

**IDENTIFICATION AND CHARACTERISATION
OF TRANSCRIPTIONAL REGULATORY
PROTEINS THAT MODULATE ESTROGEN
RECEPTOR α ACTIVITY**

Dr.Eliyaz Ahamed

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**Department of Oncology, Division of Surgery, Oncology,
Reproductive Biology and Anaesthetics,
Imperial College London**

ABSTRACT

Breast cancer is the most common malignancy in women in the Western world, accounting for 18% of all female cancers. Two-thirds of breast cancers express Estrogen receptor- α (ER α) and ER α is a predictive and positive prognostic marker. Estrogens stimulate breast cancer growth, primarily through activation of ER α and hormonal therapies used in breast cancer treatment either inhibit estrogen synthesis to prevent ER α activation or bind to ER α to inhibit its activation. Unfortunately resistance to these drugs is a common clinical problem.

Altered phosphorylation of ER α has been proposed as one of the mechanisms of resistance to endocrine therapy. Phosphorylation of ER α can result in its activation, both in the presence and absence of ligand. The key phosphorylation sites have been mapped to transcription activation function AF1. In particular, phosphorylation of serine residues at amino acid positions 104, 106 and 118 in AF1 results in stimulation of ER α activity. A wealth of evidence demonstrates an important role for the ERK1/2 MAPK signal transduction pathway in non-responsiveness to endocrine therapies. However, it remains unclear as to how phosphorylation potentiates the activity of AF1. It is likely that phosphorylation of AF1 stimulates the recruitment of transcriptional coactivators that mediate the transcriptional activity of AF1. Identification and characterisation of such coactivators would further our understanding of the role of phosphorylation in the molecular mechanism of transcriptional regulation by ER α

Two approaches were used to identify coactivators that may mediate the effects of ER α phosphorylation on its activity. In the first approach, bacterial two-hybrid system was used to screen a breast cDNA expression library. In the second, p68 and p72 RNA helicases, two known transcriptional coregulators, previously reported to interact with AF1 of ER α , were characterised as coactivators of ER α . Both proteins stimulated activity of ER α and show synergism with the well-characterised nuclear receptor coactivator, SRC-1. Surprisingly, reporter gene assays showed that phosphorylation of ER α is not essential for the coactivator function of p68. Importantly, RNA interference studies revealed complex effects of p68 and p72 on ER α activity and consequently on estrogen-regulated gene expression. p72 knockdown reduced the expression of the estrogen-regulated genes, pS2, Cathepsin D and GREB1,

and inhibited the growth of the ER α -dependent MCF-7 breast cancer cell line. Surprisingly, p68 knockdown did not affect ER signalling. Data are also presented to suggest that the balance and interplay between p68 and p72 may be responsible for the previously described regulation of ER α activity by p68. Collectively, the data presented show that p72 may be more important than p68 in modulating transcriptional responses of ER α 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 thesis contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma of any university or other institute of higher learning, except where due acknowledgement has been made in the text.

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ABBREVIATIONS

AF1/AF2	Activation function 1/Activation function 2
AIs	Aromatase Inhibitors
AIBI	Amplified In Breast cancer1
AR	Androgen Receptor
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CARM1	Coactivator-Associated Arginine methyltransferase 1
CBP	cAMP response element Binding Protein
DBD	DNA Binding Domain
DMEM	Dulbecco's Modified Eagle's Medium
DPE	Downstream promoter element
DRIP	Vitamin D Receptor Interacting Proteins
E2	17 β -Estradiol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ER	Estrogen Receptor :
ERE	Estrogen Response Element
ERK1/2	Extracellular regulated kinases1 and 2
FCS	Fetal Calf Serum
GRIP 1	Glucocorticoid Receptor Interacting Protein 1
GST	Glutathione- S- Transferase
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
HER2	Human epidermal growth factor receptor 2
His	Histidine
HMT	Histone Methyl Transferase
LB	Luria-Bertani
LBD	Ligand Binding Domain
LCoR	Ligan dependent Co repressor

LH-RH	Luteinising Hormone Releasing Hormone
MAPK	Mitogen Associated Protein Kinase
ml	Millilitre
μl	Microlitre
nM	nanomolar
ng	nanogram
NCoA	Nuclear Receptor Coactivator
NCoR	Nuclear Receptor Corepressor:
OHT	4-hydroxytamoxifen
PAGE	Polyacrylamide gel Electrophoresis
PCAF	p300/CBP Associated Factor
PCR	Polymerase chain reaction
PBS	phosphate Buffered Saline
PI3K/Akt	Phosphatidylinositol-3- kinase
PPAR	Peroxisome Proliferator Activated Receptor
PR-DMEM	Phenol Red Free Dulbeccos Modified Eagle's Medium
PIC	Pre-initiation complex
PR	Progesterone Receptor
P/S/G	Penicillin/Streptomycin/L-glutamine
RAR	Retinoic Acid Receptor α
RT	Reverse Transcriptase
RXR α	Retinoid X Receptor α
S118	Serine 118
SDS	Sodium dodecyl sulphate
SERM	Selective Estrogen Receptor modulator
siRNA	small interfering RNA
SMRT	Silencing Mediator for Retinoic acid and Thyroid hormone Receptors
SRA	Steroid receptor RNA Activator
SRC-1	Steroid Receptor Coactivator 1
TBP	TATA binding Protein
TBE	Tris borate/EDTA

TEMED	N, N, N', N'-tetramethylethylenediamine
TIF2	Transcription Intermediary Factor 2
TR	Thyroid hormone Receptor
TRAP	Thyroid hormone Receptor Associated Protein
VDR	Vitamin D Receptor

1. INTRODUCTION

1.1. Estrogens in health and disease

Estrogens including other steroid hormones such as androgens, mineralocorticoids, and glucocorticoids are derived from cholesterol. In females, 17 β -estradiol (E2), the most abundant circulating form of estrogens is produced in the granulosa cells of ovary before menopause, and in adult men, in the adrenals, testes and adipose tissue. A small proportion of estrogen is also produced in skeletal muscle, skin, adipose tissue, brain, and bone (Simpson and Davis, 2001). The estrogen synthesis in the ovary is under the control of pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Russo and Russo, 1998) which influence the reproductive cycles. They exert their effects on target tissues by binding to their cognate receptors, the estrogen receptors α and β (ER α and ER β), which function as transcription factors in the nucleus.

Estrogens play a crucial role in the development and function of both female and male reproductive system and in the development of the mammary gland where they stimulate the growth and differentiation of the ductal epithelium and connective tissue. In the central nervous system, they have neuroprotective actions and are involved in memory. Estrogens reduce vascular tone and cause vasodilatation by increasing the formation and release of nitric oxide. They are considered to be vasoprotective and have beneficial effect on lipids and protect against atherosclerosis. Estrogens inhibit the osteoclastic activity and are antiresorptive. Estrogen therapy reduces bone loss and reduces risk of fracture in women with osteoporosis (Auchus and Fuqua, 1994; Mendelsohn and Karas, 1999; Hall *et al.*, 2001; Gruber *et al.*, 2002).

While they play an important role in the physiological functions noted above, they have also been implicated in the pathogenesis of diseases like osteoporosis (Horowitz, 1993), breast, endometrial and ovarian cancers, (Henderson *et al.*, 1988; Deroo and Korach, 2006), arteriosclerosis (Rackley, 2004), cardiovascular disease (Mendelsohn and Karas, 1999), and Alzheimer's disease (Honjo *et al.*, 2001) (Figure 1.1).

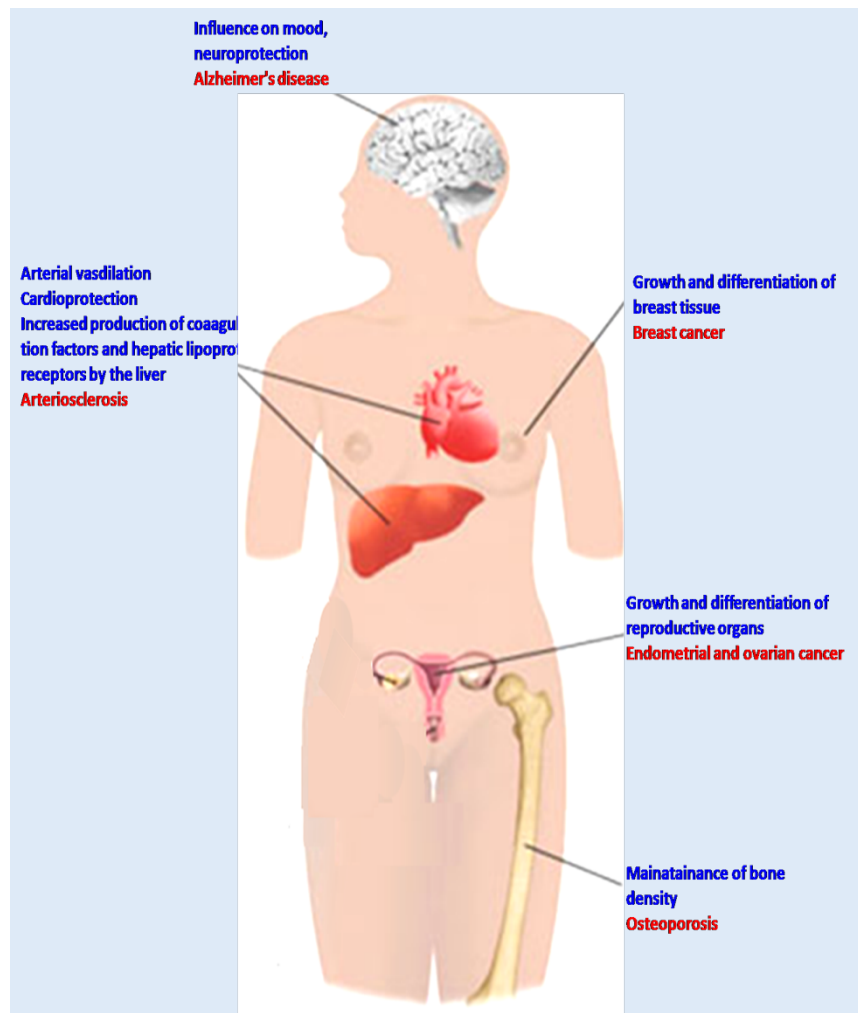


Figure1.1 Estrogens in health and disease

The physiological role of estrogens in different organ systems is highlighted in blue and the diseases linked to estrogens are highlighted in red (adapted from Gruber et al., 2002).

1.2 ER α and its role in breast cancer

Breast cancer is the most common malignancy in women in the western world, accounting for 23% of all cancers with an estimated 1.15 million new cases in 2002, with the highest rates in the developed world and the lowest rates in Africa and Asia (Parkin *et al.*, 2005). In 2004, in the UK, there were 44,659 new cases of breast cancer diagnosed and the estimated figure for USA in 2006 was 212,920 (Office for National Statistics, UK, 2004; Jemal *et al.*, 2006). The incidence of breast cancer is increasing, partly due to the screening programs and the lifetime risk of developing breast cancer is 1 in 9. However, the mortality from breast cancer has fallen over the last one or two decades due to early diagnosis and the use of adjuvant therapy (Jatoi and Miller, 2003).

Two-thirds of breast cancers express estrogen receptor α (ER α) and ER α is a predictive marker of response to endocrine therapies in breast cancer as well as a positive prognostic marker (Fisher *et al.*, 1998). The estrogen dependent nature of breast cancer was first demonstrated when in 1896, Beatson reported regression of the breast cancer after surgical oophorectomy (Beatson, 1896). Clinical studies have identified prolonged estrogen exposure through early menarche, late menopause, nulliparity, late age at first pregnancy and use of oral contraceptive hormones, as risk factors for breast cancer. Drugs to block the effects of estrogens have been used successfully to inhibit the growth of breast cancer as well as prevent the development of breast cancer, further supporting the role of estrogens and estrogen receptor in development and progression of breast cancer (Clemons and Goss, 2001; Jordan, 2003).

1.3 Endocrine therapy of breast cancer

The key principle of hormonal treatment of estrogen-responsive breast cancer is based on targeting the ER signalling and thus preventing the hormonal stimulation of malignant cells by estrogen. This is accomplished by a) decreasing the production of estrogens by the ovary, by surgical or chemical means b) competing with the binding of the hormone to its receptor with anti-estrogens such as tamoxifen or the newer generation receptor modulators such as fulvestrant or raloxifene, or c) by

interfering with the production of estrogens from the steroid precursors, such as androgens, in post-menopausal women via inhibition of the converting enzyme, the aromatase. The endocrine therapies can be classified according to the mechanism of action (Osborne and Schiff, 2005) (Table 1.1).

1.3.1 Ovarian ablation or oophorectomy

Ovarian ablation or oophorectomy can be achieved through pharmacological, radiation or surgical means. Luteinizing hormone-releasing hormone agonists such as goserelin suppress ovarian function by reducing the estrogen levels to menopausal levels through suppression of pituitary secretion of FH and LSH. Goserelin has been shown to produce equivalent results to oophorectomy in terms of response rates and survival times (Nicholson *et al.*, 1985; Taylor *et al.*, 1998). However, laparoscopic oophorectomy remains an option for women who like to avoid monthly/injections. Some centres also practice radiation induced ovarian ablation although this is much less common now.

1.3.2 Selective estrogen receptor modulators (SERMs)

Tamoxifen, a nonsteroidal anti-estrogen is the prototype drug from the class of SERMs which also include toremifene, and raloxifene. They are so named because they can either stimulate or inhibit ER activity depending on the target organ. Tamoxifen, for example, has antagonist action in breast, while in the endometrium, bone and liver it has agonist activity mimicking the estrogen effects (MacGregor and Jordan 1998). Tamoxifen competes with endogenous estrogens for receptor binding and inhibit the activation of a ligand-dependent activation function of ER α , AF-2, thereby inhibiting activation of estrogen regulated genes in the breast epithelium, where most activity of ER α is dependent on AF-2. However, these compounds do not inhibit activation of a second, non-ligand dependent, activation function, AF-1, which accounts for the agonist activity in other tissues, such as the uterus (Berry *et al.*, 1990; Osborne, 1998). Tamoxifen is currently the most widely used drug for the treatment of all stages of endocrine-responsive breast cancer and has also been investigated for its

Table 1.1 Classification of endocrine therapies
(Taken from Osborne and Schiff, 2005)

Selective estrogen receptor modulators (possess both estrogen antagonist and agonist activities)
Tamoxifen, toremifene, raloxifene
Estrogen deprivation therapy
Ovarian ablation, LHRH agonists
Aromatase inhibitors (steroidal and nonsteroidal)
ER down regulators/complete antagonists

utility prophylactically in the treatment of women at high risk of developing breast cancer (Fisher *et al.*, 1998). In the adjuvant setting, tamoxifen therapy for 5 years, results in a 40-50% reduction in the annual odds of recurrence and leads to prolonged disease-free and overall survival. Additionally, adjuvant tamoxifen therapy reduces the risk of death by 26% and nearly halves the 10-year recurrence risk (EBCTCG, 1998). A recent Oxford meta-analysis has shown similar data confirming the benefits of adjuvant tamoxifen (EBCTCG, 2005). In patients with advanced disease tamoxifen therapy resulted in response rates of 16- 56 % with overall mean time to disease progression of about 6 months and duration of response between 12 and 18 months. In some patients the responses may persist for a number of years (Osborne, 1998). Tamoxifen is generally well tolerated but adverse effects such as thromboembolism, cerebrovascular accident, cataract and endometrial cancer have been associated with its usage (Osborne, 1998).

Tormifene has shown similar efficacy as Tamoxifen and has been approved for treatment of advanced disease. Raloxifene has been used for osteoporosis and has been evaluated for the primary prevention of breast cancer (Powles, 2006). The main clinical problem apart from the adverse effects associated with tamoxifen therapy is the development of resistance. Newer agents have been developed to circumvent these problems. Fulvestrant is a pure anti-estrogen which on binding the receptor, inhibits dimerization of ER and promotes rapid degradation of the receptor and unlike tamoxifen, it has no agonist activity (Howell, 2002). In clinical trials it has shown equivalent results to AIs in tamoxifen-resistant disease. In the first line treatment of advanced breast cancer, fulvestrant showed no superiority in terms of response rates and time to progression, compared to tamoxifen. Currently it is used in sequencing treatment in patients who have failed prior endocrine therapy (Perey *et al.*, 2004; Perey *et al.*, 2007; Robertson *et al.*, 2003).

1.3.3 Aromatase Inhibitors

The Aromatase inhibitors (AIs) act by inhibiting the cytochrome P450 aromatase enzyme that converts androgens to estrogens (Brueggemeier *et al.*, 2005).

The steroidal AIs such as exemestane, bind irreversibly to the enzyme while the non-steroidal compounds such as anastrozole and letrozole, bind reversibly. The newer third generation AIs (exemestane, anastrozole and letrozole) are more potent and highly selective compared to the first-generation (testolactone, aminoglutethimide) and second-generation AIs (formestane, fadrozole). In clinical trials the third generation AIs showed superior results compared to the first and second generation agents in the treatment of advanced hormone responsive breast cancer (Smith and Dowsett, 2003; Mauri *et al.*, 2006).

In first-line therapy of postmenopausal women with advanced breast cancer, anastrozole, letrozole and exemestane, have all shown superior efficacy over tamoxifen in terms of response rates and time to progression. Following on the success and superior efficacy of AIs in the advanced breast cancer in postmenopausal women, a number of randomized control trials were initiated comparing the adjuvant therapy with tamoxifen to AI. Three modes of comparisons have been performed against 5 years of tamoxifen which had been the standard treatment: 5 years of AIs (upfront strategy), 2–3 years of tamoxifen followed by 2–3 years of AIs (switch/sequencing strategy) and 5 years of AIs after 5 years of tamoxifen (extended strategy).

Upfront strategy: Two major international trials established the superiority of AIs over tamoxifen in the post menopausal women in the adjuvant setting. The ATAC study randomized women to tamoxifen or anastrozole either alone or in combination. The combination arm was closed after initial analysis showed no benefit. The results after a median follow up of 68 months showed superiority of anastrozole over tamoxifen with improved disease free survival(DFS) (hazard ratio(HR) 0.87, $p=0.01$), time-to-recurrence (HR 0.79, $p=0.0005$), and distant metastases DFS(HR 0.86, $p=0.04$). The contra lateral breast cancers were reduced in the anastrozole arm compared to tamoxifen (42% reduction, $p=0.01$). The overall survival, however was equivalent between the two arms(HR 0.97, $p=0.7$) (Howell *et al.*, 2005). Updated results after a median follow up of 100 months have been published recently and these again showed improved DFS, distant DFS, time to recurrence and incidence of contra lateral breast cancer in the anastrozole arm(The ATAC Trialists' Group, 2008).

BIG 1-98(Breast International Study Group), another large randomized control study randomised post-menopausal ER positive breast cancer patients to one of four treatment arms: (1) 5 years of letrozole, (2) 5 years of tamoxifen, (3) 2 years of letrozole followed by 3 years of tamoxifen, or (4) 2 years of tamoxifen followed by 3 years of letrozole. The results were comparable to ATAC study with superior efficacy of letrozole compared to tamoxifen. At a median follow-up of 51 months, letrozole showed significantly improved DFS (HR 0.82; P=0.007) and time to distant metastases (HR 0.81; P =0.03) compared to tamoxifen. The overall survival was again equivalent (Coates et al., 2007).

Switch/Sequencing strategy: The largest sequencing trial, Intergroup Exemestane Study (IES) randomised postmenopausal women with ER positive (or unknown) breast cancers, who were disease-free after 2–3 years of tamoxifen therapy, to switch to exemestane or remain on tamoxifen for a total period of 5 years. Analysis of data after a median follow-up of 55.7 months showed benefit in switching from tamoxifen to exemestane at 2-3 years versus 5 years of tamoxifen, in terms of DFS (HR 0.76 P=0.0001), breast cancer specific survival and distant recurrence. An updated sub group analysis showed OS advantage for the exemestane group after exclusion of patients with estrogen-receptor-negative disease (HR 0.83 p=0.04) (Coombes et al., 2007).

The ITA trial, a smaller study, randomized women with ER positive breast cancer, who were on adjuvant tamoxifen for 2- 3 years, to receive anastrozole or continue tamoxifen for a total of five years. At a median follow up of 64 months, switch to anastrozole was associated with a significantly longer DFS (HR 0.57; P= 0.005) and distant metastases-free survival. There was no overall survival advantage (Boccardo et al., 2006).

Austrian Breast and Colorectal Cancer Study Group (ABCSG)-8 trial and Breast Cancer Group's Arimidex/Nolvadex (ARNO)-95, trial were designed similar to ITA trial. There were differences in the tamoxifen dose in these two trials, with ABCSG using 20 mg while the ARNO-95 used 20-30 mg of tamoxifen. The ARNO-95 study randomised patients who were disease free after 2 years of tamoxifen to either continue or switch to anastrozole where as the ABCSG 8 study randomized women prior to starting adjuvant tamoxifen. In a combined analysis of the Arimidex-Nolvadex (ARNO) 95 and Austrian Breast Cancer Study Group (ABSG) 8 studies, 3224 women receiving tamoxifen for 2 years, were randomized to switch to anastrozole for a further

3 years or to continue on 5 years of tamoxifen. A switch was associated with improved event-free survival (EFS) (HR 0.60 $p=0.0009$) and distant recurrence-free survival (HR 0.61 $p=0.0067$) but no difference in overall survival (Jakesz et al., 2005). A meta-analysis of the ABCSG-8, ARNO-95, and ITA trials showed improved DFS (HR 0.59, $p < 0.0001$), distant DFS (HR 0.61, $p=0.002$) and OS (HR 0.71, $p = 0.04$) with the switching strategy (Jonat et al., 2006).

In all the major trials comparing tamoxifen and AIs, the AIs were associated with lower incidence of thromboembolism and endometrial cancer compared to tamoxifen. However, the AIs were associated with increased skeletal events with changes in bone mineral density, osteoporosis and fractures. Further, there is concern about changes in lipid profile and cardiovascular events with AIs.

The data from these studies show modest but statistically significant benefit for AIs over tamoxifen with a suggestion that a switch strategy may be superior to upfront AI.

Although the above studies appear similar, there were many differences that might affect the outcome. ARNO, ITA and IES trials randomised patients after 2-3 years of tamoxifen to switch or no switch, therefore excluding those with early relapse. ITA patients were mostly node positive while the ARNO 95/ABCSG 8 patients were mostly node negative. ARNO and ABCSG 8, excluded patients who had prior chemotherapy or radiotherapy and in the other studies, the proportion of patients who received chemotherapy was not uniform. Therefore, it could be argued that a group of patients with favourable prognosis are selected who would have done better with any endocrine therapy.

Extended strategy: In the extended strategy, studies were done to determine the benefit of continuing the endocrine therapy beyond 5 years. Two international trials, ATLAS (Peto et al., 2007) and aTTom (Gray et al., 2008), randomized women to 5 and 10 years of adjuvant tamoxifen treatment. The emerging data show benefit in favour of extended therapy but further follow up is needed. The MA.17 trial randomized patients to 5 years of letrozole versus placebo after completion of 5 years of tamoxifen. The trial was unblinded after nearly 2.5 years of follow up as the data showed improved DFS (HR 0.58, $p>0.001$) and overall survival (HR 0.61, $p=0.040$) in favour of letrozole. When this trial was unblinded, patients receiving placebo were offered letrozole. In a cohort analysis, patients who continued letrozole demonstrated superior DFS (HR,

0.37; $p < 0.0001$) and distant DFS (HR, 0.39; $p = 0.004$) compared to those who opted to remain off letrozole (Goss, 2007).

High dose estrogens, progestins and androgens are rarely used now as third line or fourth line treatment in view of the toxicities associated with these agents (Osborne and Schiff, 2005).

1.4 Resistance to endocrine therapy in breast cancer

Despite considerable improvements in the developments of endocrine therapy for breast cancer, resistance to endocrine agents is a key clinical problem. In the case of tamoxifen, half of all patients do not respond, and of those that do, a large proportion eventually develop resistance, leading to tumour re-growth. The patterns of resistance can be illustrated from the analysis of the data from a clinical trial that compared letrozole with tamoxifen in advanced breast cancer in post-menopausal women. The trial showed superiority of letrozole over tamoxifen (Mouridsen *et al.*, 2003). Analysis of the time to disease progression curves reveal three groups of patients based on the response to treatment (Figure 1.2). Arrow 1 indicates the subgroup of patients who did not respond to treatment either with tamoxifen or letrozole with rapid disease progression within 3 months. This pattern of resistance is pan-endocrine resistance or *de novo* resistance. Between 3 and 6 months, the two curves separate, indicating a group who show intrinsic resistance to tamoxifen but are sensitive to Letrozole (arrow 2). After 6 months, the two curves continue to separate during the 5 year follow-up period and on further follow-up, tumours progress despite continued endocrine therapy in both groups. This is secondary or acquired resistance (arrow 3) (Ellis, 2004). Acquired resistance to tamoxifen is not due to loss or alteration of ER expression as these resistant tumours often respond to second line endocrine therapy, such as the potent anti-estrogen, Fulvestrant or other aromatase inhibitors (Ali and Coombes, 2002). Several mechanisms have been put forward for explaining Tamoxifen resistance, resulting from increased ER α activation, rather than inhibition of ER α when bound by tamoxifen. These include mutations in the ER α gene, loss of expression of

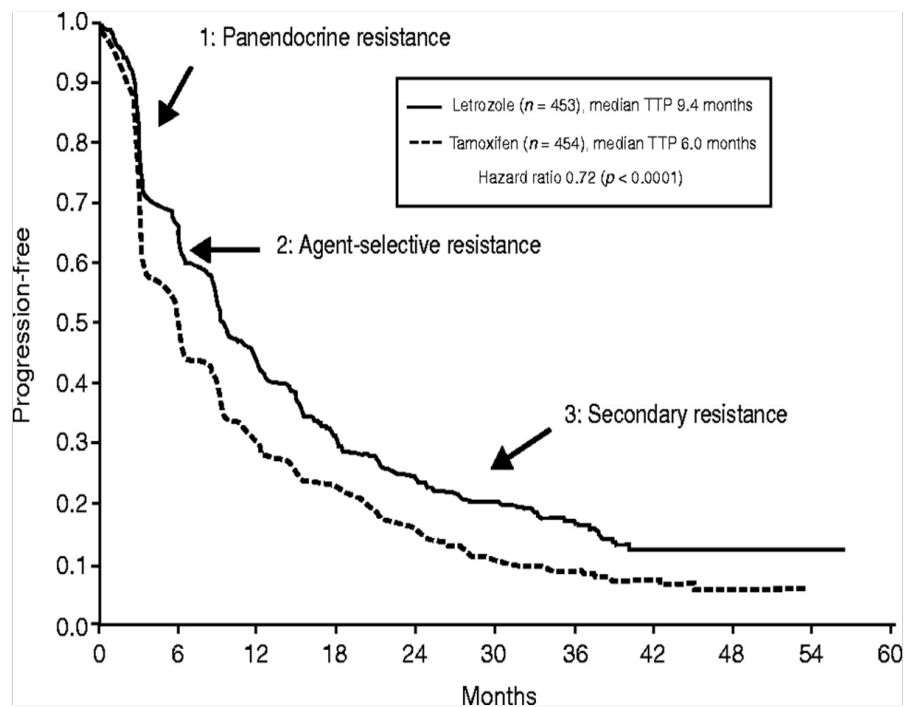


Figure 1.2 Patterns of endocrine resistance. Time to disease progression curves from a phase III study of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer showing pan endocrine resistance (arrow 1), agent-selective resistance (arrow 2), and secondary resistance (arrow 3)(Taken from Ellis, 2004)

ER α , aberrant growth factor signalling, phosphorylation of ER, alteration in the coregulatory proteins and altered expression of ER β .

1.4.1 Mutations in the ER α gene

Dysfunctional forms of ER with mutations have been described *in vitro* and in breast cancer cell lines that would make these constitutively active. However there is little clinical evidence for such mutations in breast cancer patients and therefore this does not support as the main mechanism of resistance. In fact, studies have shown that only 1% of primary breast cancers showed ER mutation in the coding regions, most of which did not result in alteration in the protein (Ali and Coombes, 2002). Some mutations have been identified in small series of breast cancer samples which could contribute to resistance. One such mutation with substitution of tyrosine-537 in the ligand-binding domain for asparagine was found in metastatic breast cancer. This mutation conferred constitutive estradiol-independent transcriptional activity, uninhibited by tamoxifen (Zhang et al., 1997). Another mutation, lysine to arginine at amino acid 303, was found in hyperplastic breast lesions. This mutation produced a hypersensitive receptor, with enhanced binding of co-activators in the presence of low estrogen levels (Ring and Dowsett, 2004; Herynk and Fuqua, 2004).

1.4.2 Loss of ER α expression/function

One of the mechanisms of *de novo* resistance is the lack of ER expression as these tumours do not respond to treatment. Therefore, loss of expression could lead to acquired resistance. However clinical studies suggest that ER continue to be functional and drive the tumour growth and loss of expression has been demonstrated only in 17-28 % of patients resistant to tamoxifen. Furthermore, nearly 20% of patients with tamoxifen resistance respond to AIs implying that ER is continued to be expressed in majority of patients resistant to tamoxifen (Osborne., 1998; Howell *et al.*, 1995; Ring and Dowsett, 2004).

1.4.3 Alterations in co-regulatory proteins

Coactivator and corepressor proteins have important roles in mediating transcriptional activation or repression by the ER. The agonist or antagonist effects of SERMs like tamoxifen may be determined by the balance of coactivator and corepressor proteins in a cell. Changes in the levels of coactivator and corepressors can lead to resistance by modifying the agonist and antagonist properties of SERMs such as tamoxifen. High levels of coactivator expression may enhance the agonist activity of tamoxifen, thus contributing to endocrine resistance. The SRC family member, AIB1 (Amplified in Breast Cancer 1), is over expressed in 50% of the breast tumours, and the AIB1 gene is amplified in 5-10% of breast tumours (Anzick *et al.*, 1997; Bautista *et al.*, 1998). Over expression of the coactivator, SRC-1 enhances the ER agonist response to tamoxifen and over expression of AIB1 together with HER-2 in breast tumours is associated with tamoxifen resistance (Schiff *et al.*, 2003; Nicholson *et al.*, 2004; Osborne *et al.*, 2003; Shou *et al.*, 2004). Corepressors are recruited to ER when bound by an antagonist such as tamoxifen. Recruitment of corepressors in the multiunit repressor complex to ER leads to repression of transcription as the corepressors which include the HDACs facilitate chromatin compaction and inhibition of transcription. In a mouse model of tamoxifen resistance, nuclear-receptor corepressor 1 (NCOR1) levels were decreased and expression of dominant negative NCOR in MCF7 cells resulted in enhanced transcriptional activity of tamoxifen bound ER. Furthermore, in a study, patients who had low levels of NCOR1, treated with tamoxifen had significantly shorter relapse free survival (Clarke *et al.*, 2003; Ring and Dowsett, 2004). Alteration of cofactor dynamics has been found in estrogen resistant MCF-7 cell line with loss of coactivator recruitment and loss of requirement of these factors for cell growth (Naughton *et al.*, 2007).

1.4.4 Cross talk between ER and growth factor signalling

In addition to the classical mode of ER signalling or genomic pathway, ER is also involved in signalling through a small proportion of ERs in the cytoplasm and plasma membrane. Some of the rapid effects seen in response to estrogens are mediated by these receptors without direct involvement of ER in transcription. This is referred to as non-genomic or membrane initiated signalling. ER can interact with the insulin-like

growth factor (IGFR), epidermal growth factor (EGF), c-Src, shc and p85 subunit of phosphatidylinositol- 3-OH kinase (PI3K) and the net result is activation of secondary signalling messengers and downstream kinase pathways such as p21 ras/p42/44 mitogen-activated protein kinase (MAPK) and AKT, leading to activation of various cellular processes such as proliferation, growth and survival. The kinase pathways can in turn activate the nuclear activity of ER, establishing a link between the genomic and non-genomic pathway. The non genomic signalling can be activated both by estrogen and SERMS such as tamoxifen. There is growing evidence, both from preclinical and clinical studies linking the cross talk between ER and growth factor pathways in resistance to endocrine therapy (Schiff *et al.*, 2005; Osborne *et al.*, 2005; Massarweh and Schiff, 2006). Over expression of EGFR and its member HER2 potentiates the non genomic activity in response to estrogen and tamoxifen. While the membranous ER activates HER2 signalling, the kinase cascade downstream can phosphorylate ER and its coregulatory proteins. ER itself is the substrate for a number of kinases in the growth factor pathway, including ERK 1/2 and p38 MAPKs, cyclin-dependent kinase 2, cyclin-dependent kinase 7, c-Src, protein kinase A, pp90rsk1, and AKT. These kinases phosphorylate ER at specific sites increasing the transcriptional activity of ER even when bound by tamoxifen and therefore, it may play a role in resistance to endocrine therapy. Phosphorylation of coactivators such as AIB1 (SRC3) increases ER-dependent transcription. In MCF-7 cells, over expression of HER2 converts tamoxifen to agonist, promoting the growth of cells. Inhibition of cross talk between HER 2 and ER by TK inhibitor gefitinib could restore the antagonist activity of tamoxifen (Shou *et al.*, 2004). Furthermore, tumours over expressing HER2 and ER coactivator AIB1, treated with adjuvant tamoxifen have been shown to be associated with poor disease free survival. It is known that kinases downstream of HER2 pathway phosphorylate AIB1 and these signalling molecules reduce the antagonist activity of tamoxifen, contributing to resistance (Nicholson *et al.*, 2004; Osborne *et al.*, 2003; Schiff *et al.*, 2003; Shou *et al.*, 2004).

Another signalling pathway that might have an important role in ER activity is the PI3K signalling pathway in response to growth factor stimulation including IGF-1R, EGFR and HER2. The serine/threonine protein kinase AKT (or PKB) is one of the downstream targets of PI3K. Its activation promotes cellular proliferation and anti-

apoptotic responses. ER α can bind in a ligand-dependent manner with the p85 α regulatory subunit of PI3K, leading to the activation of AKT. PI3K activates AKT which phosphorylates the ER at serine-167 resulting in ligand-independent activation. The expression and activity of all three AKT family members is increased in breast cancers, as well as other cancers, and often involves gene amplification. Moreover, the tumour suppressor PTEN, a negative regulator of AKT, is frequently inactivated in breast cancer. So, altered activity of the PI3K–AKT pathway could contribute to resistance to endocrine therapy (Campbell *et al.*, 2001; Jordan *et al.*, 2004). Collectively these studies support the role of cross talk between ER and growth factor signalling in endocrine resistance. This knowledge has been utilised in combining hormonal therapy with the signal transduction inhibitors to overcome resistance (Nicholson *et al.*, 2004).

Over expression of HER2 has also been shown to be involved in *de novo* resistance. Breast cancer patients with higher levels of HER2 have low levels of ER than those with lower levels of HER2. Since absolute levels of ER correlate with response to endocrine therapy, reduced expression of ER may be a mechanism of resistance in those with co expression of HER2 and ER. Preclinical data suggest that increased growth factor signalling in response to ligands like EGF, IGF-1, TGF- β and heregulin can down regulate ER expression leading to estrogen- independent phenotype. Transfection of constitutively active GF receptors or signalling molecules such as HER2, EGFR, MEK1 and Raf-1 led to down regulation of ER expression and genomic signalling. It is debated whether ER negative and HER 2 positive tumours are as a result of down regulation of ER by the increased signalling from HER2. If this is indeed true, then HER2 therapy can restore the expression of ER and can reverse resistance to endocrine therapy with combination therapy (Moy and Goss, 2006; Masserweh and Schiff, 2006; Nicholson *et al.*, 2004).

In the case of acquired resistance to AIs, certain adaptive changes occur as a result of sustained low estrogen environment conferring a supersensitive phenotype. This may be due to an increase in ER protein or to relocation of ER to the plasma membrane in association with Shc and insulin-like growth factor-1 receptor. Increased

activation of Src and RAS/RAF/MEK/MAPK signalling may also result in enhanced sensitivity to estrogen. Although xenograft tumour models exposed to AIs display some initial up-regulation of the ER, it seems that increased growth factor pathway signalling, particularly the mitogen-activated protein kinase cascade, is critical to the process of adaptation and tumour resistance. This suggests that tumour cells adapt to estrogen deprivation during treatment with AIs by the activation of alternate signalling pathways. Abrogation of alternative growth factor signalling, therefore, may restore sensitivity to endocrine therapy (Nicholson *et al.*, 2004; Ring and Dowsett, 2004, Schiff *et al.*, 2005). In cell culture models, MCF-7 cells grown in the absence or low estrogen levels, show increased expression of ER and increased transcription of estrogen regulated genes such as pS2 and PgR. In cell lines resistant to fulvestrant there was also reduced phosphorylation of ER α (Kuske *et al.*, 2006).

1.4.5 Phosphorylation of ER α

Phosphorylation has been shown to modulate the activity of many nuclear receptors. Being a phosphoprotein ER activity is also regulated by phosphorylation. The role of phosphorylation in resistance to endocrine therapy is discussed later under 'phosphorylation of ER α .

1.4.6 Altered expression of ER β

ER β has been shown to be expressed in the normal and malignant breast but its role in breast cancer and/or resistance to endocrine therapies is unclear. ER β expression is decreased in premalignant lesions compared to normal or benign lesions suggesting that it might have a protective role (Harris, 2007; Bardin *et al.*, 2004).

1.5 Nuclear receptor superfamily

Nuclear hormone receptors are ligand-activated transcription factors which form a superfamily of evolutionally conserved proteins that regulate gene expression in response to lipophilic molecules. They are essential for a variety of processes involved in development, growth, procreation, cell differentiation, proliferation, and apoptosis, and the maintenance of homeostasis. Abnormal function of nuclear receptor signalling has been implicated in proliferative, reproductive and metabolic diseases such as cancer, infertility, obesity and diabetes (Gronemeyer *et al.*, 2004; Germain *et al.*, 2006). Agonists/antagonists for a number of these receptors have been used in the treatment of these disorders. Examples of these include tamoxifen in breast cancer targeting ER, androgen antagonists in prostate cancer targeting Androgen receptor, thiazolidinediones in type II diabetes targeting peroxisome proliferator-activated receptor - γ (PPAR γ) and dexamethasone in inflammatory diseases targeting the glucocorticoid receptor (Gronemeyer *et al.*, 2004). Forty eight nuclear receptors have been identified in humans and based on sequence alignment and phylogeny they are classified into six groups. The first group contains the TRs, RARs, VDR, and PPARs, as well as orphan receptors such as RORs, Rev-erbs, CAR (NR1I3), PXR (NR1I2), LXRs, and others. The second group consists of RXRs, COUP-TF, and HNF-4. The third subfamily includes the steroid receptors with ERs, GRs, PRs, and ARs as well as the ERRs. The fourth family contains the nerve growth factor-induced clone B group of orphan receptors [NGFI-B, NURR1, and NOR1. The fifth is small family that includes the steroidogenic factor 1 (NR5A1) and the receptors related to the Drosophila FTZ-F1. The sixth subfamily contains only the GCNFI receptor (NR6A1), which does not fit well into any other subfamilies (Germain *et al.*, 2006). The orphan receptors refer to a family of nuclear receptors which share the homology with the other nuclear receptors for which no ligand has been identified yet (Robinson-Rechavi *et al.*, 2003). The nuclear family can also be divided into four classes based on the dimerisation and the DNA binding properties. Class I receptors include the steroid hormone receptors which bind as homodimers and bind to DNA half-sites organized as inverted repeats. Class II receptors heterodimerize with RXR and characteristically bind to direct repeats. This group contains all known ligand dependent receptors excluding the steroid receptors. Class III receptors bind primarily to direct repeats as homodimers. Class IV receptors

typically bind to extended core sites as monomers. Most of the orphan receptors fall into class III and IV categories (Mangelsdorf *et al.*, 1995).

1.5.1 The estrogen receptors

The effects of estrogen are mediated by two receptors, ER α and ER β , products of two separate genes located on two different chromosomes. The ER α gene is located on chromosome 6(6q25.1) and consists of 8 exons spanning more than 140 kb on chromosome 6, encoding a 595 amino acid protein (~66.2 kDa) (Walter *et al.*, 1985; Green *et al.*, 1986). The human ER β gene (ESR2) is located on chromosome 14 spanning ~61.2 kb and encodes 530 amino acids (Enmark *et al.*, 1997; Ogawa *et al.*, 1998).

Both receptors share the modular structure, characteristic of the NR superfamily which includes six functional domains. DBD of both receptors shows 97% similarity in the sequences while the LBD and AB regions show 55 % and less than 20 % similarity, respectively (Figure 1.3). When bound to estradiol, ER α and ER β can form homo and heterodimers. Although ER α and ER β have similar binding affinities for estrogen, they show some differences in the regulation of gene expression. For example, at AP-1 sites when bound by estrogen, ER β inhibits transcription while ER α activates it (Peach *et al.*, 1997; Webb *et al.*, 1999). ER α and ER β also exhibit different transcriptional effects in regulation of the cyclin D1 promoter (Liu *et al.*, 2002a). ER α mediates the stimulatory effect of estrogen on cyclin D1 expression whereas ER β has a repressive effect. However, both ER α and ER β induce the expression of cyclin D1 in response to anti-estrogens.

ER α and ER β are widely distributed throughout the body showing distinct but overlapping tissue distribution. ER α is expressed predominantly in the uterus, liver, kidney, and heart, whereas ER β is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, and the hematopoietic and central nervous systems. However, they are co-expressed in a number of tissues including the mammary gland, epididymis, thyroid, adrenal, bone, and certain regions of the brain (Pettersson & Gustafsson, 2001; Couse & Korach, 1999). Loss of ER β expression has been shown in breast, ovarian and prostate cancer suggesting a protective function of ER β (Pasquali *et*

al., 2001; Roger et al., 2001; Konstantinopoulos et al., 2003; Lazennec, 2006). The relative importance of each receptor in various tissues and the consequences of lack of functional receptor has been studied by development of ER α , ER β and double knock out models in mice (Couse & Korach, 1999; Curtis et al., 2000). The phenotypes exhibited by mice lacking ER α , ER β and both receptors and their effects in different organs systems is summarised in the Table 1.2.

Organ system	α ERKO	βERKO	$\alpha\beta$ ERKO
Male reproductive system	Normal pre- and neonatal development; progressive testicular atrophy, decreased sperm count and function	Normal fertility	Both sexes are infertile
Female reproductive system	Normal pre- and neonatal development but is insensitive to estradiol during adulthood; no ovulation; immature follicles and develop hemorrhagic cysts due to elevated LH; loss of mitogenic actions of EGF 30-40% incidence of ovarian tumours by 18 months	Ovaries undergo normal pre- and neonatal development, reduced and inefficient ovulation; sub fertile	Resemble α ERKO phenotype and insensitive to estradiol
Mammary gland	Undergoes normal prenatal development but is insensitive to estrogen-induced development during puberty and adulthood with rudimentary ductal system	Normal differentiation and lactation during pregnancy and motherhood	Immature and resemble α ERKO phenotype
Neuroendocrine system	Elevated LHb, FSHb and gonadotrophin transcripts and reduced Prolactin transcripts; elevated serum levels of estradiol Normal sexual behaviour but lack of intromission and ejaculation in males; reduced aggression	Normal serum levels of estradiol No defects in sexual behaviour	Similar to α ERKO phenotype Complete disruption of sexual behaviour
Cardiovascular system	Reduced estradiol induced angiogenesis and reduced basal levels of vascular Nitric oxide; increased expression of L-type calcium channels; retain estrogen protection in vascular injury model	No effect	Estrogen protection lost in vascular injury model
Bone	Growth arrest of longitudinal bones	Normal	Both sexes are shorter than wild type

ERKO, Estrogen receptor knockout; FSH, follicle-stimulating hormone; LH- Luteinising hormone

Table 1.2 Phenotype of ER α , ER β and double knock out in mice (adapted from Couse & Korach, 1999; Curtis et al., 2000 and Hewitt *et al.*, 2005).

1.5.2 Structure and function of estrogen receptors

ERs are characterised by six regions of differing amino acid sequence homology, designated A to F (Figure 1.3) based on the sequence alignment of human and chicken ER α (Kumar *et al.*, 1987).

AB region

The N-terminal A/B region is the least conserved domain between ER α from different species and is poorly conserved between ER α and ER β . The A/B region encodes transcriptional activation function AF1, which, when linked to a heterologous DNA-binding domain, can activate transcription in a constitutive manner. The AF-1 region contains phosphorylation sites for a number of kinases including MAPK, AKT and cyclin A/cdk2 (Kato *et al.*, 1995; Rogatsky *et al.*, 1999; Campbell *et al.*, 2001), resulting in ER activation in the absence of ligand or when the receptor is bound by tamoxifen.

The DNA binding domain (C-domain)

The C domain is the most conserved among nuclear receptors and is 97% identical between human ER α and ER β . This domain contains the DNA-binding domain (DBD), which is comprised of two zinc-binding Cys2-Cys2 sequence motifs, known as zinc fingers. ER α and ER β bind to palindromic DNA sequences, known as estrogen response elements (ERE), in the promoters of the estrogen responsive genes (Schwabe *et al.*, 1993) as homodimers, or as heterodimers (Cowley *et al.*, 1997; Pace *et al.*, 1997). The consensus ERE sequence is a 13bp palindromic inverted repeat: 5' - AGGTCA $\overline{\text{nnn}}$ TGACCT- 3', where n = any nucleotide. However, EREs within most estrogen-regulated genes are imperfect palindromes (Driscoll *et al.*, 1998).

The hinge region (D-domain)

The hinge region joins the DBD and the ligand binding domain, allowing the receptor to change its conformation upon ligand binding. It harbours the nuclear localization signals and is involved in nuclear localization, dimerisation and in the interaction of unliganded ER α with the hsp90 complex that maintains ER α in an inactive state (Klinge, 2000).

The ligand binding domain (E-domain)

This region contains not only the ligand-binding site but also the ligand-dependent transactivation function (AF-2), and possesses interaction surfaces for multiple partners, including the homo-/heterodimeric partner, corepressors and coactivators. Crystallographic studies of LBD of ER bound by different ligands have revealed mechanism of agonist and antagonist nature of ligands. The LBD is composed of 12 α helices with the amino acids in helix 3, 4, 5, and 12 forming the AF2 interaction surface. Binding of ligand leads to conformational change that alters the position of the H12 and the positioning of the helix 12 is important in discriminating the agonist or antagonist nature of the ligands. When bound by agonist such as estrogen, the helix 12 is positioned over the ligand-binding pocket and forms the surface for recruitment and interaction of coactivators. In contrast, when bound by antagonist such as tamoxifen, helix 12 is displaced from its agonist position over the ligand-binding cavity and occupies the hydrophobic groove formed by helix 3, 4, and 5 preventing the recruitment of coactivators (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998; Wurtz *et al.*, 1996).

The F-domain

The role of the C-terminal region F is unclear, but it may facilitate coactivator recruitment to the E domain and in determining the specificity of the LBD coactivator interface as well as modulating the response to ER α antagonists (Peters and Khan., 1999; Sladek *et al.*, 1999; Montano *et al.*, 1995).

Transcriptional activation by ER α is mediated by AF-1 and AF-2, which can activate transcription independently of each other, and/or in a synergistic manner. The relative activities of the two AFs are dependent on promoter and cell type (Tora *et al.*, 1989; Laszio *et al.*, 1989). AF-2 activity requires agonist binding whereas the activity of

AF-1 is mediated via phosphorylation of specific residues by growth factors acting through the MAPK pathway (Bunone *et al.*, 1996; Kato *et al.*, 1995; Katzenellenbogen, 1996; Kato *et al.*, 2000). The activity of ER α AF-2 is dependent on a hydrophobic interaction surface generated by conserved amino acids in helix 3, helices 4/5 and helix 12 (Danielian *et al.*, 1992; Henttu *et al.*, 1997; Mak *et al.*, 1999). On binding estrogen, ER α dissociates from HSP90 (Sabbah *et al.*, 1996), HSP70 and other proteins, inducing a conformational change and homodimerization of the receptor (Cowley *et al.*, 1997; Pace *et al.*, 1997; Ogawa *et al.*, 1998). Dimeric ER α binds to EREs in the promoters of

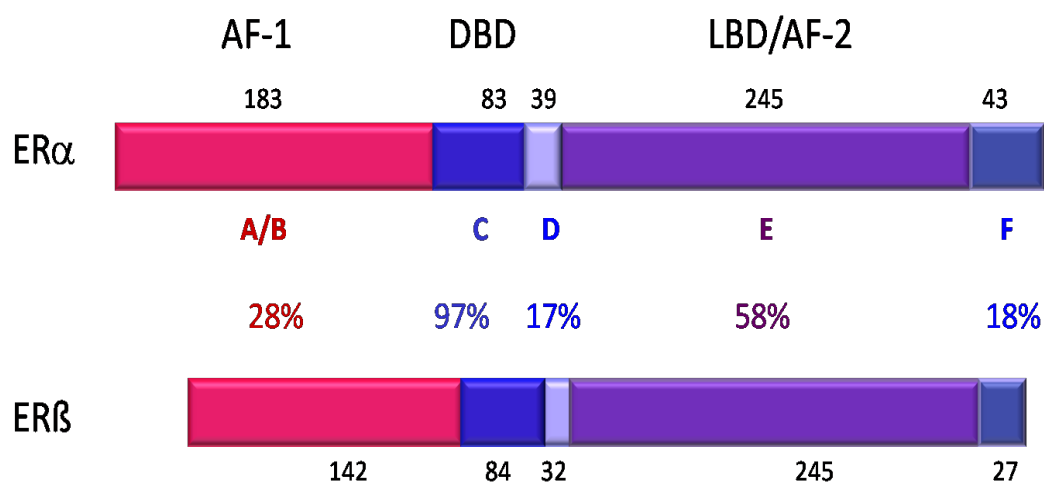


Figure 1.3 Schematic representation of the two human estrogen receptors, ER α and ER β , showing functional domains. The numbers correspond to the number of amino acids in each domain. The numbers shown between the schematics of the two ER subtypes, indicate the percentage amino acid sequence homology between the two receptors in the different functional domains.

estrogen-regulated target genes. Direct binding of the ER α homodimer to EREs result in bending of the DNA towards the major groove. This is thought to facilitate interaction with the basal transcription complex and enhance transcription from the near by promoter (MacGregor and Jordan, 1998; Kerppola and Curran, 1997). Estrogen binding to the LBD results in a receptor conformation that facilitates co activator recruitment. Key to transcriptional activation by ER α is the recruitment of coactivator complexes that remodel and modify core histones in the chromatin at gene promoters and protein complexes that facilitate RNAPolymerase recruitment to the transcription initiation site (Figure 1.4) (Rosenfeld and Glass, 2001; Metivier *et al.*, 2003). In contrast when bound by ER antagonist, tamoxifen, ER recruits corepressors that mediate repression by recruiting histone deacetylase complexes leading to chromatin compaction and repression of transcription (Figure 1.4).

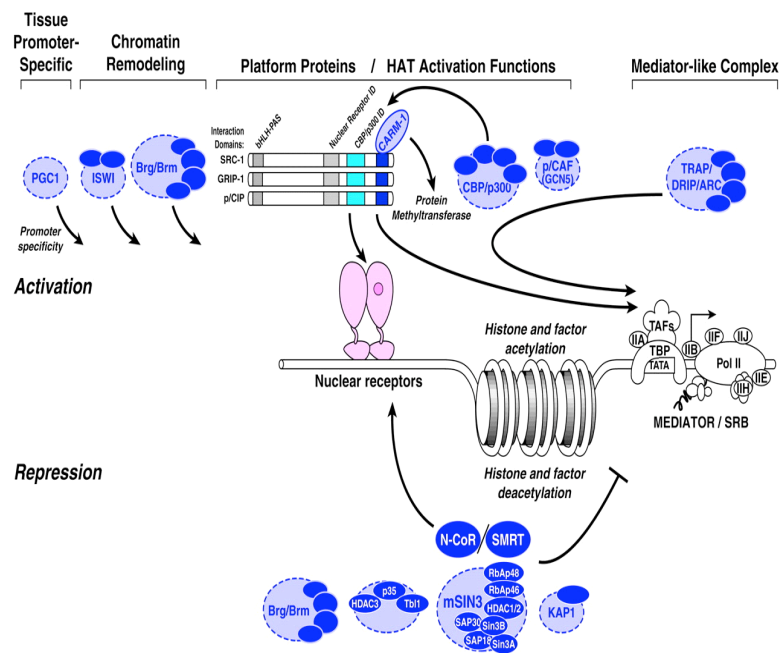


Figure 1.4 Mechanisms of transcriptional activation and repression by the nuclear receptors. When bound by activating ligand nuclear receptors recruit various coactivator complexes each of which have distinct activities that contribute to decompaction of chromatin, enabling initiation of transcription. These complexes are

assembled and disassembled in an ordered and cyclical manner at the promoters of target genes. In the absence of ligand, or when bound by antagonist, nuclear receptors recruit corepressors that mediate repression by recruiting histone deacetylase complexes that contribute to chromatin compaction and repression of transcription (Taken from Rosenfeld and Glass, 2001).

1.6 Transcriptional regulation in eukaryotes

Expression of genes in eukaryotes is a tightly regulated process that enables an organism to respond to environmental stimuli and for its development. The regulation of switching on and off, of specific genes is critical for the proper functioning and survival of an organism and it ensures that only those genes in a given context are expressed. Transcription is a multistep process which starts with the initiation of transcription by RNA polymerase II at specific DNA sites called promoters. This is followed by transcription elongation and termination, and by processing of RNA which eventually leads to the production of a messenger RNA. The messenger RNA is then transported out of the nucleus for translation. While all these steps can be specifically regulated the majority of regulatory events occur at the initiation of gene transcription (Mitchell and Tjian, 1989). The regulatory factors of this process are *cis*-acting factors (DNA and chromatin), *trans*-acting factors (transcriptional activators and associated complexes), and the basal transcription machinery (including RNA polymerase II (Pol II) and TATA-binding protein (TBP). The *cis*-acting elements are DNA sequences that can influence transcription of a gene and these are subdivided into promoter and enhancer elements. Initiation of transcription requires the assembly of a pre-initiation complex (PIC) composed of RNA polymerase II and general transcription factors. This multi-protein complex binds to a short DNA sequence called the minimal promoter, which often contains a conserved DNA sequence motif called the TATA box, situated 20–30 nucleotides upstream of the

transcription start site. Not all promoters contain TATA box sequences. In *Drosophila*, roughly half of all core promoters contain a TATA box, 25-30 nucleotides upstream of the transcription start site combined with an initiator element (Inr) overlapping the start site. The other half contain an Inr element combined with a downstream promoter element (DPE) which is located around 30 nucleotides downstream of the start site. All three of these elements act as recognition sites for subunits of TFIID, which contains the TBP and several TBP-associated factors. Many promoters seem to lack all three of these core elements and these have a GC-rich sequence in the promoter, 20-50 nucleotides 5' of the initiation site. Enhancers are *cis*-acting elements that can be located within several hundred or thousand base pairs of DNA, situated 5' or 3' of the initiation site. Enhancers act to stimulate the activity of certain promoters. In common with upstream promoter elements they can be active in all tissues or can display tissue-specificity. Many enhancers seem to contain multiple binding sites for transcription factors and often these are sites which are also found in promoter sequences. Enhancers are thought to act by binding transcription factors, bringing factor binding sites together, and its essential role may be to increase the efficiency of recruitment of transcription factors to the promoter (Levine and Tjian, 2003).

1.6.1 General transcription factors

The PIC is formed by step wise association of several transcription factors with the RNA polymerase II on the minimal promoter. The general transcription factors are designated as TFIIA, TFIIB, TFIIC, TFIID, TFIIIE, TFIIF, TFIIG, and TFIIH (Lee and Young, 2000). The initial step in the initiation involves binding of TFIID to TATA box. The TFIID itself is composed of TBP and a group of eleven subunits called TBP associated factors. TFIIA binds subsequently to stabilise the TFIID-DNA complex followed by binding of TFIIB. The TFIIB makes contacts with the DNA, both upstream and down stream of TATA box and form a platform for the assembly of Pol II enzyme complex. This is followed by the recruitment of TFIIF, TFIIIE and TFIIH to form the transcription initiation complex which catalyses the synthesis of mRNA from DNA. TFIIH plays a crucial role in transcriptional initiation by phosphorylating the C-terminal domain of RNA Pol II at serine 5. Phosphorylation of CTD results in

recruitment of elongation factors, which facilitate the Pol II to transcribe along the gene (Orphanides and Reinberg, 2002). TFIIF is a multisubunit complex consisting of 9 subunits with helicase, ATPase and kinase activities. Some of these subunits are also essential for the mechanisms of DNA repair coupled with transcription

1.6.2 Trans- acting factors

Trans-acting elements are proteins (activators and repressors) that bind specific cis-acting elements to activate or repress transcription by interaction with regulatory DNA sequences and other proteins. Eukaryotic transcriptional activators are modular proteins that are typically composed of a sequence-specific DNA binding domain and an activating domain. Activation domains could work by recruiting or accelerating the assembly of the general transcription factors on the promoter, but their mode of action remains unclear. The majority of transcription factors bind DNA as homo- or heterodimers, and thus have a dimerization domain. Some transcription factors also have ligand binding domains, such as hormone binding domains, which are essential for controlling this activity. Comparisons of DNA binding domains of many transcription factors show distinct motifs that define a protein structure capable of binding DNA. The four well-described motifs are the basic helix-loop-helix (HLH) motif, the basic leucine zipper (LZ) motif, the zinc finger (ZF) motif, and helix-turn-helix (HTH) domains (Tupler *et al.*, 2001). The exact way regulatory transcription factors affect the level of transcription has not been clearly demonstrated but they probably work by the recruitment and/or stabilization of general transcription factors, induction of a conformational change or the stimulation of an enzymatic activity of the basal transcription machinery. Moreover, some trans-acting factors may be implicated in chromatin remodelling to permit enhanced accessibility to general transcription factors for specific activators. These different roles can be promoted directly via protein–protein interactions with the basal transcription machinery, or via interactions with other trans-acting factors. In addition, some regulatory transcription factors do not contact the basal machinery directly, but instead bind co-activators that in turn contact the basal apparatus (Pabo and Sauer 1992). Coactivators may be regarded as transcription factors whose specificity is conferred by the ability to bind to DNA-

binding transcription factors instead of directly to DNA. The formation and dissociation of trans-acting factor complexes are an integral part of the regulation of many cellular processes. The combinatorial action of trans-activators confers specificity and synergistic activation of gene expression (Ptashne, 2003).

1.6.3 Role of chromatin in gene expression

The entire length of the DNA is tightly packaged within the nucleus as chromatin. The basic structural unit of chromatin is the nucleosome (Figure 1.5A). Nucleosomes comprise of around 147 base pairs of DNA wrapped in a left-handed superhelix, 1.7 times around a core histone octamer, composed of four highly conserved histones, H2A, H2b, H3 and H4 (Luger *et al.*, 1997). These proteins carry a net positive charge which neutralises the net negative charge on the DNA, allowing folding to occur (Khorasanizadeh, 2004). The degree of compaction or condensation of chromatin has been characterised as heterochromatin and euchromatin. Heterochromatin represents highly condensed state with low gene density or genes that are repressed while the euchromatin represents more open structure containing genes that are active or potentially active. The result of this organisation is not only that it provides genomic structure but it also help to regulate gene expression by restricting access to the transcription factors. Changes in local chromatin structure, including post-translational modification of histones (discussed below), would cause an opening up of the promoter DNA to allow transcription complexes access to the template, to activate gene expression or block access, to cause gene repression, depending on the type of modification (Figure 1.5B).

Chromatin remodelling is an ATP dependent process which involves the disruption and re-formation of histone–DNA contacts to activate or silence gene expression. Chromatin remodelling factors comprise an ATPase subunit along with other polypeptides that are responsible for the regulation, efficiency, and functional specificity of each complex. The ATPase subunit belongs to the Snf2 (Sucrose Nonfermenting in Yeast) super family of proteins, which includes the SWI2/Snf2 group and the imitation SWI (ISWI) group. The SWI2/Snf2 group includes yeast SWI/Snf (ySWI/Snf), yeast RSC, the Drosophila Brm (Brahma) complex, and the hBrm (human

Brm) and BRG1 (Brm/SWI2 Related Gene-1, hBRG1 in humans) complexes. There are many other proteins that are closely related to the ATPase subunits of chromatin remodelling complexes. In order to remodel chromatin, remodelling complexes recognize and bind to their substrate. The SWI/Snf, NURF, and RSC complexes act as ATP-dependent motors that track along the DNA strands and pull them away from the histone octamer cores. During this shift of histone-DNA contact points, the DNA presumably becomes accessible to the transcriptional machinery. These complexes serve to maintain chromatin in a repressive configuration by dissociating other

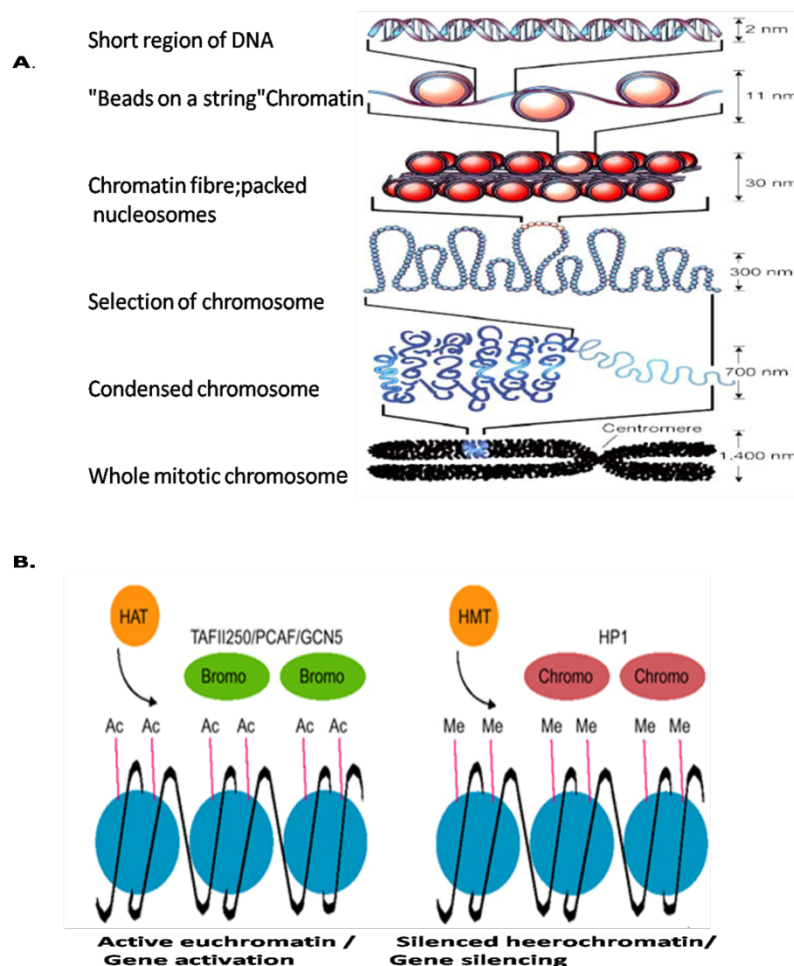


Figure 1.5 (A) Chromatin structure: organisation of DNA in the nucleus into nucleosomes, chromatin and chromosomes (taken from Horn and Peterson, 2002). (B) A model for gene activation and repression by post translational modification of histones. Acetylation of histones by Histone acetyltransferases (HATs) generate

patterns of acetylation (Ac), recognised by other transcriptional regulators, such as the bromodomain-containing (Bromo) factors TAFII250, PCAF and GCN5, leading to 'open' chromatin and gene activation. Conversely, the histone methyltransferase (HMT), SUV39H1 methylates (Me) histone H3, which is then bound by the heterochromatin-associated chromodomain (Chromo) protein HP1/Swi6, leading to a silent chromatin and gene repression (taken from Grant, 2001).

chromatin-associated proteins from the DNA, such as the TATA-binding protein (Studitsky *et al.*, 1995; Vignali *et al.*, 2000; Narlikar *et al.*, 2002).

The amino termini of histones that protrude from the nucleosome (termed as histone tails) are subjected to post translational modifications which allow access to the transcription factors. These modifications include acetylation, phosphorylation, ubiquitination, ADP-ribosylation, and methylation (Strahl and Allis, 2000; Kouzarides, 2007). These modifications are mediated by histone acetyl transferases (HATs), the histone deacetylases (HDACs), the histone methyl transferases (HMTs) and the histone kinases. The outcome of these modifications is either gene silencing or activation. Specific modifications on the histone tails act as a marker for other proteins and produce distinct biological effects. This has been referred to as 'histone code' (Jenuwein & Allis, 2001). Acetylation, which adds an acetyl group to an amino acid on the histone tail, has been linked to both gene activation and silencing, depending on which amino acid is modified. Methylation (addition of a methyl group to the histone tail) has also been linked to gene activation and repression (Paterson and Laniel, 2004).

Acetylation was the first and most extensively studied modification in transcriptional regulation. Increased acetylation of lysines in histones tails has been correlated with transcriptional activity. Acetylation neutralizes the positively charged lysine residues of the histone NH₂ termini, decreasing their affinity for DNA, which causes nucleosome to unfold and can increase access of transcription factor to the promoter. It is also probable that the acetylation of specific lysine residues in the core histones provides a platform to promote the association of other regulators in the

transcription process (Havas *et al*, 2001). HAT proteins form a part of large complexes and a number of coactivator proteins are associated with HAT activity. Since the first identification of GNAT (Gcn5-related *N*-acetyltransferases) superfamily, there are several reported families of acetyl transferases, consisting of over twenty enzymes. The GNAT family includes Gcn5 and PCAF, Elp3) and Hat1. The p300/CBP HAT family consist of the highly related p300 and CBP proteins, which function as coactivators for several transcription factors. The MYST family of HATs include MOZ and its yeast homologue, Sas3, Sas2 and Tip60. Other acetyltransferases are grouped as basal transcription factors, such as the TAFII250 component of the TFIID complex, or as nuclear hormone-receptor cofactors, such as SRC1 and SRC3 (Struhl, 1998; Sterner and Berger, 2000; Grant, 2001). While the histone acetylation is associated with gene activation, the opposite, deacetylation, catalysed by HDACs results in repression and silencing of genes. HDACs remove acetyl groups from lysine residues of core histones H3 and H4, increasing ionic interactions between positively charged lysines of histones and negatively charged DNA, resulting in a more compact nucleosome structure that limits gene transcription. In humans at least eighteen different HDACs have been reported and are sub-divided into class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, and 9), and class III (SIRT 1 to 7) (Thiagalingam *et al.*, 2003; de Ruijter *et al.*, 2003).

Methylation plays an important role in maintaining the state of chromatin. Histones can be methylated at arginine and lysine residues. Histone arginine methylation is generally associated with transcriptionally active chromatin, whereas histone lysine methylation is found in both silenced and active regions of chromatin. In general, methylation of histone H3 at Lys-4, 36, and 79 is correlated with euchromatin and transcriptional activation, whereas methylation of histone H3 at Lys-9 and 27 and histone H4 at Lys-20 is associated with heterochromatin and transcriptional repression (Rice and Allis, 2001; Zhang and Reinberg, 2001). Unlike many other histone modifications, methylation does not alter the overall charge of the lysine or arginine residues and therefore is not thought to directly alter interactions between histone molecules and DNA. Several enzymes that mediate the arginine or lysine methylation have been identified. Protein arginine methyltransferases (PRMT1ases) and Cofactor associated arginine methyltransferase 1(CARM1), have been demonstrated to have

histone methyltransferase activity and both have been shown to act as co-activators in transient transfection assays, and their co-activator potential is dependent on an intact methyltransferase domain (Chen *et al.*, 1999a ; Koh *et al.*, 2001; Wang *et al.*, 2001).

Phosphorylation of serine 10 in histone H3 has been shown be associated with gene activation. Phosphorylation of serine 10 in H2A has been correlated with chromosome condensation during mitosis .However the mechanism by which phosphorylation contributes to transcriptional activation is not clear. Addition of negatively charged phosphate groups to histones neutralises the charge reducing the affinity to DNA. Further phosphorylation of serine 10 seems to enhance the HAT activity of several acetyl transferases. Thus phosphorylation may contribute to gene activation through stimulation of HAT activity. Phosphoacetylation of histone H3 on *c-fos*- and *c-jun*-associated nucleosomes has been demonstrated upon gene activation. Phosphorylation of serine 139 in histone H3 is also known to occur after activation of DNA-damage signalling pathways and is important in DNA repair (Grant, 2001).

The consequence of chromatin remodelling leading to either activation of transcription or repression is dependent on the context of nucleosomes at a given promoter. In addition to catalyzing nucleosome mobility, chromatin-remodelling factors can enhance the access of DNA-binding factors and nucleases to DNA packaged into chromatin (Vignali *et al.*, 2000).

1.7 Mechanism of estrogen receptor signalling

Genome wide transcriptome analyses have identified a large number of possible E2 target genes in a variety of cell types (Inoue *et al.*, 2002; Frasor *et al.*, 2003; Bourdeau *et al.*, 2004). Nearly half of these are induced and the others are repressed in response to estrogen. There are three different mechanisms by which the ERs modulate the expression of these genes (Figure 1.6). In the classical or genomic action, the ERs control the expression of genes either directly, by binding to EREs or indirectly, through interaction with other transcription factors without binding to DNA. In the non-genomic signalling estrogenic effects are mediated by membrane bound ERs without affecting the transcription (Hall *et al.*, 2001).

1.7.1 Classical or Genomic action

In the absence of hormone, ER is associated with a multiprotein inhibitory complex within the nucleus. Binding of hormone induces conformational change within the ER and promotes dimerisation and high affinity binding to EREs in promoters of estrogen responsive genes. Key to transcriptional activation by ER α is the recruitment of coactivator complexes that remodel and modify core histones in the chromatin at gene promoters and protein complexes that facilitate RNA polymerase recruitment to the transcription initiation site (Rosenfeld and Glass, 2001; Metivier *et al.*, 2003). A large number of proteins that modulate transcription by nuclear receptors have now been identified, the most important of which are the SWI/SNF chromatin remodelling complexes, the SRC/CBP/PCAF histone acetyl transferase (HAT) complex, which also includes PRMT1 and CARM1, histone arginine methylases (HMT) and the TRAP/DRIP/Mediator complex that interacts directly with RNA polymerase II and associated factors (Rosenfeld and Glass, 2001). The conformational change induced by ligand binding to the LBD enables interaction of transcriptional co-activators with the LBD through simple α -helical motifs in coactivators, having the sequence LXXLL (where L is leucine and X is any amino acid). Chromatin immunoprecipitation experiments show that transcription factors, coregulators and the RNA Polymerase II cycle on the promoter in an ordered and sequential manner (Metivier *et al.*, 2003). The sequential recruitment of these complexes has been studied in detail, in particular for the pS2 gene. In the absence of ligand, a transcriptionally unproductive cycle is followed by recruitment of the ATP-dependent remodeling factor SWI/SNF, on addition of E2, which is then followed by the recruitment of HATs and HMTs. The action of these complexes in chromatin remodeling is followed by the recruitment RNA polymerase II and the TRAP/DRIP/Mediator complex. After transcription initiation, ER α and chromatin remodeling complexes are removed from the promoter, with proteosomal degradation of the DNA-bound ER α . New cycles of ER α and coactivator recruitment is preceded by histone deacetylation and demethylation (Figure 1.4 and Figure 1.5) (Metivier *et al.*, 2003; Reid *et al.*, 2003).

1.7.2 Indirect modulation of transactivation through protein-protein interactions

ER α can also modulate gene transcription indirectly without binding to EREs through protein/protein interactions with DNA bound transcription factors such as AP1, Sp1 and NF-kB. The estrogen bound ER can activate the expression of collagenase and IGF-1 through the interaction with Fos and jun at AP1 binding sites. The estrogen induction of Cyclin D is through the interaction with Sp1. Similarly genes such as TNF α and other cytokines are modulated through interaction with NF- kB transcription factor (Hall *et al.*, 2001; Kushner *et al.*, 2003).

1.7.3 Non-genomic activity of ER α

Certain effects of estrogen are apparent within seconds or minutes. The examples include rapid rise in the calcium observed in cultured endometrial cells, maturing oocytes and granulosa cells and vasodilator effects of estrogen on coronary arteries and aorta. These effects cannot be blocked by transcriptional or translational inhibitors, implying that these are independent of transcription. This rapid signalling is referred to as non-genomic activity, also called membrane-initiated steroid signalling (MISS). These effects are thought to be due to a small pool of ERs localised in the cytoplasm or plasma membrane. ER, in response to estrogen, interact with several growth factor tyrosine kinase receptors such as HER2 and IGFR and activate their signalling pathways. ER also directly associates with the key signal transduction adaptors and kinases such as Shc, the p85 regulatory subunit of phosphatidylinositol-3-OH kinase and c-Src. These interactions also trigger activation of crucial secondary downstream kinases such as p21Ras/ p42/44 MAPK and PDK1/Akt. ER predominantly residing at the membrane caveolar domains can act as a G-protein-coupled receptor. GPR30, a member of the G-protein coupled receptors is seven-transmembrane-spanning (7TM) receptor shown to mediate the MISS. Binding of GPR30 to estrogen results in intracellular calcium mobilization and synthesis of phosphatidylinositol 3, 4, 5-trisphosphate in the nucleus (Revankar *et al.*, 2005). The GPR30 is linked to the estrogen induced activation of adenylyl cyclase, release of epidermal growth factor (EGF) related ligands and the activation of growth factor -MAPK pathway and these

effects of GPR 30 are independent of, ER α and ER β . In MCF-7 cells that express GPR30, estrogen stimulates adenylyl cyclase and MAPK activation but this is not seen in MDA-MB 231 cells that express ER β but not GPR30 (Filardo et al., 2002). The antiestrogens bind GPR 30 and mimic estrogen actions in breast cancer cell lines and this may have a role in resistance to endocrine resistance (Thomas et al., 2005). Kinase cascade signalling induced by non-genomic ER activity can impinge on the ER genomic pathway, by phosphorylating and activating the various components of the ER pathway as well as other components of the transcriptional machinery, resulting in potentiation of nuclear ER transcriptional activity (Levin, 2003; Losel and Wehling, 2003; Bjornstrom and Sjoberg, 2005). The non-genomic effects of ER, as with genomic pathway are also modulated by coregulatory proteins. Several ER coregulatory proteins for non-genomic ER activity have been recently identified. The ER-interacting protein, modulator of non-genomic activity of estrogen receptor (MNAR)/PELP1 stimulates both genomic ER activity on gene transcription and non-genomic ER activity via the c-Src/p42/44 MAPK pathway by facilitating ER interaction with c-Src. The nuclear MTA1 factor is a member of the metastasis-associated gene (MTA) family of ER coregulators. It acts as corepressor of genomic ER activity but increases non-genomic ER activity. Non-genomic activity is dependent on ligand for its activation but unlike genomic activity is not inhibited by SERMs such as tamoxifen. On the other hand, the non-genomic activity can even be stimulated by the SERMs, implying a role of this pathway in endocrine resistance (Losel and Wehling, 2003; Bjornstrom and Sjoberg, 2005).

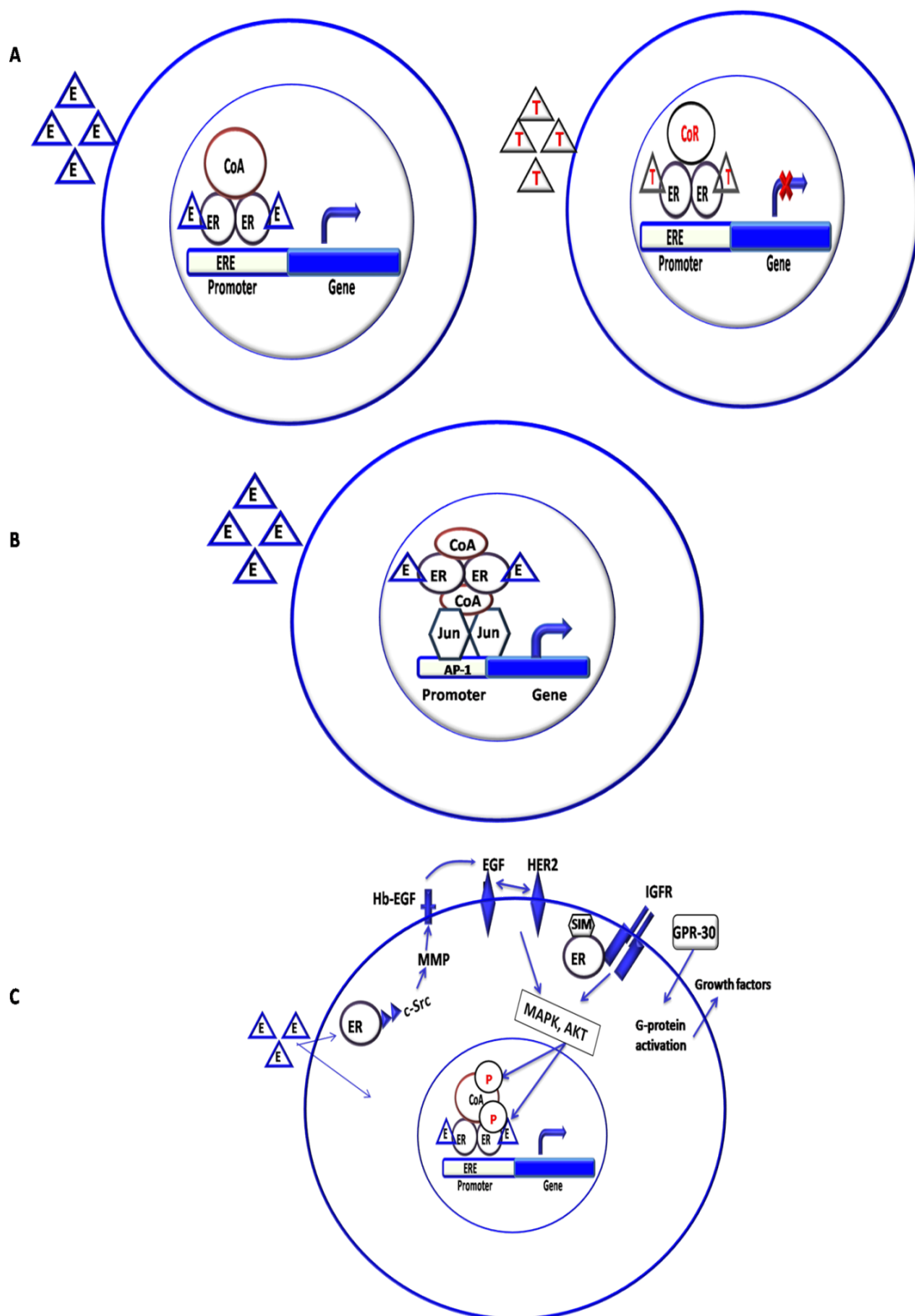


Figure 1.6 Mechanisms of ER signaling; Genomic and non genomic signaling

(A) ER, in response to estrogen (E) or tamoxifen (T) binds to estrogen response elements (EREs) in the promoter region of target genes and recruits coactivator complexes or corepressor complexes, activating or repressing the transcription **(B)** ER also modulates genomic activity, indirectly by via protein–protein interaction (e.g., with the Fos/Jun family members) at promoters responsive to other transcription factors such as AP-1. Both these activities are termed genomic signalling **(C)** In nongenomic or membrane-initiated steroid signalling (MISS), a small subset of the cellular pool of ER, localized near the plasma membrane, can associate in response to estrogen (E) with several growth factor tyrosine kinase receptors such as HER2 and IGFR and with signalling intermediate molecules (SIM). This activates protein kinase cascades involving MAPKs and AKT. ER also directly associates with the key signal transduction adaptors and kinases such as Shc, the p85 regulatory subunit of phosphatidylinositol-3-OH kinase and c-Src which activates metalloproteinases (MMPs), which then cleave and liberate heparin-binding EGF (HB-EGF). These interactions also trigger activation of crucial secondary downstream kinases such as p21Ras/ p42/44 MAPK and PDK1/Akt. MISS is also mediated by G-protein-coupled receptor, GPR30 which in turn is linked to the activation of growth factor-MAPK pathway. Kinase cascade signalling induced by nongenomic ER activity can impinge on the ER genomic pathway, by phosphorylating and activating the various components of the ER pathway as well as other components of the transcriptional machinery, resulting in enhancement of genomic activity.

1.8 Phosphorylation of ER α

Phosphorylation has been shown to modulate the activity of many nuclear receptors. All aspects of receptor function can be regulated, including DNA binding and dimerisation, transcriptional activity, interaction with cofactors and ligand-binding affinity (Smith, 1998; Shao and Lazar, 1999). ER α is phosphorylated in the absence of ligand as well as on binding to estrogen and anti-estrogens (Ali *et al.*, 1993). Several phosphorylation sites have been mapped and these include serine 104, 106, 118, 167, 236, 305, threonine 311 and tyrosine 537 (Figure 1.7) (Ali *et al.*, 1993; Arnold *et al.*, 1994; Arnold *et al.*, 1995a; Castoria *et al.*, 1993; Joel *et al.*, 1995; Le Goff *et al.*, 1994; Lee and Bai, 2002, Wang *et al.* 2002; Michalides *et al.* , 2004). The major ER α phosphorylation sites on serine residues at 104, 106 and 118 have been mapped to AF1, and serine 118 is the major site phosphorylated in response to estrogen as well as partial antagonist tamoxifen. Mutation of this residue to alanine reduces the ER transactivation while substitution of this residue by glutamic acid enhances its activity in reporter gene assays mimicking phosphorylation (Legoff *et al.*, 1994, Joel *et al.*, 1995 Ali *et al.*, 1993). These residues are the target for different kinases. Cyclin-A-cyclin dependent kinase 2 (Cdk2) complexes phosphorylates Ser-104 and Ser-106 but not Ser-118 (Rogatsky *et al.*, 1999) while Serine 118 is the major site of phosphorylation by the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2, in response to growth factor induced activation of MAPK pathway (Joel *et al.*, 1998; Kato *et al.*, 1995). Ras/Raf/MEK/MAPK signal transduction pathway results in ER α activation through phosphorylation of S104, S106, and S118 (Thomas *et al.*, 2008). The kinase responsible for ligand induced phosphorylation remains unclear and one of the kinase phosphorylating serine 118 in response to estrogen binding is CDK7, the cyclin-dependent kinase that is associated with the basal transcription factor TFIIF cyclin dependant kinase (Chen *et al.*, 2000).

Serine 167 is another important phosphorylation site which is phosphorylated by p90 ribosomal S6 kinase, a kinase downstream of MAPK pathway (Joel *et al.*, 1998). It is also phosphorylated by AKT (protein kinase B) and protein kinase A (Campbell *et al.*, 2001; Martin *et al.*, 2000).

Protein kinase A (PKA) has been shown to phosphorylate serine-305 in the hinge region of ER and converts tamoxifen from an antagonist to agonist of ER. Protein kinase A also phosphorylates Ser-236, which is located in the DNA binding domain, involved in dimerisation and ER binding to EREs (Chen *et al.*, 1999b).

Tyrosine 537 is also phosphorylated and this residue is thought to be important in the interaction of ER with the kinases containing SH2 domains. This residue is important for AF2 function and some mutations of tyrosine 537 make the receptor constitutively active with the recruitment of coactivators seen even in the absence of ligand (Lannigan, 2003).

1.8.1 Role of phosphorylation in resistance to endocrine therapy

As discussed above, phosphorylation can increase the activity of ER both in the presence and absence of ligand. Growth factor induced signalling activate a number of kinase cascades which phosphorylate ER α and this “cross talk” of ER α with the growth factor signalling pathways, has been implicated in endocrine resistance. In particular serines 104, 106 and 118 are phosphorylated by the p42/p44 mitogen-activated protein kinases (MAPK). This is particularly interesting, as a proportion of breast cancers show increased expression of MAPK activity (Sivaraman *et al.*, 1997) and its over-expression in breast tumours correlates with poor response to tamoxifen (Gee *et al.*, 2001). Serine 167 is phosphorylated by ribosomal S6 kinase, which in turn is activated by MAPKs, ERK1 and ERK2 (Joel *et al.*, 1998). Serine 167 is also phosphorylated by AKT (Campbell *et al.*, 2001). There is evidence for increased activity of the AKTs in breast cancer (Faridi *et al.*, 2003). PTEN, a tumour suppressor gene is a negative regulator of AKT, and has been shown to be frequently mutated in breast cancer (Testa and Bellacosa, 2001). Phosphorylation of serine-305 in the hinge region of ER by protein kinase A (PKA) converted tamoxifen from an antagonist to agonist of ER. In breast tumours PKA-R1 α , a negative regulator of PKA has been shown to be down regulated, contributing to tamoxifen resistance (Michalides *et al.*, 2004). Thus, phosphorylation of ER α could be one of the key mechanisms of resistance to endocrine therapy.

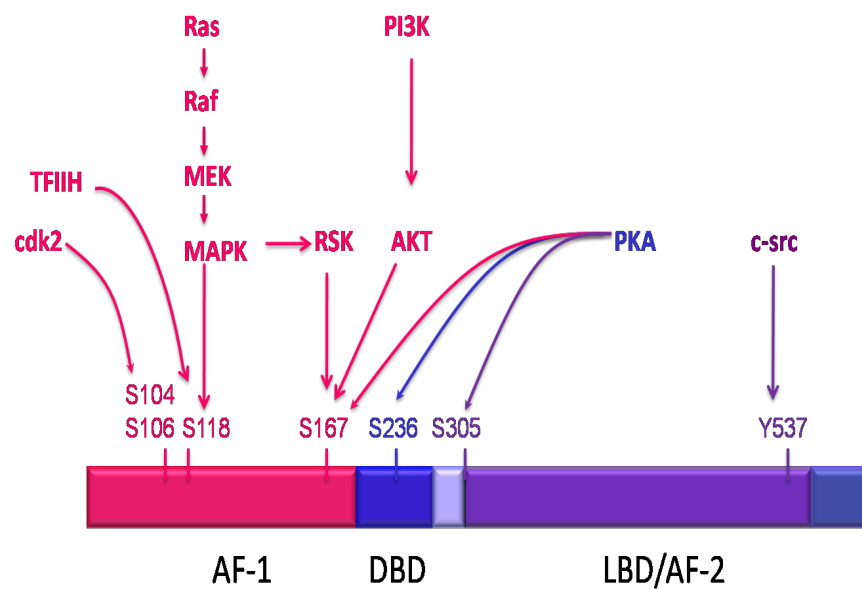


Figure 1.7 Phosphorylation of ER α

Schematic diagram showing the different sites of phosphorylation in ER α . The kinases and the kinase pathways that phosphorylate these sites are also shown.

Though the *in-vitro* data support the role of phosphorylation in endocrine resistance, there is little clinical evidence to corroborate this. Reports from the clinical studies, in fact suggests that the phosphorylation status is associated with good prognosis and response to endocrine therapy (Murphy *et al.*, 2004; Sarwar *et al.*, 2006).

1.9 Coregulatory proteins

Coregulatory proteins are a class of proteins which modulate the activity of receptors without directly binding to the DNA. The indication for the existence of these came from the phenomenon of squelching seen in transient transfection assays, in which the activated nuclear receptor inhibited transcriptional activation by another receptor. This was thought to be due to competition by the receptors for a limiting pool of cellular factors (Meyer *et al.*, 1989). Subsequent biochemical and expression cloning approaches identified a large number of factors that were capable of interacting with steroid/nuclear receptors in a ligand-dependent manner. As explained above the classical mode of action of estrogen receptor involves ligand and DNA binding and sequential recruitment of coregulator proteins for transcriptional initiation. The coregulatory proteins which enhance the transactivation are termed as coactivators while those that inhibit it are referred to as corepressors. These coregulatory proteins possess enzymatic activities such as acetylation, deacetylation, methylation, ubiquitination, and kinase activity that are important in modulating the transactivation by nuclear receptors. The well characterised coactivators and the corepressors with relevance to ER α are discussed in the following section and a brief description of others is given in the Table 1.3.

1.9.1 The p160/ steroid receptor coactivator (SRC) family of Coactivators

The p160/SRC family is a group of structurally and functionally related proteins that act as coactivators for several nuclear receptors to increase ligand - dependant transactivation. Members of this family include (i) SRC-1 (or NcoA-1) (ii) SRC-2 (also known as TIF-2 or GRIP1, NcoA-2) and (iii) SRC-3 (also known as RAC3, ACTR, AIB1, P/CIP and TRAM) (McKenna and O'Malley BW, 2002; Glass

and Rosenfeld, 2000). They have characteristic conserved LXXLL signature motifs (where L is leucine, X is any amino acid), termed the nuclear receptor (NR) box which is necessary and sufficient to mediate binding of the coactivators to liganded nuclear receptors. Three such motifs are conserved in SRC family members, and an additional NR box is present in the extreme C terminus of SRC-1 (Voegel *et al.* 1996, Heery *et al.* 1997). Core helix 12 in AF2 undergoes conformational change forming a charged clamp that accommodates the p160 coactivators within the hydrophobic cleft of the receptor LBD, through direct contacts with the LXXLL motifs (Nolte *et al.*, 1998; Darimont *et al.*, 1998). The SRC family possess two intrinsic transcriptional activation domains (AD1 and AD2). The AD1 is involved in the interaction with coactivators, CBP/p300 with HAT activity while AD2 is involved in the interaction with histone methyltransferases, CARM1 and PRMT1. The SRC proteins are thought to function in part by recruiting the general coactivators, CBP and p300, which possess histone acetyltransferase activity and interact with other components of the transcriptional machinery. Weak intrinsic HAT activity has been reported with the p160 family, suggesting that they may also have a role in chromatin remodelling, enabling the transcription factors to access the transcriptional machinery (Chen *et al.*, 1997; Spencer *et al.*, 1997; Klinge, 2000).

1.9.2 The TRAP/DRIP complex

TRAPS and DRIPS are homologous multi-protein complexes which interact with the ligand bound nuclear receptors. TRAP were purified from HeLa cells cultured in the presence of thyroid hormone and the DRIPs were similarly identified with vitamin D receptor in the presence of the ligand (Fondell *et al.*, 1996; Rachez *et al.*, 1998). These complexes enhance the transactivation of nuclear receptors in response to the ligand and contain a distinct set of 14-16 proteins that range in size from 70 to 230 kDa excluding the p610 family and the other coactivators. These complexes are recruited at the LBD/AF2 on ligand binding through a component of this complex, PBP/TRAP220/DRIP205 which has the nuclear receptor interacting signature motif, LXXLL. Several DRIP/TRAP components are similar to the components of other coactivator complexes including CRSP (coactivator required for Sp1 activation), NAT

(Negative regulator of activated transcription) and SMCC (SRB and MED containing cofactor complexes (Hampsey and Reinberg, 1999). These components associate with the RNA polymerase II and the DRIP/TRAP complex may function to enhance RNA polymerase recruitment to the target promoter (Rosenfeld and Glass, 2001).

1.9.3 The SWI/SNF family

The SWI/SNF is a family of ATP-dependent chromatin-remodelling proteins. SWI/SNF is a 2 Mda, multisubunit, DNA-dependent ATPase that facilitates the access of the transcription factors to the nucleosomal DNA through chromatin remodelling. These proteins are conserved from yeast to humans and are involved in the regulation of transcription via remodelling of chromatin structure. Mammalian SWI/SNF complexes consist of about 15 subunits composed of hBrm or BRG1 and several BRG1-associated factors (BAFs) (Mohrmann and Verrijzer, 2005). These complexes along with BRG1 have been shown to be recruited to endogenous ER α promoters in a ligand-dependant manner (Chiba *et al.*, 1994; Ichinose *et al.*, 1997).

1.9.4 Cointegrators- CBP/p300

CREB-binding protein (CBP) and p300 are structurally and functionally related proteins that serve as coactivators for different types of transcription factors. CBP was initially identified as a coactivator for the cAMP-response element-binding protein (Kwok *et al.*, 1994). Subsequent studies have shown that it acts as coactivator for a number of transcription factors including nuclear receptors, p53, NF- κ B (Shikama *et al.*, 1997). In addition, it also interacts with the SRC family of coactivators and enhances the transcriptional activation by ER and PR (Smith *et al.*, 1996). Being involved as coactivators for a number of transcription factors, they serve to integrate multiple signalling events at the transcriptional level (Kwok *et al.*, 1994). They possess intrinsic HAT activity and are involved in chromatin remodelling of nucleosomes (Bannister and Kouzarides, 1996). They serve to link the transcription factors to the basal transcriptional machinery by interaction with TBP and TFIID. In addition, it co-purifies with RNA polymerase and exists in complex with RNA polymerase suggesting

its role in recruitment of RNA polymerase to the promoters (Cho *et al.*, 1998; Nakajima *et al.*, 1997).

1.9.5 Corepressors

Nuclear receptors such TR and RAR repress transcription in the absence of hormone. The repression domains were mapped to the LBD of TR and RAR. Over expression of the repression domains was able to inhibit the repression suggesting the existence of a limited pool of repressors that mediate the repression. Yeast two hybrid screens led to the identification and cloning of two repressors proteins. Nuclear receptor corepressor (NCoR) and Silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Horlein *et al.*., 1995; Chen and Evans 1995). These are structurally related molecules which bind to NRs in the absence of ligand and harbour autonomous, transferable repression domains. NCoR and SMRT are large proteins, whose NR binding and repression functions are mediated by the carboxyl and amino terminal halves of the molecules, respectively. Similar to the coactivator binding, the corepressors bind to surface formed by the helices 3, 4 and 5, through a motif in the corepressor, CoRNR box, similar to the NR box in the coactivators (Hu and Lazar, 1999; Nagy *et al.*, 1999; Xu *et al.*, 2002). Loss of the CoRNR box led to loss of repression by unliganded TR suggesting that this motif is essential for interaction with nuclear receptor for repression (Horlein *et al.*, 1995). The aminotermminus contains several interaction domains that mediate the formation of complex containing WD-40 proteins TBL1/TBLR-1, GPS2 and histone deacetylase 3. SMRT and N-CoR both exist in repression complexes with HDAC enzyme activity, and HDAC3 is largely responsible for this activity (Guenther *et al.*, 2000; Li *et al.*, 2000; Zhang *et al.*, 2002). The deacetylation of lysine residues in the N-terminal tails of histone proteins by the HDACs leads to closed nucleosomal structure and repression.

1.9.6 AF 1 interacting proteins

While the mechanisms of AF2 mediated transcriptional activity in response to ligand binding is well characterised (McKenna and O'Malley, 2002; Gronemeyer *et al.*, 2004), the mechanisms of AF1 mediated transcriptional activity remains less well defined. It also remains unclear, how the phosphorylation potentiates the AF1 activity. This is important given that the key phosphorylation sites are mapped to AF1 domain and the phosphorylation of key residues is important in modulating the response. Moreover, the phosphorylation plays an important role in the cross talk between ER α and growth factor mediated kinase signalling. Attempts to investigate the AF1 transcriptional activity through recruitment of coregulatory proteins has led to identification of co-regulatory proteins for AF1. These include SRC1, CBP, SRC1-associated Steroid receptor RNA activator (SRA), p68 RNA helicase, stromelysin-1 platelet-derived growth factor responsive element-binding protein (SPBP) and spliceosome component splicing factor (SF) 3a p120. Among these SRC1, CBP and SRA have been shown to interact with AF1 and stimulate ER α activity in reporter gene assays but are not phosphorylation dependant (Lanz *et al.*, 1999; Watanabe *et al.*, 2001; Smith *et al.*, 1996). The other three co-regulator proteins, p68RNA helicase, SPBP and SF3a p120 have been shown to be recruited preferentially by ER α phosphorylated at serine 118. The first, p68 RNA helicase has been shown to preferentially interact with AF1 phosphorylated at serine 118 but the stimulation of ER α activity by p68 is relatively weak and cell specific and it is not strictly S118 dependent (discussed below). The second, stromelysin-1 platelet growth factor responsive element-binding protein (SPBP), acts not as a coactivator, but as a corepressor for ER α . It inhibited triple glutamic acid mutant but not that of triple alanine mutant in the presence of OHT in HepG2 cells. It also repressed AF1 activity in response to EGF stimulation and inhibited both basal and E2 induced proliferation of MCF-7 cells when cotransfected with SPBP (Gburcik *et al.*, 2005). Finally, another protein that interacts preferentially with ER α phosphorylated at S118 is the component splicing factor (SF) 3a p120. SF3a p120 was identified by far western blotting analysis of nuclear extracts from MCF-7, HeLa and COS-1 cells, using *in-vitro* phosphorylated ER AF1 as a probe. It interacted with AF1 on GST pull down assays and enhanced ER α transactivation in reporter gene

assays. Transactivation and binding was stimulated in response to Growth factor mediated phosphorylation of ER at serine 118 and inhibited by mutation of serine 118 to alanine. Furthermore, it was shown that the SF3a p120 potentiated the ER mediated RNA splicing which was dependant on Ser-118 phosphorylation. Thus the coactivator function of SF3ap120 is mediated through its effect on enhancement of ER α mediated RNA splicing on phosphorylation of ER (Masuhiro *et al.*, 2005).

Coregulator	Coregulatory function	References
TIF2/SRC-2 / GRIP-1	Interacts with and coactivates nuclear receptors; interacts with CBP.	Hong et al., 1996; Leers et al., 1998; Voegl et al., 1998
SRC3	Interacts with nuclear receptors, interacts with CBP/p300 and PCAF. Over expressed in breast tumours, SRC-3 preferentially coactivates ER over ERβ	Anzick et al., 1994; Chen et al., 1997; Li et al., 1997; Suen et al., 1998
RIP-140	Interacts with and coactivates ER; acts as a corepressor for TR2 orphan receptor.	Cavaillès et al., 1995; Christian et al., 2004; Lee et al., 1998; Miyata et al., 1998; Subramaniam et al., 1999
PCAF	Possesses intrinsic acetyltransferase activity; interacts with PR, TR, SRC-1, CBP/p300	Blanco et al., 1998; Ogryzko et al., 1998; Yang et al., 1996;
TBP/TAF _{II} s	Interact with and specifically coactivate nuclear receptors.	Jacq et al., 1994; Schulman et al., 1995; Ing et al., 1992; May et al., 1996; Mengus et al., 1997
PRMTs	Interacts with nuclear receptors and p160 family	Chen et al., 1999; Koh et al., 2001; McBride and Silver et al., 2001; Qi et al., 2002
E6-AP	E3 ubiquitin-protein ligases; interacts with and coactivates AR, ER, PR and GR, Sp1 and CREB	Nawaz et al., 1999
SRA	Functions as RNA transcript; selectively coactivates AF-1 of steroid receptors; present in SRC-1 complex.	Lanz et al., 1999
NSD-1	SET domain-containing protein; interacts with liganded and unliganded nuclear receptor LBDs.	Huang et al., 1998
PGC-1	Expressed in brown adipose tissue and skeletal muscle; interacts with PPAR-γ and RXR α	Puigserver et al., 1998
HMG-1	PBP binds to PPAR, RAR, RXR and TRβ-1 and coactivates PPAR; originally isolated as a TR-binding protein, TRIP2.	Boonyaratanakornkit et al., 1998
NCoA-62	Interacts with VDR, RAR and RXR; coactivates VDR strongly, GR, RAR and ER weakly.	Baudino et al., 1998
PBP	PBP binds to PPAR, RXR and TR and coactivates PPAR; originally isolated as a TR-binding protein, TRIP2.	Zhu et al., 1997; Yuan et al., 1998; Lee et al., 1995; Treuter et al., 1999
ADA	Yeast acetylase complex which mediates AF-2 dependent activation by RXR and ER.	Pina et al., 1993; Vom Baur et al., 1998
SMCC	Human SRB/mediator-containing complex; contains components homologous to TRAPs/DRIPs.	Gu et al., 1999
LCoR	Interacts with and inhibits nuclear receptor ligand induced activity; Also interacts with HDACs and CtBP	Fernandes et al., 2003
SNURF	Interacts with AR in yeast and mammalian cells; coactivates AR, PR, GR, Sp1 and AP-1	Moilanen et al., 1998
ARA-70	Ligand dependant coactivation of AR, PR, GR, Sp-1 and AP-1	Yeh and Chang, 1996
BRAC1	Blocked E2 stimulated reporter gene when over expressed in cultured human breast and prostate cells	Fan et al., 1999
SHP (short heterodimer partner)	Interacts with helix 12 of ERα through its LXXLL motif and inhibits the E2 dependent ER activation.	Seol et al., 1996; Johansson et al., 1999
REA (repressor of estrogen receptor activity)	Interacted with ERα in the presence of E2 or 4-OHT but not in the absence of ligand. enhanced the antiestrogenic activity of 4-OHT, ICI 182,780	Montano et al., 1999

Table 1.3 List of a selection of nuclear receptor coregulatory proteins
Adapted from McKenna *et al.*, 1999 and Klinge, 2000.

1.10 RNA helicases

Helicases are highly conserved enzymes that utilize the energy derived from nucleotide triphosphate hydrolysis to unwind DNA and/or RNA duplexes. Based on the sequence homology helicases are grouped into helicase super families 1-4, which include both DNA and RNA helicases (Gorbalenya and Koonin, 1993). They share a highly conserved helicase domain consisting of several motifs which are involved in ATP binding, hydrolysis, and nucleic acid binding and unwinding (Tanner and Linder, 2001). Most RNA helicases belong to helicase super family 2(SF2) and they are also referred to as 'DExD/H family' based on the presence the characteristic 'DExD/H' sequence (where x can be any amino acid) in the central domain. The amino acid sequence of RNA helicases is characterized by the presence of eight conserved motifs (Figure 1.8) (Tanner and Linder, 2001; Fuller-Pace, 2006). They have a high degree of homology in the central conserved helicase domain but show divergence in the N-terminal and C-terminal sequences flanking the conserved domain which is thought to confer specific function to these proteins. The RNA helicases are found in almost all organisms, from bacteria to human and are involved in diverse biological processes that involve RNA, including transcription, splicing, transport, translation, decay and ribosome biogenesis (de la Cruz *et al.*, 1999; Tanner and Linder, 2001; Jankowsky *et al.*, 2001). They have been implicated in growth regulation and have been shown to be involved in both pre-mRNA and pre-rRNA processing. They modulate the rearrangement of RNA-protein interactions and regulate gene expression by their role in splicing. They are required for pre-mRNA splicing and alternative splicing, and are components of spliceosome (Liu, 2002b; Lin *et al.* 2005; Guil *et al.* 2003). They are also involved in the processing of rRNA and miRNAs (Fukuda *et al.* 2007). Though the principal function of RNA helicases is to unwind double-stranded RNA, not all RNA helicases show unwinding activity on duplex RNA molecules *in vitro*.

While the function of these proteins in various aspects of RNA metabolism has been well characterised, a number of these proteins recently have been shown to play important roles in transcriptional regulation (Fuller-Pace, 2006). The RNA helicases involved in transcriptional regulation and their functions are summarised in table 1.4.

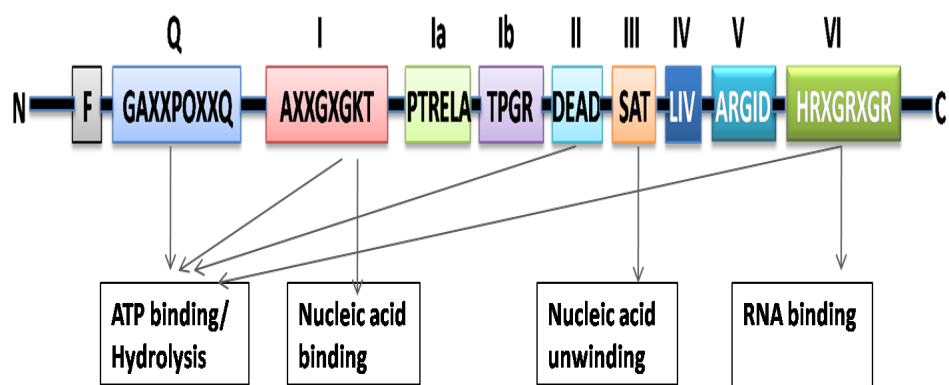


Figure 1.8 Schematic diagram showing the conserved motifs in the DEAD box proteins and their functions (Adapted from Rocak and Linder, 2004).

1.10.1 p68 and p72 RNA helicases

p68 RNA helicase is the prototype member of the DEAD box family which was initially discovered by a cross-reaction with an antibody against simian virus (SV) 40 large T proteins (Lane and Hoeffler, 1980). p72 RNA helicase is another member of DEAD box family of proteins, closely related to p68 with 90 % homology in the central helicase domain and a 60% and 30% similarity in the N- and C-terminal domain, respectively. Both are nuclear proteins with ATPase, RNA binding and helicase activities. p68 and p72 can interact with each other, as well as self-associate, and form heterodimers in cells (Lamm et al, 1996; Rossler et al., 2001; Lee, 2002; Ogilvie et al., 2003). The high homology in the central domain could explain their functional redundancy while the divergence in the N and C terminal might account for the differences in their functions.

p68 and to some extent p72 have been shown to be involved in transcriptional regulation by acting as cofactors for a number of transcription factors such as p53, ER α , MyoD, Runx2 (Fuller-Pace and Ali, 2008) and AR (Clark et al., 2008). They also interact with other coactivators such as SRC-1, CBP and SRA and associate with RNA polymerase II (Watanabe *et al*, 2001; Rossow and Janknecht, 2003; Caretti *et al.*, 2006).

p68 and p72 are coactivators of p53 which is a tumour suppressor activated in response to several cellular stresses including DNA damage. p68 depletion by RNA interference leads to inhibition of p53 target gene expression in response to DNA damage, as well as p53-dependent apoptosis. p53 and p68 interact *in vivo* and p68 is recruited to the promoters of the p53 regulated genes (Bates et al, 2005). Thus, p68 in conjunction with p53 acts a tumour cosuppressor.

Ddx/Dhx nomenclature	Other names	Role in transcription	References
Dhx9	RNA Helicase A (RHA) Nuclear DNA Helicase II (NDHII) Drosophila maleless (MLE)	Transcriptional coactivator-acting as bridging factor between transcription factors/co-factors Binding to promoters	Nakajima et al., 1997 Aratani et al., 2001 Anderson et al., 1998
Ddx20	DP103 Gemin 3	Transcriptional repressor/corepressor-interacts with repressors	Ou et al., 2001 Yan et al., 2003 Klappacher et al., 2002 , Gillian et al., 2004
Ddx 5	p68 Dbp2(yeast homologue of p68/72) RM62/Dmp68(drosophila homologue)	Coactivator for p53, ER, MyoD, Runx2, AR Interacts with SRC1, CBP, RNA polymerase and SRA Transcriptional deactivation Transcriptional corepressor Associates with HDAC1	Bates et al, 2005 Endoh et al, 1999 Watanabe et al, 2001 Rossow and Janknecht, 2003 Carette et al, 2006 Jenson et al, 2008 Clark et al., 2008 Buszczak and Spradling, 2006 Wilson et al, 2004
Ddx 17	p72(Dbp2-yeast homologue)	Coactivator of p53, ER, Myo D Interacts with SRC1, CBP and SRA	Bates et al, 2005 Watanabe et al, 2001 Carette et al, 2006 Shin and Janknecht, 2007

Table 1.4 A brief summary of RNA helicases involved in transcriptional regulation.
Adapted from Fuller-Pace, 2006.

p68/p72 also coactivates ER α by interacting with AF1 domain of ER α and acts in synergy with SRC1 and SRA to enhance ER α activity (Endoh et al., 1999; Watanabe et al., 2001). It was reported that the interaction of p68 with ER was enhanced by phosphorylation of serine 118 in AF1 of ER. It was reported to be specific coactivator of ER with no interaction with other nuclear receptors. However, a recent report suggests that p68 also acts as a coactivator of androgen receptor (Clark et al., 2008).

p68/p72 and SRA have been shown to be coactivators of MyoD, which is a transcription factor that regulates myogenesis. Depletion of p68/72 through RNAi leads to inhibition of activation of genes involved in myogenesis and skeletal cell differentiation. Furthermore, p68/72 depletion impaired the recruitment of the TATA binding protein, TBP, RNA polymerase II and the catalytic subunit of the ATPase SWI/SNF complex, Brg-1, suggesting that these proteins play an important role in formation of the transcription initiation complex and chromatin remodelling (Carette et al., 2006).

More recently p68 has been reported as a coactivator of Runx2, a transcription factor essential for osteoblast development. Interestingly, Runx2 itself regulates the expression of p68 and their complex interaction seem to control the osteoblast maturation and differentiation (Jensen et al., 2008).

p68 has been shown to be phosphorylated at multiple amino-acid residues, including serine/threonine and tyrosine (Yang et al., 2005). In response to platelet derived growth factor (PDGF) stimulation, p68 is phosphorylated at Tyrosine 593. Phosphorylated p68 is involved in transcriptional regulation of cyclin D1 and c-Myc genes and is recruited to the promoters of cyclin D1 and c-Myc. Phospho p68, through the activation of cyclin D1 and c-Myc genes mediates the stimulatory effects of PDGF on cell proliferation. Interestingly, the helicase activity was required for this function (Yang et al., 2007). Furthermore, phosphorylation of p68 promotes epithelial-mesenchymal transition via promoting β catenin nuclear translocation (Yang et al., 2006).

While the above reports highlight their role in transcriptional activation, they have also reported to act as repressors. p68/p72 can also act as corepressors depending on the context of the promoter and the transcriptional complex in which they

exist (Wilson et al, 2004). Both proteins interact with histone deacetylase 1 (HDAC1) which is a transcriptional repressor. Furthermore, Yeast homologue of the p68 has been shown to be involved in clearing of the nascent transcripts from the promoter leading to transcriptional deactivation (Buszczak and Spradling, 2006).

From the above data, the role of p68/p72 in transcriptional regulation seems to be complex. At least with some transcription factors such as ER α and MyoD, a model of p68/72 in transactivation is emerging, where they serve as linking or bridging molecules between transcription factors and coactivators. Interestingly, the helicase activity does not appear to be required for the coregulatory function. The interactions of these proteins with corepressors argue for a complex role, in which these proteins act to enhance or repress transcription depending upon the promoter and cellular context.

p68 and p72 share some but not all functions. Both p68 and p72 have been shown to be coactivators of ER α , p53 and MyoD and associates with SRC1 and SRA (Bates et al., 2005; Watanabe et al., 2001; Caretti et al., 2006). Like p68, p72 forms complexes with the coactivators p300 and CBP *in vitro* and *in vivo* (Rossow and Janknecht, 2003; Shin and Janknecht, 2007). Both are involved in pre-mRNA processing and alternative splicing (Liu et al., 2002b; Honig et al., 2002). There are, however, some subtle differences in their function which could be due to the divergence in their sequences in the N and C- terminal domains. The expression of these proteins is growth and developmentally regulated with differences in their expression patterns. p68 is up regulated, correlating with organ maturation and differentiation, where as p72 appeared to be down-regulated during development (Fuller-Pace, 2006). Further more, p68/p72 mice knock out studies show that the p68 disrupted embryos do not survive beyond 11.5 days, while the p72 knock out mice survived only for 2 days after birth. Embryos with double knock out led to earlier death than the p68 knock out alone. These studies imply that both proteins are essential for growth and development (Fukuda et al., 2007). In some contexts their function could be redundant or complimentary while in others one protein may be more important than the other. For example, with regards to coactivation of p53, both p68 and p72 augment p53 activity in reporter gene assays. However, knockdown of p68 but not p72, affected the p53 regulated genes in response to DNA damage, implying that p68 might be more important for p53 function than p72 (Bates et al., 2005).

1.10.2 p68/p72 RNA helicases as coactivators of ER α

p68 RNA Helicase was isolated from MCF-7 nuclear extracts by GST pull-down assays using ER α AF1-GST fusion protein as a probe. Endoh *et al* showed that the interaction between ER α AF1 and p68 was enhanced by in-vitro phosphorylated ER α AF1 at serine 118 by MAPK. Substitution of Serine 118 by alanine inhibited this interaction. In mammalian two-hybrid assays p68 stimulated reporter gene activity of ER α AF1 and full length ER α in the presence of estrogen but not of ER α - AF2/LBD. It stimulated the tamoxifen bound ER but not ICI 164,384 confirming the specificity of p68 to AF1. p68 did not show interaction with the other nuclear receptors (Endoh *et al.*, 1999). p68 is recruited to the promoter of the ER target gene, pS2, confirming a direct role for p68 in ER α transactivation (Metivier *et al*, 2003). p68 RNA helicase also interacts with the other ER α coactivators such as SRC1, CBP and SRA. It is therefore thought that the p68 might mediate the interaction between AF1 and AF2 of ER α and act as bridging factor between transcription factors and coactivators (Watanabe *et al.*, 2001). Interestingly the RNA helicase activity was dispensable for the ER α AF-1 coactivator activity in these experiments. In the original report, p68 was shown to be ER α specific coactivator. However, according to a recent report, p68 also coactivates androgen receptor. It regulates the androgen dependant gene expression by its role in RNA processing in AR-regulated genes, coupling the process of transcription and splicing (Clark et al., 2008). p72 RNA helicase which shows striking similarity to p68 also functions as a coactivator of ER α and like p68, shows interaction with the other coactivators such as SRC1, SRA and CBP.

1.10.3 Role of RNA helicases in cancer

RNA helicases play an important role in transcription, translation and splicing. A number of RNA helicases are dysregulated in cancer and have been implicated in cancers such as lung, colorectal, hepatocellular, gastric, murine sarcoma, melanoma, neuroblastoma and retinoblastoma (Abdelhaleem, 2004).

p68/p72 has been found to be over expressed in colon cancer cell lines and the expression of these proteins was found to be progressively increased in the specimens of colonic polyp, adenoma and carcinoma, which is the sequence of events in colon carcinogenesis. Down regulation of p68/p72 lead to inhibition of the colon cancer cell proliferation and inability of these to form tumours *in vivo* (Shin et al., 2007). p68 has been shown to be poly-ubiquitylated, suggestive of defect in proteasomal degradation of p68, leading to over expression in tumour cells (Causevic et al., 2001). p68 was also found to be phosphorylated in different cancer cell lines including breast cancer cell line but not in the corresponding normal tissues. Phosphorylated p68 is involved in the regulation of cyclin D1 and c-Myc genes, promoting abnormal cell proliferation. Phosphorylation of p68 therefore, might be involved in carcinogenesis (Yang et al., 2005; Yang et al., 2007). Collectively, these studies suggest that altered expression or posttranslational modification of p68/p72 might be involved in cancer development or progression.

1.11 Hypothesis and aims

As described in the introduction, the estrogen-dependent growth of breast tumours is underpinned by ER α . The last decade has seen considerable advances in understanding the molecular mechanisms of transcriptional regulation by the nuclear receptors, mainly through the identification and characterization of a number of transcriptional regulatory proteins. While the mechanism by which LBD/AF2 activates transcription by recruitment of transcriptional regulatory proteins upon ligand binding, has become much clearer, the mechanism(s) by which AF1 activates gene expression remains unclear. This is an important question, given that the agonist activity of tamoxifen in some tissues derives from its ability to inhibit AF2, but allow activation of AF1. Further, as described above, phosphorylation within AF1 can result in ER α activation in a ligand-independent manner, with potential for tumour growth in the absence of ligand or in the presence of anti-estrogens. Moreover, EGFR and HER2 expression in breast tumours correlates with poor response to endocrine therapies and ERK1/2 MAPK is often over expressed in breast cancer, it's over expression correlating with reduced likelihood of response to tamoxifen. As EGF signalling

through MAPK phosphorylates ER α at S104, S106 and S118 in AF1, resulting in ligand independent ER α activation, phosphorylation is likely to be involved, at least in part for the emergence of endocrine resistance in breast cancer (Sivaraman et al., 1997; Gee et al., 2001; Ali and Coombes, 2002).

I hypothesised that stimulation of AF1 activity by phosphorylation is likely to be due to the preferential recruitment of transcriptional coactivators by ER α . Further, it is possible that such coactivators may be important in breast cancer progression. In order to test this possibility I proposed a screen to identify coactivators that preferentially interact with ER α phosphorylated within AF1. As p68 RNA helicase has previously been identified on the basis of its preferential interaction with ER α phosphorylated within AF1 at S118, I also wanted to investigate the importance of p68 RNA helicase, as well as the highly related p72 RNA helicase in regulating ER α activity and in determining their importance in breast cancer progression.

In order to identify phosphorylation-specific coactivators, I established a bacterial two-hybrid system to screen a human breast cDNA expression library, using the AF1 region of ER α in which serine residues at positions 104, 106 and 118 had been substituted by glutamic acid, as a bait. The aim of the screening was to identify novel ER α coactivators. A number of potential interactants were found which on further analysis turned out to be false positives.

The second aim of the project was to understand the mechanisms by which p68 RNA helicase acts as a coactivator for ER α . Over-expression, interaction assays and RNA interference studies were carried out to map the interaction domains between p68 and ER and to determine the importance of p68 RNA helicase in transcription activation by ER α . p72 RNA helicase, another DEAD box RNA helicase is closely related to p68 and shares some of the functions of p68 including coactivation of ER α . Studies were done in parallel with p68 to study the interaction of p72 with ER α . While the p68 and p72 RNA helicases show similarity in some functions, the data presented here show important differences in the transcriptional regulation by ER α and estrogen dependant growth of breast cancer in vivo. Collectively, these studies show complex

role for these proteins in ER function and more studies are needed to understand the relevance of these to the development and progression of breast cancer.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General chemicals and reagents

All chemicals were obtained from Sigma-Aldrich (UK) or BDH/Merck (UK) with the exception of the His Dropout Supplement (BD/Clontech, UK). Molecular biology reagents such as restriction endonucleases, Taq polymerase and DNA ladders were obtained from Mbi Fermentas/Helena Biosciences (Sunderland, UK) and Roche Diagnostics (UK). AnalaR or molecular biology grade reagents were used, unless otherwise stated. Kits for large-scale DNA preparation or RNA preparation were obtained from Qiagen (West Sussex, UK). Small-scale DNA preparation kits were obtained from Promega (Hants, UK).

2.1.2 General Stock Solutions

Stock solutions were made by dissolving appropriate amounts of the chemicals in distilled, de-ionised water (ddH₂O). Solutions were autoclaved and stored at room temperature, unless otherwise stated.

Table 2.1 General stock solutions

10X TBE	89mM Tris base, 89mM Boric acid, 2mM Na EDTA, pH 8.0.
TE Buffer	10mM Tris-HCl, 1mM Na EDTA, pH 8.0.
Sodium ethylene diamine tetra-acetate (Na EDTA)	0.5M Na EDTA, pH 8.0.
10% Lauryl sodium dodecyl sulphate (SDS)	10% w/v SDS, pH 7.0. Not autoclaved.
3-amino-1,2,4-triazole (3-AT) (1 M)	840.8 mg of 3-AT was dissolved in 10 ml of DMSO. Aliquoted and stored at –20°C for use up to one month
10X phosphate buffered saline (PBS)	136mM NaCl, 3mM KCl, 8mM Na₂HPO₄, 1.5mM KH₂PO₄.
3 M Sodium Acetate	3M sodium acetate dissolved in H₂O, pH adjusted to 5.2 with glacial acetic acid

Table 2.2 SDS- PAGE and western blotting solutions

2 X Laemmli buffer	125mM Tris pH 6.8, 20% Glycerol, 4% SDS, 1mg/ml bromophenol dye, 200mM DTT
4 X Separation gel buffer	1.5 M Tris- HCl pH 8.8, 0.4% SDS
10 X Stacking gel buffer	1.25 M Tris-HCl pH 6.8, 1% SDS
10 X running buffer	0.25M Tris base, 1.92 M Glycine, 1 % SDS, pH 8.3
50 X Electro transfer buffer	0.25M Tris base, 1.92M Glycine 0.03% SDS, pH 8.3
Wash buffer	1 x PBS containing 0.1 % (v/v) Tween 20

Table 2.3 SDS- PAGE gels

	separating gel		stacking gel (5%)
	10%	15%	
Separation gel buffer	8ml	8ml	-
Stacking gel buffer	—	—	2.5 ml
30 % w/v Acrylamide (0.8% w/v bis-acrylamide stock solution 37.5 :1) (National diagnostics)	10 ml	15 ml	1.5 ml
ddH₂O	11.7 ml	6.7 ml	6ml
10% fresh ammonium sulphate (Sigma Aldrich)	300 µl	300ul	50 µl
Temed (Sigma Aldrich)	30 µl	30ul	20 µl

2.1.3 Bacterial media and plates

Bacterial medium was prepared using ddH₂O. Medium was autoclaved and stored at 4°C. Selective antibiotic(s) was added fresh prior to inoculation with bacteria. L-Broth and L-Broth agar were obtained from BIO 101 systems as capsules.

Table 2.4 Bacterial media and plates

L-Broth (LB) (Luria-Bertani)	Per litre: 16g Tryptone-B, 10g yeast extract-B and 5g of NaCl.
SOB Medium	20 g of Tryptone-B, 5 g of yeast extract, 0.5 g of NaCl added to 1 liter of deionized H ₂ O Autoclaved and 10 ml of filter-sterilized 1M MgCl ₂ and 10 ml of filter-sterilized 1M MgSO ₄ were added prior to use
SOC Medium	Prepared immediately before use. 2 ml of filter-sterilized 20% (w/v) glucose added to SOB medium (autoclaved) to a final volume of 100 ml
LB Agar	Per litre: 16g Tryptone-B, 10g yeast extract-B, 5g of NaCl and 15g of Agar-B.
LB plates	LB Agar was melted in microwave and cooled to 50°C prior to addition of selection antibiotic(s). Poured into petri dishes.

Table 2.5 Special media used for the BacterioMatch II Two-Hybrid Assays

M9 Media Additives	Solutions I and II were prepared separately and mixed together by adding solution II to solution I Solution I: 10 ml of 20% glucose (filter-sterilized), 5 ml of 20 mM adenine HCl (filter sterilized), 50 ml of 10× His dropout amino acid supplement (autoclaved) Solution II: 0.5 ml of 1 M MgSO₄, 0.5 ml of 1 M Thiamine-HCl (filter sterilized), 0.5 ml of 10 mM ZnSO₄ (autoclaved), 0.5 ml of 100 mM CaCl₂ (autoclaved), 0.5 ml of 50 mM IPTG (isopropyl-beta-D-thiogalactopyranoside)
M9+ His-dropout Broth	In a 500-ml flask, the following were combined and mixed well: 380 ml of sterile, deionised H₂O 50 ml of 10× M9 salts 67.5 ml of M9 Media Additives Stored at 4°C for use up to one month
Nonselective Screening Medium	In a 500-ml flask, the following were combined: 380 ml deionised H₂O and 7.5g Bacto agar Autoclaved at 120°C for 20 minutes Cooled the agar to 70°C, and then added 50 ml of 10× M9 salts The mixture was cooled to 50°C and added 67.5 ml of M9 Media Additives mixed with 0.5 ml of 25 mg/ml chloramphenicol, 0.5 ml of 12.5 mg/ml tetracycline The mixture was poured into Petri dishes (~25 ml/100-mm plate) After the agar was solidified the plates were wrapped in aluminium foil. Stored plates at 4°C for use up to 1 month
Selective Screening Medium (5 mM, 3-AT)	In a 500-ml flask, the following were combined: 7.5g Bacto agar and 380 ml H₂O Autoclaved at 120°C for 20 minutes the agar was cooled to 70°C and added 50 ml of 10× M9 salts The mixture was cooled to 50°C and added 67.5 ml of M9 Media Additives mixed with 0.5 ml of 25 mg/ml chloramphenicol, 0.5 ml of 12.5 mg/ml tetracycline, 2.5 ml of 1 M 3-AT The mixture was poured into Petri dishes (~25 ml/100-mm plate) After the agar was solidified the plates were wrapped in aluminium foil. Stored plates at 4°C for use up to 1 month
Dual Selective Screening Medium (5mM,3-AT+ streptomycin)	Same as above plus 0.5 ml of 12.5 mg/ml streptomycin

2.1.4 Antibiotics

All antibiotics were obtained as solids from the Sigma Aldrich, (Dorset, UK).

Table 2.6 Antibiotics

Ampicillin:	Supplied as the sodium salt, ampicillin was dissolved in ddH₂O at a concentration of 100mg/ml and stored in 1 ml aliquots at -20°C. Typically, ampicillin was used at a final concentration of 100 µg /ml.
Tetracycline	Supplied as the hydrochloride, tetracycline was dissolved in ddH₂O at a concentration of 12.5 mg /ml and stored in the dark at -20°C. Typically, tetracycline was used at a final concentration of 12.5 µg / ml.
Chloramphenicol	Dissolved in ethanol at a concentration of 34ug/ml and stored at -20°C. Used at 34ug/ml.
Kanamycin	Obtained as monosulphate and dissolved in ddH₂O at a concentration of 30mg/ml and stored in 1 ml aliquots at -20°C. It was used at a final concentration of 50 µg /ml.
Streptomycin	Dissolved in ethanol at a concentration of 12.5mg/ml and stored at -20°C. Used at 12.5µg/ml.

2.1.5 Bacterial strains

BacterioMatch II Electro competent Reporter Cells: (Δ (*mcrA*) 183 Δ (*mcrCB-hsdSMR-mrr*) 173 *endA1 hisB supE44 thi-1 recA1 gyrA96 relA1 lac* [*F'* *laqIq HIS3 aadA Kanr*) and *XL1-Blue MRF' Kan* (Δ (*mcrA*) 183 Δ (*mcrCB-hsdSMR-mrr*) 173*endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [*F'* *proAB lacIqZ* Δ M15 *Tn5* (*Kanr*) were obtained from Stratagene.

The *Escherichia coli* bacterial strain *DH5 α* : (*supE44* Δ *lacU169* (Φ 80 *lacZ* Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*). This strain was used for transformation and preparing plasmid DNA.

The *Escherichia coli* bacterial strain *BL21 (DE3) pLysS*: (*F-* *ompT hsdSB (rb- mB-) gal dcm* (*DE3*) *pLysS* (*CmR*)) (Studier et al., 1990). This strain was used for the preparation of GST proteins.

2.1.6 Cell Culture Reagents

Cells were cultured at 37°C in a humidified 5% (v/v) CO₂:air atmosphere. Cell manipulations were carried out in a NuAIRE class II, ducted, laminar flow safety cabinet (NuAIRE DH Auto flow, Oxon, UK). Dulbecco's Modified Eagle's Medium (DMEM), 0.02% Na-EDTA solution, 10X trypsin solution and 5mg penicillin/streptomycin/L-glutamine solution was supplied by Sigma-Aldrich (Dorset, UK). DMEM lacking phenol red and foetal calf serum (FCS) were supplied by Gibco-BRL Life Technologies (Paisley, UK). Dextran-coated charcoal-treated FCS (DSS) was supplied by Globepharm (Surrey, UK). 17 β -estradiol (E2) and 4 hydroxytamoxifen (OHT) were purchased from Sigma-Aldrich (Dorset, UK). ICI 182, 780 (ICI) was purchased from Tocris Bioscience (Bristol, UK).

2.1.7 Cell Lines

COS-1: African Green Monkey Kidney fibroblast-like CV-1 cell line was transformed with SV40 to give the COS-1 cell line (ATCC cat no.: CRL-1650). This cell line does not express ER α .

MCF-7: Human cell line obtained from a pleural effusion derived from a breast carcinoma (ATCC cat. No.: HTB-22). This cell line is estrogen-dependent for its growth and expresses ER α (Soule et al., 1973).

MELN: MCF-7 derived cell line established by transfecting MCF-7 cells with the ERE- β -globin-luc-SV-Neo plasmid that express luciferase in an estrogen-dependent manner (Pons et al., 1990).

MVLN: MCF7 cell line transfected with a reporter gene whereby the vitellogenin promoter regulates the expression of luciferase in an estrogen-dependent manner (Demirpence et al. 1993). Both MELN and MVLN cell lines are a kind gift from Dr Patrick Balaguer (Montpellier, France).

2.1.8 DNA size Markers

DNA fragments in the range 2- 25 Kb were sized against a Hind III digest of λ bacteriophage DNA (λ Hind III marker; MBI - Fermentas Enzymes, distributed by Helena Biosciences, Sunderland, and Tyne & Wear). The fragment sizes in this digest are (base pairs): 23 130, 9416, 6557, 2322, 2027, 562, 125. DNA fragments in the

range 1 Kb - 10 kb was achieved by using a “1kb ladder” (MBI - Fermentas Enzymes). Fragment sizes (base pairs): 10 000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 500.

2.1.9 Protein size markers

The molecular weights of the protein bands were identified by comparison with the Full Range Rainbow markers (MW 250000, 160000, 105000, 75000, 50000, 35000, 30000, 25000, 15000, 10000Da) (Amersham Life Science, Bucks, UK).

2.1.10 General Equipment

Centrifugation of volumes greater than 1.5 mls was carried out in disposable 30ml centrifuge tubes (Sarstedt, Leicester, UK), using a Sorval SS34 rotor on a Sorval RC-5B refrigerated centrifuge (Du Pont Instruments (UK) Ltd, Stevenage, UK). Centrifugation of sample volumes < 1.5 mls was carried out using a bench top micro centrifuge (Heraeus' Biofuge Pico, DJB lab care, UK) at 13,000 rpm.

Samples were incubated at constant temperatures in water baths (Techne, Cambridge, UK) and incubator ovens (LEEC, Nottingham, UK; Fisons Scientific Instruments, Loughborough, UK), which were also used for growth of bacteria on agar plates. Liquid bacterial cultures were grown in a shaking incubator (New Brunswick Scientific Company Incorporated, Edison, NJ, USA). Samples were mixed using an autovortex (Stuart Scientific, London, UK).

Polymerase Chain Reactions (PCR) were carried out using GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems, Cheshire, UK). For Real-Time PCR the ABI-Prism 7900 sequence detection system (Applied Bio systems) was used.

Spectrophotometric measurements were carried out on a Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Tokyo, Japan).

For nucleotide separation mini horizontal agarose gel electrophoresis tanks from Cambridge Electrophoresis (Cherry Hinton, Cambridge, UK) were employed. Western blotting was carried out using vertical gel electrophoresis tanks and transfer apparatus from (Amersham Pharmacia Biotech, St Albans, UK) and Bio-Rad (Hemel Hempstead, UK). Power packs used were purchased from Bio-Rad, UK.

2.1.11 Sundries

Polypropylene microfuge tubes were obtained from Sarsted (Leics, UK). Thin-walled PCR tubes were obtained from Advanced Biotechnologies Ltd (Surrey, UK). Parafilm, laboratory film was obtained from American National Can (CT, USA). Whatman filter paper was obtained from Whatman International Ltd (Kent, UK). Tissue culture plastics were obtained from Triple Red (Oxon, UK).

2.1.12 Plasmids

The mammalian expression vector pSG5 (Green et al., 1988) encoding human ER α (HEGO), HE15 (encoding amino acids 1-281 of human ER α and lacking the LBD), HE19 (encoding amino acids 180-595 and lacking lacking AF1) have been described previously (Tora et al., 1989a), as have HEG0 in which serine 118 has been substituted by alanine (HEG0-118A, HE15-118A), or glutamic acid (HEG0-118E, HE15-118E) (Ali et al., 1993). HEG0 in which serine 104, 106 and 118 have been substituted by alanine or by glutamic acid (HEG0-104A/106A/118A, HEG0-104E/106E/118E) were kindly provided by Dr Ross Thomas. HEG0 containing mutations in the LBD were kindly provided by Dr. Sylvie Mader (University of Montreal, Canada). The ERE-3-firefly luciferase reporter gene (pXP-ERE3) contains three copies of the ERE and the adenovirus major late promoter TATA box cloned in the polylinker of the luciferase gene containing vector pXP2 has previously been described (Nordeen, 1988). 5Gal-TK-Luc, a luciferase reporter gene which has 5 Gal4 binding sites upstream of the Thymidine kinase promoter, was a kind gift from Dr.Charlotte Bevan (Department of Oncology, Imperial College, London). pRLTK, encoding the Renilla luciferase gene under the control of a constitutive thymidine kinase promoter, was obtained from Promega, UK. pBluscript (BSM), used as a carrier DNA for transient transfection of mammalian cell lines, was purchased from Stratagene Ltd (Europe). GST tagged ER α deletion and substitution mutants were generated in our laboratory by Dr. Ross Thomas. p68 and p72 cloned into a myc-tagged derivative of pSG5, pcDNA encoding Gal4-p68 deletion constructs and pGEX-p68 deletion constructs were kindly provided by Dr F. Fuller-Pace (University of Dundee).

Recombinant plasmids used in the bacterial two-hybrid system: DNA sequences encoding the human ER α coding sequence or ER α functional domains were

cloned into the Bacteriomatch II pBT vector (Stratagene, UK), whilst human SRC1, TIF2 and p68 RNA helicase were cloned into the pTRG vector (Stratagene, UK) following PCR amplification from the mammalian expression plasmid pSG5 encoding the human ER α (HEG0), SRC1, TIF2 or p68 RNA helicase. HEG0 and TIF2 were kindly provided by Prof. P. Chambon, pSG5-SRC1 by Prof. M. Parker. ER α substitution mutants were similarly cloned into pBT following PCR amplification of HEG0 encoding these substitutions. The recombinant plasmids used in the bacterial two-hybrid system and the substitution mutants in HEG0 were generated by Dr Ross Thomas and Mr Paul Pace. Positive control plasmids pBT-LGF2 and pTRG-Gal11P were from Stratagene.

2.1.13 Antibodies

The antibodies used for immunoprecipitation and western blotting are listed in Table 2.7.

2.1.14 Oligonucleotides

The oligonucleotides are listed in Table 2.8. The p68 and p72 siRNAs were from Dharmacon (USA) and primers and probes used for Real-Time PCR were obtained from Applied Biosystems. All other oligos were obtained from MWG Biotech AG, Ebersberg, Germany.

Table 2.7 Antibodies

Antibody	Type/Source	Manufacturer	Dilution used
ER α – 6F11	Mouse monoclonal	Novacastra	1:2000
ER α (HC-20)	Rabbit polyclonal	Santacruz	1:2000
GST - B-14	Mouse monoclonal	Santacruz	1:2000
p68 –Pab 204	Mouse monoclonal	Gift from Dr.F. V. Fuller-pace, University of Dundee	1:100
P68-Pab 2906	Rabbit polyclonal	Gift from Dr.F. V. Fuller-pace, University of Dundee	1:1000
p72 –SQQ-K14	Rabbit polyclonal	Gift from Dr.F. V. Fuller-pace, University of Dundee	1:1000
Cathepsin D	Mouse monoclonal	Abcam	1:1000
PR	Rabbit polyclonal	Santacruz	1:300
pS2	Rabbit polyclonal	Santacruz	1:300
Lamin	Mouse monoclonal	Santacruz	1:500
Actin	Mouse monoclonal	Santacruz	1:2000 0
Polyclonal goat Anti-Mouse immnuoglobulins HRP		DAKO	1:2000
Polyclonal goat Anti-Rabbit immnuoglobulins HRP		DAKO	1:2000

Table 2.8 Oligonucleotides

Primers for RT- PCR		
p68	Forward	5'- GTG TCA TCG GTG TCC TTC CT -3'
	Reverse	5'- TGC CCT ACT TCC AAA TC -3'
p72	Forward	5'- GTG GAG GAT TTG GAG CAA GA3'
	Reverse	5'- CTT CTT TCG GCG TAG CTC AT -3'
pS2	Forward	5'-TTG GAG CAG AGA GGA GGC AAT G -3'
	Reverse	5'-TTA GGA TAG AAG CAC CAG GGG ACC-3'
Cathepsin D	Forward	5'-CAT TCC CGA GGT GCT CAA GAA C -3'
	Reverse	5'-TCT TCA CGT AGG TGC TGG ACT TG -3
ER α	Forward	5'-CAG ATG GTC AGT GCC TTG TTG G -3'
	Reverse	5'-CCA AGA GCA AGT TAG GAG CAA ACA G -3'
GAPDH	Forward	5'-TTC CAT CAC CAT CTT CCA -3'
	Reverse	5'-CAT CAC GCC ACA GTT TCC -3'
Lamin	Forward	5'-GCGTACGGCTCTCATCAACT-3'
	Reverse	5-GACACTGGAGGCAGAAGAGC-3

2.2 METHODS

2.2.1 Preparation of plasmid DNA

2.2.1.1 Miniprep plasmid DNA preparation

Small-scale (5-10 µg) plasmid preparations were prepared from 1.5 ml cultures using the Wizard *Plus* SV spin column kit (Promega, Hants, UK), following the manufacturer's instructions. A disposable plastic bacterial loop was used to inoculate 5ml aliquots of LB bacterial medium containing the appropriate antibiotic, with a single bacterial colony transformed with the desired plasmid and cultures were incubated overnight at 37°C in a shaking incubator. 1.5ml of each culture was transferred to microfuge tubes and centrifuged at 13000g for 5 minutes and the supernatant was discarded. The pellets were resuspended in 250µl of Cell resuspension solution (50mM Tris-HCl pH 7.5, 10mM Na-EDTA pH 8.0, 100µg/ml RNase A). Cells were lysed with 250µl of Cell Lysis Solution (0.2M NaOH, 1%SDS); tubes were inverted four times to mix and incubated at room temperature for 5 minutes. Ten µl of Alkaline protease solution was then added and mixed and incubated at room temperature for a further 5 minutes. 350µl of Neutralization solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid pH 4.2) was added to neutralise the reaction, tubes were inverted four times to mix thoroughly and samples were incubated at room temperature for 10 minutes. The samples were centrifuged for 10 minutes at 13,000rpm in a micro centrifuge and the supernatants were transferred to a Wizard plus SV Miniprep Spin Column by decanting and centrifuged at 13,000rpm for 1minute at room temperature. Flow throughs were discarded from the collection tube and the spin column was reinserted. Columns were washed by adding 750µl of Wash solution (60mM potassium acetate, 10mM Tris-HCL pH 7.5, 60% ethanol) and centrifuged at 13,000rpm for 1 minute at room temperature and flow through was discarded. A second wash was performed with 250µl of Wash solution by centrifugation at 13,000 for 2 minutes to ensure complete removal of the solution. The columns were transferred to new sterile microfuge tubes and DNA was eluted by adding 100µl of Nuclease-Free water and centrifugation at 13,000 rpm for 1 minute at room temperature. The DNA concentration of the plasmid DNA was estimated against

a known amount of control, plasmid DNA following agarose gel electrophoresis. Typically, yields of 5-10 μ g of plasmid DNA were obtained.

2.2.1.2 Large scale plasmid preparation (maxi prep)

Large scale plasmid DNA preparation was carried out using the Qiagen-tip500 maxi-prep kit as per the manufacturers' protocol (Qiagen Ltd, West Sussex, and UK). A single bacterial colony transformed with the appropriate plasmid was picked using the disposable plastic bacterial loop and inoculated into 5ml of LB supplemented with the appropriate antibiotic. The culture was incubated at 37°C for 8 hours in a shaking incubator. This starter culture was diluted 1/500 into selective LB medium and incubated overnight at 37°C with shaking. The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. The pellet was resuspended in 10 ml Buffer P1 (50mM Tris-HCl, pH 8.0, 10mM Na EDTA pH 8.0, 100 μ g/ml RNase A). 10 ml of lysis Buffer, P2 (0.2M NaOH, 1% SDS) was added and mixed thoroughly by inverting the sealed tube 6 times, and incubated at room temperature for 5 min. 10 ml of neutralisation Buffer, P3 (3.0M K acetate, pH 5.5) was then added and mixed by inverting the tube 6 times, and the tube was placed on ice for 20 min. The resulting precipitate was centrifuged at 20,000 x g for 30 min at 4°C. The supernatant containing plasmid DNA was transferred to a new 30ml centrifuge tube and the supernatant was centrifuged again at 20,000 x g for 15 min at 4°C. QIAGEN-tip 500 columns were equilibrated by applying 10 ml of Buffer QBT (0.75M NaCl; 50mM MOPS pH 7.0; 15% isopropanol). The supernatant was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 30 ml of Buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% isopropanol) and the DNA was eluted with 15 ml Buffer QF (1.25M NaCl, 50mM MOPS pH 7.0, 15% isopropanol). Eluted DNA was precipitated by adding 10.5 ml (0.7 volumes) of isopropanol and centrifuged at 15,000 x g for 30 min at 4°C. The DNA pellet was washed with 5 ml of 70% ethanol, and centrifuged at 15,000 x g for 10 min. The supernatant was discarded and the pellet was air-dried for 10 min., resuspended in 1X TE buffer (pH 8.0) and transferred to microfuge tubes. The final plasmid concentrations were determined by UV spectrophotometer at an OD of 260 nm using a quartz cuvette (Shimadzu, Milton

Keynes, UK). An Absorbance at 260 nm of 1.0 corresponds to a concentration of 50µg/ml for double-stranded DNA. The readings were taken at 260nm and 280 nm and the ratio for the absorbance at 260 nm and 280 nm gives an estimate of purity of the DNA, which is 1.8, with values below this suggestive of protein contamination.

2.2.2 Restriction endonuclease digestion of DNA

For mapping and analysis of DNA with restriction endonucleases, 0.5-3µg of DNA was typically digested for 2-4 hours in a total volume of 30-50µl, with a 5-10-fold excess of enzyme. Digests were carried out in the buffers recommended by the manufacturer. The total volume of enzyme did not exceed 10% of the total digest volume.

2.2.3 Agarose gel electrophoresis of DNA

Nucleic acids were separated by gel electrophoresis using agarose gels. DNA samples were mixed with 0.1 volumes of DNA loading buffer (40% sucrose, 10mM Tris-HCl pH 8.0, 1mM Na EDTA pH 8.0, 2% w/v SDS, 0.5% w/v bromophenol blue, 0.5% w/v xylene cyanol) prior to electrophoresis in EM100 gel tanks (Cambridge Electrophoresis, Cambs, UK). Restriction digests of plasmids and genomic DNA fragments between 1-50kb in size were resolved in 0.8-1.5% agarose-TBE gels containing 0.25µg/ml ethidium bromide, run at 75mA for 30-45 minutes. Small PCR products and small digestion fragments were resolved on 1-2% agarose-TBE gels.

2.2.4 Preparation of electrocompetent cells

10 ml of 2X YT media was inoculated with *E.Coli* (XL Blue reporter strain. Strategene) and grown overnight in a 50ml tube at 37°C with shaking. The cells were diluted 1:1000 in 2, 200ml 2X YT media and grown until the optical density (OD) reached ~ 0.8. The cultures were incubated on ice for an hour. The cells were harvested by centrifugation at 3000 rpm at 4°C for ten minutes, in a Sorval High speed centrifuge. The cell pellet was resuspended in ice cold wash buffer (10% ultra-pure glycerol in ultra-pure deiodinised water). The cells were centrifuged at 3000 rpm at 4°C for thirty

minutes. The cell pellet was resuspended again in wash buffer and harvested by centrifugation at 3000 rpm at 4°C for thirty minutes. The cell pellet was resuspended in 10 ml ice-cold wash buffer and harvested by centrifugation at 300 rpm at 4°C for 10 minutes. The cell pellet was resuspended in 0.5 ml of ice cold wash buffer and was aliquoted in 50µl volumes in sterile screw- cap microfuge tubes. The aliquots were frozen in liquid nitrogen and stored at – 80°C.

2.2.5 Bacterial Transformation by Electroporation method

All transformations in the BacterioMatch II Two-Hybrid assays were carried out by electroporation method using the Bio-Rad electroporator (Bio-Rad, UK). Fifty ng each of the bait and target plasmid DNA was added to 40 ul of electrocompetent cells in a pre-chilled 0.1 cm cuvette and mixed gently by inverting the cuvette. Control transformations were carried out with 50 ng each of the positive control plasmids, pBT-LGF2 and pTRG-Gal11^P and negative control plasmids pBT and pTRG and the target plasmid with the empty bait. The cuvette was placed in the electroporation chamber and electroporated with the electroporator set to voltage of 1.5kv, resistance to 400 Ohms and the capacitance to 25 µF. The cells were resuspended in 1 ml of pre-warmed (37°C) SOC medium and incubated with shaking at 225 at 37°C rpm for 90 minutes. The cells were centrifuged at 2000 × g for 10 minutes. The supernatant was discarded and the rich media was removed by washing the cells in 1ml of M9+ His-dropout broth and centrifugation at 2000 × g for 10 minutes. The cells were resuspended in 1 ml of M9+ His-dropout broth and incubated at 37°C with shaking at 225 rpm for 2 hours. The cotransformation mixture was plated on to the 150mm plates containing non selective medium and selective medium (5mM 3-Amino-124Triazole). The plates were incubated at 37°C for 24 hours. The colonies were picked and patched on to 100mm plates with selective medium and incubated at room temperature for 16 hours for enrichment of the cotransformants. The cells were picked and repatched on to dual selective medium plates containing the 3-AT and streptomycin and LB agar plates containing tetracycline and chloramphenicol. The colonies grown on the dual selective medium plates indicate the true interactors and were analysed further as described under the library screening.

2.2.6 Chemical Transformation of bacterial competent cells with Recombinant DNA

For transformation of DNA, DH5 α cells were generally used. 100 μ l of competent cells were aliquoted into pre-chilled 1.5 ml Eppendorf tube and 1-10 ng of the DNA was added to the cells. The cells were then incubated on ice for 30 minutes after which they were heat-shocked at 42°C for 45 seconds and then placed back on ice for 2 minutes. 1 ml of LB medium was added to each transformation and incubated at 37°C with shaking at 150 rpm for 1 hour to allow cells to recover and start expressing the corresponding antibiotic resistance gene. Cells were centrifuged briefly and the medium was removed, leaving 100-200 μ l of LB medium. The cells were re-suspended in the remaining medium, which was spread on an LB agar plate made up with the appropriate antibiotic and incubated overnight at 37°C.

2.2.7 Spectrophotometry

Concentrations of nucleic acids were determined photometrically at a wavelength of 260 nm using quartz cuvettes (Shimadzu, Milton Keynes, UK). An optical density (OD) of 1 corresponds to a concentration of 50 μ g/ml for double-stranded DNA and 40 μ g/ml for RNA (Sambrook et al. 1989). The ratio between the readings at 260 nm and 280 nm (A_{260}/OD_{280}) provides an estimation of the purity of the nucleic acid preparation. Highly pure DNA or RNA is characterized by ratios between 1.8 and 2.0 respectively.

The bacterial culture growth was assessed in disposable polystyrene cuvettes at a wavelength of 600nm using the same spectrophotometer.

2.2.8 Ethanol Precipitation of Nucleic acid

DNA was precipitated from aqueous solution by the addition of 0.1 volume of 3M sodium acetate, pH 5.2 and 2.5 volumes of absolute ethanol. After mixing, the tube was placed at -70° C for 30 minutes. Precipitated DNA was pelleted by centrifugation at 13,000 rpm for 10 minutes. The supernatant was removed and the

nucleic acid pellet washed carefully with ice-cold 70% ethanol. After washing, all ethanol was removed and the DNA pellets vacuum-dried and resuspended in the appropriate volume of 1 X TE buffer or autoclaved ddH₂O.

2.2.9 Automated sequencing of double-stranded plasmid DNA

All the plasmids used in the experiments were sequenced to verify the inserts by automated DNA sequencing using an ABI3700 DNA sequencer and ABI BigDye chemistry version3 terminator mix (Applied Bio systems, Cheshire, UK) at the DNA Sequencing Service, Genetics Core Facility, ICSM, Hammersmith Campus, London. Sequencing reactions were set up in 10 µl, containing the DNA to be sequenced (200–500ng per 3kb) and the primers (3.2 pmol) and was submitted to the sequencing lab. The sequencing technique is based on the dideoxynucleotide chain termination method (Sanger et al., 1977). Chain termination sequencing involves the synthesis of new strands of DNA complementary to a single-stranded template. The template DNA is supplied with a mixture of all four deoxynucleotides, four dideoxynucleotides, each tagged with a different fluorescent dye. As all four deoxynucleotides are present, chain elongation proceeds until, by chance, DNA polymerase inserts a dideoxynucleotide. The result is a new set of DNA chains all of different lengths. The fragments are then separated by size using gel electrophoresis. As each labelled DNA fragment passes a detector at the bottom of the gel, the colour is recorded. The DNA sequence is then reconstructed from the pattern of colours representing each nucleotide sequence. The sequence was analyzed using Vector NTI programme (Invitrogen, UK).

2.2.10 Bacteriomatch II cDNA Expression Library Screening

The bait construct, pBT encoding AF1 of human ERα, in which serine residues at positions 104, 106 and 118 have been substituted by glutamic acid (AF1-EEE) was used as a bait in two-hybrid screening of the human breast cDNA library (BacterioMatch Two-Hybrid System XR Plasmid cDNA Library) (Stratagene, UK), according to manufacturer's instructions using the electroporation method. The bait and target plasmid were co transformed in an *E.coli* reporter strain that carries a reporter

cassette consisting HIS 3 and *aadA* gene conferring streptomycin resistance. Putative positive clones were isolated by growth medium containing 5mM, 3-amino triazole (3-AT) which is competitive inhibitor of His3 enzyme. These clones were retested for interaction by their ability to grow in the presence of streptomycin and are considered as true positives. DNA from these clones was isolated based on tetracycline and chloramphenicol selection. The target DNA was then separated from the bait, based on the tetracycline resistance gene encoded by target plasmid but not the bait plasmid. The isolated target DNA was then retransformed with the bait plasmid in the reporter cell along with empty bait as a control. Inserts of target plasmids of positive clones were sequenced. The sequences of the target plasmids were compared to the Gene bank database using the BLASTn search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.2.11 Site directed mutagenesis

Site directed mutagenesis is technique to introduce a mutation at a desired position site within the target DNA. The mutant strand synthesis reaction was set up in thin-walled 0.5 ml tubes as listed below

- 2.5 µl of 10X reaction buffer
- 100ng of the DNA template
- 100 ng of the primer
- 1 µl of the dNTP mix
- 1 µl of the Quick change multi enzyme blend
- ddH₂O to final volume of 25 µl

The following cycling parameters were used for the reaction:

Segment 1	95°C for 1 min.
Segment 2	95°C for 1 min.
	55°C for 1min.
	65°C for 10 min. for 16 cycles

Following the completion of the reaction 1 µl of *DpnI* restriction endonuclease was added to the reaction mixture and incubated at room temperature for one hour. *DpnI* digests methylated and hemimethylated DNA, but not *in vitro* synthesised

unmethylated DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore gets digested. 1.5 µl of the mutagenesis reaction was used to transform XL-10 Gold ultracompetent cells and plated on LB agar plates with appropriate antibiotic selection. Miniprep DNAs were screened for successful mutagenesis by automated DNA sequencing. The primers used for the mutant p68LSYAA are shown below.

Primers for generating p68 LSYAA mutant by mutagenesis		
p68LSYAA	Forward	GGATCTGGGAAAACATTGTCTTATGCGGCTCCTGCCATTGTCCACATCAATC
	Reverse	GATTGATGTGGACAATGGCAGGAGCCGCATAAGACAATGTTTTCCAGATCC

2.2.12 Cell Culture

Frozen cells were thawed in a 37°C water bath, resuspended in 20ml DMEM, supplemented with 10% FCS and PSG and pelleted by centrifugation at 1200 rpm for 5 minutes in a bench top centrifuge. The pelleted cells were washed with DMEM and resuspended in 10ml DMEM containing 10% FCS in a T25 tissue culture flask and incubated at 37°C in a humidified, 5% (v/v) CO₂: air atmosphere incubator.

Cultures grown to 70-80% confluency were trypsinised (10% trypsin in 0.02% Na EDTA) for 3-5 minutes to detach the cells from the plastic. The trypsin reaction was terminated by adding DMEM supplemented with 10% FCS and the cells were passaged into new flasks. All mammalian cell lines were routinely maintained in DMEM with either 5% or 10% FCS and PSG.

Aliquots of cells were frozen at regular intervals to have a supply of stocks. For this, cells from healthy confluent culture were trypsinised and pelleted by centrifugation at 1200 rpm for 5 minutes. The cell pellet was resuspended in 1 ml freezing medium (40% DMEM, 10% DMSO, and 50% FCS). Cells were aliquoted into 2 ml cryovials, placed at -80°C overnight and then transferred to a liquid nitrogen container for long-term storage.

2.2.13 Mammalian Cell Transfections

2.2.13.1 Transient transfection of COS-1 cells for reporter gene assay

COS-1 cells were maintained in DMEM supplemented with 5% FCS. For transient transfections, COS-1 cells were cultured for three days in DMEM lacking phenol red and supplemented with 5% DSS. Cells were seeded at 5×10^4 cells per well in 24 well plates. The following day the cells were transiently transfected using Fugene 6 (Roche diagnostics, UK), a lipid based multicomponent transfection reagent, which complexes with the DNA and transports the complex into cells. Per transfection, 3 μ l of Fugene 6 was diluted in 50 μ l of DMEM without serum and mixed with the 50 μ l of DMEM containing the DNAs. The mixture was incubated for 20 min. at room temperature. The Fugene-DNA mixture was added drop-wise to the cells. The DNA mix comprised 100 ng of the estrogen-responsive luciferase reporter gene, 100 ng of renilla control reporter gene (RLTK), 110 ng of the carrier DNA (BSM), together with the expression plasmids (up to a total of 250 ng), to give a total amount of DNA of 1 μ g. Four hours post-transfection, the medium was replaced with fresh medium (DMEM-PR, 5% DSS) containing 17 β -estradiol (E2; 10 nM) or 4-hydroxytamoxifen (OHT; 100 nM), as appropriate. Since E2 and OHT were prepared in ethanol, an equal volume of ethanol was added to the no ligand controls. The cells were harvested after a further 24 hours using the passive lysis buffer (Promega, UK) and assayed for firefly and renilla luciferase activities.

For the mammalian 2-hybrid assay, COS-1 cells were transfected as above, using 100 ng of 5Gal-tk-luc, 100 ng of RLTK, 100 ng of the Gal4-p68 fusion constructs and 50 ng of the ER α expression plasmid HEG0 or VP16-fused to the DBD and LBD of ER α (VP16-HEG19). The cells were harvested and processed for determination of luciferase activities 48 hours following the addition of E2.

2.2.13.2 Transient transfection of COS-1 cells for preparation of whole cell extracts

For whole cell extract preparation following transient transfection of COS-1 cells, 3×10^5 cells were plated in each well of 6-well plates, and transfected with 0.5 μg of each expression plasmid, up to a total of 1 μg of DNA, using 4 μl of Fugene 6, essentially as described in section 2.2.11.1. Four hours following transfection the medium was replaced with fresh medium and a further 48 hours later the cells were washed twice with PBS and lysed by the addition of 100 μl of 2xLaemmli buffer (0.12 M Tris HCl, pH 6.8, 4% w/v SDS, 20% glycerol, 0.2 M DTT, 0.008% bromophenol blue), heated to 100°C. The cells were scraped using a rubber policeman and the lysates collected in 1.5 ml microfuge tubes. The samples were heated to 100°C for 10 minutes, cooled on ice and stored at -80°C.

2.2.13.3 Transfection of MCF-7 cells for over expression of p68 and p72

For over-expression studies, MCF-7 cells were transfected in 6 well plates using the transfection reagent, Fugene 6 (Roche, UK) as per the manufacturer's instructions. Prior to transfection MCF-7 cells were grown in DMEM containing 5%DSS for three days. The cells were seeded in 6 well plates at a density of 3×10^5 and transfected 16-24 hours later with 0.5 μg of expression plasmid and 4 μl of Fugene 6. The cells were harvested into hot lysis buffer as described for COS-1 cells (Section 2.2.13.2).

2.2.13.4 RNA Interference

RNA interference was carried out using double-stranded RNA oligonucleotides (Table 2.9) purchased from Dharmacon (USA). MCF-7 cell were transfected using lipofectamine siRNAMAX (Invitrogen, UK), as per the manufacturer's "reverse transfection" protocol for transfecting MCF-7 cells. MCF-7 cells were routinely cultured in DMEM, supplemented with 10% FCS. Three days prior to transfection the medium was changed to DMEM-PR containing 5% DSS. MCF-7,

MELN or MVLN cells were trypsinised and resuspended in DMEM-PR containing 5% DSS (no antibiotics), at a cell number per ml, as listed in Table 2.10. Double-stranded RNAi oligonucleotides diluted in optiMEM-I medium (Invitrogen) were added to the plates, followed by the addition of the appropriate volume of Lipofectamine RNAiMAX, as listed in Table 2.10. Transfection complexes were allowed to form in the well by incubating at room temperature for 20 minutes. MCF-7 cells were added to the wells containing the transfection complexes and returned to the CO₂ incubator. Cells were harvested 48 hours post-transfection, with E2 (10n M) being added 4 or 8 hours prior to harvesting for preparation of total RNA, protein lysates, or in the case of MELN and MVLN cells, to perform the firefly luciferase reporter gene assay. RNA interference was carried out in 10 cm plates for RNA preparation, in 6-well plates for protein lysates and in 24-well plates for luciferase assays.

In order to determine the effect of RNA interference on MCF-7 cell growth, the transfections were carried out in 96-well plates. 16-24 hours after transfection the medium was replaced with fresh medium containing estrogen (10nM), or an equal volume of ethanol (vehicle). Cell numbers were estimated using the SRB growth assay.

Table 2.9 p68 and p72 siRNA oligos

p68 and p72 siRNA Sequences
p68
AACUCUAAUGUGGAGUGCGAC
p72
AACAAGGGUACCGCCUAUACC

Table 2.10 Quantities of siRNAs and transfection reagents used for transfections of MCF-7 cells

Culture vessel	Volume of plating medium	Cells seeded/well	RNAi oligo Dilution medium (Volume)	RNAi duplex (pmol)/well	Lipofectamine RNAiMAX/well
96-well	200 μ l	1.3×10^4	20 μ l	4	0.3 μ l
24-well	500 μ l	5.0×10^4	100 μ l	6	1 μ l
6-well	2 ml	3.0×10^5	500 μ l	100	5 μ l
10 cm plate	10 ml	2.4×10^6	1 ml	1200	40 μ l

2.2.14 Luciferase reporter assay

The Firefly and renilla luciferase activities were determined using Dual-Glo Luciferase Assay system (Promega LTD, UK). Media was removed from the wells and the cells were washed twice with PBS. Then 50 μ l of passive lysis buffer (PLB, Promega LTD, UK) was added to each well and the plates left on a rocking tray for 30 minutes at room temperature. Lysates were then transferred to reading plates, to which 50 μ l of the Firefly reagent was added to initiate Firefly bioluminescence. The plates were read by TopCount NXT Microplate Scintillation and Luminescence Counter (Parkard Bioscience). Then 50 μ l of Stop-Glo buffer was added, to detect the Renilla luciferase activity, the plate was read again and Firefly luciferase readings were then normalised against the Renilla luciferase.

2.2.15 Determination of protein concentration in whole cell lysates

The amount of protein in bacterial and cellular lysates was determined using Bio-Rad protein assay kit (Herts, UK). The Bio-Rad Protein Assay is a dye-binding assay in which a differential colour change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. A standard curve was generated using serial dilutions (100-5000 μ g/ml) of bovine serum albumin (BSA). The Bio-Rad reagent was diluted 1:5 in ddH₂O and

990 µl of the diluted reagent was mixed with 10 µl of each sample and BSA standards in 1 ml plastic cuvettes (Sigma, Dorset, UK). The samples were mixed and incubated at room temperature for 10 minutes. The optical density was measured at a wavelength of 595 nm. The OD of the standards was plotted as a line graph and the protein concentration of the samples was determined from the standard curve.

2.2.16 SDS- Polyacrylamide gel electrophoresis and Immunoblotting

SDS-PAGE and western blotting was carried out using a Bio-Rad mini gel system (Herts, UK). Protein samples were mixed with an equal volume of 2X Laemmli buffer (50 mM Tris.HCl pH 6.8, 15% Glycerol, 2% SDS, 10 mg bromophenol blue dye, 1 mM DTT). All lysates in Laemmli buffer were heated to 100°C on a heat block for 5 minutes prior to loading on a gel. Lysates were fractionated on 10%-15% SDS-polyacrylamide gels. The molecular weights of the protein bands were identified by comparison with the Full Range Rainbow markers (Amersham Life Science, Bucks, UK). Gels were electrophoresed in 1x SDS-PAGE running buffer (25mM Tris base; 192mM glycine; 0.1% SDS) at 80 - 100V for 1 – 1½ hours until the dye front reached the bottom of the gel. The gel was blotted onto Hybond ECL nitrocellulose membrane (Amersham Biosciences, Bucks, UK) at 90V for 1½ hours in 1 x transfer buffer (5mM Tris base; 38mM glycine; 20% methanol). Successful transfer was indicated by the presence of the coloured molecular weight markers on the membrane. In order to prevent non-specific binding of antibody, the membrane was placed into blocking solution (3% Marvel/PBS-0.05%Tween20) for 30–60 minutes at room temperature, prior to incubation with the appropriate primary antibody (diluted in 0.3% Marvel/PBS-0.05%Tween20) for 1 hour at room temperature. The blot was then washed 3 x in PBS-0.05%Tween20 (wash buffer) for 15 minutes per wash, blocked in blocking solution for a further 30 minutes, then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody in 0.3% Marvel/PBS-0.05%Tween20 for 1 hour. The membrane was then washed 4 times in wash buffer, again for 15 minutes per wash. Bands were visualised using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Perbio Sciences UK LTD). Signals were detected by exposing X-ray film

(BioMax 10 X-ray film, Kodak UK Ltd, Cambridge, UK) to the membrane and films were developed with a Konica SRX-101A X-ray developer.

2.2.17 *In vitro* transcription and translation

In vitro transcription and translation was performed using the TNT T7 Quick Coupled Transcription/Translation System kit from Promega (# L1170). 40 µl of TNT master mix, 1 µg of plasmid DNA, 2 µl of ³⁵S-labelled methionine (1,000Ci/mmol) and nuclease-free ddH₂O to a final volume of 50 µl were assembled in a 1.5 ml tube and incubated for 90 minutes at 30°C in a water bath. Two µl of the reaction was used to analyse the translation product by SDS-PAGE.

2.2.18 *In vitro* Phosphorylation of ERα by MAPK

Purified GST proteins GST-ERα, GST-AF1 were incubated with 20µM ATP with or without 50 ng of MAPK, 10 ul of 7 X protease inhibitor cocktail and 50µl of MAPK buffer for 30 minutes. The reaction was stopped by adding 40µl of 2 X SDS buffer.

2.2.19 GST pull-down assay

E. coli BL21 (DE3) cells were transformed with pGEX constructs for expression of GST-fusion proteins. A single colony was picked into 5 ml LB broth containing ampicillin and chloramphenicol and incubated overnight in a shaker at 37°C. The cultures were diluted 1:10 in LB medium and grown for 1 hour and protein expression was induced by adding IPTG to a final concentration of 0.1 mM and grown for a further three hours. Cells were pelleted by centrifugation at 4000 x g for 10 minutes at 4°C, the pellets were resuspended in 10 volumes of NETN buffer and sonicated four times for 30 seconds. The samples were centrifuged at 15000 rpm for 15 minutes at 4°C. The supernatants were collected and protein concentration estimated using the Bio-Rad assay. Fifty µg of crude bacterial GST-fusion protein was added to 100 µl of glutathione-sepharose beads (Amersham biosciences) as 50% slurry in NETN

buffer. NETN buffer was added to a total volume of 1 ml and the samples were incubated, with shaking, at 4°C for 1 hour. The samples were centrifuged and unbound protein was cleared by washing 4 times with 1 ml NETN buffer. Twenty µl of ³⁵S labelled *in vitro* translated protein was added, the volume made up to 1 ml with NETN buffer and the samples incubated at 4°C with shaking, for an hour. The samples were centrifuged and washed 4 times with 1 ml NETN buffer, followed by the addition of 40 µl of 2 X Laemmli buffer to the beads and eluted proteins analysed by SDS- PAGE. The gels were fixed, dried and exposed on Kodak BioMax Light 1 film for visualisation of ³⁵S-labelled proteins.

2.2.20 Immunoprecipitations

COS-1 cells cultured in DMEM-PR supplemented with 5% DSS for three days were seeded in 10 cm dishes at a density of 7.5×10^4 . Sixteen to 24 hour later, the cells were transfected with 10 µg each of expression plasmid (ERα, p68, p72, as appropriate), using Fugene 6 (Roche, UK). 48 hours following transfection the cells were lysed in 0.5 ml of High salt lysis buffer (330mM NaCl, 0.1% NP-40 and 10 mM Tris-HCl (pH7.5) containing protease inhibitors. Lysates (1 mg) were immunoprecipitated using an anti-ERα mouse monoclonal antibody (6F11). Control immunoprecipitations were carried out using mouse immunoglobulins (Sigma-Aldrich, UK). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted using rabbit polyclonal antibody to ERα (HC20, Santa Cruz) and rabbit polyclonal antibody to p68 RNA helicase (pAb 2906) or a rabbit polyclonal antibody for p72 (Gift from Dr FV Fuller Pace).

For the co immunoprecipitation of endogenous p68/72 and ER proteins in MCF cells, nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, # 78833) as per the manufacturer's instructions. MCF-7 cells were grown in 5%DSS medium for 3 days and were seeded in 10 cm dishes at a concentration of 2.4×10^6 . Cells were grown in the absence or presence of 10nM of estrogen for 24 hours. After washing the cells with 1% PBS cells from 4 plates were pooled and harvested by centrifugation at 5000 rpm for 2-3 minutes. The cell pellet was mixed with 1ml of ice cold CER I (composition withheld by the

manufacturer). The sample was vortexed vigorously for 15 seconds to fully resuspend the pellet and was incubated on ice for 10 minutes. 500ul of CER II was added, vortexed for 5 seconds and incubated on ice for 1 minute. The sample was vortexed again for 5 seconds and centrifuged in a micro centrifuge at 13000 rpm for 5 minutes. The supernatant containing the cytoplasmic extract was discarded and pellet containing the nuclear extract was resuspended in 250 ul of ice cold NER (composition withheld by the manufacturer). The tube was vortexed every 15 seconds for a total of 40 minutes keeping the sample on ice in between the vortex. The sample was centrifuged at maximum speed of 13000 rpm for 10 minutes. The supernatant was transferred to a pre-chilled eppendorf tube and placed on ice. The samples were stored at -80°C. Immunoprecipitations were carried out as described for COS-1 cells.

2.2.21 RNA Extraction

Total RNA was extracted from MCF-7 cells using the RNeasy mini-kit (Qiagen) as per the manufacturers' instructions. The cells were washed with PBS and harvested by adding 600 µl of RLT buffer containing β mercaptoethanol (composition withheld by the manufacture). The lysate was loaded on to "QIA shredder" column placed in a 2 ml collection tube. The lysate was centrifuged at 13000 rpm for 2 minutes. To this, one volume of 70% ethanol was added and mixed well by pipetting. The sample was transferred to RNeasy mini column placed in a 2 ml collection tube and centrifuged at 10000 rpm for 15 seconds and the flow-through was discarded. To remove genomic DNA contaminating the lysate DNase digestion was performed by washing first with 350 µl of Buffer RW1 (composition withheld by the manufacturer) followed by addition of DNase incubation mix (10ul of DNase stock solution plus 70 µl of Buffer RDD (composition withheld by the manufacturer) and incubation for 15 minutes on a bench top. A further wash was performed by adding 350 µl of Buffer RW1 and centrifuging for 15 seconds. The flow-through was discarded and the column washed twice with 500 µl buffer RPE (composition withheld by the manufacturer), centrifuging for 15 seconds for first wash and for 2 minutes for the second wash. The RNA binds to the RNeasy column membrane and the washes remove the contaminants. The column was transferred to a new collection tube and centrifuged for 1 minute,

placed in a new 1.5 ml collection tube and RNA eluted by adding 40 µl of RNase free water to the column and centrifuging for 1 minute at 10000 rpm. The RNA concentration was determined using a spectrophotometer.

2.2.22 cDNA preparation by Reverse Transcription (RT)

RNA was converted to cDNA using reverse transcriptase. The reaction was set up in a thin-walled PCR tube with the following components.

- 4 µl 5x RT buffer (containing DTT - Mbi Fermentas)
- 2 µl dNTPs (10nM stock)
- 1 µl random hexamers Pd (N) 6 (200ng)
- 2 µg total RNA
- 1 µl Reverse transcriptase stock (Revert Aid M-MuLV - Mbi Fermentas)
- Sterile water to a total volume of 20µl

The reaction was incubated at 37°C for 1 hour and then heat inactivated at 65°C for 10 minutes. The product was diluted in 80 µl of water and 2 µl of cDNA was subsequently used for each PCR reaction.

2.2.23 Polymerase chain reaction (PCR)

The PCR reactions were set up in thin-walled PCR tubes containing 2 µl of cDNA, 12.5 µl of PCR master mix (Reddy Mix, ABgene, Epsom, UK), 2.5 µl each of forward and reverse primers, and 5.5 µl of water in a 25 µl reaction volume. The samples were centrifuged briefly and loaded onto a Gene Amp 9700 PCR machine (Applied Bio systems, Cheshire, UK). The PCR primers used are listed in Table 2 .8 and the cycling parameters are as given below.

- | | | | |
|--------------|------|---|--------------|
| Hot start | 94°C | 5 minutes | 1 cycle |
| Denaturation | 95°C | 1 minute | |
| Annealing | X°C | 1 minute (where X is the optimal annealing temperature) | |
| Extension | 72°C | 1.5 minutes, | 22-28 cycles |
| Extension | 72°C | 5 minutes | 1 cycle |

The optimal primer annealing temperature ($X^{\circ}\text{C}$) was typically calculated using the following equation:

$$T_m = (4 \times G + C) + (2 \times A + T)$$

PCR products were visualised using an UV transilluminator following electrophoresis on 0.25 $\mu\text{g/ml}$ ethidium bromide-agarose gels. The concentration of the gel was dependent on the expected size of the PCR product to be resolved.

2.2.24 Real-time RT-PCR

Real-time RT-PCR, also known as kinetic PCR, qRT-PCR or RT-qPCR, is a quantitative PCR method for the determination of copy number of PCR templates such as cDNA in a PCR reaction. Real-Time PCR was performed using the ABI-Prism 7700 sequence analyser system (Applied Bio systems). A special thermo cycler equipped with a sensitive laser that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction. It requires a pair of PCR primers as in standard PCR with an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. The primers and the probes used are shown in Table 2.8. The reactions were set up in 20 μl volume containing 2 μl of cDNA, 1 μl of primers (Custom TaqMan Gene Expression Assays, Applied Bio systems) 10 μl of 2 x Taqman Fast universal PCR mix (Applied Bio systems) and 7 μl of RNase free water, in Optical 96-Well Fast Thermal Cycling Plate (Applied Bio systems), in triplicates. The level of expression of a gene was quantitated using the comparative C_T (threshold cycle) method. The level of expression of a target gene was expressed as fold difference relative to the reference gene, GAPDH.

2.2.25 SRB Growth assay

Sulphorhodamine B (SRB; Sigma, UK) is a pink aminoxanthene dye which binds to protein basic amino acid residues in TCA-fixed cells and provides a sensitive index of cellular protein content which reflects cell growth. Cells were fixed by adding ice-cold 40% trichloroacetic acid (TCA) and incubating for 1 h at room temperature. The plates were washed with distilled water, air-dried and stained with SRB solution

(0.4%, w/v, in 1% acetic acid) for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid and the plates were air-dried. The bound SRB stain was solubilised with 10mM Tris buffer, and the optical density was read on a spectrophotometric plate reader at a wavelength of 492 nm.

3. RESULTS

3.1. Bacterial 2-hybrid approach for the identification of AF1-interacting proteins

The functional reconstitution of a transcription factor through protein-protein interactions between two proteins of interest allows functional studies on the nature of the interaction. Such an approach has been exploited in the generation of screening strategies, particularly in yeast, whereby a protein of interest is expressed as a fusion protein with a DNA binding domain, allowing for screening for interacting partners using cDNA expression libraries in which the proteins are expressed as fusions with a transcriptional activation domain (Fields and Song, 1989). Although this method has allowed the identification of numerous interacting proteins it is difficult to use if the protein of interest is a transcription factor capable of activating the reporter gene. In this context ER α and the AF1 region of ER α are both transcriptionally active in yeast cells (Metzger et al., 1988; Metzger et al., 1992). A technique that is based on the same principle as yeast two-hybrid system but uses a bacterial host, was developed by Dove et al (Dove *et al.*, 1997). This bacterial two-hybrid approach is more amenable for the study of mammalian transcription factors, as mammalian transcription activation domains are unlikely to activate gene expression in bacterial cells. Further, the system offers a number of advantages over the yeast 2-hybrid system, which include higher transformation efficiency and faster growth of *E. coli*, allowing a reduction in the time required for screening cDNA expression libraries for interacting partners. I used the BacterioMatch II Two-Hybrid system (Stratagene, UK) based on the above methodology to screen for ER α interacting proteins. In the Bacteriomatch II system the bacterial hisB gene is mutated, but the strain has stable integration of the yeast His3 gene, which can complement for the bacterial hisB gene. The λ operator sequence has been engineered in the yeast His3 gene promoter, so that growth of this strain on medium lacking histidine requires the λ cI repressor-mediated recruitment of bacterial RNA polymerase. This is achieved by fusion of the λ cI repressor to the protein of interest. The interacting partner is fused to the α subunit of bacterial RNA polymerase, such that a productive interaction between the proteins of interest results in the

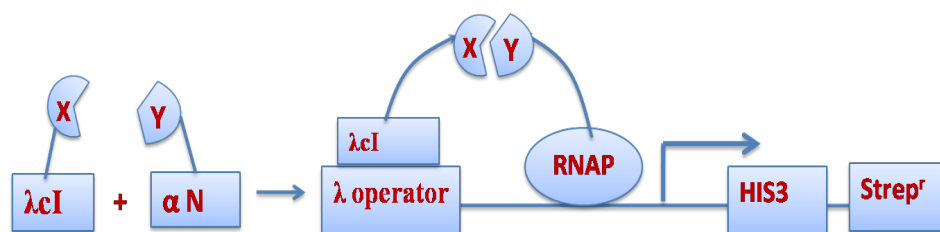


Figure 3.1 Schematic diagram of the bacterial two hybrid system. Bait (X) protein fused to full length bacteriophage repressor protein, λ cI and the Target (Y) protein fused to subunit (α N) of *E.coli* RNA polymerase (RNAP), are expressed in a reporter strain. When the hybrid bait and target proteins interact, the RNA polymerase is recruited to the promoter activating the transcription of $HIS3$. The system has a second reporter that confers streptomycin resistance ($Strep^r$), useful in verifying the bait and target protein interactions.

recruitment of RNA polymerase to the His3 gene promoter, enabling gene expression and consequent growth on medium lacking histidine (Figure 3.1).

To assess the efficacy of this system for screening a cDNA expression library for ER α interacting proteins, a panel of known ER α interacting proteins was tested for interaction using the system. The AF1 and LBD/AF2 domains of ER α were cloned into the bait vector, pBT, in fusion with λ cI coding sequences. Previous work in our laboratory has shown that substitution of the key AF1 phosphorylation sites, serines 104, 106 and 118 to glutamic acid stimulates ER α activity in a ligand-independent manner, potentially by “mimicking” phosphorylation at these sites, whereas substitution by alanines reduces AF1 activity. The ER α AF1 region in which the serine residues at these positions were substituted by glutamic acid (AF1-EEE) or by alanines (AF1-AAA) were also generated, in order to identify ER α -interacting proteins that are preferentially recruited to the phosphorylated AF1 region. Finally, an AF2 mutant that does not interact with coactivators and is transcriptionally inactive, AF2-L539A/L540A (AF2-LLAA) was also constructed, as a negative control for interaction with AF2. The known ER α coactivators SRC1 and TIF2, which interact with AF2 in a ligand-dependent manner, were cloned into the target vector, pTRG, in frame with α unit of RNA polymerase. Additionally, p68 RNA helicase, which preferentially interacts with AF1 phosphorylated at serine 118, as well as with AF1 in which serine 118 has been replaced by glutamic acid, but not with AF1 in which serine 118 has been substituted by alanine, was cloned into pTRG. pBT-LGF2 and pTRG-Gal11p are the bait and target plasmids encoding yeast transcriptional activator Gal4 and a domain derived from a mutant form of Gal11 protein, called Gal11p, respectively. These have been shown to interact in *E. coli* cells and are used as positive controls for interaction in the Bacteriomatch system. The respective bait and target plasmids were cotransformed into the reporter strain and were plated into selective and non-selective medium. Growth was observed in nonselective medium lacking histidine under all conditions (Figure 3.2). This growth is likely to be due to leaky expression of the His3 gene, as has been described (Stratagene manual). 3-amino-1, 2, 4-triazole (3-AT), is a competitive inhibitor of the yeast His3 enzyme and is added to reduce background growth due to leaky expression of the His3 gene. In the presence of 10 mM 3-AT in the medium, no

BAIT	TARGET				
	Gal11	SRC-1	TIF2	p68	3AT (mM)
LGF2					0
					5
					10
AF2WT					0
					5
					10
AF2 LLAA					0
					5
					10
AF1WT					0
					5
					10
AF1AAA					0
					5
					10
AF1 EEE					0
					5
					10

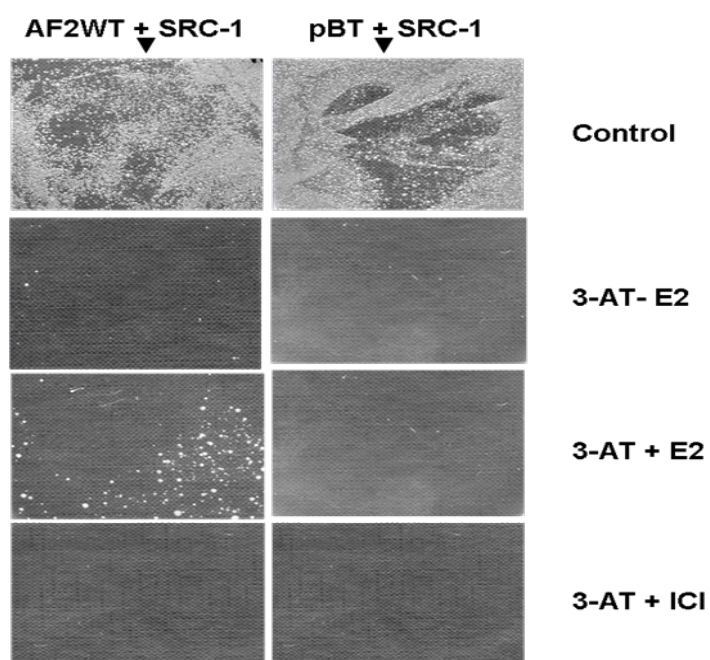
Figure 3.2 Bacterial growth spot assay showing interaction between ER α and ER α coactivators. Bait plasmids encoding the ER α LBD/AF2 (AF2WT), AF2 with alanine substitution of residues 539 and 540 (AF2LLAA), the ER α AF1 region (AF1WT), AF1 in which serines 104, 106 and 118 have been substituted by glutamic acid (AF1-EEE) or alanines (AF1-AAA) or control bait plasmid LGF2, were cotransformed with the target plasmids encoding the steroid receptor interacting domain of SRC1, p68 RNA helicase (p68), the region of TIF2 shown to interact with the AF1 region of ER α or the control target plasmid Gal11. The co-transformants were plated on minimal media with or without 3-AT(5-10mM).

growth was observed for pBT-AF2 co-transformed with pTRG-Gall11p, in the absence or presence of 17 β -estradiol (Figure 3.2). As expected, growth was also not observed for pBT-AF2 co-transformed with pTRG-p68, since p68 interacts with AF1. pBT-AF2 and pTRG-SRC1 did, however, interact, as evidenced by growth in the absence of histidine and in the presence of 10 mM 3-AT. This growth was not observed for AF2-LLAA, indicative of a productive interaction between AF2 and SRC1. Some growth was also observed for AF1, when cotransformed with SRC1 or TIF2, but not with p68. Similar growth was observed for AF1-AAA, although not for AF1-EEE. Whilst interaction of AF1 with SRC1 and TIF2 has been described, but the lack of interaction between p68 and AF1-EEE was unexpected, since interaction of p68 RNA helicase with AF1 in which S118 has been substituted by glutamic acid has been described (Endoh et al., 1999), and may be a reflection of incorrect folding of AF1.

The ligand-dependence of the interaction between AF2 of ER α and SRC1 was further demonstrated by cotransforming target vector encoding SRC1 with pBT or pBT-AF2 and assaying for growth in the presence or absence of ligands (Figure 3.3). When AF2 and SRC1 were cotransformed and plated on minimal medium lacking histidine and containing 5 mM 3-AT, 300 colonies were obtained in the presence of estrogen, whereas only 50 colonies were observed in the presence of no ligand. In the presence of the pure estrogen antagonist ICI182, 780 (ICI) no colonies were observed, indicating that ICI blocks AF2 interaction with SRC1 in bacterial cells, as it does in mammalian cells. When SRC1 was cotransformed with the empty bait plasmid there was no growth in the presence or absence of ligands. These data indicate that SRC1 and the ER α LBD interact in bacterial cells in a ligand-dependent manner.

3.2 cDNA Expression Library Screening for proteins that interact with the human ERAF1 domain

Numerous transcriptional coactivators that are recruited through interaction with the LBD/AF2 region of ER α , as well as other nuclear receptors, have been described in the literature. To date, however, very few AF1-interacting transcriptional coregulator proteins have been described. I therefore, wished to determine if the bacterial 2-hybrid system could be used to identify AF1-interacting proteins.



Transformations	Control	3-AT		3-AT
		+E2	-E2	+ICI
AF2WT+SRC1	>800	300	50	0
SRC1+pBT	>800	0	0	0

Figure 3.3 Ligand–dependent interaction of the ER α AF2 domain with the steroid receptor interacting domain of SRC1 in bacterial cells. The reporter strain co-transformed with pTRG-SRC1 and pBT or pBT-AF2 was plated on minimal media containing 5mM 3-AT. Cells were also plated in the absence of 3-AT to determine total colony number, as a measure of transformation efficiency (control). 100 nM E2 or ICI were added, as shown. Plates were incubated for 24 hours and growth was assayed by colony count. The table shows the colony count for each transformation.

Furthermore, as AF1 phosphorylation stimulates ER α activity, I wished to see whether the bacteriomatch system could be used to identify proteins that interact preferentially with ER α phosphorylated at serines 104, 106 and 118, by using the glutamic acid substitutions at these positions. I therefore screened a human breast cDNA expression library using AF1-EEE as bait. The human breast cDNA expression library from Stratagene was reported as being prepared from an oligo d(T)-primed normal human mammary gland RNA derived from 10 pooled samples of women, aged 33-80 years and containing 2.5×10^6 independent clones. Prior to screening the cDNA library, the expression of the bait was verified by Western blotting. Lysates were prepared from Bacteriomatch II reporter strain transformed with the pBT-AF1-EEE and immunoblotted and probed with antibodies specific for AF1 and λ cI. Immunoblotting demonstrated IPTG-induced expression of the bait protein, with the detected polypeptide having the expected molecular weight (Fig. 3.4).

An overview of library screening using the BacterioMatch II Two-Hybrid system is shown in the in Fig. 3.4. The BacterioMatch Two-Hybrid system reporter strain, a derivative of Stratagene's XL1-Blue MR, was transformed with the bait AF-1EEE and electrocompetent cells were prepared, as described in the Materials and Methods chapter. These cells were transformed with the pTRG-cDNA expression library using the electroporation method. The transformed cells were plated on medium containing 5 mM 3-AT, together with the antibiotic streptomycin. Control plating was carried out in the absence of 3-AT and/or streptomycin. The transformation yielded 7.5×10^5 clones, with 644 colonies being obtained on plates containing 3-AT. The BacterioMatch Two- Hybrid system reporter strain has a second λ cI-regulated reporter gene, *aadA*, which confers streptomycin resistance. Of the 644, 3-AT-resistant clones, 104 were able to grow in the presence of streptomycin. The pTRG plasmids were recovered from the 104 colonies by growth of the 3-AT and streptomycin-resistant colonies on tetracycline, as the pTRG plasmid, but not the pBT plasmid, encodes the tetracycline-resistance gene. All pTRG plasmids were sequenced and the sequences were compared to the Genbank database using BLASTn search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

A significant proportion (44%) of the positives encoded mitochondrial genes, various immunoglobulin gene sequences and sequences mapping to several chromosomes, at regions which did not correspond to any predicted genes. The remaining sequences corresponded to genes encoding proteins involved in signal transduction, cytoskeleton and membrane proteins. Multiple independent clones for some genes, Surf4 (13 clones), hARF5 (8 clones), ATP binding cassette (6 clones) and cyclophilinB (4 clones) were obtained. However, for all but two cases, the protein-coding sequences were in the wrong reading frame relative to the α subunit of RNA polymerase, which would prevent correct translation. Regardless of whether they were in frame, the positive clones were analysed further to confirm the interaction, although the clones encoding mitochondrial genes, immunoglobulins, as well as the chromosomal sequences with no clear open reading frame were not included in the further analysis. The remaining positive clones were re-transformed to reconfirm the interaction by testing the positive clone for its dependence on the bait plasmid in activating the reporter genes. Each positive cDNA target plasmid was cotransformed with either the empty bait or the bait encoding AF1-EEE and plated on to non-selective and selective medium containing 3-AT. However, none of the positive clones showed interaction with AF1-EEE, following re-transformation, indicating that these clones do not encode genuine AF1-interacting proteins.

The bacterial two-hybrid has been reported to give fewer false positives compared to the yeast two- hybrid system. Our screen yielded 104 potential interacting proteins out of the 7.5×10^5 primary transformants, all of which proved on further analysis to be false positives. Such a high number of false positives indicated that it was unlikely that further screening would successfully lead to the identification of AF1-interacting proteins. Given the disappointing nature of these results I decided not to persevere with the Bacteriomatch methodology.

PROTEIN	ACCESSION NUMBER
SURF4	NM033161
ADP-Riibosylation Factor 5	M57567
Basic Transcription Factor 3	NM001207
Coronin 1b variant	AB209579
Actin, alpha 2, smooth muscle, aorta	NM001613
S100 calcium binding protein A16	NM080388
Rho GTPactivating protein19 GTPase activating protein	NM032900
Cystien and Glycine rich Protein1	NM004078
Peptidylprolyl isomerase B (cyclophilin B)	NM000942
Myofibrillogenesis regulator 1	BCO 21118
ATP binding cassette, subfamily member 3	NM018358
Tweety homolog 1 (Drosophila) (TTYH1), transcript variant 1	NM020659
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit	NM001697
Retinol dehydroginase 11	NM016026
Regulator of G-protein signalling 5	NM003617
Amphiregulin (schwannoma-derived growth factor)	NM001657
Protein tyrosine phosphatase, receptor type, C-associated protein	NM005608
NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	NM005003
Glutamate-ammonia ligase (glutamine synthase)	NM001033044

Table 3.1 -List of genes identified by screening the human breast cDNA library with AF1-EEE as the bait using the bacterial two-hybrid system. The gene accession numbers for all genes is also shown.

4. RESULTS:

Characterisation p68 RNA helicase as a coactivator of ER α

4.1 Reporter gene assays show that p68 and p72 RNA helicases are coactivators for ER α

As discussed in the Introduction, p68 RNA helicase was previously shown to interact with ER α (Endoh et al., 1999). In the absence of significant findings from the Bacteriomatch II hybrid screening and because of *in vitro* studies showing that p68 binds preferentially to the ER α AF1, when it is phosphorylated at serine 118 by MAPK or to the ER α AF1 when S118 was substituted by glutamic acid, I decided to further characterise p68 as an ER α coactivator, and to investigate its importance for ER α function using over expression and RNA interference in breast cancer cell lines. Reporter gene assays were carried out using ER α and deletion and substitution mutants of ER α (Figure 4.1). Firstly, I confirmed the previously published findings showing that p68 acts as a coactivator for ER α , using reporter gene assays. COS1 cells were co-transfected with increasing amounts of ER α , p68 and/or the well-characterised ER α coactivator, SRC1, along with an estrogen responsive luciferase reporter gene encoding three copies of estrogen responsive elements upstream of the thymidine kinase promoter and were assayed for luciferase activity (ERE-luc). Stimulation of ER α activity by SRC1 and p68 was observed when low amounts (0.5-2.5 ng) of ER α were used, coactivation not being evident if higher amounts of ER α were transfected (Figure 4.2). Synergy between p68 and SRC1 was also evident in the presence of low levels of ER α . The lack of coactivation by known ER α coactivators unless low amounts of ER α are co-transfected, is in agreement with a previous study by Lee et al., who showed that synergy between coactivators is best seen when small amounts of ER α are transfected (Lee et al., 2002).

Having established that p68 stimulates ER α activity and that p68 synergises with SRC1, ER α was co-transfected with increasing amounts of p68, in order to determine the amount of p68 required for optimal coactivation. Increasing the amount of p68 transfected did not significantly increase the coactivator activity of p68, and indeed, at the highest concentration, coactivation by p68 was slightly inhibited (Figure 4.3). Hence, optimal coactivation by p68 and synergism with SRC1 was

obtained following transfection with 1 ng of ER α , 20 ng of SRC1 and 250 ng of p68. These data are in agreement with a recent report showing that 250-500 ng of p68 provide the highest level of coactivation of p53 in reporter gene assays (Bates et al., 2005). The need for such high amounts of the p68 expression plasmid may be due to the high levels of p68 present in most cell types, together with the difficulty in achieving good expression of exogenous p68 (F.V. Fuller-Pace, personal communication). Therefore 1 ng of ER expression vector and 250 ng of p68 were used in reporter gene assays for further experiments.

4.2 p68 RNA helicase is a coactivator of ER α and shows synergism with SRC1

As shown in figure 4.4, p68 stimulated ER α activity by about 1.5-fold, and synergised with SRC1, stimulating ER α activity by about 7-fold. These findings are similar to those previously reported for p68 stimulation of ER α activity (Endoh et al., 1999). Another DEAD box protein, named p72 RNA helicase, is very closely related to p68 (Lamm et al., 1996), and in reporter gene assays, p72 stimulated ER α activity at a level similar to that observed for p68 (Watanabe et al., 2001). Further, p72 also showed synergy with SRC1 (Figure 4.4). Similar results have been reported for p53, although p68 was found to be a more potent coactivator of p53 than p72 (Bates et al., 2005).

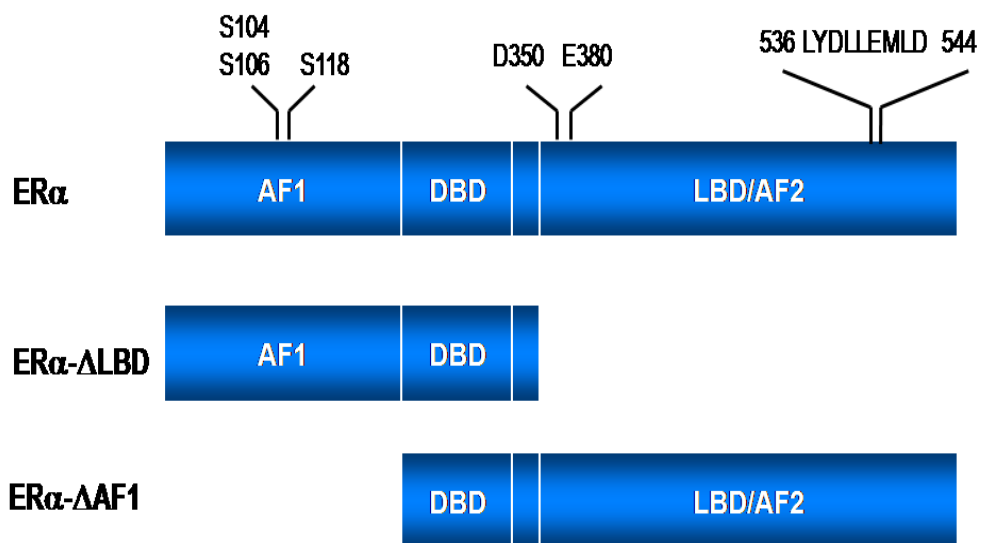


Figure 4.1 Schematic diagram depicting the domains of ERα and the constructs used in the transient transfection experiments. ERα, LBD-deleted ERα (ERα-ΔLBD) and AF1-deleted ERα (ERα-ΔAF1) are shown. The location of serines 104, 106 and 118 is shown, as is the position of amino acids in the ligand binding domain (LBD), whose mutation either stimulates ERα in a ligand-independent manner, or the mutation of which prevents coactivator recruitment by the LBD.

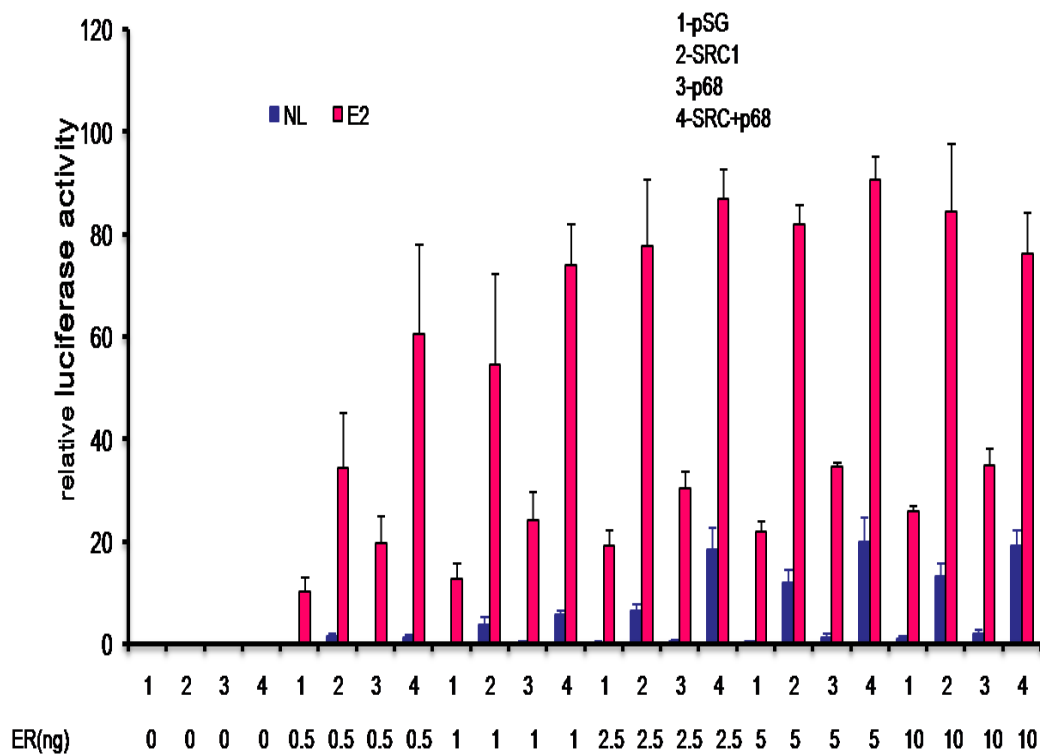


Figure 4.2 p68 RNA helicase (p68) is a coactivator for ER α in reporter gene assays. COS-1 cells, grown in medium lacking estrogen, were transfected with increasing amounts of expression vectors of ER α (as shown), 20ng of SRC1, 250ng of p68, 100ng of ERE-luc. A thymidine kinase (tk)-regulated renilla luciferase reporter (RLTK; 100 ng) was cotransfected, as a control for transfection efficiency. 17 β -estradiol (E2; 10 nM) was added, as shown, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls, Shown are the ERE-luc activities, with the luciferase activity obtained for pSG5 (lane 1) being taken as 1. All other activities are shown relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

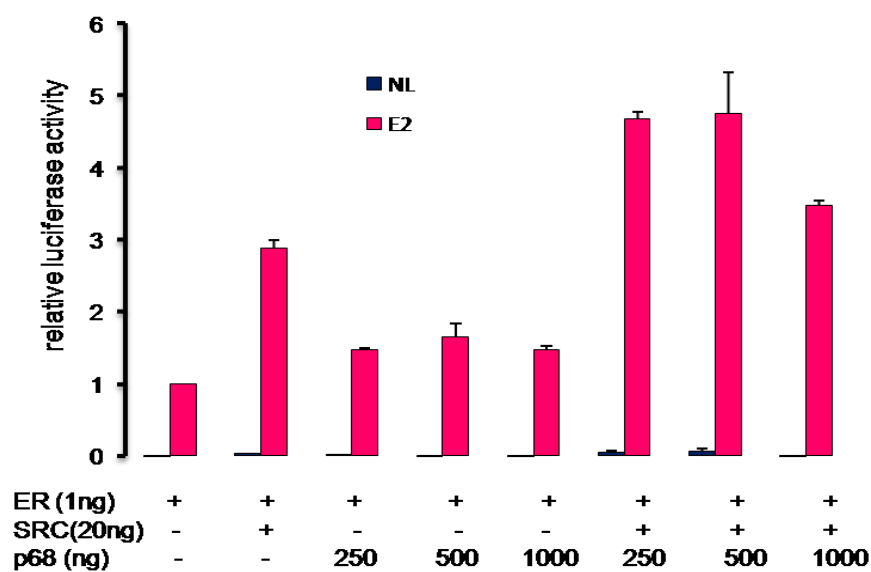


Figure 4.3 Stimulation of ER α activity by p68. COS-1 cells were transiently transfected with expression vectors encoding ER α (1ng), 20ng of SRC1 and varying amounts of p68 as indicated, together with 100ng of ERE-luc and RLTK. As shown for Figure 4.2, 17 β -estradiol (E2; 10 nM) was added, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls, The reporter gene activity obtained with 1ng of ER α in the presence of E2 and absence of coactivator was set at 1 and other data is expressed relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

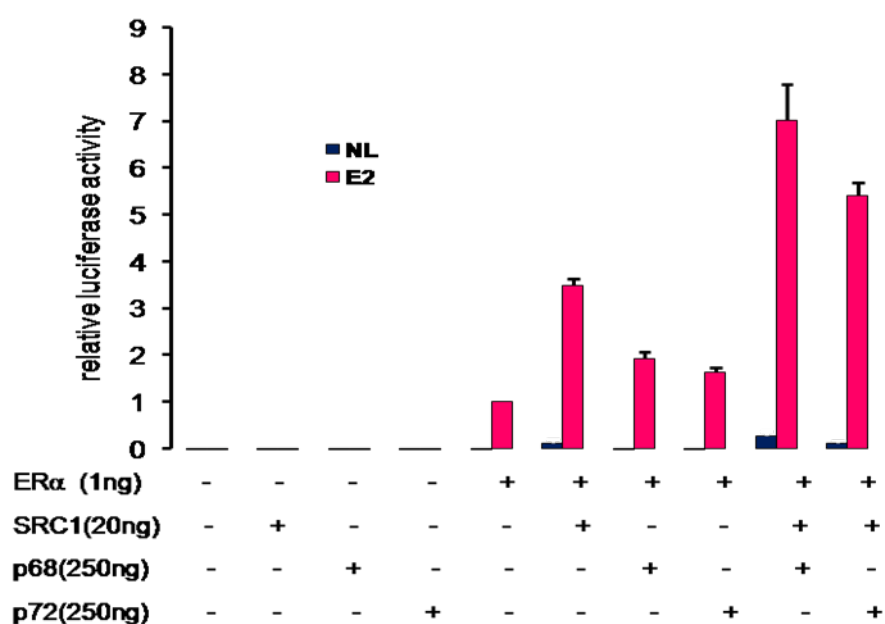


Figure 4.4 p72 RNA helicase is an ERα coactivator. COS-1 cells were transiently transfected with expression vectors encoding ERα (1ng), 20ng of SRC1 250 ng p68 and 250 ng p72, as indicated, together with 100ng of ERE-luc and RLTK. As shown for Figure 4.2, 17β-estradiol (E2; 10 nM) was added, as shown, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls, The reporter gene activity obtained with 1ng of ERα in the presence of E2 and absence of coactivator was set at 1 and other data is expressed relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

4.3 p68 modulates the activity of both AF1 and AF2 of ER α

p68 has been reported to be an AF1 specific coactivator of ER α (Endoh et al., 1999). In order to investigate this further, reporter assays were carried out using deletion mutants of ER α (Figure 4.1) in COS-1 cells. As expected and in agreement with the published data, p68 stimulated the activity of ER α - Δ LBD, which lacks the LBD and contains the AF1 and DBD domains (Figure 4.5). In this context p68 and SRC1 also demonstrated some cooperativity in stimulating the activity of AF1. Interestingly, p68 also stimulated the activity of ER α - Δ AF1, which encodes the DBD and LBD/AF2 but lacks AF1 (Figure 4.6).

The above data suggest that p68 potentiates the activity of the ER α LBD. As this has not previously been described, further reporter gene assays were carried out so as to confirm a role for p68 as a coactivator for the LBD/AF2. In order to do this, p68 was co-transfected with ER α in which key residues in the α helices 3, 5 and 12 had been substituted. These residues in α -helices 3, 5 and 12 form the AF-2 surface that is important in the ER α LBD interaction with coactivators (Feng et al 1998; Mak et al 1999). COS-1 cells were transiently transfected with expression plasmids encoding ER α containing amino acid substitutions, together with p68 and SRC1. Substitution of D351 affects ligand binding by ER α , particularly anti-estrogen binding (Wolf and Jordan, 1994). D351Y mutant has reduced basal activity that is stimulated to 40-fold by the addition of tamoxifen, in the presence of the SRC1 homologue, TIF2 (Anghel et al., 2000). In agreement with this report, high reporter activity of D351Y mutant was seen with SRC1 in the presence of tamoxifen. p68 also stimulated the activity of this mutant and increased activity was also seen in the presence of tamoxifen (Figure 4.7).

Amino acids in α -helix 12 in the ER α LBD form the interaction surface with the coactivators and the positioning of helix 12 is key to discriminating the agonist and antagonist nature of ligands (Norris et al., 1998). Estrogen binding results in conformational change in the LBD, which allows co-activator recruitment to the LBD. In the tamoxifen-bound LBD, helix 12 takes up position in the co-activator binding groove, thereby blocking coactivator recruitment by ER α (Shiau et al, 1998). Mutations in helix 12 prevent coactivator binding, presumably by altering the structure of helix 12.

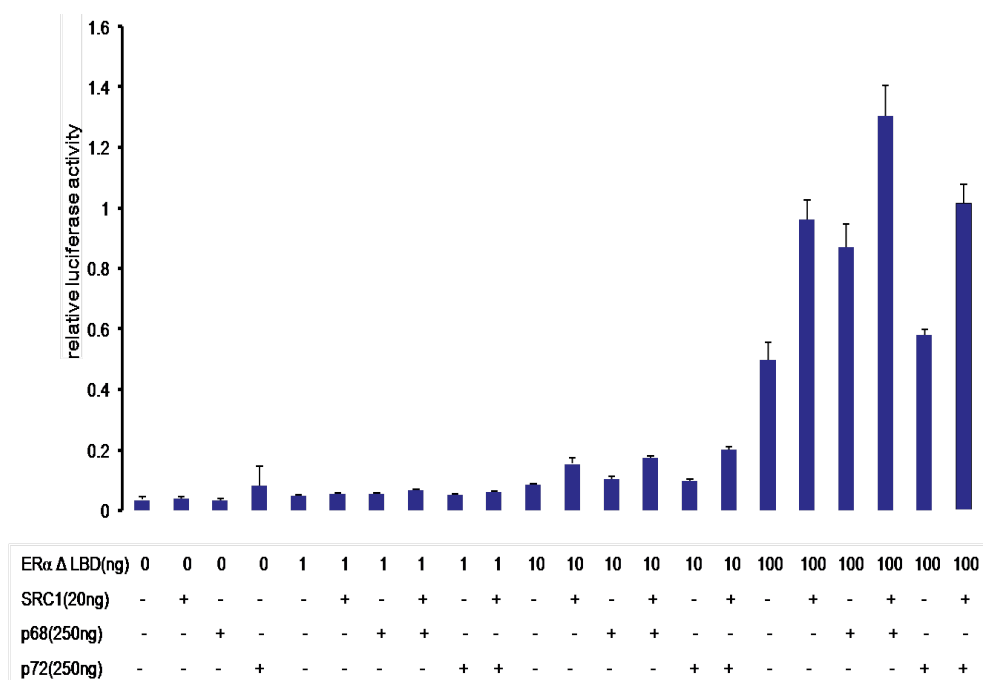


Figure 4.5 p68 enhances ERα AF1 activity. COS-1 cells, grown in medium lacking estrogen, were transfected with increasing amounts of expression vectors of ERα ΔLBD (as shown), 20ng of SRC1, 250ng each of p68 and p72, together with 100ng of ERE-luc and RLTK. Shown are relative ratios of firefly to renilla control luciferase activities. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

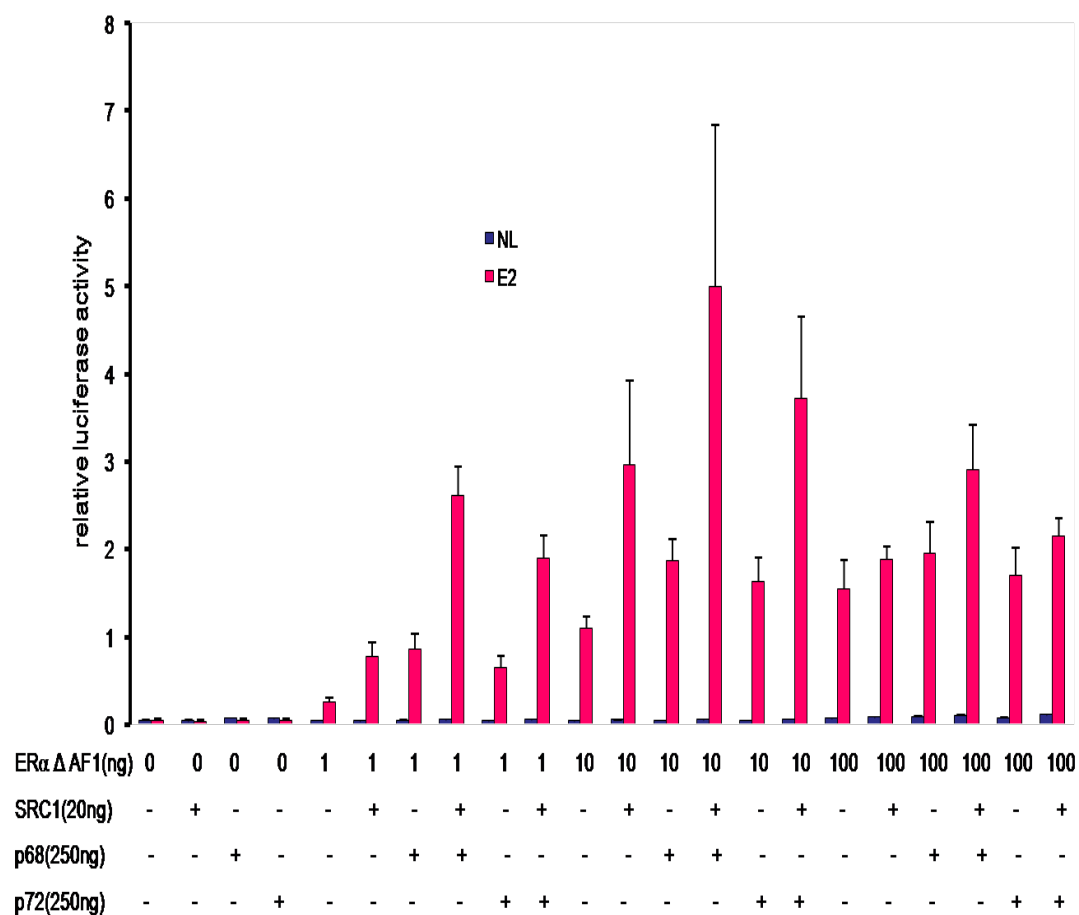


Figure 4.6 p68 modulates ERα AF2 activity. COS-1 cells, grown in medium lacking estrogen, were transfected with increasing amounts of expression vectors of ERα ΔAF1 (as shown), 20ng of SRC1, 250ng each of p68 and p72, together with 100ng of ERE-luc and RLTK. 17β-estradiol (E2; 10 nM) was added, as shown, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls. Shown are relative ratios of firefly to renilla control luciferase activities. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

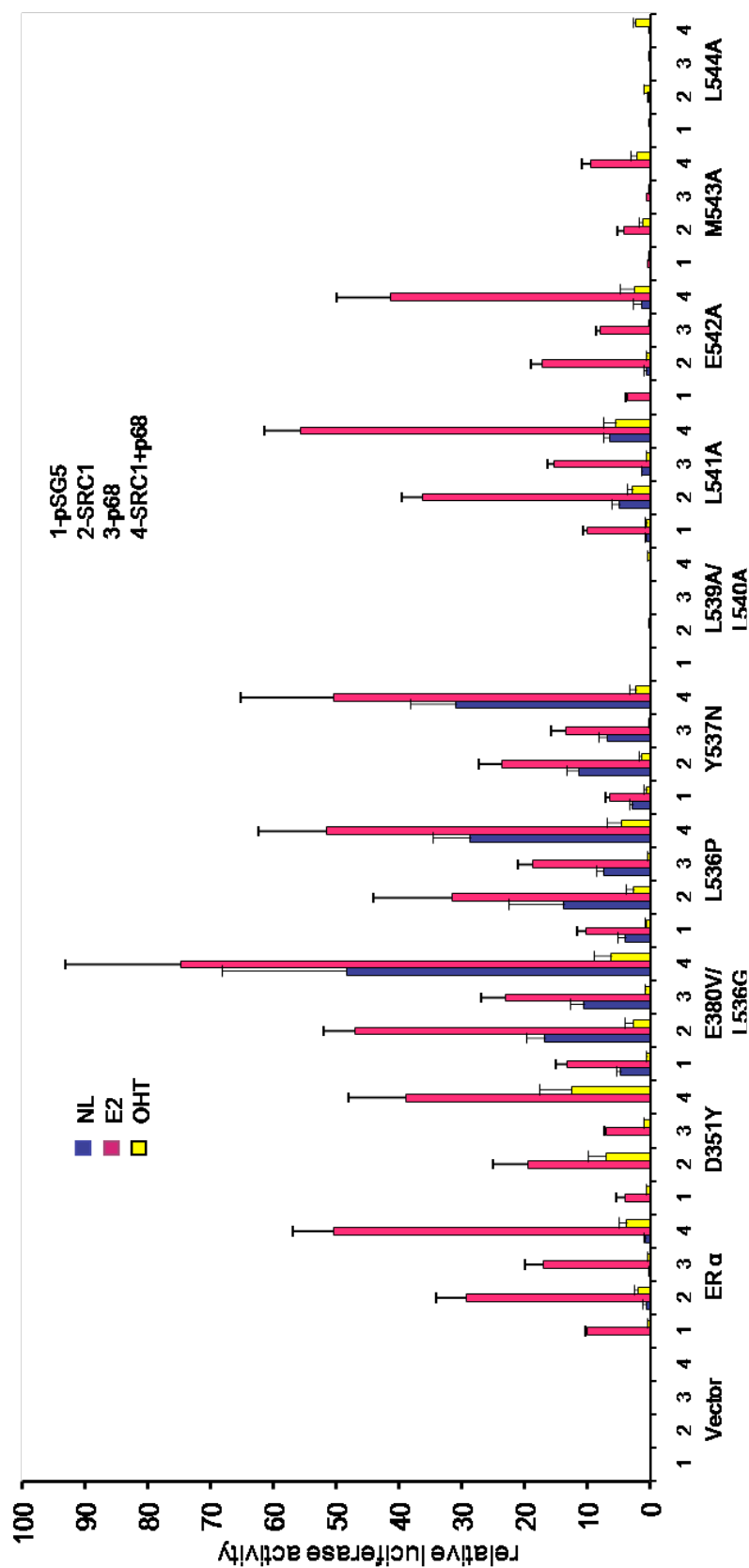


Figure 4.7 p68 modulates both AF1 and AF2 activity of ERα . COS-1 cells, grown in medium lacking estrogen were transfected with 1ng each of expression vectors of ERα and the mutants (as shown), 20ng of SRC1, 250ng of p68, together with 100ng of ERE-luc and RLTK. 17β-estradiol (E2; 10 nM) was added, as shown, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls, Shown are the ERE-luc activities, with the luciferase activity obtained for ERα in the presence of E2 and absence of coactivator being taken as 1. All other activities are shown relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

This is particularly seen for leucine 539, leucine 540, methionine 543 and leucine 544, whose substitution by alanine, blocks coactivator recruitment and prevents ER α activation (Danielian et al., 1992; Danielian et al., 1993; White et al., 1997). Neither SRC-1 nor p68 stimulated the activities of these mutants (Figure 4.7). The mutants E542A or M543A transactivate less efficiently than wild-type ER α and this was reflected in the lower reporter gene activities, although the extent of coactivation by SRC1 and p68 was similar to that for wild-type ER α , again in agreement with the published reports (Hong et al., 1997; Valentine et al., 2000). Conversely, substitution of leucine 536 and tyrosine 537 can lead to ligand-independent coactivator recruitment and ER α activation (Eng et al., 1997; White et al., 1997; Zhao et al., 2003; Zhong and Skafar, 2002). p68 was able to stimulate the activities of these ER α mutants in a ligand-independent manner, as also observed for SRC-1. Together, these results indicate that coactivation of ER α by p68 is similar to coactivation by SRC1, further supporting the idea that p68 modulates the activity of LBD/AF2, as well as that of AF1 of ER α .

4.4 Synergism between p68 and SRC1 in coactivation of ER α

p68 has shown to interact with other coactivators such as the p160 coactivators SRC1 and AIB1, the histone acetyltransferase CBP and with the RNA coactivator, SRA (Watanabe et al., 2001; Rossow & Janknecht, 2003). The interaction of p68 with SRC1 requires activation domain 2 (AD2) of SRC1 (Watanabe et al., 2001). In order to determine whether the cooperativity of p68 and SRC1 in regulating ER α activity is mediated by p68 recruitment by AF1 and SRC1 recruitment by AF2, the ability of p68 and SRC1 to cooperate, was assessed using mutants of SRC1 in which the LBD-interacting motifs and a mutant in which AD2 was deleted. SRC-M123 is a mutant in which the key leucine residues in the three NR boxes (LXXLL) of SRC1 have been substituted by alanine (LXXAA). This mutation impairs the ability of SRC1 to interact with AF2 of ER α and therefore is functionally inactive in coactivator assays (Kalkhoven et al., 1998). As expected, SRC-M123 failed to stimulate ER α activity

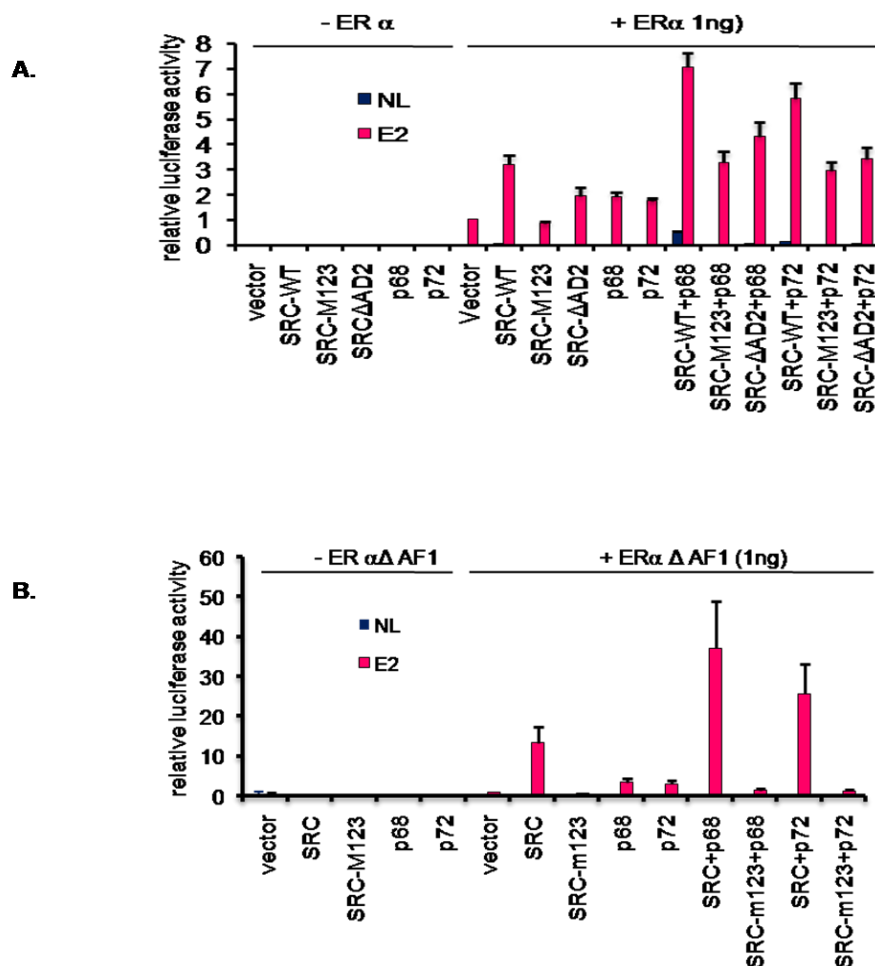


Figure 4.8 Synergistic coactivation of ERα by SRC1 and p68. COS-1 cells were transfected with 1 ng of ERα (A), or ERα ΔAF1 (B), 250ng each of p68 and p72, together with 100ng of ERE-luc and RLTK and 20ng each of SRC1 and the indicated SRC1 mutants. 17β-estradiol (E2; 10 nM) was added, as shown, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls. The reporter gene activity obtained with 1ng of ERα (A), or ERα ΔAF1 (B) in the presence of E2 and absence of coactivator was set at 1 and other data is expressed relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

(Figure 4.8A). On cotransfection with p68, however, the reporter activity observed was higher than that associated with p68 alone, which may result from SRC-M123 recruitment to ER α through interaction with p68. This synergism was not observed, however, for ER α - Δ AF1, (Figure 4.8B), suggesting that the synergistic coactivation of SRC1 and p68 requires an intact AF1. Cooperativity between p68 and SRC1 was maintained when the AD2 domain of SRC1 was deleted (SRC- Δ AD2), although the stimulation of ER α activity was additive, rather than synergistic, suggesting that both p68 and SRC1 were recruited to ER α , but did not interact with each other. Clearly it would be interesting to determine whether the cooperativity would be observed for ER α - Δ AF1.

4.5 Phosphorylation of Serine 118 is not required for p68 interaction with ER α

Phosphorylation of ER α enhances its activity and the key phosphorylation sites are mapped within AF1 of ER α at serines 104, 106 and 118. The mechanisms by which phosphorylation enhances its activity remain unclear and it has been postulated that the increase in ER α activity as a result of AF1 phosphorylation is due to recruitment of specific coactivators (Lannigan, 2003). p68 RNA helicase was originally identified in an *in vitro* screen for proteins that interact with AF1 phosphorylated at S118 (Endoh et al., 1999). However the reported stimulation of ER α activity by p68 was rather weak and has not been confirmed by other groups. The data shown above provide the first confirmation of the original description of p68 as a coactivator for ER α . The results described above also show, however, that p68 appears to be a coactivator for the ER α LBD, in addition to stimulating AF1 activity. In order to examine the importance of S118 phosphorylation for interaction with p68, COS-1 cells were transiently transfected with p68, ER α and ER α in which S118 has been substituted by alanine, or by glutamic acid. A glutamic acid substitution at this position has been shown to stimulate p68 interaction with ER α (Endoh et al., 1999). As shown in figure 4.9, p68 enhanced the activity of ER α by 1.5 fold. There was an increase in the reporter gene activity when S118 was substituted by glutamic acid (S118E), whereas substitution by alanine (S118A) reduced ER α activity, as has previously been

described (Ali et al., 1993). As expected, p68 stimulated the activities of S118E (Figure 4.9), although the stimulation was no greater than that observed for the wild-type ER α . Further, S118A activity was stimulated to a similar extent, indicating that S118 is not important for ER α interaction with p68. Other work from our laboratory has shown that ERK1/2 MAPK can phosphorylate S104 and S106, in addition to S118 (Thomas et al. 2008). It is possible, therefore, that p68 interaction involves these residues, in addition to S118. Substitution of S104 and S106, singly, doubly, or in combination with S118, however, gave the same result, namely a similar level of activation by p68. These results suggest that the serine residues at 104, 106 and 118 are not important for coactivation of ER α by p68.

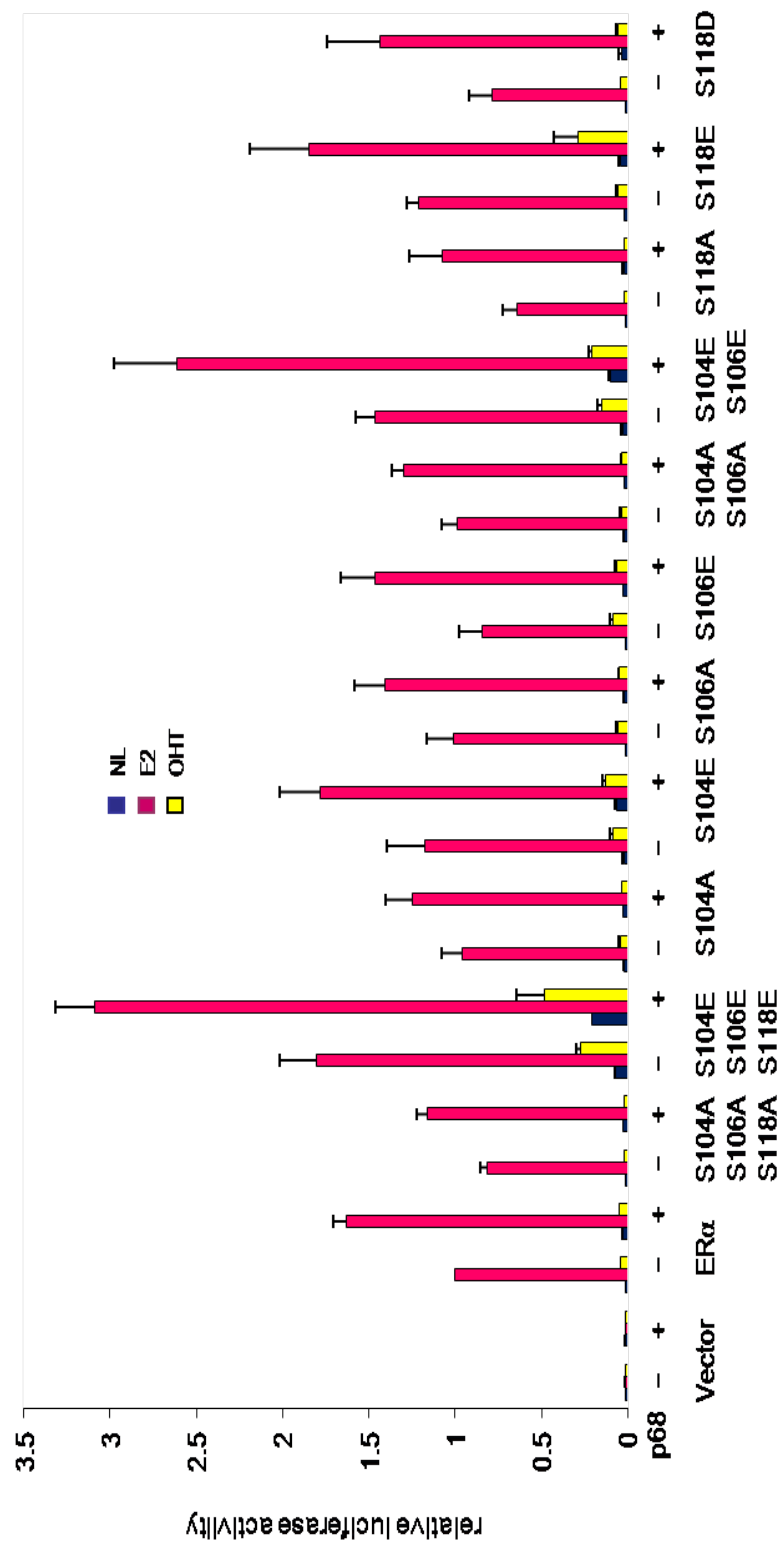


Figure 4.9 Mutation of serine residues of ER α at 104, 106 and 118 does not affect p68 coactivation of ER α . COS-1 cells were transfected with 1ng each of expression vectors of ER α , the phosphorylation mutants of ER α (as shown), 250ng of p68, together with 100ng of ERE-luc and RLTK. Shown are the ERE-luc activities, with the luciferase activity obtained for ER α in the presence of E2 and absence of coactivator being taken as 1. All other activities are shown relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

4.6 The interaction of p68 with ER α does not require the LXXLL-type sequence in p68

Ligand-dependent transactivation by nuclear receptors involves the sequential recruitment and dissociation of a number of coactivators. Many of these proteins are recruited to nuclear receptor LBDs through α -helical motifs containing the sequence, LXXLL where L is Leucine and X is any amino acid (Heery et al, 1997). Scanning the amino acid sequence of p68 RNA helicase shows the presence of the sequence 145-TLSYLL-150 (Figure 4.10). As the previous experiments suggested that the p68 RNA helicase modulates the activity of AF2 and hence may bind to the ER α LBD, we speculated that p68 might interact with ER α through this potential LXXLL motif. Therefore we generated a LXXLL motif mutant, p68 LSYAA, in which the leucine residues were replaced by alanine by site directed mutagenesis and examined the effect of this mutant on transactivation of ER α . However, stimulation of ER α activity by this mutant was similar to that seen with wild-type p68 (Figure 4.11B), suggesting that any interaction between p68 and ER α is independent of this LXXLL motif. The expression of the mutant and the wild type proteins in COS1 cells were confirmed by immunoblotting with p68 antibody (Pab 204) (Figure 4.11C).

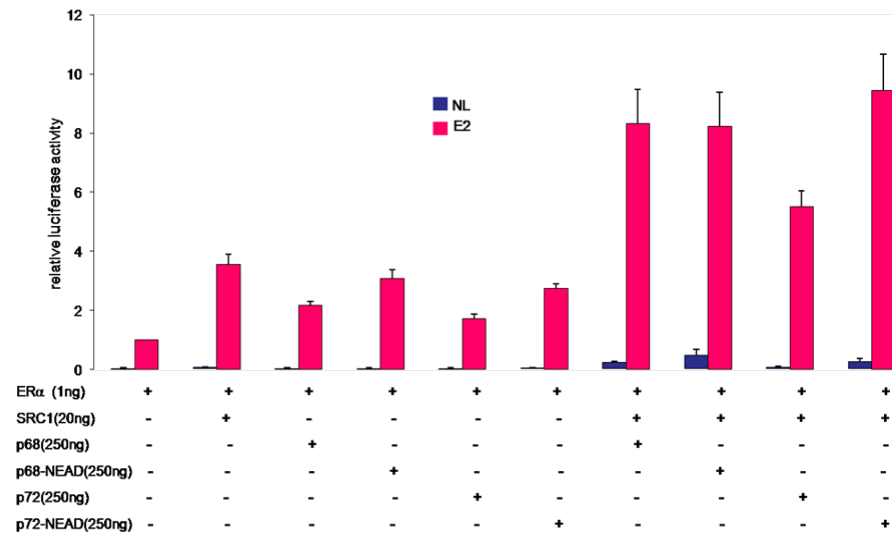
4.7 The helicase activity of p68 is not required for the ER α coactivator activity of p68

DEAD box proteins are characterized by the presence of a central conserved helicase motif. These proteins have multiple functions, not of all which require the helicase activity (Fuller-Pace, 2006). The requirement for the helicase activity for its coactivator functions is variable, being dispensable for coactivator function of p53. The role of helicase activity of p68 for coactivator function of ER α was assessed using a DEAD box mutant in which the first aspartate (amino acid 240) of the DEAD box in p68 was substituted by asparagine to generate p68-NEAD. p68-NEAD and p72-NEAD stimulated ER α activity as well as wild-type p68 and p72. Nor did the NEAD mutants affect synergism of p68 and p72 with SRC-1 (Figure 4.11A). The expression of the helicase mutants were confirmed by immunoblotting using a p68 antibody (Figure 4.11C).

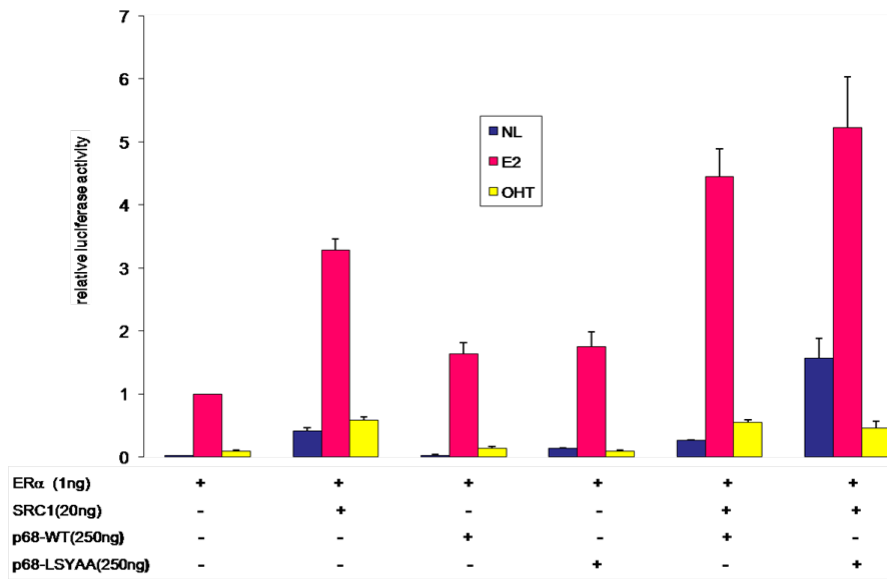
MSGYSSDRDR	GRDRGFGAPR	FGGSRAGPLS	GKKFGNPGEK	LVKKKWNLDE	LPKFEKNFYQ	60
EHPDLARRTA	QEVETVRRSK	EITVRGHNCP	KPVLNFYEAN	FPANVMDVIA	RQNFTEPTAI	120
QAQGWPVALS	GLDMVGVAQT	GSGKT <u>LSYLL</u>	PAIVHINHQP	FLERGDGPIC	LVLAPTRELA	180
QQVQQVAAEY	CRACRLKSTC	IYGGAPKGPQ	IRDLERGVEI	CIATPGRLLD	FLECGKTNLR	240
RTTYLV <u>DEA</u>	<u>DRMLDMGFEP</u>	QIRKIVDQIR	PDRQTLMWSA	TWPKEVRQLA	EDFLKDYIHI	300
NIGALELSAN	HNILQIVDVC	HDVEKDEKLI	RLMEEIMSEK	ENKTIVFVET	KRRCDDELTRK	360
MRRDGWPAMG	IHGDKSQQER	DWVLNEFKHG	KAPILIAITDV	ASRGLDVEDV	KFVINYDYPN	420
SSEDYIHRIG	RTARSTKTGT	AYTFFTPNNI	KQVSDILSVL	REANQAINPK	LLQLVEDRGS	480
GRSRGRGGMK	DDRRDRYSAG	KRGGFNTFRD	RENYDRGYSS	LLKRDFGAKT	QNGVYSAANY	540
TNGSFGSNFV	SAGIQTSFRT	GNPTGTYNQNG	YDSTQQYGSN	VPNMHNGMNQ	QAYAYPATAA	600
APMIGYPMPT	GYS					614

Figure 4.10 Amino acid sequence of human p68 RNA helicase. Position of the LXXLL sequence and the DEAD box are underlined.

A.



B.



C.

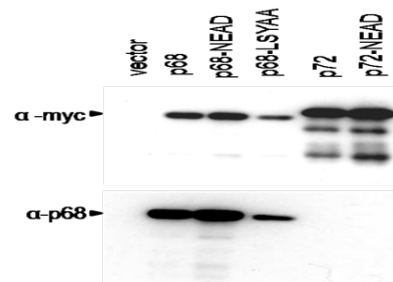


Figure 4.11(A) The LXXLL sequence in p68 not required for coactivation of ER α .

COS-1 cells were transfected with expression vectors of ER α (1ng), 20ng of SRC1, 250 ng each of p68, and LXXLL mutant of p68, p68LSYAA, together with 100ng of ERE-luc and RLTK. 17 β -estradiol (E2; 10 nM) was added, as shown, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls. Shown are the ERE-luc activities, with the luciferase activity obtained for ER α in the presence of E2 and absence of coactivator being taken as 1. All other activities are shown relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM). **(B) Helicase activity is not required for the stimulation of ER α activity by p68 and p72.**

COS-1 cells were transfected with expression vectors of ER α (1ng), 20ng of SRC1, 250 ng each of p68, p72, helicase mutants of p68/72, p68NEAD and p72NEAD, together with 100ng of ERE-luc and RLTK. 17 β -estradiol (E2; 10 nM) and 100nM of tamoxifen (OHT) was added, as shown, with an equal volume of vehicle (ethanol), being added to the no ligand (NL) controls. Shown are the ERE-luc activities, with the luciferase activity obtained for ER α in the presence of E2 and absence of coactivator being taken as 1. All other activities are shown relative to this. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM). **(C)**

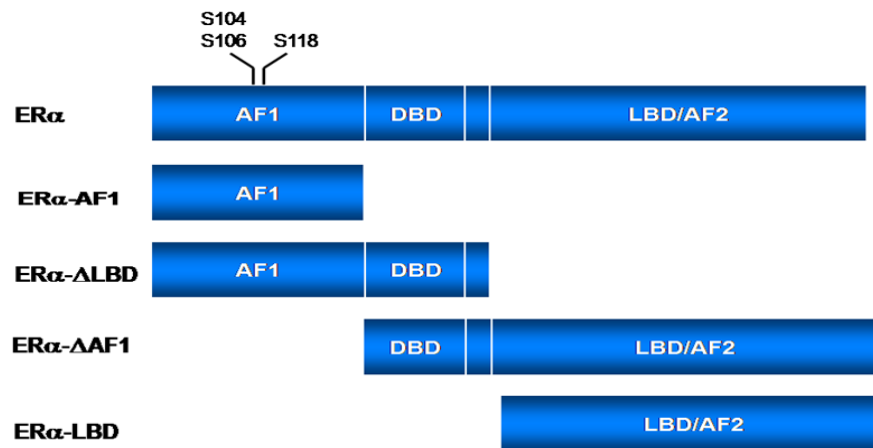
Immunoblotting showing the expression of p68/72 and the mutants. Cells were transfected with 500ng each of expression vectors for myc tagged p68, p68NEAD, p68LSYAA, p72 and p72NEAD. Lysates were immunoblotted with anti-cmyc and anti-p68 antibody.

These findings are in accordance with the findings of Endoh et al who showed that a mutation in the ATP binding domain (p68 K144R), which abolishes p68 helicase activity stimulated ER α activity as well as wild-type p68 (Endoh et al., 1999).

4.8 Interaction of p68 and ER α *in vitro*

The reported studies of p68 and ER α have demonstrated a direct interaction between p68 and the AF1 region of ER α , with the interaction being regulated by phosphorylation at S118. As the reporter gene studies described above provide clear evidence that p68 influences AF2 activity in addition to its action on AF1, GST pull down assays were performed in order to determine whether regions of ER α other than AF1 interact with p68. GST fusion proteins encoding full-length ER α , or small regions of ER α (Figure 4.12A) were expressed in *E.coli* and the expression was confirmed by immunoblotting using GST antibody (4.12B). Purified GST fusion proteins were incubated with *in vitro* translated ³⁵S-methionine-labelled p68. p68 interacted with ER α , ER α - Δ LBD and ER α - Δ AF1, but there was little interaction of p68 with AF1 or LBD, suggesting that the interaction of p68 may involve the ER α DNA binding domain. The interaction was similar in the absence or presence of E2, and the antagonists OHT and ICI, demonstrating that the interaction of p68 with ER α is ligand-independent (Figure 4.13A). Similar results were obtained when the pull downs were carried out using GST-p68 (Figure 4.13B), whereas the interaction of the ER α LBD with SRC1 showed estrogen regulation, indicating that the lack of ligand regulation for the interaction of p68 and ER α is not due to an artefact of the *in vitro* binding assay. As these results suggest an interaction of p68 with the ER α DBD, we tested whether p68 interacts with ER β , since ER α and ER β show considerable divergence in AF1, whereas

A.



B.

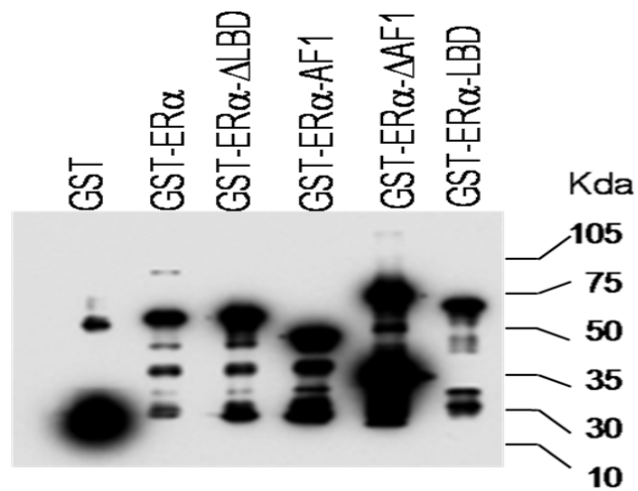
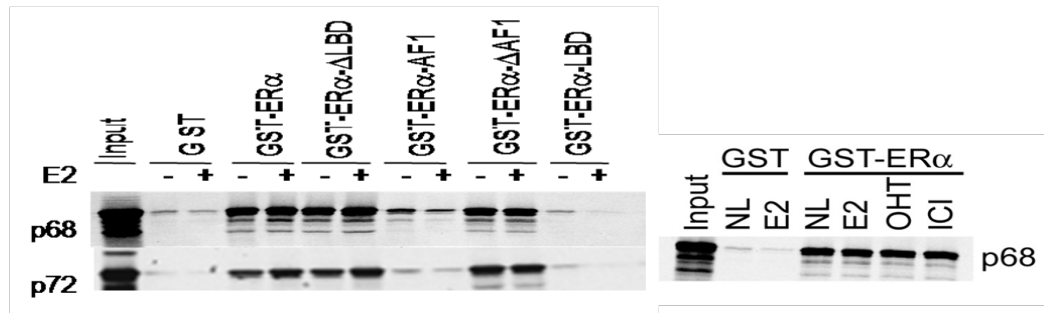
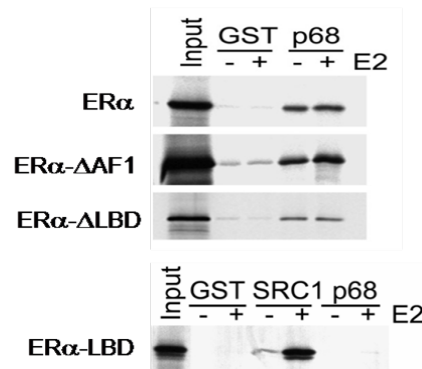


Figure 4.12 (A) Schematic representation of ER α and the deletion mutants used in the GST pull down assays (B) Purified GST-ER α fusion proteins were immunoblotted using an antibody for GST. The positions of the molecular weight markers are depicted on the right.

A.



B.



C.

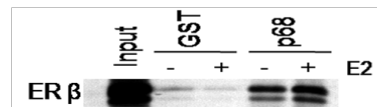


Figure 4.13 p68 interacts with ERα *in vitro*. (A) GST-ERα proteins were expressed in *E.Coli* and purified on glutathione beads. Purified proteins were incubated with *in vitro* translated, ³⁵S-methionine-labelled p68 in the presence or absence of 17β-estradiol (E2; 100 nM), 4-hydroxytamoxifen (OHT; 100 nM) or ICI 182, 780 (ICI; 100 nM). An equal volume of the vehicle (ethanol) was added to the no ligand controls. (B-C) *In vitro* translated ³⁵S-labelled ERα or ERα deletion mutants, (B) or *in vitro* translated human ERβ (C) were used in GST pulldown assays using GST-p68, in the presence or absence of 100 nM E2. Input refers to 10% of the *in vitro* translation reaction used in the pull down.

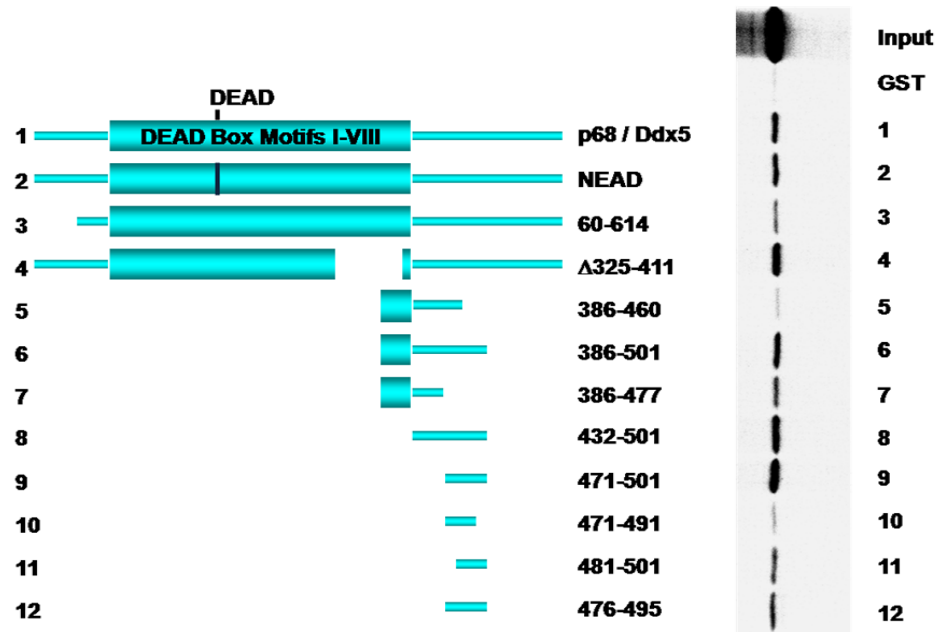
the DBD is 97% identical between ER α and ER β (Mosselman et al., 1996). Indeed, ER β also bound p68, both in the presence and absence of estrogen (Figure 4.13C). These findings show that p68 interacts with ER α *in vitro* and suggest that the interaction is mediated by the ER α DBD, although there was evidence for weak interaction of p68 with the AF1 region.

4.9 Mapping the region(S) of p68 that mediate interaction with ER α *in vitro*

To determine the sequences in p68 required for interaction with ER α , a series of GST fusion proteins containing different regions of p68 (Figure 4.14 A) were expressed in *E. coli* and the expression was confirmed by immunoblotting the purified extracts with a GST antibody (Figure 4.14 B). Purified proteins were assayed for interaction with *in vitro* translated ³⁵S-methionine-labelled ER α . As shown above, ER α interacted with full-length p68 (Figure 4.14A, construct 1). Substitution of the DEAD box motif by the sequence NEAD did not prevent interaction with ER α . Deletion of amino acids 1-59 appeared to reduce interaction of ER α with p68, suggesting that this region plays a role in the interaction. However, p68 amino acids 386-501 were sufficient for interaction with ER α , whereas amino acids 386-460 did not interact strongly with ER α , suggesting that amino acids 460-501 are important for interaction of ER α with p68. This was confirmed by the finding that amino acids 432-501 bind ER α strongly, as do amino acids 471-501. Further deletions did not allow finer mapping of the region of interaction, suggesting that amino acids 471-501 may represent the smallest region of p68 required for interaction with ER α .

The results shown here are contradictory to the results reported by Endoh et al who mapped the p68 interacting region in AF1 of ER α , to amino acids 56-127 and the interaction domain in p68, to amino acids 300 to 400. In their interaction assays deletion of amino acids 200-400, 200-614 and 1-400 resulted in loss of binding while in our assays the binding was seen with these deletions. Further our assays show that p68 binds to the ER α AF1 region and to the ER α DBD. The reasons for the disparity in the findings presented here and those of Endoh et al. may be due to the fact that the GST

A.



B.

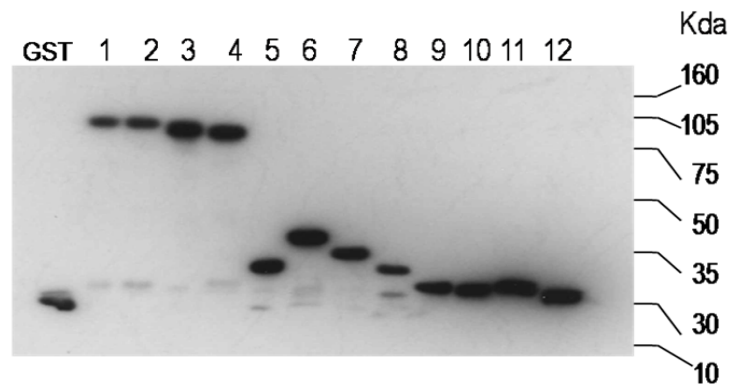


Figure 4.14 Detailed mapping of the regions of p68 required for interaction with ER *in vitro* (A) Schematic representation of GST-p68 fusion proteins p used in the GST pull down assays. The right hand panel shows the results of the pull down assay, using *in vitro* translated ³⁵S-labelled ERα. 10% of the *in vitro* translated ERα used in the pull downs is shown as input. (B) Immunoblotting showing the expression of GST fusion proteins with p68 and the deletion constructs. Positions of the molecular size markers are shown on the right.

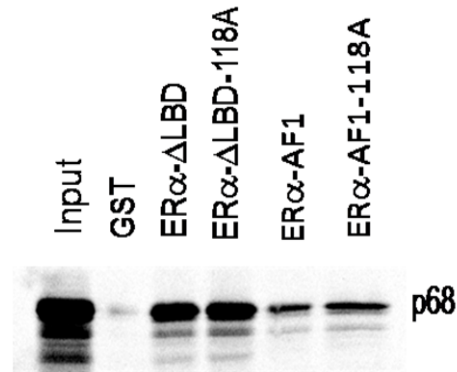
pull down assays they described were carried out with the AF1 region of ER α , and not with full-length ER α or ER α containing the DBD, although as suggested by the fact that deletion of amino acids 1-59 reduce interaction between ER α and p68, support the possibility that multiple regions of p68 can interact with ER α .

4.10 The *in vitro* interaction of p68 and ER α does not require phosphorylation at Serine 118

ER α is phosphorylated at S118 by MAP kinase *in vitro* and *in vivo* (Chen et al., 2002; Kato et al., 1995; Arnold et al., 1995; Bunone et al., 1996), as are S104 and S106 (Thomas et al., 2008). The results of the reporter gene assays indicate that S104, S106 and S118 are not required for co-activation by p68, suggesting that phosphorylation of these residues is not involved in recruitment of p68 by ER α . In agreement with these findings, in GST pull down assays, mutation of S118 to alanine did not affect interaction of the AF1 or the AF1-DBD regions of ER α , with p68 (Figure 4.15A). In the reciprocal pull down assay, mutation of S118, or all three MAPK phosphorylation sites in AF1 (S104, S106, S118) did not influence interaction of full-length ER α with p68 (Figure 4.15 B). It is possible that the lack of requirement for these residues is due to their mutation in these studies, so recombinant ER α was phosphorylated by ERK2 MAPK *in vitro* and subjected to pull down assays with p68, in order to investigate whether direct phosphorylation at these sites influences p68 recruitment *in vitro*. Purified GST-ER α and GST-ER α -AF1 were phosphorylated *in vitro* by incubation with purified ERK2 MAPK, followed by incubation of the GST proteins with *in vitro* translated p68. Immunoblotting with an antibody specific for ER α phosphorylated at S118 showed ER α phosphorylation occurred following incubation with ERK2 MAPK (Figure 4.16). However, there was no difference in p68 interaction with ER α or with the AF1 region following phosphorylation, compared with the unphosphorylated ER α .

Taken together, the GST pull down assays confirm the reporter gene assay findings, showing that phosphorylation of ER α does not influence the interaction of p68 with ER α *in vitro*.

A.



B.

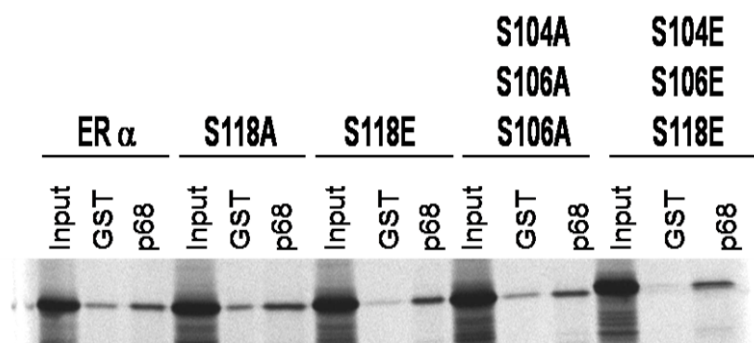


Figure 4.15 Mutation of phosphorylation sites of ER α at serine 104, 106 and 118 does not affect the interaction of ER α with p68. (A) GST fusion proteins with the ER α lacking the LBD (ER α - Δ LBD), ER α - Δ LBD in which S118 was substituted by alanine (ER α - Δ LBD-118A), the ER α AF1 region (ER α -AF1) and ER α -AF1-118A were used to pull down *in vitro* translated p68. (B) GST-p68 was used in pull down assays using ER α , or mutants of ER α in which the indicated serine residues were substituted by alanine or glutamic acid.

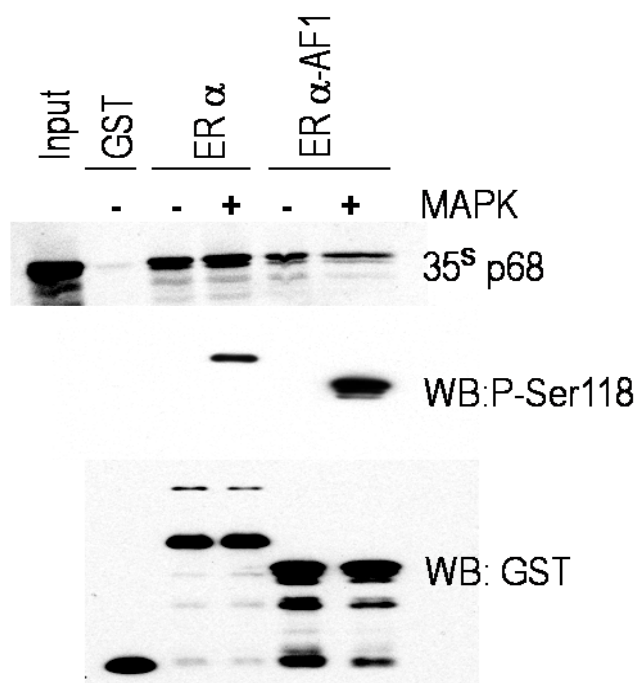


Figure 4.16 Phosphorylation of ER α by MAPK does not influence p68 binding *in vitro*. GST-ER α and GST-AF1 purified following expression in *E.coli* were phosphorylated by incubating with purified MAPK. The phosphorylated proteins were incubated with *in vitro* translated ^{35}S -methionine-labelled p68. The autoradiograph of p68 interaction with ER α (top panel) is shown. Immunoblotting using antibodies to GST (bottom panel) show the amounts of GST-ER α and GST-AF1 present in the pull downs, whilst immunoblotting using an antibody specific for ER α phosphorylated at S118 is also shown (middle panel).

4.11 Co-immunoprecipitations show that p68, p72 and ER α interact *in vivo*

Co-immunoprecipitations were carried out in order to show direct interaction between ER α and p68 *in vivo*. Whole cell extracts prepared from COS-1 cells transiently transfected with ER α and p68 were immunoprecipitated using antibodies for ER α . Immunoprecipitated proteins were analysed by Western blotting with antibodies for p68 and ER α (Figure 4.17A). Control immunoprecipitations were carried out using mouse immunoglobulins (IgG). As shown in figure 4.17A, p68 co-immunoprecipitated with ER α , indicating that p68 interacts with ER α *in vivo*. The interaction of p68 with ER α was not influenced by estrogen, in agreement with the GST pull down results, indicating that the interaction of p68 and ER α is ligand-independent. Similar results were obtained for p72 when whole cell lysates of COS-1 cells transfected with ER α and p72 were immunoprecipitated using the ER α antibody (Figure 4.17B). As for p68, the interaction with ER α was independent of ligand.

The above immunoprecipitation was carried out following over-expression of ER α and p68 in COS-1 cells. In order to examine whether endogenous ER α and p68 interact, nuclear extracts prepared from MCF-7 cells treated with E2 for 24 hours were immunoprecipitated using an ER α antibody. As observed in the over-expression, p68 co-immunoprecipitated with ER α , and as seen for the over-expression, the interaction was not stimulated by ligand (Figure 4.17C). Together, these results indicate that ER α interacts with p68 and p72 *in vivo*, confirming the *in vitro* findings presented earlier in this chapter.

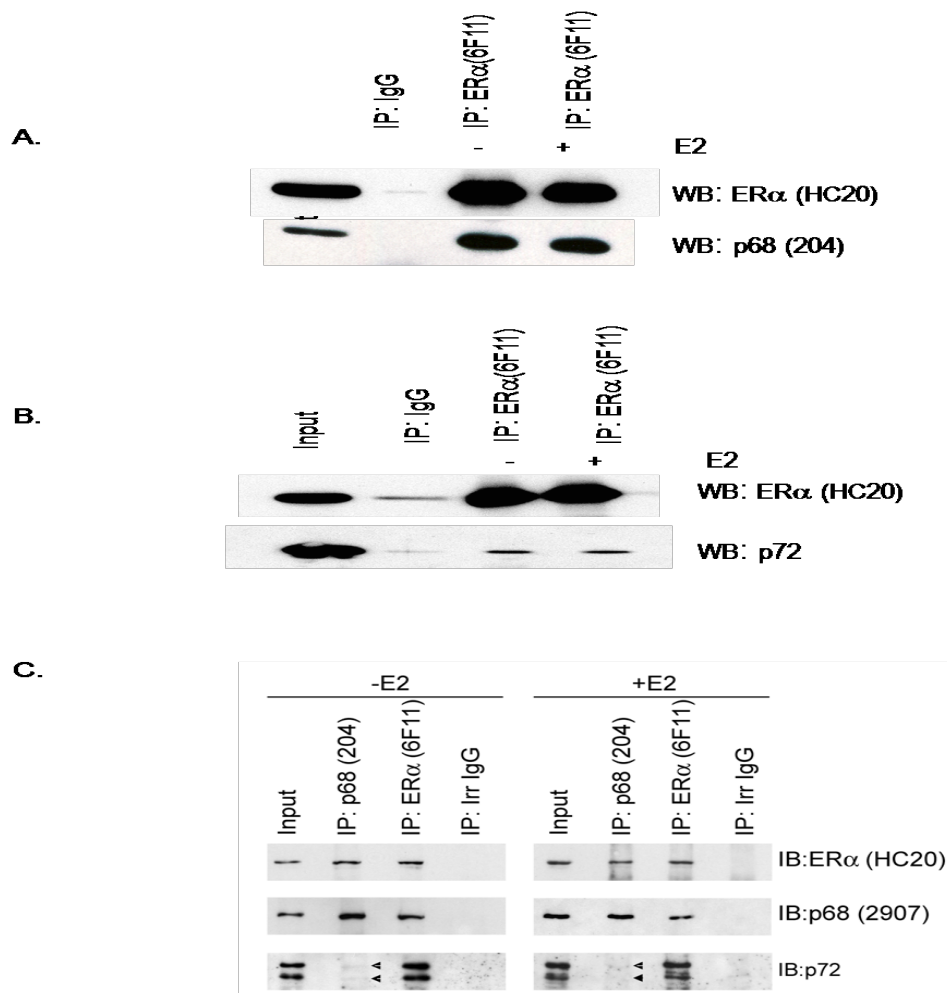


Figure 4.17 ERα interacts with p68 and p72 *in vivo*. (A) Whole cell extracts prepared from COS-1 cells transiently transfected with ERα and p68 were immunoprecipitated using an ERα antibody or using mouse immunoglobulins (IgG) as a negative control. Shown are immunoblots for ERα and p68. E2 (10 nM) was added 30 mins before cell harvesting. An equal volume of ethanol was added to the vehicle control. (B) Immunoprecipitations were performed as in (A), following transfection of ERα and p72. (C) Nuclear extracts prepared from MCF-7 cells treated with 10 nM E2 for 24 hours were immunoprecipitated with an ERα antibody, followed by immunoblotting for p68 and ERα, as in (A). The input lane represents 10% of the lysate used for the immunoprecipitation.

4.12 Effect of over-expression of p68 and 72 in MCF7 cells

The results described above show that p68 and p72 can both act as co-activators for ER α in reporter gene assays. In order to address whether p68 and p72 also stimulate ER α activity in breast cancer cells, MCF-7-derived cells were transiently transfected with c-myc-tagged p68 and/or p72, in order to determine if their over-expression can affect the expression of endogenous estrogen-regulated genes. Transfection of p68 resulted in increased levels of the estrogen-responsive pS2 and cathepsin D (CTD) proteins (Figure 4.18), in agreement with the reporter gene data. Interestingly, however, p72 transfection did not increase pS2 or CTD levels, nor was an increase observed when p68 and p72 were co-transfected. Both p68 and p72 transfections worked, as seen with immunoblotting with antibodies to the c-myc tag.

4.13 p72 knock down inhibits the transactivation of ER α while p68 knock down has no effect.

In the over expression studies, in COS-1 cells both p68 and p72 enhanced the transcriptional activity of ER α . We wished to examine the effects of endogenous p68 and p72 on the transcriptional activity of endogenous ER α in MCF -7 cells by RNA interference. For this we chose two MCF-7 derived cell lines, MELN and MVLN, stably transfected with estrogen responsive luciferase reporter genes (Demirpence et al., 1993; Balaguer et al., 2001). The advantage of these cell lines is that the estrogen induced transcriptional activity can be measured directly without the need for transfection of exogenous estrogen responsive reporter gene. The cells were transfected with the siRNA for p68, p72 and control siRNA, treated with estrogen for 4 and 8 hours and assayed for luciferase activity. The efficiency of knock down of both p68 and p72 was confirmed by immunoblotting of lysates from cells transfected with siRNA (Figure 4.19B). p68 knock down had no effect on transcriptional activity of ER α while p72 knock-down inhibited the transcriptional activity by nearly 40% compared to the untransfected control (figure 4.19 A and B).

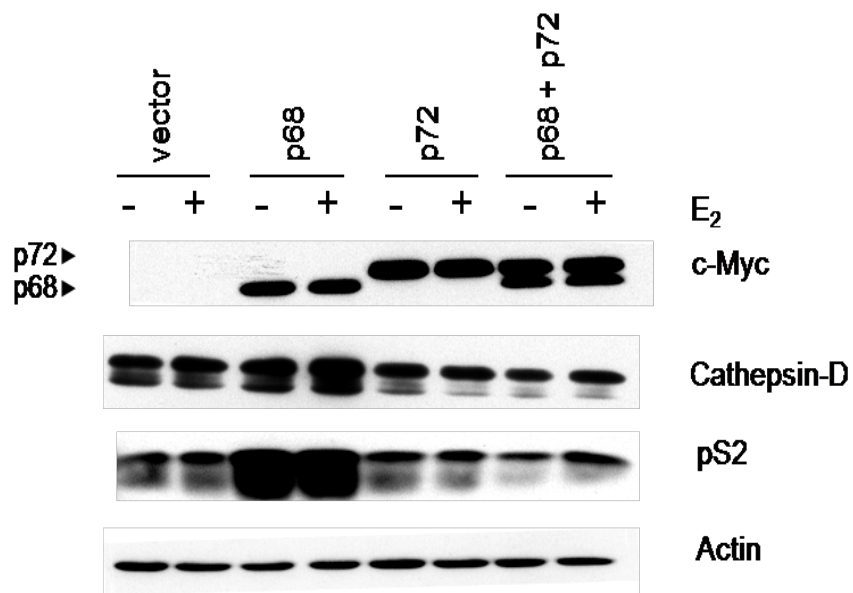


Figure 4.18 Transfection of MCF-7 cells with p68 increases the levels of pS2 and cathepsin D. MCF-7 cells were grown in double stripped serum for 3 days and transfected in 6-well plates with vector, c-myc-tagged p68 and c-myc-p72, singly or in combination. Following transfection the cells were treated with 10nM E₂ for 8hrs, prior to preparation of whole cell lysates. Immunoblotting for c-myc, cathepsin D and pS2 are shown. Immunoblotting for β -actin served as a control for protein loading.

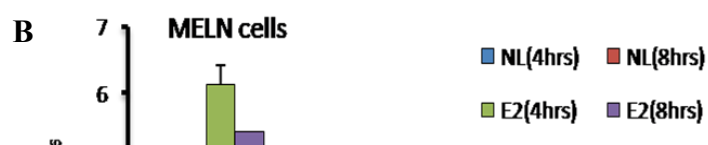


Figure 4.19 (A) p72 knock down inhibits the transactivation of ER α . MELN and MVLN cells were grown in double stripped serum for 3 days and transfected in 24 well plates with indicated siRNA. The cells were, treated with 10nM of E₂ for 4/8hrs and harvested 48 hrs post transfection and assayed for luciferase activity. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM). The bottom panel shows immunoblotting of extracts used in the experiment for p68 and p72 and β -actin as control for protein loading.

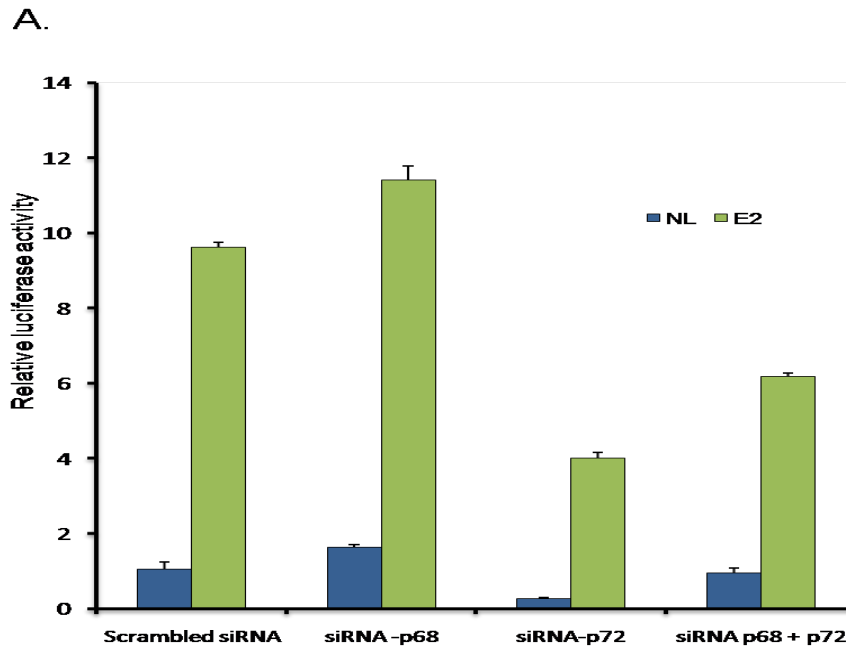


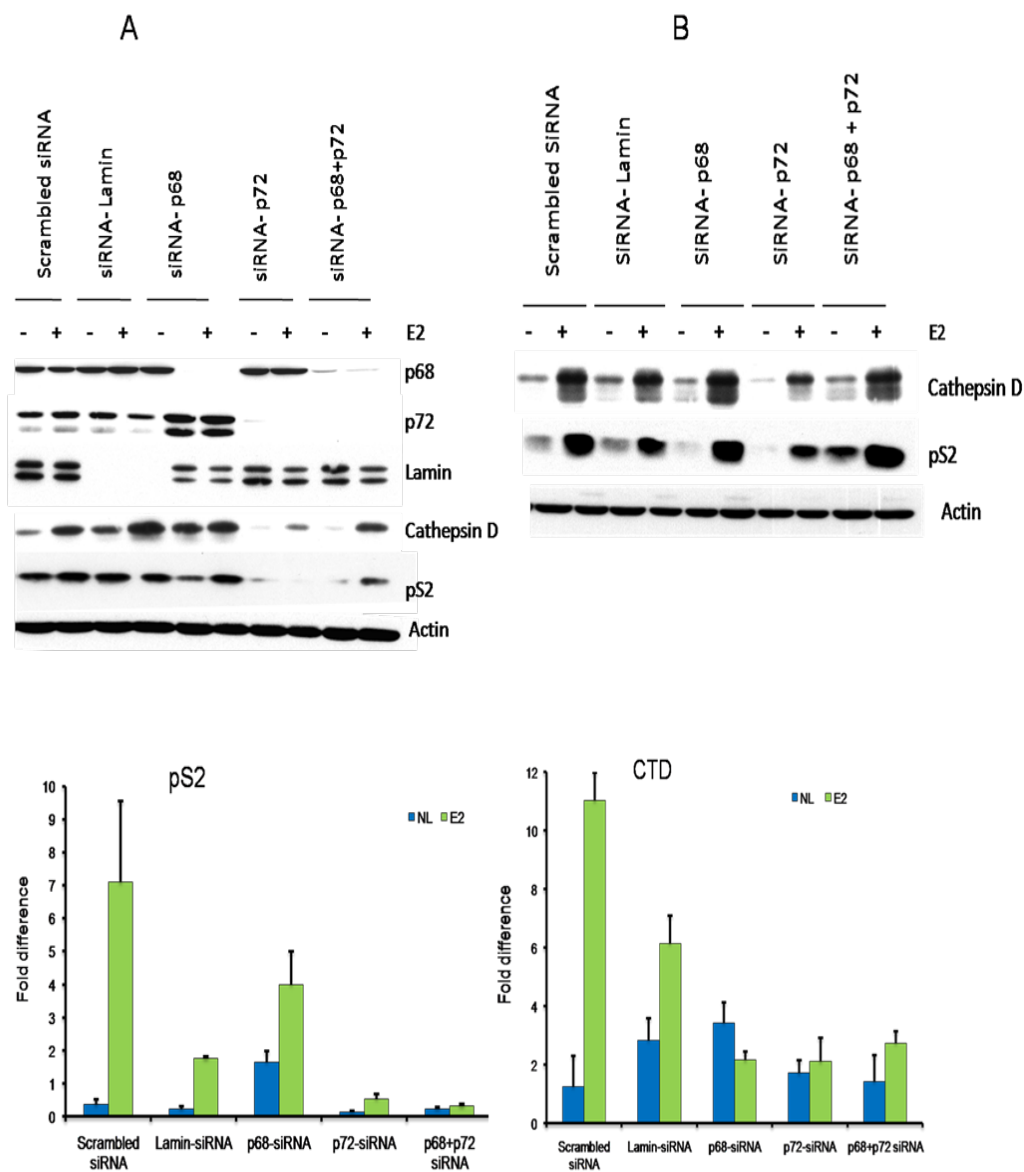
Figure 4.19 (B) p72 knock down inhibits the transactivation of ER α . MCF-7 cells were grown in double stripped serum for 3 days and transfected in 24 well plates with indicated siRNA. The following day they were transfected with 100ng each of the estrogen responsive reporter gene and renilla reporter gene used as control for transfection efficiency. The cells were, treated with 10nM of E₂ for 24 hrs, harvested 48 hrs post transfection and assayed for luciferase activity. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM).

The lack of any effect on the ER transactivation with knock down, might be due to compensation by the over expression of p72 which is also a coactivator of ER α , in p68 depleted cells. Surprisingly, the double knock down marginally affected the transcriptional activity. Perhaps this was a result of rescue by another yet unknown helicase or another unknown factor. The control scrambled siRNA consistently gave higher reporter activity.

4.14 RNA interference-mediated knock-down of p68 and p72 RNA helicases in MCF-7 cells suggests that p72 is important for ER α function in breast cancer cells

The above findings show that p68 and p72 RNA helicases can function as co-activators for ER α , although in MCF-7 cells, overexpression of p68, but not of p72 stimulated the expression of estrogen-responsive genes. RNA interference mediated gene knockdown was carried out in order to more fully determine the importance of p68 and p72 for the regulation of gene expression by ER α . MCF-7 cells were transfected with double-stranded RNA (dsRNA) oligonucleotides for p68 and p72 that have previously been described (Bates et al., 2005; Jalal et al., 2007). Transfection with dsRNA for Lamin A/C was used, together with a scrambled dsRNA, as positive and negative controls for the RNA interference, respectively. Transfection with the lamin A/C siRNA resulted in down regulation of Lamin A/C protein. Knockdown of p68 was also successful, with little p68 protein being detectable (Figure 4.20). P68 knockdown did not, however, result in reduction in the expression of CTD or pS2, and there was an increase in p72 levels. The increase in p72 levels following siRNA for p68 has previously been observed, with no effect on p68 levels following p72 knockdown (Bates et al., 2005; Jalal et al., 2007). However, p72 knockdown resulted in reduced levels of CTD and pS2, with a similar down regulation being observed when both p68 and p72 were simultaneously knocked down. Another interesting finding was that in the double knock down, the down regulation of pS2 and CTD was not as severe as observed for p72 alone, despite the marked depletion of both p68 and p72 in these cells. This suggests that there may be a compensatory mechanism involved that rescues the cells from the effects of depleting the cells of both p68 and p72.

To confirm these findings at the mRNA level, RNAi was performed in MCF-7 cells and total RNA was prepared from cells treated with estrogen for 4 hours. The levels of mRNA of pS2 and CTD were determined by quantitative real time RT-PCR and were expressed relative to the levels of reference gene GAPDH. As observed above, p72 knockdown inhibited pS2 and CTD expression (Figure 4.20), as did the double knockdown. There was again significant reduction in the induction of pS2 with p72 knock down while p68 knock down had no effect. However, similar degree of inhibition was not seen with p72 knock down in the induction of cathepsin D (Figure 4.20).



4.15 p72 knock down affects the growth of MCF-7 cells

The ER α is a key player in estrogen-dependant growth and progression of breast cancer. The knock down experiments consistently showed that p72 plays an important role in modulating ER transcriptional activity and on the expression of estrogen regulated genes. We investigated the effect of depleting p68 and p72 on the estrogen dependent growth of the breast cancer cells.

The MCF-7 cells were transfected with siRNA for p68 and p72, singly and in combination and treated with or without estrogen. Growth was assayed by SRB colorimetric assay over 6 days. p68 knock down had no effect on the growth but p72 depletion resulted in inhibition of growth compared to the untransfected cells (Figure 4.21). The lack of effect on growth in p68 depleted cells could be due to the over expression of the p72 in these cells which might compensate for p68. Depletion of both p68 and p72 resulted in inhibition of growth but not more than that seen with p72 depletion (Figure 4.21). This might be due to perhaps another unknown helicase or unknown factor rescuing the cells from the effects of double knock-down.

Overall the results of the p68 and p72 knock down on growth mirrors the pattern shown with the siRNA experiments on estrogen regulated genes and ER transactivation.

Our data somewhat contradict the results reported by Jalal et al who reported that the growth inhibition was seen only with the double knock down (Jalal et al., 2007). This is probably due to the fact that in the double knock down they titrated the siRNA oligos to prevent the over expression of p72 with p68 knock down.

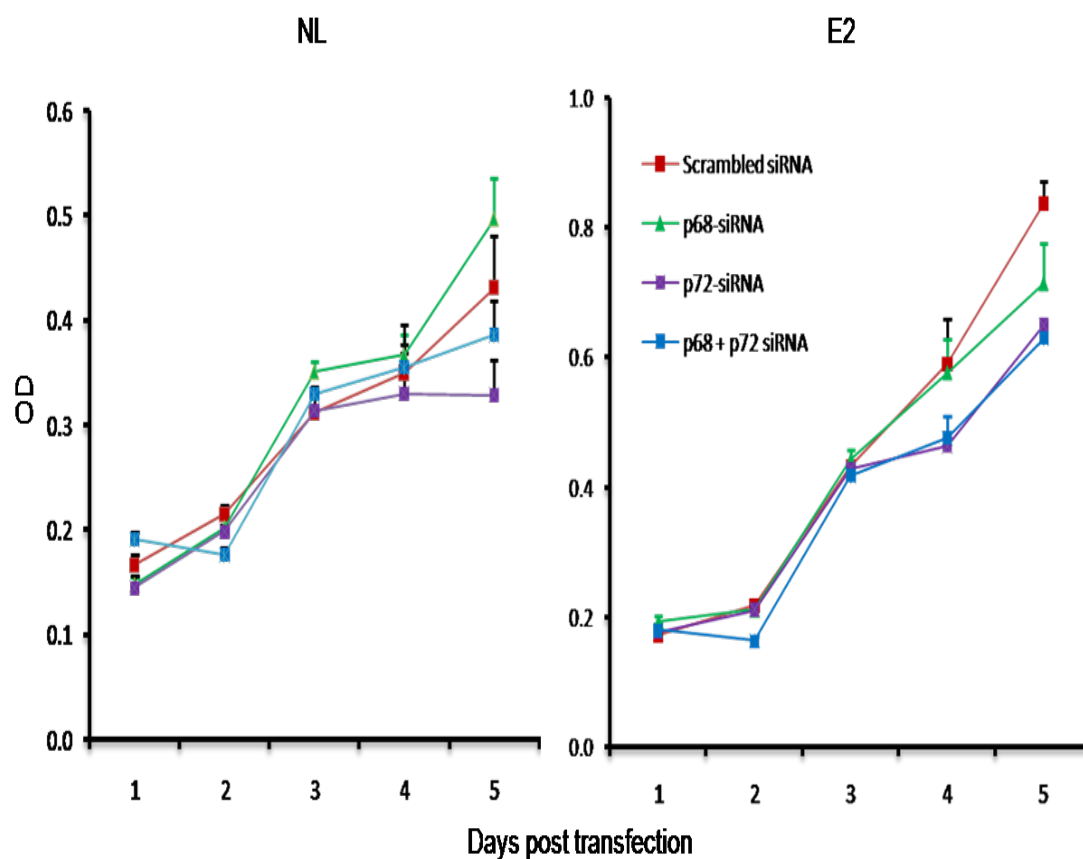


Figure 4.21 Effect of p68 and p72 knock down on the growth of MCF 7 cells. MCF 7 cells were transfected with the indicated siRNAs and treated with or without estrogen (NL) and growth was assayed over 5 days following transfection by SRB colorimetric assay. The results show the means of three independent experiments, with the error bars depicting the standard errors of the mean (SEM)

5. DISCUSSION

5.1 Identification and characterisation of AF1 interacting proteins

Transcriptional activation by ER α is mediated by two activation domains, AF1, whose activity is potentiated by phosphorylation at specific residues, and AF-2 whose activity requires ligand binding. Although the mechanisms by which the LBD/AF2 recruits transcriptional coactivator protein complexes to regulate gene expression are well-defined, the mechanisms by which phosphorylation of AF1 stimulate ER α activity are unclear. Phosphorylation of ER α can result in its activation in both ligand dependant and ligand-independent manner and the key phosphorylation sites have been mapped to transactivation function AF1. Determination of the mechanisms by which AF1 phosphorylation stimulates ER α activity may be particularly pertinent as the phosphorylation mediated cross talk between ER α and growth factor induced signalling pathways has been implicated in endocrine resistance (Masserweh and Schiff, 2006; Clarke et al, 2003; Nicholson et al ., 2004; Ring and Dowsett, 2004; Shou et al., 2004). Though the exact mechanism by which phosphorylation stimulates AF1 activity is unclear, it is likely that phosphorylation of AF1 facilitates the recruitment of transcriptional coactivators that mediate the transcriptional activity of AF1. Among the known coactivators, CBP/p300 and SRC1 are known to interact with AF1 but phosphorylation of ER α is not a requirement for these interactions. Therefore it was thought that there must be ‘unknown’ coactivators that mediate stimulatory AF1 activity of ER α upon phosphorylation (Kato, 1999). Three potential co-regulator proteins recruited preferentially by ER α phosphorylated at serine 118 (S118) have been described to date. The first, p68 RNA helicase has been shown to preferentially interact with AF1 phosphorylated at serine 118 (Endoh et al., 1999), but the stimulation of ER α activity by p68 is relatively weak and cell specific and it is not strictly S118 dependent. The second, stromelysin-1 platelet-derived growth factor responsive element-binding protein (SPBP) acts not as a coactivator, but as a corepressor for ER α (Gburcik et al., 2005). Finally, another protein that interacts preferentially with ER α phosphorylated at S118 is the spliceosome component splicing factor (SF)3a p120, which stimulates alternative splicing by ER α (Masuhiro et al.,

2005). Collectively, these data argue for additional coactivator proteins that facilitate transcription activation by ER α phosphorylated at S118. The aim of this work was to identify and characterise AF1 interacting proteins in order to understand the role of phosphorylation in ER transactivation, through recruitment of activators. Two approaches were used to identify and characterise coactivators recruited preferentially by ER α phosphorylated at sites in AF1. In the first approach, I screened a breast cDNA expression library using the bacterial two-hybrid system and in the second, p68 and p72 RNA helicases, two known coregulators, previously reported to interact with AF1 of ER α , were characterised as coactivators of ER α .

5.2 Bacterial two-hybrid for identification of AF1 interacting proteins

The functional reconstitution of a transcription factor through protein-protein interactions between two proteins of interest allows functional studies on the nature of the interaction. Yeast two hybrid based on transcriptional activation of a reporter gene has been a powerful tool for studying protein-protein interactions and mapping the protein interaction network. Bacterial two hybrid system which is analogous in principle to the yeast two hybrid system was used to screen a human breast cDNA expression library (Dove et al., 1997). The bacterial two hybrid was used in preference to the yeast two hybrid because of a number of reported advantages with this system including faster screening of cDNA libraries and lower false positive results. Additionally, ER α and ER α -AF1 are transcriptionally active in yeast which would cause reporter gene activation and therefore it will be problematic to use a yeast two hybrid approach. The bacterial two hybrid system has been compared to the yeast two hybrid in screening the cDNA libraries and the results are comparable (Saito et al., 2004; Serebriiskii et al., 2005). The efficacy of this system was initially established to detect the interaction between ER α and the known coactivators. The system was able to readily show the ligand dependant positive interaction between ER LBD and SRC1. No interaction was demonstrated when SRC1 was cotransfected with an ER LBD mutant with alanine substitution at residues 539 and 540 which abolish coactivator interaction. There was also no interaction when pure estrogen antagonist ICI 182, 780 was added to the system. An AF1 construct encoding glutamic acid in place of serine 104,106 and

118 was used as bait since these substitutions may mimic phosphorylation (Ali et al., 1993). The library screen was fraught with problems. All positives obtained from screening of a breast cDNA expression library turned out, upon careful characterisation to be false positives. The reasons for the large number of false positives are unclear, but are presumably due to leaky expression of the selective marker genes, perhaps due to the considerable mutability potential of bacterial cells. Indeed, this problem has been recognised by the manufacturers, Stratagene Ltd., who attempted to improve the Bacteriomatch system resulting in the generation of Bacteriomatch II, which was used here. Another problem appears to be the high percentage of mitochondrial sequences and immunoglobulin gene sequences represented in this library, suggestive of contamination with genomic DNA. One possible approach for the latter problem would be to construct a cDNA expression library. However, given the problems with selection, I decided that whilst improving the cDNA expression library, this may not resolve problems with selection. The likelihood of high false positive results still remains and the screening process would still require considerable time. I have therefore decided not to pursue this.

5.3 p6 modulates the activity of both AF1 and AF2 of ER α

In the original report by Endoh et al, p68 was shown to be an AF1 specific coactivator of ER α . p72 has similar properties as a co activator of ER α and both proteins potentiate ER activity in a synergistic manner with SRC1, in reporter gene assays. Furthermore, helicase activity was dispensable for the coactivator function of both proteins (Endoh et al., 1999; Watanabe et al., 2001). In agreement with these reports, we confirmed that the p68/72 stimulate the activity of ER α and shows synergistic enhancement of ER α activity with SRC1. We also confirmed that the helicase activity is required, neither for the transactivation of ER α nor for the synergism with SRC1. However, in contradiction with the published results, we have shown that p68 also, stimulates the activity of ER α Δ AF1 encoding DBD and LBD, in the reporter gene assays. To further establish the effect of p68 on AF2 activity, we have examined the p68 coactivation of a number of mutants of key residues in α -helices 3, 5 and 12 which form the AF-2 surface, important in the ER α LBD interaction with

coactivators. In these reporter gene assays, the coactivation of p68 paralleled that of SRC1. Taken together, these results supports that the p68 modulate the activity of AF2 which is consistent with the recruitment of p68 to promoter of estrogen regulated gene, pS2, in response to estrogen. p68 has a nuclear receptor interacting signature motif, LXXLL in the coding sequence. We probed whether p68 influence the AF2 activity by interaction with ER through the LXXLL motif. Replacement of leucine residues by alanine in the LXXLL motif in p68 had no effect on the coactivation of ER α in the reporter gene assays which suggest that the p68 influences the AF2 activity independent of LXXLL motif.

5.4 Interaction domain of p68 in ER α is mapped to the AF1 and DBD and the interaction domain of ER α in p68 is outside the helicase domain

We have shown that p68/72 interact with ER α *in vitro* and confirmed these results *in vivo* by over expression of these proteins in COS-cells. We also confirmed the interactions with endogenous p68/72 and ER α in MCF-7 cells. Endoh et al mapped the p68 interacting region in AF1 of ER α , to amino acids 56-127 and the interaction domain in p68, to amino acids 300 to 400. In their interaction assays deletion of amino acids 200-400, 200-614 and 1-400 resulted in loss of binding while in our assays the binding was seen with these deletions. We have mapped the interaction domain in p68 to the C-terminal region, outside the helicase domain. The helicase domain itself, as shown by the reporter gene assays and the *in vitro* binding assays, is not essential for the interaction which suggests that the C-terminal extension is important for coactivator function of p68

Our findings were also different to those reported by Endoh et al with respect to the p68 interaction domain in ER. Our assays show that p68 binds to the ER α AF1 region and to the ER α DBD. In fact, in our binding assays the DBD containing constructs showed stronger interaction compared to AF1 alone. Furthermore, p68 also bound ER β , which shares 97% homology in the DBD with ER α , which further supports that the p68 interacting domain in ER spans the DBD.

The results shown here are contradictory to the results reported by Endoh et al and the reason for this disparity may be due to the fact that the GST pulldown assays

Endoh et al described were carried out with the AF1 region of ER α , and not with full-length ER α or ER α containing the DBD. The mapping of different regions of interaction between p68 and ER α by Endoh et al and us and the fact that p68 modulates the AF2 activity, argue for the possibility of multiple points of contact between p68 and ER α . Furthermore, p68/72 have also been shown to harbour activation and repression domains (Wilson et al., 2004) and collectively these findings indicate that p68/72 may cooperate or operate separately but in different contexts, such as with ER α bound by different ligands or promoter context. A recent report suggest a role for p68 as a coactivator of Androgen receptor which adds to the growing number of transcription factors and repertoire of interactions that p68/72 are involved with and their role as multifunctional proteins.

5.5 Phosphorylation of AF1 is not required for p68 interaction with ER α

The interaction of p68 was previously reported be potentiated by the phosphorylation of ER at serine 118 (Endoh et al., 1999). We have shown in the reporter gene assays that the substitution of serine 118 by alanine or glutamic acid in ER had no effect on the p68 coactivation. Our group has shown ERK1/2 MAPK also phosphorylate S104 and S106, in addition to S118 (Thomas et al. 2008). It was possible, therefore, that the p68 interaction might involve these residues. However, substitution of these serine residues, singly, doubly, or in combination with S118 by glutamic acid or alanine showed that it had no effect on the p68 coactivation. These results were further confirmed by *in vitro* binding assays where *in vitro* phosphorylation of ER by MAPK did not affect the p68 interaction with ER. Collectively these studies show that the phosphorylation is not important for the recruitment of p68 RNA helicase. It might be that the phosphorylation of p68 itself is important in its interaction with ER. Yang et al have shown that the p68 is phosphorylated at multiple amino acid residues and phosphorylated p68 activates the transcription of cyclin D1 and c-Myc genes, promoting cell proliferation. Furthermore, phosphorylated p68 was shown to be associated with promoters of cyclin D1 and c-Myc suggestive of direct involvement in transcriptional regulation (Yang et al., 2007).

They have also reported that p68 was phosphorylated at tyrosine residue(s) in different cancer cell lines but not in the corresponding normal tissues (Yang et al., 2006).

5.6 Complex role of p68 and p72 in regulation of ER activity: p72 more important than p68?

p68 and p72 have high degree of homology in the central helicase domain with divergence in the N and C terminal domains. p68 and p72 share several functions showing some functional redundancy. These proteins exist as heterodimers in cells and present in complexes of varying sizes with the potential of interaction with other cellular factors (Ogilvie et al., 2003). As a coactivator of ER, p53 and MyoD, p72 is analogous to p68 with some subtle differences (Fuller-Pace, 2006; Shin and Janknecht, 2007). The expression of these proteins is developmentally regulated with the differences in the expression of these genes. Interestingly, p68 may auto regulate its own expression which has been shown with its yeast homologue Dbp2p (Barta and Iggo, 1995). Furthermore, we and others have observed negative regulation of expression of 72 by p68 (Bates et al., 2005; Jalal et al., 2007). The over expression of p72 on depletion of p68 by siRNA but not vice versa might be a compensatory phenomenon and this has been seen with the SRC family of coactivators, reflecting the functional redundancy of these proteins. In SRC1 knock out mice, there was up regulation of TIF2 with relatively subtle defects in the development of estrogen-dependent tissues, including the uterus and breast (Xu and Li, 2003). This complex regulatory mechanism on the expression of p68/72 might have consequences in their overall function in the cells.

We have examined the functional consequences of the coactivator function in MCF-7 cells by siRNA mediated knock down of these proteins. In our initial experiments, we found that p68 depletion in MCF-7 cells had no effect on ER function which was surprising, given the findings from over-expression in reporter gene assays. However, in subsequent experiments siRNA mediated knockdown of p68 and p72,

Function	siRNA		
	p68	p72	p68 + p72
Transactivation	↔	↓↓	↓
Gene expression (pS2 and CTD)	↔	↓↓	↓
Growth	↔	↓↓	↓↓

Table 5.1 Effect of p68/p72 knock down on ER function in MCF-7 cells

singly and in combination, showed striking differences in ER transactivation and the regulation of estrogen dependant genes, pS2 and Cathepsin D (CTD). p72 but not p68 depletion resulted in marked reduction of ER transactivation. Interestingly, the combined knock down did not reduce the ER activity more than the p72 knock down.

Similar effects were seen on the expression of estrogen target genes, at both the protein and mRNA level. p68 knock down had little effect on the expression of these genes while p72 depletion resulted in a marked down regulation of both baseline and estrogen stimulated-expression of these genes. The consequences of these on the growth of MCF-7 cells were examined by siRNA mediated depletion of p68 and p72. The results again mirrored the pattern shown with the siRNA experiments on estrogen regulated genes and ER transactivation (Table 4.1). In the above experiments the lack of any effect on ER responses with the p68 knock down may be due to the compensatory over expression of p72 in the p68 depleted cells. The effects of double knockdown were also less pronounced than those seen with the p72 knock down. This suggests that there may be a compensatory mechanism involved that rescues the cells from the effects of depleting the cells of both p68 and p72. Our findings on the growth are some what contradictory to the results reported by Jalal et al. who reported that the growth inhibition was seen only with the double knock down (Jalal et al., 2007). This is probably due to the fact that in the double knock down they titrated the siRNA oligos to prevent the over expression of p72 with p68 knock down. Similar results were also reported by Shin et al. who showed that p68 and p72 double knock down in colon cancer cell lines, inhibited the cell proliferation and diminished their ability to form tumours *in vivo*. However, the data on the effect of individual p68 or p72 depletion on growth has not been reported by them given that they showed up regulation of p68 on p72 knock down and vice versa. We and others have not observed this in our experiments.

Over-expression of p68/p72 in MCF cells interestingly had different effects on the expression of estrogen target genes. p68 over-expression resulted in increased levels of pS2 and cathepsin D proteins, in agreement with the reporter gene data. However, p72 over-expression or over-expression of both p68 and p72 did not increase pS2 or cathepsin D levels.

These results show that p72 is more important in modulating transcriptional responses of ER α and it may be that the balance and interplay between p68 and p72, fine tune these responses. However, these findings should be understood in their proper context and the differences we have seen might be relevant to the role of these proteins

in the physiological and pathological contexts. Both proteins are important in the physiological context, as shown by the knock out studies in the mice embryos. In p68 disrupted embryos, the embryos did not survive beyond 11.5 days while the p72 knock out mice survived only for 2 days after birth. Embryos with double knock out led to earlier death of embryos than the p68 knock out alone (Fukuda et al., 2007). The transcription factor/ promoter context might also be important and relevant to their function. For example, p68 was shown to be more important than p72 in modulating p53 regulated genes as the depletion of p72 had no effect on the p53 regulated genes (Bates et al., 2005). The lack of any effect on ER transactivation on p68 knock down while its over expression stimulated the ER activity suggests that in the physiological context p68 may be less important than p72. However it might be different in cancer where it may play dominant role in driving ER responses.

5.7 Role of p68/p72 helicases in regulating ER mediated gene expression

The regulation of gene expression by nuclear receptors involves complex but coordinated, sequential recruitment of cofactor complexes involved in chromatin modelling along with transcription complexes at the promoters of the target genes (McKenna and O'Malley, 2002; Shang et al., 2000; Métivier et al., 2003, Reid et al., 2003). p68 is recruited to the promoter of the estrogen target gene, pS2 in response to estrogen, indicating direct involvement of p68 in transcriptional regulation of ER. In the cyclical recruitment of various cofactor complexes at the promoter of pS2, p68 is the first to be recruited in the transcriptionally productive cycle. p68 precedes the recruitment of HATs and HMTs promoting acetylation and demethylation, leading to active chromatin state. This suggests that the p68 may be required for the binding of chromatin modifying proteins in transcriptional initiation (Caretto et al., 2007). p68/p72 may be also involved in attenuating the gene responses after initial activation. p68/p72 interacts with SRA which in turn associates with a corepressor, SMRT and HDAC1-associated repressor protein (SHARP) (Shi et al., 2001). SHARP is a repressor of SRA stimulated ER transcriptional activity and the interaction between SRA and SHARP is thought to be responsible for attenuating the ER responses (Shi et al., 2001). Furthermore, *Drosophila* p68 RNA helicase has been shown to be involved in clearance

of transcripts from the promoters attenuating the gene response. p68/p72 can also repress transcription by association with HDACs which is dependant on the promoter context. These reports suggest that apart from regulating transcription initiation p68/p72 may also be involved in attenuating the transcription.

Our reporter gene assays data suggest that the p68 influences both AF1 and AF2 activation domains of ER α and, by their known interaction with the other coactivators like SRC1, CBP and SRA, might work to synergise the function of AF1 and AF2. Our *in vitro* data show that the p68 interacts with AF1 and DBD of ER α and the mapping of p68 interaction domain suggest multiple contacts between p68 and ER α .

The functional consequences of these findings *in vivo* are complex, with p72 having a more profound effect on the ER transactivation and growth of MCF-7 cells. From our *in vitro* and *in vivo* data it appears that the interplay between p68 and p72 might regulate and fine tune the ER responses. This adds another layer to the complexity of the transcriptional regulation by ER α .

5.8 Potential role of p68/p72 in breast cancer

p68 and p72 helicases have been considered as multifunctional proteins, involved in diverse biological functions (Fuller-Pace, 2006). Several reports show that these proteins act as cofactors for transcription factors such ER, p53 and MyoD. Both have been involved in regulation of p53 which is a tumour suppressor gene, mutated in more than 50 % human cancers (Greenblatt et al., 1994; Bates et al., 2005). p68 is also involved in the regulation of proto-oncogenes such as, c-Myc, cyclin D1, c-jun, and fra-1, through β -catenin (Yang et al., 2007; Shin et al., 2007). p68/p72 has been found to be over expressed in colon cancer cell lines and the expression of these proteins was found to be progressively increased in the specimens of colonic polyp, adenoma and carcinoma, which is the sequence of events in colon carcinogenesis. Down regulation of p68/p72 lead to inhibition of the colon cancer cell proliferation and inability of these to form tumours *in vivo* (Shin et al., 2007). p68 has been shown to be over expressed in colon cancer and is poly-ubiquitylated, suggestive of defect in proteasomal degradation of p68 in tumour cells (Causevic et al., 2001). p68 was found to be phosphorylated in

different cancer cell lines including breast cancer cell line but not in the corresponding normal tissues. Phosphorylated p68 was shown to be involved in the regulation of cyclin D and c-Myc promoting abnormal cell proliferation. Phosphorylation of p68 therefore might be involved in carcinogenesis (Yang et al., 2005; Yang et al., 2007).

The role of p68/72 in breast cancer is unclear. We have shown that in breast cancer cell line, MCF-7, depletion of p72 results in significant inhibition of ER transcriptional activity and down regulation of key estrogen target genes such as pS2 and cathepsin D. Furthermore, depletion of p72 inhibited the growth of MCF-7 cells. In these experiments the p72 depletion had more profound effect than the p68 or combined depletion of p68 and p72. Interestingly, p68 depletion had no significant effect either on the ER transactivation or on the expression of estrogen regulated genes, pS2 and cathepsin D. In order to further examine the role of these proteins in ER function in vivo, we plan to establish stably transfected breast cancer cell lines with short hairpin (sh) RNA targeting p68 and p72. Expression profiling of genes regulated by p68 and p72 in these cell lines, will be carried out to identify and characterise novel estrogen regulated genes modulated by p68/p72.

To study the relevance of these findings clinically, we are currently examining the expression of these proteins in patients with ER positive breast cancer. These tumours had been previously analysed for ER α phosphorylated at S118 and other proteins including Her2, PR and AIB-1. The simultaneous analysis of expression of the oncoproteins such as HER 2 in breast cancer and the correlation of these will help clarify the role of these proteins in breast cancer pathogenesis. We will use tissue microarray studies which will help to simultaneously look at the expression of ER, p68, and p72 in large number of samples utilizing very small amounts of tissue as well as saving time. The expression of p68 and p72 in the primary samples and at the time of relapse on tamoxifen will help to clarify the role of these proteins in tamoxifen resistance.

Collectively these data will enable to define the role of these proteins in breast cancer pathogenesis and potentially identify ways of targeting these in treatment of breast cancer.

5.9 Future directions

p68 and p72 seem to regulate the ER α activity in a complex manner. While they have been shown to have functional redundancy, our findings demonstrate their opposing roles. It would be interesting to clarify and refine further, the role of each protein in ER responses and to differentiate their role in the physiological and pathological context. P68 has been shown to coactivate androgen receptor and to regulate androgen dependant gene expression through its splicing function for p68/p72. It is not known whether its splicing function has any effect on its role in transcriptional regulation of ER α and whether it is coupled to transcription.

The role of these proteins in breast cancer is yet to be established though the data presented here show that p72 affect the growth of estrogen dependant breast cancer cells by modulating estrogen-dependent transcription. Further studies enlisted above will help clarify the role and the mechanism(s) by which p68/p72 contribute to development and progression of breast cancer. Hopefully, this will identify ways to target p68/p72 network in the treatment of breast cancer or preventing resistance to endocrine therapy. As RNA helicases are essential for survival of bacteria and viruses, they have been targeted for development of antibacterial and antiviral drugs. The over expression of p68 and the posttranslational modifications in colon cancer and cancer cell lines indicate their potential for use as diagnostic/prognostic markers in cancer. More studies are needed to validate these observations and for application in clinical practice.

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