 4). Q-RT-PCR showed that BCL2 expression was estrogen regulated in MCF-7 and MLET5 cells, but was dramatically elevated in MLET5 cells (Fig. 5a). Conversely, the BH3-only pro-apoptotic BIK (BCL2-interacting killer) gene was down-regulated by estrogen in MCF-7 and MLET5 cells and its expression was greatly reduced in MLET5 cells (Fig. 5b). The related gene BID, which showed estrogen regulation, was also down-regulated in MLET5 cells (Fig. 5c), whereas expression of BAD, reduced upon estrogen treatment in MCF-7 cells, was increased in MLET5 cells (Fig. 5d). Western blotting

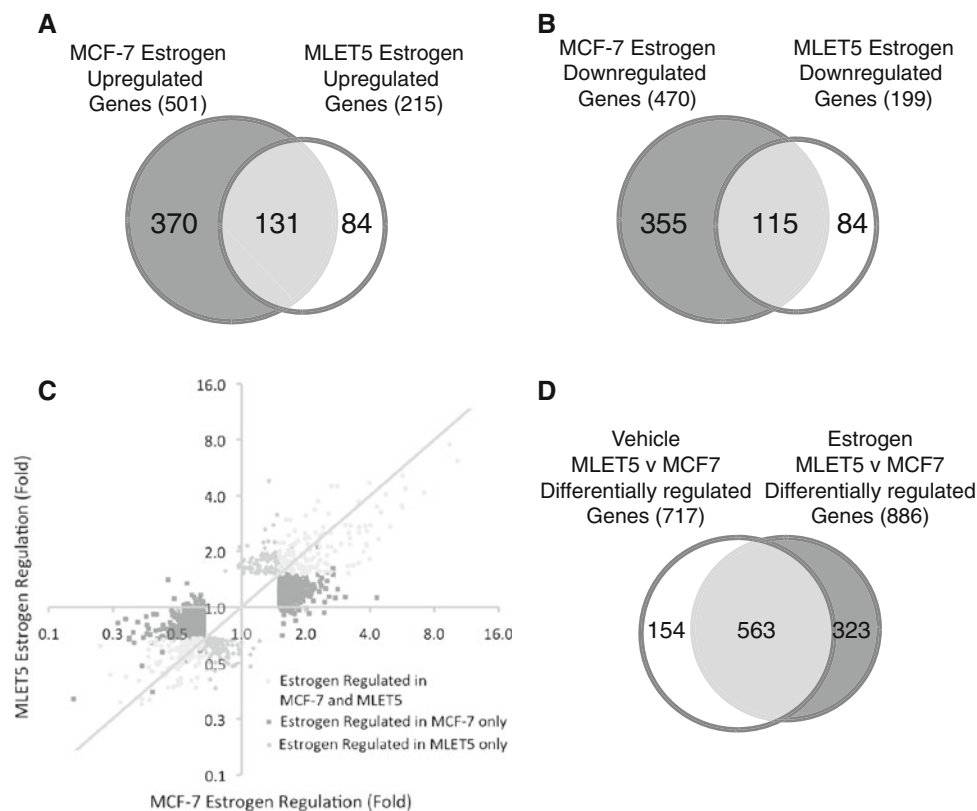


Fig. 4 Gene expression microarray profiling of MCF-7 and MLET5 cells in the absence and presence of estrogen. **a, b** Venn Diagrams to show the number of genes whose expression was up- or down-regulated ($FC \geq 1.5$, $P < 0.05$) following the addition of estrogen for 16 h in MCF-7 and MLET5 cells. **c** A scatterplot is shown for genes whose expression is altered >1.5 -fold with estrogen treatment in MCF-7 to show the fold change in expression with estrogen in

MLET5 cells, where the estrogen regulation was deemed to be significant ($P < 0.05$). Genes showing a 1.5-fold change in MLET5 treated with estrogen are similarly plotted to show genes in MCF-7 cells whose expression was significantly altered in MCF-7 cells following estrogen treatment. **d** The Venn diagram shows the number of differentially regulated genes in MCF-7 and MLET5 cells ($P < 0.05$, $FC \geq 2.0$)

Table 1 Genes associated with cell death showing differential expression in MLET5 vs. MCF-7 cells

Gene		MLET5 vs. MCF-7 Vehicle		MLET5 E2 vs. MCF-7 E2		MCF-7 E2 vs. Vehicle		MLET5 E2 vs. Vehicle	
Symbol	Description	FC	P value	FC	P value	FC	P value	FC	P value
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	8.9	0.000	10.8	0.000	0.6	0.014	0.8	0.060
BCL2	B-cell CLL/lymphoma 2	5.7	0.000	5.8	0.000	1.7	0.001	1.7	0.032
IFI6	Interferon, alpha-inducible protein 6	4.8	0.000	5.1	0.000	0.7	0.042	0.8	0.042
RTKN	Rhotekin	3.6	0.001	3.3	0.001	1.3	0.104	1.2	0.105
TSPO	Translocator protein (18 kDa)	3.1	0.000	3.4	0.001	0.8	0.111	0.9	0.308
BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	2.9	0.000	2.7	0.000	0.9	0.003	0.8	0.033
INHA	Inhibin, alpha	2.7	0.000	2.2	0.001	1.1	0.169	0.9	0.103
BAD	BCL2-antagonist of cell death	2.6	0.000	2.5	0.000	0.9	0.013	0.8	0.047
CSE1L	CSE1 chromosome segregation 1-like (yeast)	2.1	0.001	2.1	0.000	1.2	0.026	1.2	0.038
DFFA	DNA fragmentation factor, 45 kDa, alpha polypeptide	1.9	0.001	2.1	0.001	1.1	0.241	1.2	0.058
PIM1	pim-1 oncogene	0.8	0.010	0.4	0.001	2.3	0.001	1.2	0.088
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.5	0.001	0.5	0.000	1.0	0.904	0.9	0.396
BIRC3	Baculoviral IAP repeat-containing 3	0.5	0.004	0.4	0.001	1.8	0.002	1.7	0.038
TNF	Tumour necrosis factor (TNF superfamily, member 2)	0.4	0.000	0.4	0.000	1.3	0.008	1.2	0.032
BIK	BCL2-interacting killer (apoptosis-inducing)	0.4	0.001	0.4	0.001	0.6	0.006	0.6	0.030
ZAK	Sterile alpha motif and leucine zipper containing kinase AZK	0.4	0.001	0.5	0.002	0.8	0.071	1.0	0.888
PEA15	Phosphoprotein enriched in astrocytes 15	0.4	0.000	0.4	0.001	1.0	0.700	1.1	0.112
SOCS2	Suppressor of cytokine signaling 2	0.2	0.000	0.4	0.000	0.6	0.002	0.9	0.051
BNIP1	BCL2/adenovirus E1B 19 kD interacting protein like	0.2	0.000	0.2	0.000	0.6	0.001	0.7	0.031
SCIN	Scinderin	0.2	0.000	0.2	0.000	0.8	0.069	0.9	0.236
IGF1R	Insulin-like growth factor 1 receptor	0.1	0.000	0.2	0.000	1.0	0.302	1.7	0.034
ANXA1	Annexin A1	0.1	0.000	0.2	0.000	0.6	0.003	0.9	0.150

confirmed Q-RT-PCR data, with reduced BID and BIK and elevated BCL2 and BAD (Fig. 5e).

These findings suggest that altered cell survival is a key feature in the progression of the estrogen-independent MLET5 cells. To test this, the response of MCF-7 and MLET5 cells to the pro-apoptotic drug, etoposide was compared. MLET5 cells were considerably less sensitive to etoposide, with a $GI_{50} = 19.3 \mu M$, compared with a $GI_{50} = 4.6 \mu M$ for MCF-7 (t test: $P < 0.0001$) (Fig. 6a, b). Cell cycle analysis performed with etoposide at concentrations ranging from 1 nM to 100 μM showed an accumulation of MCF-7 and MLET5 in G2/M (Fig. 6c). There was also an accumulation of MCF-7 cells in the sub-G1 phase, indicative of cells undergoing apoptosis, which was confirmed with direct determination of cells in apoptosis following staining for Annexin V, with 60% of the MCF-7 cells undergoing apoptosis in the presence of 100 μM etoposide (Fig. 6d). However, even at a concentration of 100 μM , the proportion of MLET5 cells in

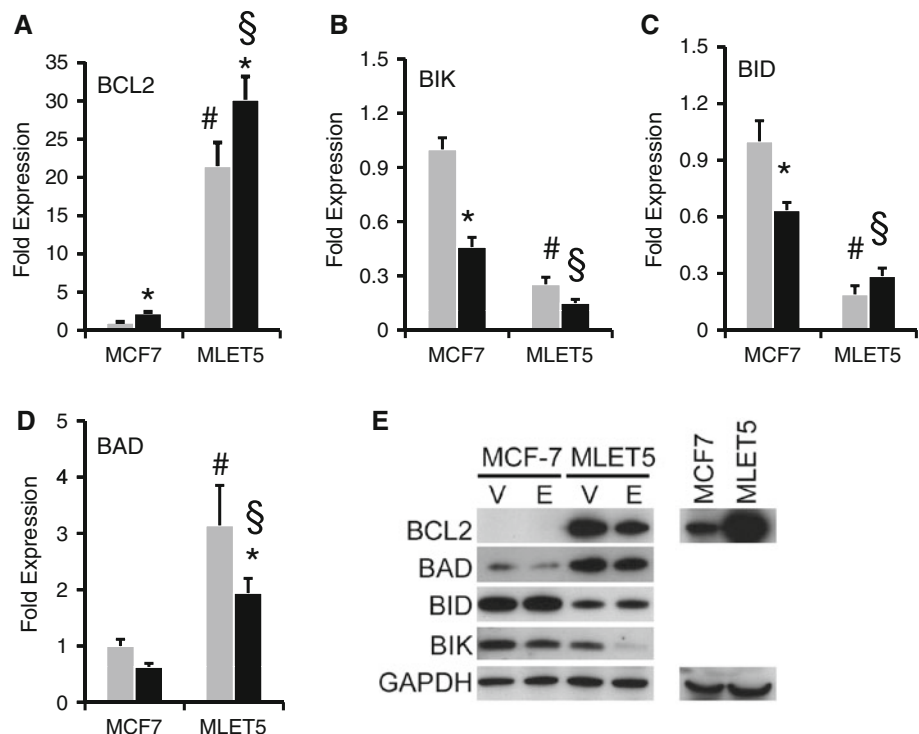
apoptosis is similar to that seen for vehicle (DMSO) treated cells.

Discussion

Estrogen receptor- α over-expression by adenoviral transduction results in ligand-independence in MCF-7 cells

In the normal breast, ER α expression is restricted to a small proportion of epithelial cells, with ER α expression being associated with a lack of estrogen-regulated proliferation. In the majority of primary breast cancer patients, however, the cancer cells are distinguished by expression of ER α in proliferating cells, with levels of expression often being elevated. Although the presence of ER α in breast cancer is associated with the likelihood of response to endocrine therapies, the impact of ER α levels in breast cancer cells on

Fig. 5 Expression of genes associated with apoptosis is altered in MLET5 cells. **a–d** Q-RT-PCR for RNA prepared from estrogen-treated MCF-7 and the MLET5 cells. Gene expression is shown relative to the expression in MCF-7 cells in the absence of ligand. *Error bars* show the standard errors of the mean. Significance for gene expression differences was calculated using the *t* test. Statistically significant differences ($P < 0.05$) are shown for comparisons between the no ligand and estrogen-treated samples within each line (*), for the no ligand samples between lines (#) and for the estrogen-treated samples (§). **e** MCF-7 and MLET5 cells were cultured in estrogen-depleted medium and whole cell lysates were prepared 24 h following the addition of 10 nM E2 (**e**) or an equal volume of ethanol (V)



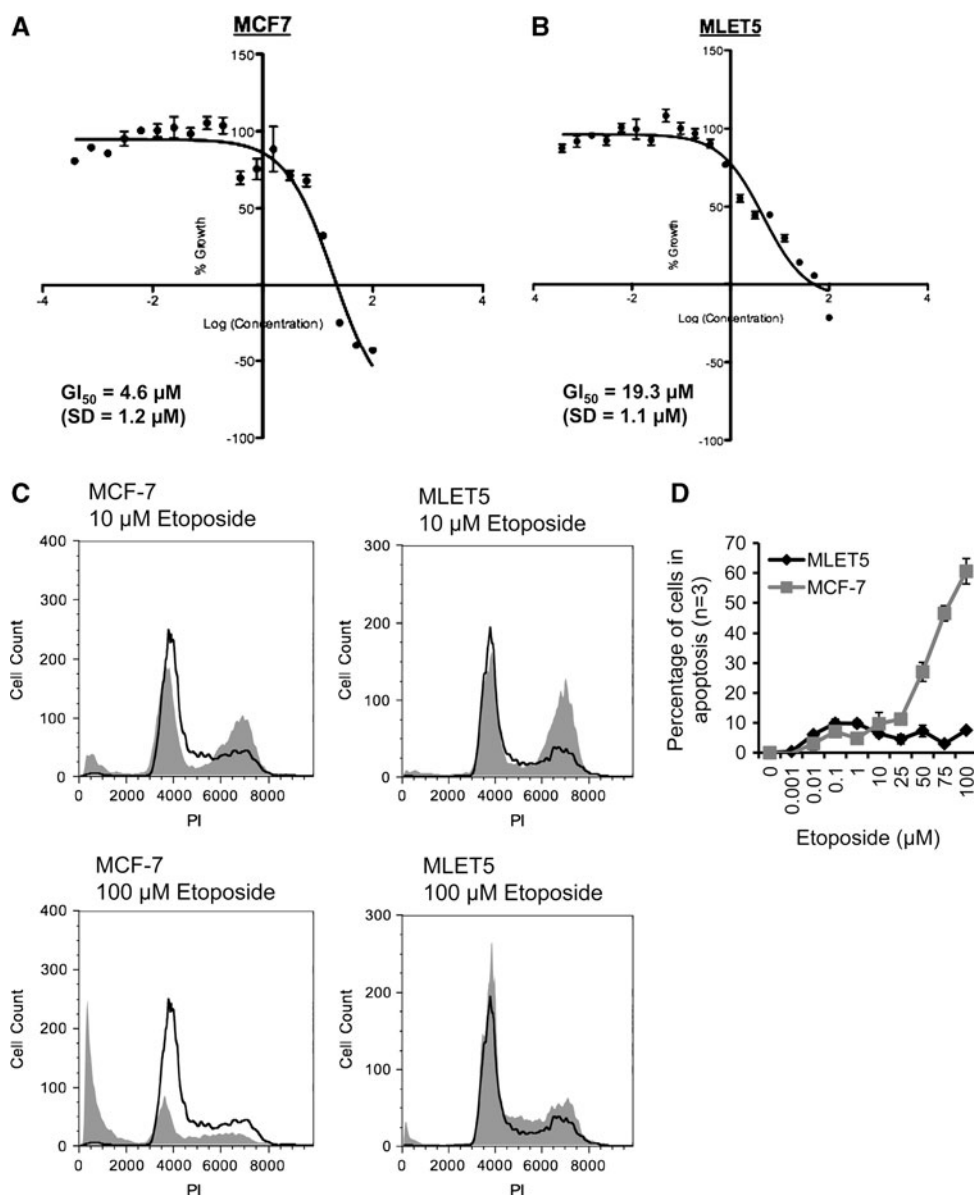
prognosis and response to endocrine treatments remain unclear. Determination of ER α levels using a ligand binding assay showed that patients with high ER α levels had a shorter relapse-free period than patients with low ER α [21]. Further, fluorescence in situ hybridization of ER α -positive breast tumours has shown that the ER α gene (ESR1) is amplified in about 20% of cases, the amplification being strongly associated with poor disease-free survival [22], whilst multiplex ligation-dependent probe amplification-based copy number analysis shows a similar percentage of ESR1 amplification in breast cancer, an association with higher mitotic index and a trend towards higher grade [23]. Other studies have shown similar levels of ER α gene amplification, but in contrast, ESR1 amplification was associated with better response to endocrine therapies [24, 25]. Further adding to the controversy are findings from other laboratories showing that the frequency of ESR1 amplification is considerably lower than 20% [26]. Additionally, there is no apparent relationship between ER α levels and response in patients with advanced disease and response in the IMPACT trial which assessed tamoxifen, anastrozole or the combination in the neo-adjuvant setting, had significantly more responders in the patient group with higher ER α levels, but only in the combination arm [27]. Therefore, it remains unclear as to whether ER α levels are related to clinical response.

In order to better understand the consequences of high ER α expression in breast cancer cells, we over-expressed ER α in the MCF-7 ER α -positive breast cancer cell line.

Interestingly, the transient over-expression of ER α led to the eventual outgrowth of cells that were estrogen independent for growth. These cells were negative for GFP expression and PCR indicated that adenoviral sequences are absent from these lines. Further, in the generation of LTED cells, Chan et al. [28] noted a transient over-expression of ER α levels in MCF-7 cells within 3 weeks of culturing in an estrogen deprived environment. This transient over-expression preceded subsequent changes leading to estrogen-independent growth, in which ER α over-expression was again seen. Taken together, these findings suggest that transient over-expression of ER α could play a role in the acquisition of an estrogen-independent phenotype by breast cancer cells. The mechanisms by which ER α over-expression may promote the development of resistant cells are unclear, but one possibility is that transient ER α over-expression provides a survival advantage that allows estrogen-independent cells to emerge. The minimum period of ER α over-expression that would be sufficient for establishment of the resistant lines is also unclear. However, at an MOI of 2,000 particles/cell, 90% of the cells were GFP-positive on day 3 following infection, falling to 48% on day 9 and no GFP expression was detectable after 12 weeks in culture (data not shown; and see Fig. 1f). Hence continued over-expression of transduced ER α was not required for the emergence of the MLET cell.

The MLET5 line was not responsive to estrogen for growth but was partially sensitive to anti-estrogens, indicating the continued importance of ER α in MLET5.

Fig. 6 MLET5 cells are resistant to apoptosis. MCF-7 and MLET5 cells were treated with etoposide at concentrations ranging from 1 nM to 100 μ M. **a, b** Cell growth was determined using the SRB assay. Shown are the results of three independent experiments. GI_{50} values were determined as the concentration of etoposide that inhibited growth by 50%. *SD* standard deviation. **c** MCF-7 cells were treated with etoposide at the concentrations shown, or with vehicle (DMSO) for 48 h, prior to fixation, staining with propidium iodide (PI) and flow cytometric analysis. Shown are representative FACS profiles, with the *black line* representing the profile obtained for DMSO treated cells and the *grey areas* represent profiles for etoposide treated cells. **d** Cells treated for 48 h with DMSO or etoposide were stained with an antibody for Annexin V and with PI. The percentage of cells that stained positive for Annexin V is shown for three independent experiments. *Error bars* represent the standard errors of the mean (SEM). Statistically significant differences between MCF-7 and MLET5 cells were reached at concentrations of etoposide of 50, 75 and 100 μ M (*t* test; *P* < 0.05)



Although ER α protein levels were significantly higher in MLET5 compared to MCF-7 cells, E2 induction of pS2 and CTD was markedly reduced. Phosphorylation of ER α at S118 and S167 stimulates its activity [29] and phosphorylation at these sites was seen to be increased in MLET5, suggesting that the reduced apparent ER α activity is not due to a reduction in ER α phosphorylation. However, levels of NCoR and particularly SMRT were elevated in MLET5 cells, compared to MCF-7 cells. This is in contrast to a previously described MCF-7 derived line, LCC1, generated through in vivo selection under conditions of low estrogen availability, and LCC9, derived from LCC1 following selection for growth in the presence of ICI [30]. LCC1 and LCC9 remain ER α -positive, but show reduced expression of the p160 co-activators (SRC1, TIF2 and AIB1) and the co-repressors NCoR and SMRT [31]. The elevated levels of

NCoR and SMRT may explain the reduced expression of pS2, CTD and PR in MLET5 cells. The reason for increased NCoR and SMRT proteins in the absence of increases in their mRNA is unclear. However, the ubiquitin ligase mSiah2 has been implicated in regulating proteasomal degradation of NCoR [32]. Siah2 expression has been shown to be estrogen-regulated and to mediate the estrogen-stimulated down-regulation of NCoR, but not SMRT protein [33], although examination of the microarray data did not show a significant difference in Siah2 levels between MCF-7 and MLET5 cells, with 2.3 and 2.1-fold stimulation of Siah2 expression by estrogen, respectively.

High-level of AIB1 expression has been associated with a poor prognosis and non-responsiveness to tamoxifen in ER α -positive breast cancers, with AIB1-positive patients also over-expressing HER1, 2 or 3, being most likely to

relapse on tamoxifen [10, 34, 35]. These findings indicate that crosstalk between ER α , AIB1 and growth factor receptor pathways are important in determining response and resistance to tamoxifen. HER2 expression and P-MAPK levels are elevated in the LTED cells [36], similar results being obtained in other LTED cells [37]. However, there was no evidence for increased expression or activity of either EGFR or HER2 in MLET5 cells. Moreover, MAPK phosphorylation levels were comparable in MCF-7 and MLET5 cells, although there was an elevation in P-AKT levels and MLET5 cells were about 5-fold more sensitive to the PI3K inhibitor LY294002 than were MCF-7 cells (Supplementary Fig. 2).

MLET5 cells are resistant to apoptosis

The gene expression microarray analysis showed that genes involved in cell survival and apoptosis are highly differentially regulated in MLET5 cells compared with MCF-7 cells. Examination of the proposed functions of these genes showed that the majority of the anti-apoptotic genes were up-regulated, whilst pro-apoptotic genes were down-regulated in MLET5 cells compared with MCF-7 cells. Of particular note were members of the Bcl2 gene family, particularly those genes whose expression is estrogen-regulated in MCF-7 cells. Hence, expression of the estrogen-regulated, anti-apoptotic Bcl2 gene was dramatically elevated in MLET5 cells at the mRNA and protein levels, whereas expression of the pro-apoptotic Bcl2 antagonists Bik, Bid, but also BMF and BNIP1 was repressed by estrogen and reduced in MLET5 cells. Although expression of the pro-apoptotic BAD gene did not follow this trend, its expression being elevated in MLET5 cells, levels of Bcl2 expression were increased to a considerably greater extent than those of Bad. It is interesting to note that analysis of the real-time RT-PCR data of Bcl2 and Bad expression shows that expression levels were similar in MCF-7 cells in the absence of estrogen, with Bcl2 levels in the presence of estrogen being 2–3-fold greater than levels of Bad. In contrast, in MLET5 cells, Bcl2 levels were 16 and 20-fold higher in MLET cells compared with MCF-7 cells in the absence and presence of estrogen, respectively. This, taken together with down-regulation of Bid, Bik, as well as BNIP1 and BMF, is indicative of anti-apoptotic programming of MLET5 cells, a fact confirmed by the demonstration that MLET5 cells are significantly less sensitive to etoposide-induced apoptosis. Although Bcl2 over-expression has been noted in MCF-7 LTED cells [38], it is not clear if this occurs in the context of other changes in gene expression indicative of overall cell survival, as we have seen in MLET5 cells. Given that Bcl2 positivity and high level Bad expression have been associated with better outcome in breast cancer patients [39, 40], it is likely that

the reduced expression of other pro-apoptotic proteins such as Bid and Bik are important for the resistance to apoptotic agents observed in MLET5 cells. Indeed, low Bik expression, as found in MLET5, has previously been associated with resistance to anti-estrogens in MCF-7 cells [41]. Further confirmation of Bcl2 up-regulation was determined by analysing its expression in LTED cells (Supplementary Fig. 5). In these cells there was an increase in Bcl2 expression as the cells progressed from the quiescent stage (weeks 2–12) to the estrogen hypersensitive stage (weeks 21–40) [28]. As observed for MLET5 cells, Bik and Bid expression fell over this time course, levels of expression in the absence of estrogen being reduced to levels similar to, or lower than those seen in the presence of estrogen in MCF-7 cells. Interestingly, Bad expression was unaltered. These findings further confirm the association between the development of estrogen independence and altered expression of Bcl2, Bik and Bid in breast cancer cells.

Taken together, our findings indicate that increased levels of ER α and/or transient over-expression of ER α in breast cancer cells may provide a mechanism for promoting estrogen-independence in breast cancer cells by facilitating cell survival under conditions of low estrogen availability, for example in patients treated with adjuvant aromatase inhibitor therapy. Our data, together with those of other groups who have over-expressed ER α in breast cancer cell lines, would indicate that breast cancer patients with high-level ER α expression would respond less well to endocrine treatment. Indeed, other studies have shown that ER α levels are elevated in LTED cells that emerge following long-term growth in estrogen-depleted media. In any case, our data indicate that even a transient over-expression of ER α would be sufficient for the development of resistance to aromatase inhibitors. In summary, the MLET5 line described here provides a useful new model for studying the mechanisms of endocrine response and resistance in breast cancer, and address the paucity of such lines that are currently available.

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Conflict of interest The authors declare no conflict of interest.

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