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Thesis submitted:
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Regulation of estrogen responsive genes by the human Estrogen Receptor alpha (ERα)

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TABLE OF CONTENTS

LIST OF FIGURES

ZUSAMMENFASSUNG

Brustkrebs ist die häufigste Krebsart bei Frauen und verursacht die meisten krebsbedingten Todesfälle. Die molekularen Mechanismen dieser Krankheit sind vielschichtig und tragen zur Komplexität der Krankheit bei. Eine frühzeitige Diagnose und genaue Charakterisierung des Tumors verbessert die Prognose für die Patientin erheblich. Eine begrenzte Zahl von Brustkrebsmarkern wird bereits zur Diagnose, Charakterisierung und Bestimmung der vielversprechendsten Therapie herangezogen. Und obwohl neue Therapieansätze, wie zum Beispiel Herceptin-Antikörper, zur Verfügung stehen, besteht dringender Bedarf an weiteren, krebsspezifischen Markern. Ein Hauptkriterium für die Beurteilung eines Tumors ist das Vorhandensein des Östrogenrezeptors alpha (ERα). Patientinen mit ERα-positiven Tumoren haben eine bessere Prognose, da diese Tumore weniger aggressiv sind und oft auf die Anti-Östrogen Therapie ansprechen. ERα ist ein Östrogen-induzierbarer Transkriptionsfaktor, der die Expression von Östrogen-sensitiven Genen moduliert. Diese Gene spielen unter normalen physiologischen aber auch unter pathologischen Bedingungen, wie beispielsweise Brustkrebs, eine Rolle. Obwohl die Regulationsmechanismen sehr komplex sind, konnten verschiedene aktivierende Mechanismen identifiziert werden. Die Mechanismen der Genrepression werden erst langsam aufgedeckt, wenngleich etwa 50 % der Östrogen-sensitiven Gene reprimiert werden.

Das Ziel dieser Promotionsarbeit bestand in der Untersuchung der Expression und Regulation des BASE Genes (Brustkrebs und Speicheldrüsen exprimiertes Gen). Die Untersuchung dieses Genes war aus zwei Gründen interessant: Erstens, seine Expression wird durch Östrogen stark unterdrückt, was auf eine Beteiligung des ERα bei der Regulation hinweist. Und zweitens deutet eine vorausgehende Studie darauf hin, dass dieses Gen hauptsächlich in Brustkrebszellen und der Speicheldrüse exprimiert wird. Damit könnte BASE eventuell als neuer Brustkrebsmarker fungieren.

Eine Haupterkenntnis dieser Arbeit ist die strikte Trennung von Expression und Regulation von BASE. Während der Transkriptionsfaktor FoxA1 für die Expression unentbehrlich ist, spielt dieser in der Regulation keine Rolle. Diese Studie zeigt weiterhin, dass das BASE Gen in Abhänigkeit von ERα sehr schnell durch Östrogen reprimiert wird. Die direkte Bindung von ERα an die DNS scheint dabei nicht notwendig zu sein. Es ist denkbar, dass ERα auch über Protein-Protein-Interaktionen zum BASE Promoter rekrutiert wird. Aufbauend auf den Ergebnissen dieser Arbeit werden zwei Modelle für den Repressionsmechanismus von Östrogen am BASE Gen vorgeschlagen.

In Zusammenarbeit mit der Arbeitsgruppe von Prof. M. Kerin (Galway, Irland) wurden Brustgewebe-proben auf die Expression von BASE untersucht. Etwa 50 % der Proben waren postiv für BASE mRNA. Obwohl sehr geringe Mengen von BASE mRNA auch in nicht Tumorproben nachgewiesen werden konnte, bleibt BASE weiterhin ein interessanter Kandidat für einen neuen Brustkrebsmarker, da das Expressionlevel in gesundem Gewebe etwa 30-fach niedriger ist als in den Tumorproben.

In einem Nebenprojekt wurde die Aktivierung von Genen durch Östrogen am Beispiel von Cathepsin D untersucht. Neben dem schon sehr gut charakterisierten proximalen Promoter wurden in früheren Studien bereits zwei weitere Bindestellen für ERα, 9 kb und 33 kb vom Transkriptionsstart entfernt, identifiziert. Diese Arbeit bestätigt die Bindung von ERα und DNS Polymerase II an das 9 kb entfernte Motif und dessen Fähigkeit, die Stimulation durch Östrogen zu vermitteln. Ob für die transkriptionelle Aktivierung eine physische Interaktion zwischen dem proximalen Promoter und dem ERα bindenden Motif notwendig ist, wird zur Zeit noch untersucht.

SUMMARY

In women, breast cancer is the most common cancer and accounts for most cancer deaths. The molecular mechanisms underlying this pathology are diverse and contribute to the complexity of the disease. Early diagnosis and detailed molecular characterization of tumours significantly increase the prognosis for the patients. A limited number of breast cancer markers are already used for diagnosis, characterization, and determination of the most promising therapy of breast cancer tumours. Although new therapeutic approaches such as herceptin antibodies are now available on the market, new markers that are specific for a subset of breast cancer patients are urgently needed. A major criterion in breast cancer diagnosis is the presence of the estrogen receptor alpha (ERα) which is associated with better prognosis and often sensitivity to anti-estrogen therapy. ERα is a ligand-inducible transcription factor that modulates expression of estrogen responsive target genes involved in both, physiological and pathological conditions such as breast cancer. Despite the complexity of the regulatory mechanisms, a variety of mechanisms resulting in transcriptional activation of target genes have been characterised. Although about 50 % of estrogen-responsive genes are repressed in response to estrogen treatment, the mechanisms underlying this regulation are just beginning to be discovered.

This thesis aimed to study expression and regulation of the breast cancer and salivary gland expression gene (BASE). The evaluation of this gene was interesting for two reasons: firstly, its expression is strongly repressed by estrogen suggesting involvement of ERα, and secondly, previous studies indicate that the expression of this putative secreted protein is restricted to breast cancer cells and salivary gland. Therefore, BASE has the potential to function as a new breast cancer marker.

One major finding of this study is the strong separation of expression and regulation of BASE. Expression of the gene is depending on the transcription factor FoxA1, which binds in a regulatory region about 2 kb upstream of the transcription start site. Although essential for expression, FoxA1 has no function in BASE regulation.

Furthermore, this study shows that the BASE gene is rapidly repressed after estrogen-treatment and that ERα is required for this regulation. ERα can bind the BASE promoter in the same regulatory region as FoxA1, however, direct binding seems not to be a critical prerequisite. Based on the data obtained in this study, two molecular models for the mechanism of repression are proposed.

Furthermore, analysis of normal and primary breast tumour samples in collaboration with M. Kerins group in Galway confirmed BASE expression in about 50 % of the samples. Therefore, BASE remains an interesting candidate as breast cancer marker.

In a side project investigating the mechanism of estrogen mediated activation of target genes, the CTSD gene has been further characterized. Besides the well characterized proximal promoter, the functionality of two further ERα binding sites, located 9 kb and 33 kb upstream of the transcription start site, have been reported. This study confirmed binding of ERα and PolII to the 9 kb upstream enhancer and moreover, the ability of this site to convey estrogen-stimulation was confirmed. Whether the enhancer requires physical interaction with the proximal promoter to enable transcriptional activation remains to be further examined.

INTRODUCTION

1. General introduction – estrogen receptor and disease

Estrogens, such as 17ß-estradiol (E2), are primarily known as female hormones although they have many diverse functions in both men and women. They control physiological functions such as fertility, cell proliferation, fat and bone metabolism, cardiovascular and neuronal activity (for review see Norman and Litwack, 1987; Auchus and Fuqua, 1994; Mendelsohn and Karas, 1999). In addition, estrogens play a critical role in diseases like osteoporosis (Horowitz, 1993), breast and endometrial cancers (Henderson *et al*., 1988), arteriosclerosis (e.g. Rackley, 2004), cardiovascular disease (Mendelsohn *et al.*, 1999), and Alzheimer's disease (Honjo *et al*., 2001).

Estrogens belong to the steroid hormone family. These lipophilic polycyclic hormones are derived from cholesterol, and also include androgens, mineralcorticoids, and glucocorticoids. In females, estrogens are synthesized in the granulosa cells of the mature ovary before menopause, and in adult men in the adrenals, testes and adipose tissue. Minor amounts of estrogens are also produced in skeletal muscle, skin, adipose tissue, brain, and bone (reviewed in Simpson, 2000), and are transported by the blood stream to their target tissues. There, the hormones either bind to membrane-associated receptors thereby initiating signalling cascades, or freely diffuse into the cell where they bind their cognate intracellular receptors, the estrogen receptors (ERα and ERβ), which then function as transcription factors in the nucleus.

2. The nuclear receptor family

The estrogen receptors belong to the superfamily of nuclear receptors. This family comprises 48 family members which are divided into six evolutionary groups. Nuclear receptors function as transcription factors. Interestingly, despite their highly conserved structural organisation, the functions and mechanisms of action are very diverse. Their actions can be genomic or non-genomic, ligand-dependent or independent, and contribute to gene activation, gene repression, or release of gene repression (reviewed in Germain *et al*., 2006). Two receptor subtypes can be distinguished. Type 1 acts as homodimer while type 2 to acts as heterodimer after ligand binding. Type 1 comprises the steroid receptors for estrogens (e.g. estradiol; ERα (NR3A1) and ERβ (NR3A2)), glucocorticoids (e.g. cortisol; GR (NR3C3), mineralcorticoids (e.g. aldosterone; MR (NR3C2)), progestins (e.g. progesterone; PR (NR3C1)), and androgens (e.g. testosterone; AR (NR3C4)). Non-steroid hormone receptors such as vitamin D receptor (VDR (NR1I1)), thyroid hormone receptor (TR (NR1A)), retinoic acid receptor (RARα (NR1B1), RARβ (NR1B2), RARγ (NR1B3)), peroxisome proliferator-activated receptor (PPAR (NR1C)) act predominantly as heterodimers with the retinoic X receptor (RXR (NR2B)). For a number of nuclear receptors, so-called orphan receptors, the natural ligands have not been identified or may not exist (for review see Mangelsdorf *et al*., 1995).

2.1 The estrogen receptors

The effects of estrogen are mediated by its cognate receptor proteins, estrogen receptors ERα and ERβ (for review see McDonnell and Norris, 2002). Both subtypes recognize the same DNA motif, the estrogen response element (ERE), and function as ligand-inducible transcription factors. They thereby provide a direct link between signalling molecules and transcriptional response.

However, depending on the nature of ligand, posttranslational modifications, cofactor interactions or promoter response elements ERs mediate distinct functions (for review see Pelletier, 2000; Moggs and Orphanides, 2001). For example, the selective estrogen receptor modulator (SERM) tamoxifen functions as a cell- and tissue specific agonist-antagonist for ERα on ERE-based reporter genes, but as a pure antagonist for ERß (Watanabe *et al*., 1997; Barkhem *et al*., 1998; Tremblay *et al*., 1998). The opposite effect has been demonstrated with Jun/Fos on AP-1 elements. When bound to the natural hormone estradiol, ERα activated transcription while activation via ERß was inhibited. In contrast, in complex with antagonists (e.g. tamoxifen) ERß functioned as a potent transactivator (Paech *et al*., 1997). Moreover, when bound to estradiol, ERα and ERß can form homo- and heterodimers. And although ERα seems to be the functionally dominant partner in the ERalpha/beta heterodimer it is possible that combining the properties of the two partners gives rise to novel functions (Li *et al*., 2004). Other important aspects are the different tissue distribution and levels of expression of the two receptors. While ERα is mainly expressed in the anterior pituitary, uterus, vagina, testis, liver and kidney, ERß is predominant in thyroid, skin, bladder, lungs, gastro-intestinal tract and cartilage. In tissues where both receptors are present (ovary, mammary gland, testis, brain) the expression pattern observed is cell type-specific (Pelletier, 2000). Even more complexity is added by the estrogen receptor related proteins (ERRα, ERRβ, and ERRγ), which are currently classified as orphan receptors. These receptors were identified by sequence similarity to ERα and share target genes, regulatory proteins and sites of action with the ERs (for review see Giguere, 2002). Altogether, the relative ratios of ERα, ERß, and ERRs in different cell types play an important role in modulation of the cellular responses to estradiol.

2.1.1 The estrogen receptor alpha

The existence of high affinity binding proteins for estrogens was first described in the 1970's (Toft and Gorski, 1966; Jensen, 1966). However, it took another 20 years until the gene was identified and cloned (Walter *et al*., 1985; Green *et al*., 1986). The gene consists of 8 exons spanning more than 140 kb on chromosome 6 (6q25.1). Expression of the gene is controlled by one or more of 7 promoters which are regulated in a cell-specific manner. For most mRNA variants the exons associated with the different promoters are spliced to the first coding exon and thus all code for the same full-length receptor (Kos *et al*., 2001). The 1788 bp transcript, generated by sequential splicing of all 8 exons, encodes a 595 amino acid protein of about 66,2 kDa, which is considered the full-length human ERα

protein (for review see Kos *et al.*, 2001). Similar to other members of the nuclear receptor family, the estrogen receptor protein is composed of 6 domains, termed A to F (Evans, 1988; Robinson-Rechavi *et al.*, 2003), that define common structural features such as a highly conserved DNA binding domain (DBD), a hinge region, a ligand binding domain (LBD) as well as two activation functions (AF-1 and AF-2) which directly recruit transcriptional cofactors (for review see Hall *et al.*, 2001; Olefsky, 2001).

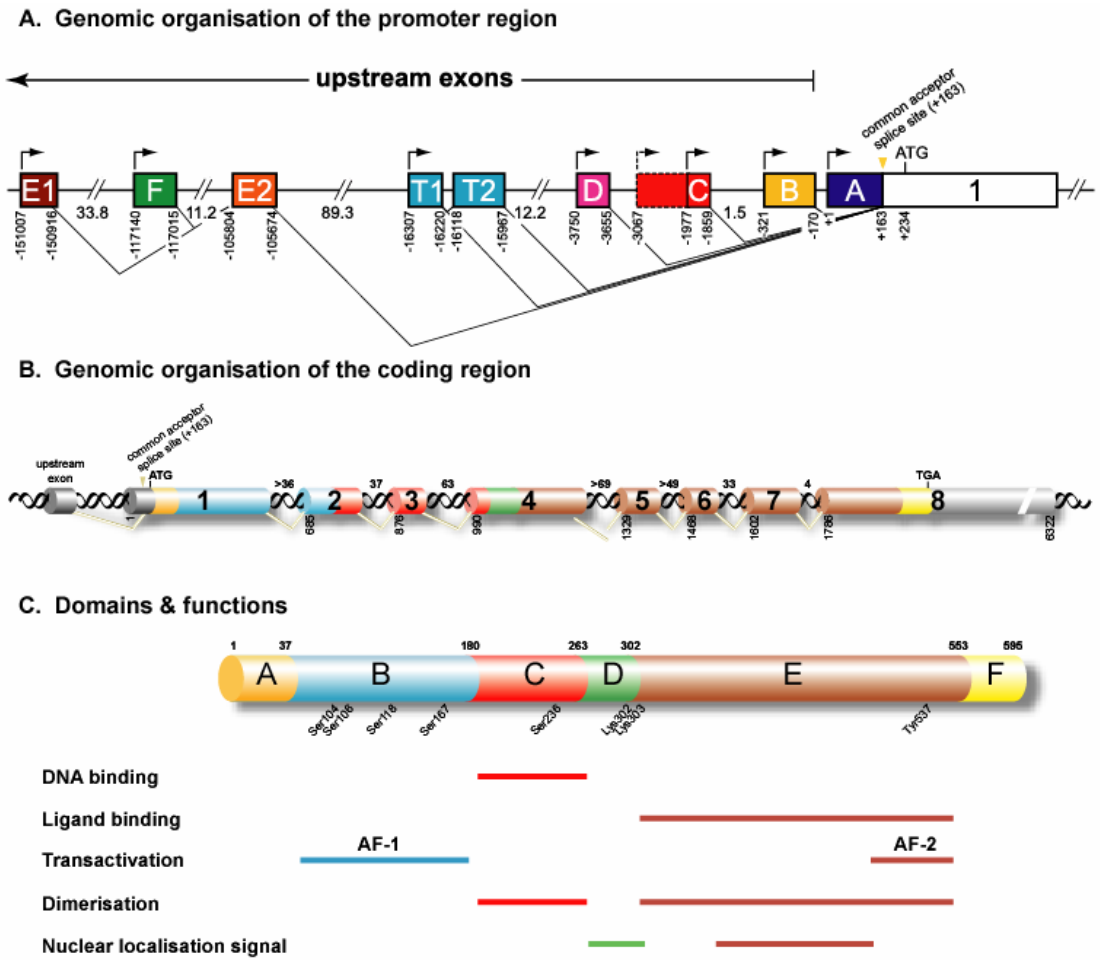


Figure I1. The estrogen receptor alpha (adapted from Reid *et al.*, 2002).
A. Genomic organization of the human ERα promoter region. The location of multiple promoters (represented as arrows) and corresponding upstream exons (depicted as shaded boxes) are shown. The size of the intronic regions is given in kb. Position of 5' start sites, or splice donor or acceptor sites, relative to the originally described start site, are indicated below the exon boxes. Furthermore, various splice variants are indicated.
B. Genomic organization of the ERα coding region including the common acceptor splicing site and the 8 coding exons of the estrogen receptor alpha gene.
C. The different domains of ERα protein are represented with their associated functions. Significant phosphorylation (Ser) and acetylation sites (Lys) are indicated below. Numbering corresponds to the position of the domains in the human receptor in amino acids.

2.1.2 The A and B domains

The N-terminal domain is highly variable both in sequence and length between the nuclear receptors. For the ERα the A/B domain encompasses the first 180 aa. The crystal structure of the A/B domain has not been resolved yet. However, structural models indicate that the A domain represses ligand-independent transactivation and transrepression through direct interaction with the E domain (C-terminal) of the receptor (Metivier *et al*., 2000; Metivier *et al*., 2002). This interaction has been shown to be important for coactivator binding.

The B domain contains one of the two activation functions (AF-1). In the presence of ligand, AF-1 synergizes with the second transactivation function (located in the ligand binding domain, see section 2.1.5). The cell type dependent effects of the partial agonist/antagonist hydroxytamoxifen (Tam) are mediated via different phosphorylation sites in AF-1 (Glaros *et al*., 2006). Also, in contrast to AF-2, AF-1 activity can be enhanced through ligand and second messenger signalling pathways, allowing the integration of alternative signalling pathways (e.g. reviewed in Lannigan, 2003). It has been shown that 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).

2.1.3 The DNA binding domain (C-domain)

The C-domain spans from amino acid 181 to 263. It is highly conserved in the nuclear receptor superfamily and serves as DNA binding domain (DBD). It forms a helix-turn-helix motif with two cystein-rich zinc finger motifs (CI and CII) which interact with alternate grooves on the DNA (Schwabe *et al*., 1990; Schwabe *et al*., 1993). The first zinc finger identifies the palindromic estrogen responsive element (ERE), while the second zinc finger stabilizes the binding by additional (unspecific) contacts with DNA, and/or dimerization with another nuclear receptor molecule (Green *et al*., 1988; Schwabe *et al.*, 1993). Three amino acids in the first zinc finger (P-box) determine the specific recognition of the ERE, with the consensus sequence A(G/A)GTCAnnnTGACC(T/C). Exchange of the P-box sequence EGckA to GSckA, changes the specificity of the receptor from an ERE to a glucocorticoid response element (GRE; Green and Chambon, 1987; Mader *et al*., 1989).

2.1.4 The hinge region (D-domain)

The D-domain, situated between amino acids 264 and 302, is not well conserved between members of the nuclear receptor superfamily and between receptors from different species (Krust *et al*., 1986; Green and Chambon, 1988; Weatherman *et al*., 2001). The hinge region joins the DNA binding domain and the ligand binding domain, allowing the receptor to change its conformation upon ligand binding without creating steric hindrance. The C-terminal part of the hinge region contains a part of the transactivation function 2 termed AF-2a, which has the potential to act autonomous in a ligand

independent manner. It might be required for the agonist activity of tamoxifen in AF-1 dominant cells (Norris *et al*., 1997). Furthermore, the D-domains of GR, PR and ERs harbour a nuclear localization signal (NLS; in human ERα amino acids 256 to 303; Picard *et al*., 1990) and provide part of the interaction interface for coactivators and -repressors (Hu and Lazar, 1999).

2.1.5 The ligand binding domain (E-domain)

The E-domain, also called the LBD, encompasses amino acids 303 to 553 and is highly conserved between species and well conserved between members of the nuclear receptor superfamily. This region harbours important features such as the ligand binding pocket (LBP), a dimerization surface which mediates interactions with partner LBDs, a coregulator binding surface, which binds to regulatory complexes, and the major part of the transcativation function 2, that is required for ligand-dependent transactivation and coactivator recruitment (Norris *et al.*, 1997). In absence of ligand, the LBD inhibits the transcriptional activity of the full-length protein through intramolecular/intermonomer interactions (e.g. Metivier *et al.*, 2002).

Crystal structure analysis revealed that binding to ligands modulates the conformation of the receptor, and consequently changes surface properties, thereby regulating the binding of coactivators (when bound to agonists) or corepressors (when bound to antagonists). More specifically, upon binding to E2, helix 12 (H12) folds into the LBD enclosing the ligand in a hydrophobic cleft (Brzozowski *et al*., 1997; Bourguet *et al*., 2000). This structural change reduces receptor - heat shock protein binding and uncovers the dimerization interface and surfaces for binding of cofactors containing an NR-box motif (Nuclear receptor interaction box, consensus sequence LxxLL, where L is a leucine and x is any amino acid). In contrast, when bound to the antagonist tamoxifen, H12 cannot fold over the LBP but instead binds the coactivator binding site, thereby preventing coactivator recruitment (reviewed in Bourguet *et al.*, 2000).

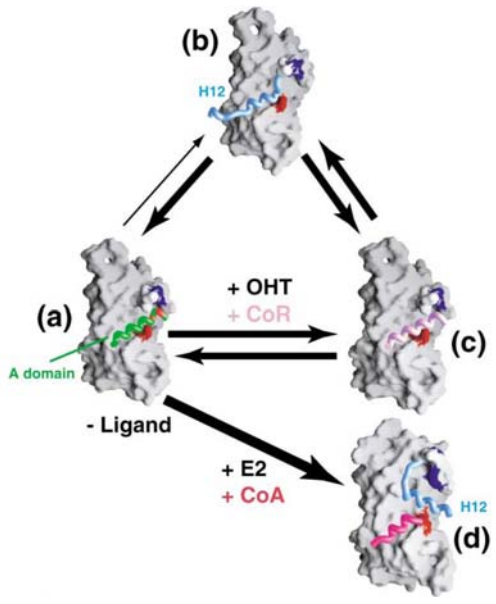


Figure I2 Model for the structural modulation of ERα activity by it's a domain (Metivier *et al.*, 2002). Depending on ligand and cofactor binding A-domain (a), ERα helix 12, and cofactors (c, d) compete for the same C-terminal binding surface.

The relative importance of the different transactivation functions (AF-1 and AF-2) depends on promoter and cell context. Three different "categories" can be distinguished: 1) AF-2 permissive cells, like the human endometrial cancer cell line HeLa, where ERα mediated transactivation depends on cofactors associating with the AF-2 surface, 2) AF-1 permissive cells, such as the human liver carcinoma cell line HepG2, where transactivation is primarily dependent on AF-1, and 3) cells with a mixed AF-1/AF-2 context such as the human breast cancer cell line MDA-MB-231 or the osteosarcoma cell line SaOS (Flouriot *et al*., 2000; Denger *et al*., 2001). The relative contribution of the two transactivation functions depends on the differentiation stage of the cell. Specifically, the more differentiated the cell is the higher its dependence on AF-1 (Merot *et al*., 2004).

2.1.6 The F-domain

The F-domain is located at the C-terminal end of the receptor starting at amino acid 554. Its function is not fully understood, but several studies show that the F-domain has a complex role in modulation of the transcriptional activation of ERα in a ligand-, promoter-, and cell-specific manner Montano *et al*., 1995; Nichols *et al*., 1998; Weatherman *et al.*, 2001; Schwartz *et al*., 2002; Kim *et al*., 2003; Koide *et al*., 2006). For example, deletion or mutation of the F-domain results in loss of agonist activity of tamoxifen in MDA-MB-321 human breast cancer cells, and also the ability of E2-bound ERα to activate transcription via interactions with SP1 is impaired. The F-domain harbours a putative PEST sequence, rich in proline, glutamic acid, serine and threonine residues. Although PEST sequences have been implicated in rapid protein turnover, the deletion of the F-domain in ERα does not change receptor stability or turnover (Pakdel *et al*., 1993; Lonard *et al*., 2000).

2.1.2 ERα isoforms

A number of ERα mRNA transcript isoforms have been reported in various tissues and organisms (Hirata *et al*., 2003). However, in addition to the full length 66 kDa protein only four smaller isoforms, hERα46, hERα36, TERP-1 and TERP-2, have been reported in humans (Castles *et al*., 1993; Friend *et al*., 1995; Flouriot *et al.*, 2000; Denger *et al.*, 2001; Wang *et al*., 2006; Bryant *et al*., 2006). Most mRNA variants are generated either by multiple promoter usage or by alternative splicing.

The 46 kDa truncated form has been identified in human breast cancer cells (Flouriot *et al*., 1998; Flouriot *et al.*, 2000), osteoblasts (Denger *et al.*, 2001; Longo *et al*., 2004), and endothelial cells (Russell *et al*., 2000; Li *et al*., 2003; Figtree *et al*., 2003). It can be produced by alternative splicing from the E and F promoters directly to coding exon 2, omitting exon 1 (Flouriot *et al.*, 2000). Alternatively, it can originate from internal ribosome entry and translation from the downstream ATG 174 in exon 2 (Barraille *et al*., 1999).

ERα46 lacks the A/B domain which harbours AF-1, and therefore, only exhibits ligand-dependent activities. Interestingly, the ERα46 homodimer shows higher affinity for the consensus ERE than the ERα66 homodimer (Denger *et al.*, 2001; Penot *et al.*, 2005). However, ERα can also heterodimerize with the full-length protein and the effect of ERα46 depends on the cell context. In a predominantly AF-2 context, ER46 functions as a ligand-inducible transcription factor. In contrast, it is an effective inhibitor for ERα66, in cells were AF-1 dominates over AF-2 (Flouriot *et al.*, 2000; Denger *et al.*, 2001; Penot *et al.*, 2005). Furthermore, full-length and truncated ERα isoforms also differ in terms of cofactor recruitment and chromatin remodelling of target promoters. More specifically, unliganded ERα66 prepares the promoter to respond to ligand, while ERα46 recruits corepressors delaying the hormone induced response (Metivier *et al*., 2004).

Another naturally occurring isoform that originates from alternative splicing is ERα36 (Wang *et al*., 2005b; Wang *et al.*, 2006). Remarkably, this ERα isoform is expressed in both ERα66-positive (e.g. MCF7 and T47D cells) and –negative cell lines (e.g. MDA-MB-231). It lacks both transactivation functions but retains DBD, partial dimerization and LBD, and can therefore inhibit genomic estrogen signalling through competition for the DNA binding elements (EREs). However, in contrast to the full-length receptor, which mainly localizes in the nucleus, the 36 kDa truncated ERα is found in association with the plasma membrane (~50 %), in the cytosol (~40 %), and in the nucleus (~10 %). Like other isoforms, hERα36 has been shown to activate the MAPK/ERK pathways in response to estrogen, referred to as "non-genomic" or "non-classic" action. Interestingly, ERα36 can also stimulate this pathway in response to anti-estrogens which could be a consequence of the altered ligand binding domain. The stimulation of signalling pathways by ERα36 in response to estrogens and anti-estrogens may play an important role in development of tamoxifen-resistance in breast cancers.

The pituitary-specific ~20 kDa isoforms TERP-1 and TERP-2 (truncated estrogen receptor product 1 and 2) are derived form an estrogen inducible promoter located between coding exons 4 and 5. The mRNAs contain a unique 5'end and exons 5 - 8 (Friend *et al.*, 1995). At low levels, TERP has been shown to enhance ERα mediated transcription on ERE containing promoters, while at higher concentrations it interferes with ERα and ERß cofactor binding and thereby suppresses their activity (Schreihofer *et al*., 1999; Resnick *et al*., 2000). The strong dependence of TERP expression on hormone levels and its ability to enhance and inhibit ERα dependent transcription suggests that it may play an important physiological role.

2.2 Posttranslational modifications of ERα

The activity of the ERα has been shown to be controlled by a number of posttranslational modifications such as phosphorylation, acetylation, ubiquitination, SUMOylation and NEDDylation. The status of the modifications influences DNA and cofactor binding, as well as dimerization and protein stability. Modifications and effects on transcription maybe cell type and promoter specific.

2.2.1 Phosphorylation

ERα and associated cofactors can be phosphorylated by various kinases in response to estrogens, growth factors, cytokines, protein kinase A (PKA)-activating agents, neutrotransmitter and cyclins (for review see (Weigel, 1996; Moggs *et al.*, 2001; Lannigan, 2003; Faus and Haendler, 2006). Thus, phosphorylation modulates both, ligand-dependent and -independent receptor activation, and thereby allows integration of different signalling pathways supporting a complex cross-talk network. ERα is phosphorylated mainly on serine residues located in the N-terminal region, where the AF-1 is situated (reviewed in Lannigan, 2003). However, while binding to estradiol induces phosphorylation on S118, S104 and S106, activation via the MAPK (mitogen activated protein kinase) pathway leads to phosphorylation of S118 and S167. Although both, estradiol binding and activation of the MAPK pathways, lead to S118 phosphorylation, the kinetics and kinases involved are different. Estradiol induces phosphorylation within 20 min while exposure to epidermal growth factor (EGF) results in rapid but transient phosphorylation. Phosphorylation of serine residues in the AF-1 domain influences coactivator recruitment resulting in enhanced ER-mediated transcription (reviewed in Lannigan, 2003). For example, S118 phosphorylation has been shown to be required for recruitment of the p68 RNA helicase and the spliceosome component (SF)3a p120, both of which interact with AF-1 and enhance ERα-mediated transcription.

Phosphorylation also plays an important role in regulation of the agonistic and antagonistic effects of tamoxifen, a selective estrogen receptor modulator used in breast cancer therapy. Tamoxifen resistant tumours show increased expression of EGFR (HER-1) and ErbB2 (HER-2), members of the epidermal growth factor receptor family. These growth factors are involved in phosphorylation of the S167 of ERα which leads to increased interaction with ER-amplified in breast cancer 1 (ER:AIB1) in the presence of tamoxifen, and consequently, resistance to tamoxifen.

Besides modulating ERα activity, phosphorylation also influences the stability of the receptor. For example, phosphorylation at S236 by PKA activates and stabilizes the receptor (Tsai *et al.*, 2004) and MAPK activation decreases ICI_{182780}-bound ERα degradation (Marsaud *et al.*, 2003).

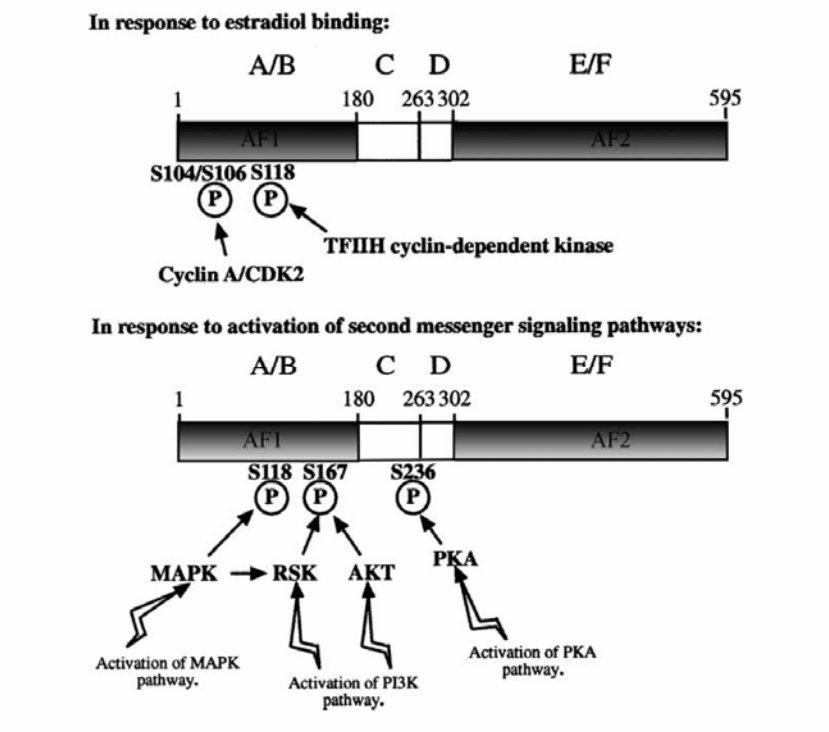


Figure I3. Pathways and kinases potentially involved in ERα phosphorylation (Lannigan, 2003).

2.2.2 Acetylation

Cofactors, like p300, CBP, SRC1 and P/CAF, which possess intrinsic histone acetylase activity, are recruited by the activated ERα where they influence chromatin structure through histone acetylation. However, p300 can also directly acetylate ERα at lysine residues (K266, K268, K299, K302 and K303) at the boundary between the hinge region and the LBD. Acetylation can stimulate DNA binding and ligand-dependent activation (K266 and K268), but also diminish ligand-induced activation (K302 and K303). Interestingly, acetylation of K303 can be prevented by phosphorylation of S305, revealing a cross-talk between different posttranslational modifications (reviewed in Faus *et al.*, 2006). Furthermore, the activity of a number of nuclear receptor cofactors is acetylation dependent (e.g. NRIP, where acetylation by p300/CBP leads to its inactivation; (Vo *et al.*, 2001)). Consequently, acetylation is likely to influence ERα mediated transcription, although the exact mechanisms remain to be elucidated.

2.2.3 Ubiquitination

The estrogen receptor has a short half-life of about 4 hours, which is further reduced to 3 hours in the presence of its ligand estradiol (Eckert *et al*., 1984). The pure anti-estrogen ICI_{182780} (Fulvestrant®, Faslodex®) further decreases the ERα half-life, while the partial antagonist tamoxifen inhibits ubiquitination and proteosomal degradation (Wijayaratne and McDonnell, 2001). Rapid turnover of ERα allows for fast changes in receptor levels and thus dynamic hormone responses. Degradation of ERα occurs mainly via the ubiquitin/proteasome pathway (Alarid *et al*., 1999; Nawaz *et al*., 1999; El Khissiin and Leclercq, 1999). Inhibition of proteasome activity stabilizes ERα but renders the protein immobile, and blocks ligand-induced transcription (Stenoien *et al*., 1998; Reid *et al*., 2003). Furthermore, proteasome activity is a requirement for cyclical ERα turnover and transcriptional activity (Lonard *et al.*, 2000; Reid *et al.*, 2003). ERα binds directly to the E3 ubiquitin ligases Mdm2 and E6AP. Interestingly, this interaction requires phosphorylation of S118 (Valley *et al*., 2005).
An inverse relation between activity and stability has been shown for PR (Syvala *et al*., 1998) and RXR (Nomura *et al*., 1999), as well as for other coactivators (Molinari *et al*., 1999; Salghetti *et al*., 2000), suggesting the existence of a link between transcriptional activity and degradation of the factors.

2.2.4 Sumoylation

Sumoylation, the covalent attachment of the small ubiquitin-like modifier (SUMO) -1, appears to be involved in the regulation of diverse cellular processes, including nuclear transport, signal transduction, and gene transcription (reviewed in Seeler and Dejean, 2003). Regulation of hormone-induced transactivation has also been shown for other nuclear receptors, including GR and PR (reviewed in Faus *et al.*, 2006). The sumoylation pathway is mechanistically similar to the ubiquitin pathway but involves a distinct set of enzymes. Unlike, ubiquitin, most targets are conjugated to only a single SUMO-1 molecule, and this modification does not target proteins for degradation, but modifies protein stability or activity (Seeler *et al.*, 2003).
Recently, ERα has been identified as a target for sumoylation by PIAS1 and PIAS3 (SUMO-E3 ligases, Sentis *et al*., 2005). ERα can be sumoylated on K266 and K268 in a strictly hormone-dependent manner. Interestingly, these residues are also subject to acetylation resulting in stimulation of DNA binding and also ligand-dependent activation of the receptor. Although, PIAS1 and PIAS3 act as SUMO-E3 ligases they regulate ERα-mediated transcription via an unknown sumoylation-independent mechanism (Sentis *et al.*, 2005).

3. Mechanisms of ERα mediated transcriptional activation

Genome wide transcriptome analysis have identified a large number of possible E2 target genes in a variety of cell types (Inoue *et al*., 2002; Frasor *et al*., 2003; Coser *et al*., 2003; Wang *et al*., 2004; Kian *et al*., 2004; Bourdeau *et al*., 2004). Roughly half of these genes are induced while the others are repressed in the presence of estrogen. Depending on the initiation step of estrogen action two modes can be distinguished: "nuclear-initiated" (also referred to as "classical or genomic action") and "membrane-initiated" (also called "non-genomic") events.

3.1 "Classical": cyclic transactivation through estrogen responsive elements (EREs)

ERs can induce transcription of target genes by directly interacting with the estrogen response element, a palindromic sequence with the consensus A(G/A)GTCAnnnTGACC(T/C) (reviewed in Klinge, 2001). The formation of transcriptional complexes is a highly ordered process which involves sequential association and dissociation of hERα (Shang *et al*, 2000; Metivier *et al*., 2003; Reid *et al.*, 2003). The kinetics of ERα-mediated transcription activation was studied in detail on the pS2 promoter (Metivier *et al*., 2003; Metivier *et al*., 2006). In the absence of ligand, ERα cycles on the pS2 promoter with a periodicity of 20 min, generating a permissive state for transcription, but without recruiting the transcriptional machinery. Upon ligand binding, the conformation of ERα changes to display coactivator-binding surfaces resulting in initiation of ordered recruitment of a number of cofactor complexes. This leads to histone acetlyation and methylation, chromatin remodeling and eventually the recruitment of the basal transcription machinery. However, after synchronization with α-amanitin, which blocks RNA polymerase II, the first cycle in the presence of hormone is transcriptionally unproductive but results in modification of histones (by histone acetyl transferases (HATs) and histone methyl transferases (HMTs)) and remodeling of the local nucleosomes (by SWI/SNF) to generate a transcriptional competent conformation (Metivier *et al.*, 2003). This first cycle is followed by two types of productive cycles that alternate with a periodicity of about 45 min (Shang *et al*., 2000; Reid *et al.*, 2003; Metivier *et al.*, 2003). During these cycles, cofactors with HAT activity (like p300, CBP, p/CAF) and HTMs (like CARM1 and PRMT1), also general transcription factors (e.g. TBP, TFIIA, TFIIB), mediator (TRAP220) and eventually RNA polymerase II are recruited. Transcriptional initiation is followed by the clearance phase. In this phase the chromatin remodelling complex SWI/SNF, HDACs, NURD, heat shock protein 70 (hsp70) and ubiquitin E3-ligases (MDM2 and E6AP; Reid *et al.*, 2003) are recruited, resetting the promoter to allow a new cycle to begin. Interestingly, some factors, like TBP and TFIIA, and modifications such as dimethylation of histone H3 persist over two cycles. Thus, the two transcriptional productive cycles differ in the clearance phase and the proteins present at the beginning of the cycles. Sequential chromatin immunoprecipitations, which detect the simultaneous presence of two proteins at one site, have

identified six ERα-containing complexes. Within these complexes functions such as HAT activity can be provided by various proteins (e.g. p300 or Tip60), reflecting functional redundancy in the system. Recruitment of hsp70 and ubiquitin E3-ligases provide two mechanisms which can limit estrogen signalling. One mechanism involves targeting ERα for degradation concomitant with transcription (Reid *et al.*, 2003; Metivier *et al.*, 2003; Metivier *et al.*, 2004). The other is based on disassembly of transcriptional complexes by molecular chaperones (Freeman and Yamamoto, 2002). Periodic assembly and disassembly of the transcription machinery on the pS2 promoter allows the continuous sampling of estradiol levels and ensures an appropriate limitation of the response to hormone (reviewed in Metivier *et al.*, 2006).

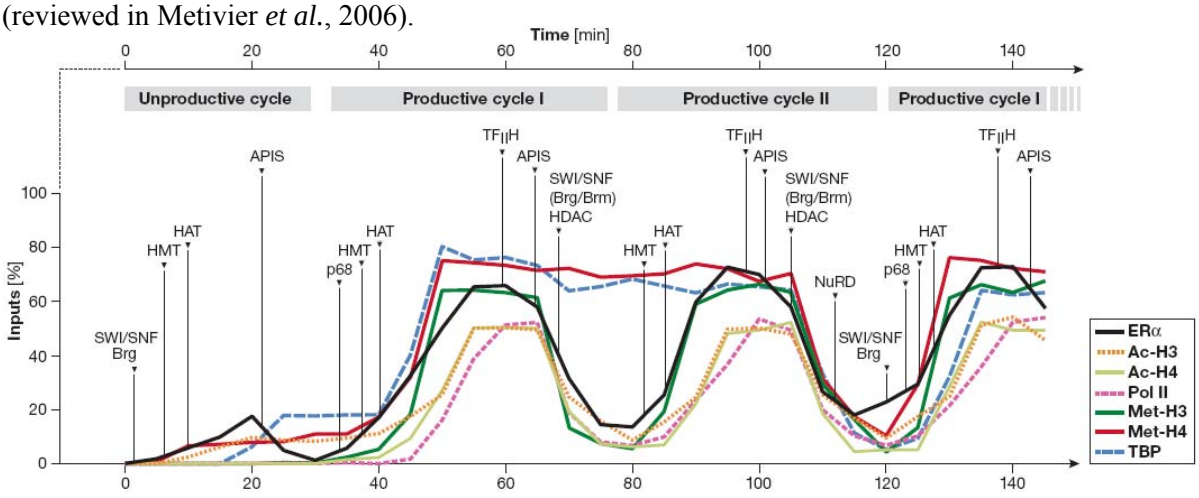


Figure I4. Cyclical recruitment of transcription factors on the pS2 promoter in MCF-7 cells in the presence of estrogen (from Metivier *et al.*, 2006). The periodic association of HATs, HDACs, HMTs and SWI/SNF (Brg/Brm), as well as other important complexes that contribute to ERα dynamics and promoter clearance are shown with arrows. Ac-H3, acetylated histone 3 (K14); Ac-H4, acetylated histone 4 (K16); APIS, AAA ATPase proteins independent of 20S; ERE, estrogen response element; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; Met-H3, dimethylated histone 3 (R17); Met-H4, dimethylated histone 4 (R3); NuRD, nucleosome remodelling and deacetylating complex ; p68, p68 RNA helicase; TBP, TATA-binding protein.

3.2 Indirect transactivation through protein-protein interaction on non-ERE elements

ERα can also regulate gene transcription in an ERE-independent manner via protein/protein interactions. This is seen on AP-1 sites where liganded ERα binds and enhances the activity of components of the coactivator complexes recruited by Jun/Fos heterodimer (reviewed in Kushner *et al.*, 2000). Two different mechanisms have been proposed for estrogen- and anti-estrogen-bound ERα. When stimulated by estrogen ERα uses the same protein-protein interactions (binding of p160 to AF-1 and AF-2) on AP-1 sites as on EREs. In contrast, when bound to tamoxifen, a selective estrogen receptor modulator (SERM), ERα adapts a conformation which allows binding of HDAC-repressor complexes, enabling ERα to titrate these complexes away from the Jun/Fos coactivator complex, resulting in increased activation from AP-1 sites (Webb *et al.*, 1999; Kushner *et al.*, 2000). ERα can also activate transcription by interacting with SP1 on motifs consisting of SP1 binding sites and either a half ERE or a full ERE, or only GC-rich regions (for review see Safe, 2001).

3.3 "Non-genomic activity" of ERα

In addition to transcriptional responses, estrogens also induce very rapid responses (within seconds or minutes) which are insensitive to transcriptional and translational inhibitors. These effects are usually referred to as "non-genomic" or "membrane-initiated signalling". They can originate from interaction of ligand-bound ERα with cytosolic or cell membrane associated regulatory proteins of second messenger signalling pathways, or from membrane-bound ERα (reviewed in Wehling, 1997; Kelly and Levin, 2001; Kousteni *et al*., 2001; Razandi *et al*., 2004; Pedram *et al*., 2006). Reported non-genomic actions of estrogens include

(1) the rapid activation of the ERK/MAPK signal transduction pathway,

(2) phospholipase-C (PLC) activation through $G\alpha_q$ resulting in PKC activation and increases in cytosolic Ca2+ pools,

(3) activation of adenylate cyclase through G-protein subunit $G\alpha_s$ resulting in cAMP-induced gene transcription,

(4) increased activities of the phosphatidylinositol-3-kinase (P13K)/Akt pathway,

(5) stimulation of growth factor receptors,

(6) activation of endothelial nitric oxide synthase (eNOS) which is believed to contribute to the cardioprotective effects of estradiol (for review see Mendelsohn *et al.*, 1999).

Non-genomic actions can converge and can furthermore influence the genomic actions of estradiol. For example, ERα and eNOS have been shown to colocalize in caveolae, specialized membrane domains enriched in the scaffold protein caveolin-1. It was suggested that E2 stimulates eNOS through regulation of the local calcium environment (Chambliss *et al*., 2000). In addition, activation of eNOS requires the PI3K/Akt pathway (Haynes *et al*., 2000) which is mediated through direct interaction of estradiol-bound ERα with the p85 regulatory subunit of PI3K. Interestingly, Akt can directly phosphorylate ERα, resulting in "ligand-independent" transcription of estrogen-responsive genes (Campbell *et al.*, 2001).

Membrane-located ERα has been reported in vascular smooth muscle, osteoblasts, and endothelial cells that express endogenous ERα (Song and Santen, 2006). ERα does not have a transmembrane domain but can be palmitoylated which allows the receptor to associate with the membrane. At present, it is not known whether ERα translocation to the membrane and activation occur sequentially or are independent of each other. In addition to the classical ERs (full-length and truncated isoforms), sex-steroid binding protein receptor (SBP-R; Catalano *et al*., 1997), as well as still unidentified proteins present in non-ER expressing cells such as CHO and COS 7 cells (Nethrapalli *et al*., 2005) could play a role in non-genomic effects of estrogen. However, the estrogen-induced rapid effects are most likely regulated through the formation of protein complexes containing ERα. A central role in these rapid effects has been proposed for the tyrosine kinase Src. It has been hypothesized that Src and

ERα associate and upon estrogen stimulation this interaction is stabilized leading to activation of Src. Several other potential components of these large complexes have been identified, including MNAR (modulator of nongenomic action of ER), p85α, Shc, G proteins and caveolin-1 (reviewed in Song *et al*., 2005). In addition, membrane growth factor receptors, such as IGF-1R (insulin-like growth factor receptor) and EGFR (epidermal growth factor receptor) are also activated in response to estrogen. Further complexity, is added by the fact that activation of signaling pathways by estrogen leads to phosphorylation and activation of coactivator proteins which in turn then modulate ER-mediated transcription (Wu *et al*., 2005).

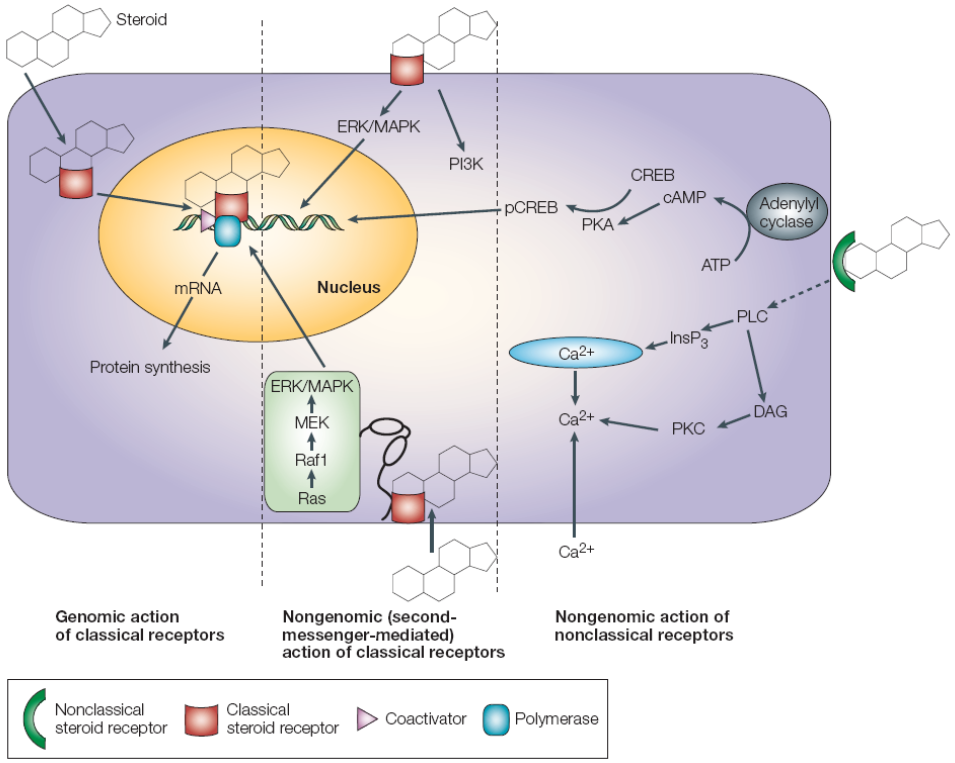


Figure I5: Different pathways of steroid mediated actions (Losel and Wehling, 2003).
This schematic representation summarizes the mechanisms of nongenomic action that can occur within a generic cell - no cell type has been shown to display all effects. The modes of action include direct transcriptional activation by classical receptors (left), kinase pathways driven by classical receptors (middle part), as well as cyclic AMP, lipase and kinase pathways, including ion fluxes, which are driven by nonclassical receptors (right). Some signalling pathways eventually lead to (indirect) modulation of gene expression by modification of transcription factors. CREB, cyclic AMP response-element-binding protein; DAG, diacylglycerol; ERK/MAPK, extracellular-signalregulated kinase/mitogen-activated protein kinase; InsP3, inositol trisphosphate; MEK, MAPK and ERK kinase; pCREB, phosphorylated CREB; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C (Losel *et al.*, 2003).

4. Mechanism of hormone-induced gene repression

E2-mediated transcriptional repression has only recently become an active field of research. Hence, it is not surprising that although equivalent numbers of genes are up- and down-regulated in the presence of estrogen, the underlying mechanisms of down-regulation are only beginning to be revealed. However, already the few genes that have been studied demonstrated a very broad and complex orchestra of mechanisms and tide regulations. Like gene activation, repression of gene expression can be achieved through direct and indirect actions in ligand-dependent and -independent manners. A number of mechanisms are conceivable (Fig. I6). ERα can interfere with association of other transcription factors (e.g factor X) at their target promoters. This can occur by preventing DNA binding through direct interaction between ERα and putative factor X (A, 4.1), or through allosteric competition, which excludes factor X from the promoter (B, 4.2). Furthermore, competition for cofactor binding could also result in estrogen-induced gene repression (C). DNA bound-ERα can also repress gene expression through recruitment of corepressors (D, 4.3). Whether ERα interacts with activators or repressors might further be defined by the DNA sequence or other transcription factors in close proximity (E, 4.4). In cases when unliganded ER enhances transcription, dismissal of ER upon hormone treatment can lead to reduced gene expression (F, 4.5). Moreover, the level of estrogen may define the availability of cofactors through stimulation of expression or degradation (G) or by influencing cellular localization. Regulatory networks in cells are a result of complex interactions involving multiple factors. Therefore, different mechanisms cannot always be separated and often combinations of several pathways apply. A few examples for estrogen-mediated gene repression will be introduced briefly in the next sections.

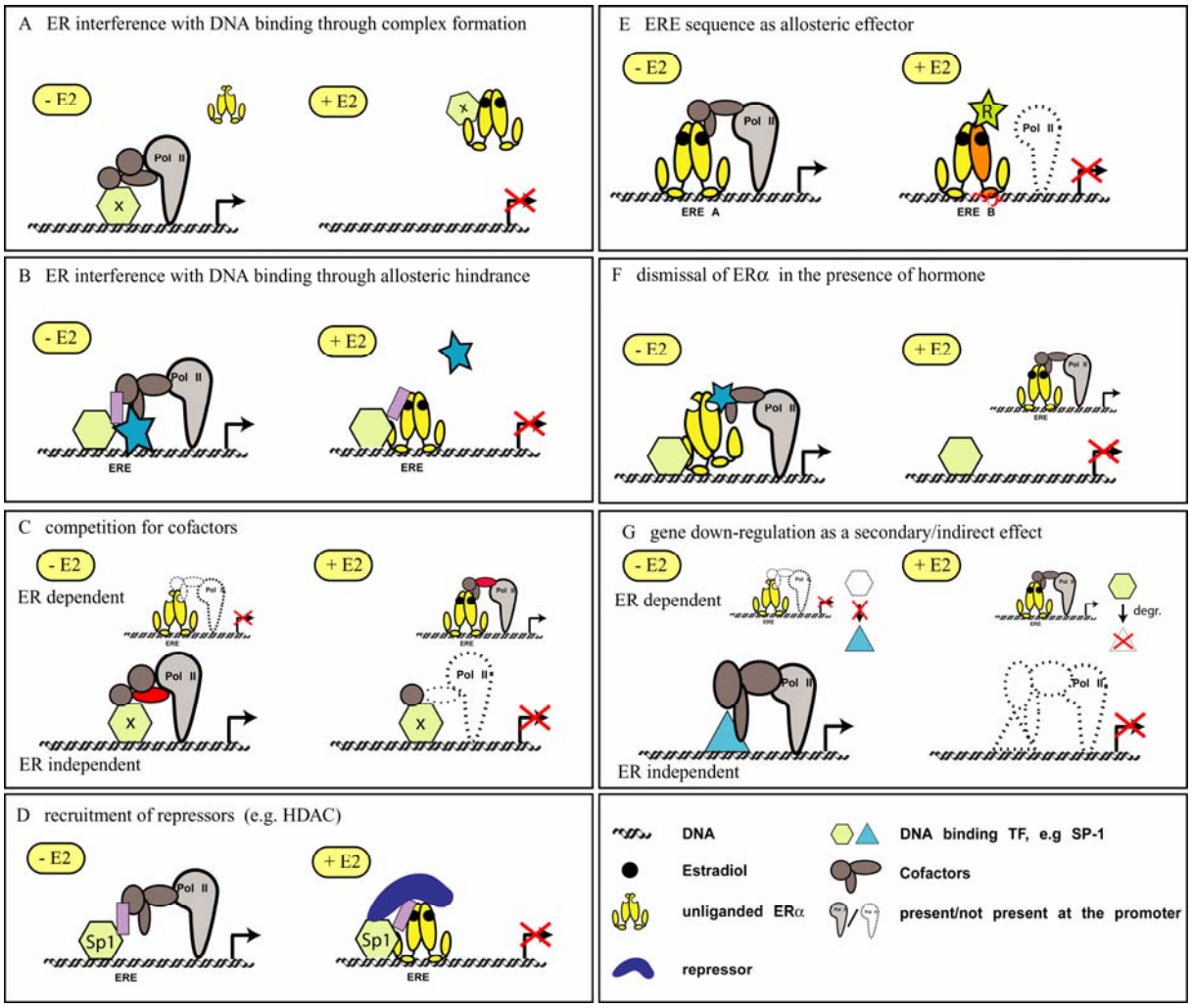


Figure I6. Conceivable mechanisms for estrogen induced gene repression mediated by ERα. For details see sections 4.1 to 4.5.

4.1 Repression of GATA-1 activity by estrogen

The transcription factor GATA-1 (ERYF1) is expressed mainly in erythroid cells and related linages such as mast cells and megakaryocytes, where it is involved in the regulation of the majority of erythroid-specific genes. Thus, GATA-1 is essential for continued survival and full maturation of erythroid precursor cells, and its inhibition drives cells into apoptosis (reviewed in Morceau *et al.*, 2004). Its transcriptional activity has been shown to be impaired by the estrogen-activated ERα which can directly interact with the zinc finger and N-terminus of GATA-1, preventing its DNA binding (Blobel *et al.*, 1995; Blobel and Orkin, 1996). Consequently, "non-classical" ERα target genes (such as erythroid kruppel-like factor (EKLF, KLF1) and ß-globin) are repressed in an estrogen-dependent manner. This interference might account for some biological effects of estrogens on eryhropoiesis like the induction of anemia in mammals treated with high doses of estrogen (Fried *et al.*, 1974) and the reduction of human bone marrow derived erythroid progenitor cells in the presence of estrogen (Blobel *et al.*, 1995).

4.2 Passive repression through allosteric hindrance

Regulation of gene expression can also be achieved through competition for binding sites. In this scenario, ERα binds to the promoter thereby blocking the (identical or overlapping) transcription factor binding site of a specific transcription factor. The factor is excluded from the promoter and the gene is no longer activated. This mechanism has been demonstrated for monoamine oxidase B, a key enzyme involved in neurotransmitter degradation. MAO-B gene expression is stimulated by ERRα and ERRγ, and repressed by ERα and ERß in a ligand-dependent and -independent manner (Zhang *et al*., 2006). ERRs (estrogen-related receptors) are orphan receptors closely related to the ERs with which they share target genes and coregulators (reviewed in Giguere, 2002). While the ERs preferentially bind as homodimers to EREs (AGGTCAnnnTGACC), ERRs recognize the ERRE with the consensus extended half-site TnAAGGTCA. At the MAO-B promoter, ERRs and ERs share proximal and distal binding sites. After hormone treatment, ERRα and ERRγ, which stimulate expression, are displaced while ERα and ERß occupancy is increased. Consequently, MAO-B expression is reduced. It is of note that the ERß was more effective than ERα in inhibiting MAO-B expression, indicating physiological consequences of the differences between the isoforms (Giguere, 2002).

4.3 Recruitment of corepressors to a half ERE in the cyclin G2 promoter by ERα

Cyclin G2 is a primary ERα target gene in MCF7 cells. Its promoter contains a half ERE embedded into a GC-rich region that functions as a Sp1 binding site. While Sp1 is associated with the promoter regardless of hormone treatment, ERα is only recruited to the half ERE after E2 treatment. Presence of liganded ERα is accompanied by recruitment of an NCoR-HDAC1 complex, resulting in histone deacetylation, release of PolII and consequently repression of cycling G2 expression (Stossi *et al*., 2006). Thus, liganded ERα exerts opposite effects on the cyclin G2 promoter in comparison to the pS2 promoter where it leads to coactivator and PolII recruitment (e.g. Shang *et al.*, 2000; Metivier *et al.*, 2003). This example emphasizes the importance of the promoter context in defining the transcriptional outcome.

4.4 DNA sequence as allosteric effector

The DNA itself also contains information which is interpreted through conformational adaptation of the DNA binding domain. The allosteric information is then transmitted via intramolecular interactions to the hinge region and ligand binding domain resulting in altered cofactor binding sites (reviewed in Lefstin and Yamamoto, 1998). Differences in ERα conformation have been observed for example between ERα bound to the consensus vitellogenin A2 ERE and bound to the imperfect ERE

of pS2 (Wood *et al.*, 2001; Klinge *et al.*, 2004). Thus, ERα may present a binding surface for activators on one gene while recruiting repressors on another gene.

4.5 Enhancement of TNFα action on the TNFα promoter through apo-ERα

TNFα (Tumor necrosis factor-α) recruits AP-1 and NF-κB to its own promoter. Subsequent association of the unliganded ERα, Hsp90 and CBP results in potentialization of the activation by TNFα. TNFα treatment does not induce phosphorylation of ERα S118 or S167, and the positive effect of ERα is not likely to be due to recruitment of p160s, SRC-1, SRC-2 (GRIP1, TIF2, NcoA2) or SRC-3 (AIB1). Upon hormone treatment, ERα is either dismissed from the promoter or masked by GRIP1, whose recruitment has shown to be crucial for the repression (Cvoro *et al.*, 2006).
In addition, estradiol has been shown to decrease the activity of the Jun NH(2)-terminal kinase (JNK) resulting in reduced phosphorylation of c-Jun and JunD which diminishes the autostimulation of c-Jun and JunD genes. Consequently, less binding of c-Jun/c-Fox and JunD/c-Fos heterodimers to AP-1 sites in the TNF promoter results in decreased transactivation of the TNF gene (Srivastava *et al.*, 1999).

4.6 Crosstalk with NF-κB – an example of complexity

The interaction between the activities exerted by ERα and NF-κB (RelA (p65), RelB and c-Rel, p50, p52) are a good example of the very complex cross-talk which can occur between transcription factors. Both factors regulate important physiological processes and can (dependent on the gene) act separately, synergistically or in an inhibitory manner (Kalaitzidis and Gilmore, 2005). Inactive NF-κB is located in the cytoplasm in complex with IκB (inhibitory protein κB). Following phosphorylation of IκB by IKK, which can be activated by numerous signals including chemokines and growth factors, NF-κB is released and translocated to the nucleus where it modulates expression of target genes. ERα can interfere with this pathway at different steps: 1) ERα can inhibit IKK activity (preventing phosphorylation of the NF-κB inhibitor), 2) it can inhibit IκB degradation (preventing release of RelA and p50), 3) it can block NF-κB DNA binding, 4) it can compete for coactivator binding (e.g. both interact with p300 and CBP), 5) and finally ERα can bind DNA bound NF-κB and prevent transactivation (reviewed in Kalaitzidis *et al.*, 2005). Interestingly, while cooperation requires the ERα AF-1 domain, repression depends on the LBD, DBD and region D of ERα.
More specifically, ERα has been shown to interfere with NF-κB transactivation on the cyctokine interleukine-6 (IL6) promoter. IL-6 is a key mediator of immune and acute phase responses. Its expression is induced in response to cytokines such as IL1, activators of protein kinase C (phorbol esters (phorbol 12-myristate 13-acetate (PMA)) and protein kinase A (forskolin), while estrogens and glucocorticoids exert inhibitory effects. The synergistic action of NF-κB and C/EBPb on the IL-6 promoter is required for its expression. However, ERα can directly interact with both factors and

prevent their binding to the IL-6 promoter (Stein and Yang, 1995; Ray *et al*., 1997). This physical interaction depends in part on the DBD of ERα and the DNA binding and dimerization domains of NF-κB and C/EBP.

In contrast, ERα and NF-κB act synergistically on the cyclin D1 promoter and promote cell cycle progression. Cyclin D1 gene expression is induced by a complex that contains the activated ERα, NF-κB, and the nuclear receptor coactivator RAC3, which can interact with both transcription factors (Rubio *et al*., 2006).

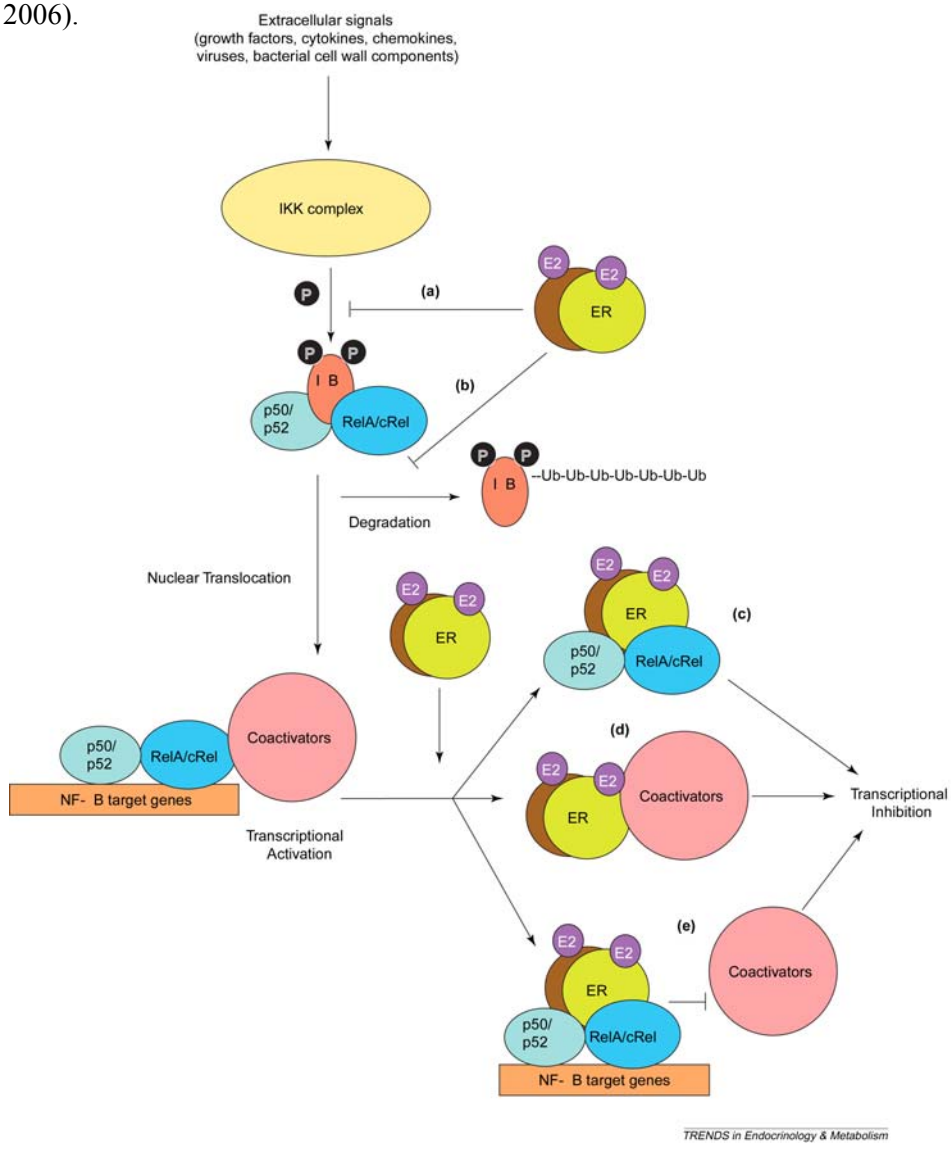


Figure I7. Inhibition of NF-κB by ERα through several mechanisms (Kalaitzidis *et al.*, 2005).
Shown is the canonical NF-κB pathway in which extracellular signals lead to activation of the IKK complex. The IKK complex phosphorylates inhibitors of NF-κB (IκBs), which usually hold the NF-κB complex (which consists of various homo- and heterodimeric NF-κB family members) latent in the cytoplasm. Phosphorylation of IκB leads to its ubiquitination and proteasome-mediated degradation and consequently to nuclear accumulation of the active NF-κB complex. NF-κB then binds to promoters in target genes and recruits coactivators to enhance transcription. ERα has been shown to block NF-κB activity at several steps: (a) it can inhibit IKK activity, (b) it can inhibit degradation of IκB, (c) it can block DNA binding by NF-κB, (d) it can bind coactivators and compete with NF-κB for coactivator binding, and (e) it can bind directly to NF-κB to inhibit NF-κB-mediated transcriptional activation.

5. The estrogen receptor in breast cancer

Estrogens play an important role in normal physiology. However, high levels of exogenous estrogens have been shown to be directly associated with an increased risk of breast cancer (London *et al*., 1992)**.** Moreover, prolonged exposure to estrogens through early menarche, late menopause, nulliparity and late age at first pregnancy are related to increased risk of developing breast cancer (Kelsey *et al*., 1993). The molecular mechanisms underlying this relationship could be the stimulation of cellular proliferation by estrogen through modulation of expression and activity of key regulatory components of the cell cycle like cyclin D1 or c-myc. The high proliferation rate increases the risk of replication errors which may result in accumulation of harmful mutations.

Estrogen receptors have proven to be one of the most important targets in breast cancer with more than 70 % of primary breast cancers in women being ERα positive and showing estrogen dependent growth (Masood, 1992). These tumours are often susceptible to anti-estrogen treatment (e.g tamoxifen) and/or hormone reduction therapy using aromatase inhibitors. In contrast, ER-negative breast cancers are more aggressive and unresponsive to anti-estrogens. Consequently, ER-positive status is generally associated with better prognosis (reviewed in Ariazi *et al*., 2006)**.**

SERMS (e.g. tamoxifen (Nolvadex)) have been successfully used in breast cancer treatment. Tamoxifen blocks estrogen action in breast cancers while exerting estrogenic effects in bone, brain and cardiovascular tissues. Thus, while preventing breast cancer and osteoporosis tamoxifen increases the risk of endometrial cancer. Also, following an initial response, long-term treatment with tamoxifen often results in development of resistance. However, most tumours, even if resistant to SERM treatment, retain ERα and therefore estrogen responsiveness which allows them to be targeted with pure anti-estrogens (e.g. Fulvestrant (ICI_{182780})) or aromatase inhibitors (AI, e.g Letrozole, Exemeestane). While Fulvestrant impairs transcriptional action and accelerates degradation of ERα, AIs decrease the availability of estrogen.

Both, polychemotherapy and hormonal therapy reduce the risk of recurrence and death from breast cancer. However, these therapies are not equally effective in all patients and they can be associated with severe side effects. Early diagnosis and detailed molecular characterization of tumours significantly increases the prognosis for the patients by minimizing unnecessary treatments. Therefore, identification of markers that are indicative of metastatic potential and tumour growth is critical since they can be used in diagnosis, therapy, and prognosis determination for women with early stage breast cancer. At the moment only a limited number of such markers are being used such as ERα, PR, HER-2 (ErbB2, neu), and BRCA1 (reviewed in Murphy *et al*., 2005). Depending on the expression status of these markers five breast cancer subtypes can be defined (Carey *et al*., 2006): luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), basal-like (ER-, PR-, HER2-, cytokeratin 5/6+, and/or HER1 (EGFR)+), HER2+/ER- (ER-, PR-, HER+), and unclassified (negative for all 5 markers). Expression of ER and PR are used to select patients for hormone therapy and generally indicate a favourable prognosis. In contrast, HER-2 positively identifies patients with metastatic disease who

have a poorer prognosis manifested by shorter disease-free and overall survival. HER-2 overexpression correlates with a lower probability to respond to hormone therapy. This might be attributed to activation of the MAPK and Akt pathways by HER-2 and the consequent activation of ERα in response to tamoxifen. However, these cancers might respond to trastuzumab, a recombinant monoclonal antibody against HER-2, either alone or in combination with cytotoxic chemotherapy (reviewed in McKeage and Perry, 2002). Nowadays, microarray gene expression profiling is increasingly used for tumour classification, prognosis and therapy prediction. This has led to identification of sets of potential predictive genes and gene expression profiles (predictive signatures) and may eventually give rise to individualized treatment of patients.

The risk to develop breast cancer can be inherited. Women who have mutations in BRCA1 or BRCA2 genes have a lifetime risk of 50 to 80% to develop breast cancer. The arising tumours are distinct from sporadic cancers and each other. While BRCA1 breast tumours are typically negative for ER and PR expression, BRCA2 tumours are likely to express both receptors (Hedenfalk *et al*., 2001; Murphy *et al.*, 2005).

Treatment of the more aggressive ER-negative tumours requires other targets. In addition to HER2, cathepsin D (CTSD), a lysosomal protease, had been identified as putative target. Cathepsin D is overexpressed in about 60 % of the ER-negative breast cancer, although it was originally identified as an estrogen-induced gene in ER-positive breast cancer cells. Its overexpression is frequently associated with shorter relapse-free and overall survival, and does not correlate with either ER levels or HER-2 amplification, thus classifying CTSD as a potential new target. Furthermore, CTSD was identified as one of the rate-limiting factors for growth of micrometastases (reviewed in Rochefort *et al*., 2003). The protease exerts a dual mode of action: As an extracellular protease CTSD can free factors that stimulated angiogenesis such as fibrioblast growth factor (bFGF). In addition, the secreted proenzyme might function as a ligand for a still unidentified cell-surface receptor and trigger mitogenic signals. In summary, CTSD has essential roles in cancer cell proliferation, fibroblast outgrowth, angiogenesis and inhibition of tumour apoptosis (for review see Liaudet-Coopman *et al*., 2006) and thus represents a interesting target for treatment of breast cancers overexpressing this protease.

6. Estrogen-stimulated gene activation – the impact of distal enhancers

A very detailed analysis on the dynamics of cofactor assembly and disassembly has previously been performed for the pS2 gene. The promoter of this gene contains an ERE in close vicinity to the transcription start site. However, many genes are regulated through distal motifs (personal communication with Jane Thomsen, GIS Singapore). To our knowledge, no studies have been performed on cyclical recruitment of complexes to these distal elements. A well known estrogen induced gene is Cathepsin D (CTSD), encoding a lysosomal aspartyl protease. Three ERα binding regions have been discovered: the proximal promoter and two distal binding sites, 9 kb and 33 kb upstream (Krishnan *et al*., 1994; Augereau *et al*., 1994; Bourdeau *et al.*, 2004; Carroll *et al*., 2006). Regulation of CTSD expression through its proximal promoter has been extensively studied and revealed a complex mode of regulation. Within the proximal region several E2-responsive motifs have been identified: 1) a SP1-ERE half site (-199 to -165; Krishnan *et al*., 1994; Krishnan *et al*., 1995), which binds an ERα/SP1 protein complex; 2) an SP1 binding site (-145 to -135; Wang *et al*., 1998), and 3) an imperfect ERE as part of the MLPE (E2 responsive major late promoter element, -119 to -107; Augereau *et al*., 1994; Wang *et al*., 1997), which binds the ERα homodimer. Estrogen induced CTSD expression can be inhibited by interaction of the aryl hydrocarbon receptor (AhR) with dioxin responsive elements (DRE) which then blocks the formation of the ERα/Sp1 complex (Wang *et al*., 2001). In 2000, Shang *et al.* showed that association of ERα with the proximal promoter of CTSD (-295 to -54) occurs in a cyclical manner with similar cycling times to that described for the pS2 promoter (80 min).

So far, studies have concentrated on the proximal promoter of CTSD neglecting the potential influence of distal ERα binding sites. Although ERα is recruited to these sites, it is still unclear whether these distal EREs can contribute to estrogen-induced activation of CTSD. It is noteworthy that cooperativity between widely spread weak binding sites for ERα binding and transactivation has been observed (reviewed in Sanchez *et al*., 2002). Therefore, the aim of a side project of this thesis was the evaluation of the regulatory potential of the 9 kb upstream binding site. For comparative reasons, the analysis was extended to the estrogen-induced genes GREB1 (gene regulated by estrogen in breast cancer protein) and the ELOVL2 (elongation of very long chain fatty acids protein 2) for which ERα binding sites were also identified using ChIP-on-Chip (our group and others, e.g. Bourdeau *et al.*, 2004; Carroll *et al.*, 2006).

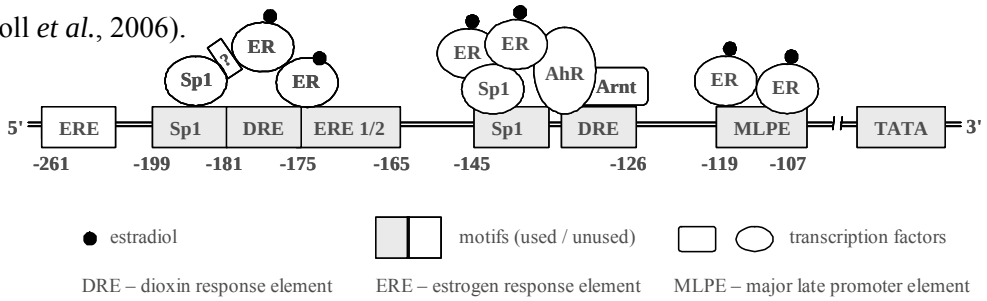


Figure I8. Proximal promoter region of CTSD (modified from Wang *et al.*, 1998).

7. The BASE gene – what is known

The Breast cancer and salivary gland expression gene (BASE) was recently identified in a screen for membrane and secreted proteins that are present in prostate or breast cancers (Egland *et al*., 2003). The gene is located on chromosome 20q11.21 and consists of 9 exons. The 1203 bp transcript encodes a putative secreted 19,5 kDa protein. The secretion of the protein would potentially allow measurements of its levels in serum, plasma or even saliva. Remarkably, BASE expression seems to be restricted to breast cancer and salivary gland tissue as assayed using a commercially available tissue array. BASE expression was also detected in 5 out of 8 breast tumours and in the breast cancer cell lines MCF7, ZR-75, and SKBR3 (Egland *et al*., 2003). With its restricted expression pattern and the possible secretion, BASE has the potential to be an easily accessible and potent diagnostic marker for breast cancer. However, its reported expression profile remains to be further examined, especially in terms of differences in expression levels between primary and metastatic tumours.

The BASE gene and protein have not been experimentally characterized previously. The protein has no predicted domains which could indicate its function. BASE does share sequence similarity with Latherin, a surfactant protein found in horse sweat. It is 42 % identical and 63 % similar to the first 178 aa of Latherin (Beeley *et al*., 1986). BASE has been assigned to the PLUNC gene family which so far consists of 4 short (SPLUNC) and 6 long (LPLUNC) proteins expressed in the upper airways (reviewed in Bingle and Craven, 2004a). These proteins contain domains predicted to be structurally similar to one (SPLUNC) or both (LPLUNC) of the domains of BPI (bacterial/permeability-increasing protein) which plays an important role in the innate immune system. PLUNC proteins may function in host defence (Bingle *et al*., 2004b).

In contrast to the other SPLUNC genes BASE contains a stop codon in exon 6 which removes the section containing a second conserved cyctein. This results in the loss of a disulphide bond essential for the correct folding of the PLUNC genes. The stop codon in Exon 6 was considered to be premature (Bingle *et al.*, 2004b) which suggests that BASE mRNA could be subject of nonsense-mediated decay (NMD), a pathway leading to degradation of mRNAs containing premature stop codons (reviewed in Wagner and Lykke-Andersen, 2002). For its potential use as a diagnostic or prognostic marker it is essential to understand the mechanism underlying BASE expression. Since the gene has only been discovered recently, no studies have been carried out so far. However, genome wide expression analysis in the human breast cancer cell line MCF7 performed in our laboratory indicated a role for ERα in BASE regulation. In this analysis BASE mRNA levels were 6 fold down-regulated in response to estradiol. The aim of this thesis is to investigate the mechanism underlying BASE gene expression and to further analyse the role of the ERα in hormone induced gene repression.

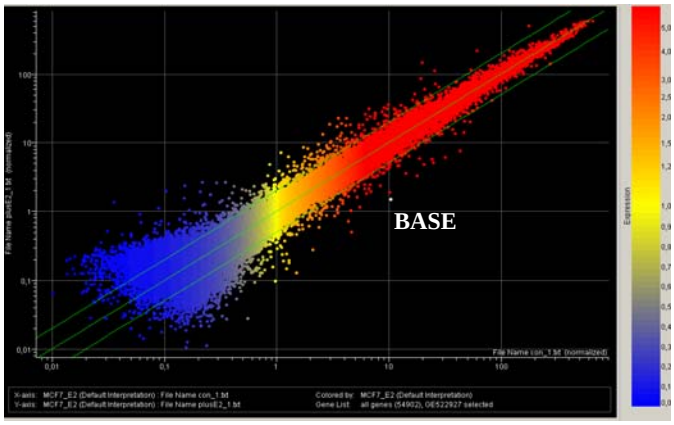
RESULTS

A Expression and regulation of the Breast Cancer and Salivary Gland Expressed gene

1. Validation of microarray analysis with qRT-PCR

BASE was identified as an estrogen responsive gene by Dr. Stefanie Denger in a comparative transcriptome analysis of untreated (EtOH) and estrogen-stimulated (E2, 10 nM) MCF7 cells using Amersham whole human genome 55 K array (Amersham, Freiburg, Germany). After 24 hour treatment, BASE mRNA levels were decreased about 6 fold while known E2 induced genes, like GREB1 and pS2 were up-regulated 6 and 2.9 fold, respectively. The first issue of this thesis was to validate the microarray data. For a number of genes that have been examined by real-time PCR for their regulation by E2 a high degree of correlation was observed with the microarray data (Fig. R1).

A



B

Gene	MA	RT-qPCR
ßactin	0,89	1,00 +/- 0,00
GREB1	6,06	16,00 +/- 6,22
ELOVL2	1,40	2,76 +/- 1,90
pS2	2,88	6,93 +/- 4,57
CTSD	2,26	2,36 +/- 0,12
ERα	0,71	0,96 +/- 0,07
MSX2	0,62	0,62 +/- 0,09
BASE	0,14	0,08 +/- 0,02
DSCR1	0,48	0,29 +/- 0,06

Figure R1. Comparative transcriptome profiling of MCF7 in absence and presence of E2.
A. Microarray analysis presented as Scatter blot. MCF7 cells were serum starved for 3 days followed by a treatment with either EtOH or 10 nM E2 for 24 hours before cell harvest. mRNA from 3 biological replicates, both for control and E2 treatment, was used to generate labeled cRNA which was then applied to individual Amersham Human 55 K arrays. Results are presented as scatter blot showing the average of the 3 replicates of control (EtOH) on the x-axis and average of E2 treatment on the y-axis.
B. Real-time PCR validation of microarray (MA) data. Total RNA was prepared from 3 biological replicates of cells treated as for the microarray experiment. cDNA was generated and the relative levels of mRNA of selected genes were analysed by quantitative RT-PCR. Samples were analysed in triplicates and normalized to ßactin. Fold change of transcript levels after E2 treatment are listed in the table.

2. BASE regulation is ERα-dependent

The microarray analysis showed a highly significant repression of BASE expression in response to estradiol treatment. However, the long exposure to hormone does not rule out regulation through secondary, ERα independent, effects. In order to assess whether the effect of hormone treatment on BASE expression is mediated via ERα several approaches have been undertaken.

2.1 BASE is rapidly down-regulated in response to E2

The microarray analysis provided only a "snapshot" of BASE regulation induced by hormone. To assess the time-dependent regulation of the BASE gene expression by estrogen, a time course experiment was performed in which BASE transcript levels were determined at different time points up to 24 hours after treatment with 10 nM E2. RNA from triplicate samples at each time point was analyzed using quantitative RT-PCR. The repression of the BASE gene was found to be very rapid, showing about 30 % decrease in transcript levels after only one hour, which then constantly decreased further until the 24 hour time point (Fig. R2 A). This indicates that BASE down-regulation after hormone treatment is a direct rather than a secondary effect. Nevertheless, a mRNA degradation dependent mechanism can not be excluded. However, it also confirms that the 24 hour treatment is a valid time point for further investigations. Time course studies have also been performed for the pure anti-estrogen ICI_{182780}. For these experiments cells were maintained in normal medium. As expected for an ERα-dependent repression, BASE mRNA levels rose after treatment with ICI_{182780}. However, the induction was delayed compared to the repression in response to E2. An increase was only detectable after 6 to 12 hours. Opposite trends were observed for the well characterized E2-inducible pS2 gene which was included as positive control (Fig. R2 B).

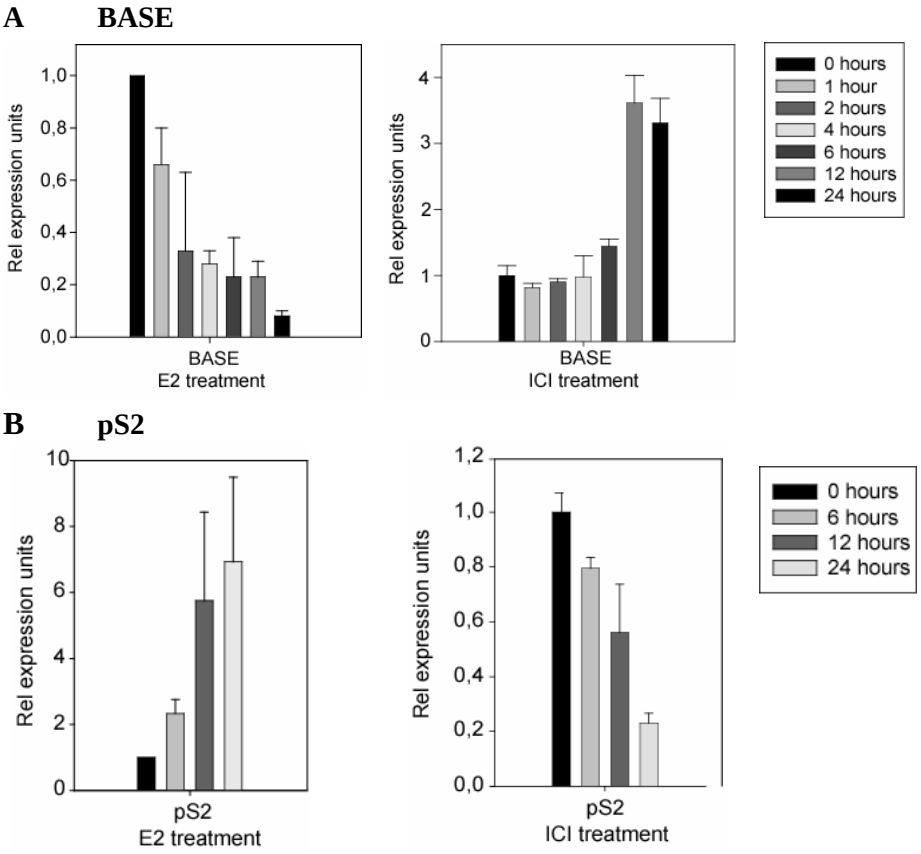


Figure R2. Time course of BASE (A) and pS2 (B) expression in MCF7 cells.

cDNA was prepared from 3 biological replicates of MCF7 cells after treatment with E2 (in stripped medium) or ICI_{182780} (in normal medium) for indicated times. Expression levels of BASE (A) and pS2 (B) were determined with qPCR and normalized to ßactin.

2.2 Knockdown of ERα induces BASE expression

siRNA against ERα was used to investigate the effect of reduction of ERα receptor levels on BASE expression without additional treatment. Transcript levels of ERα and BASE were analysed 24 hours after transfection with ERα-siRNA. The receptor mRNA levels dropped to 30 % while BASE mRNA was increased to about 2.6 fold (Fig. R3). These results indicate that activation and degradation of ERα are the main reasons for BASE regulation in response to E2 and ICI_{182780}, and not unidentified side effects of ICI_{182780}.

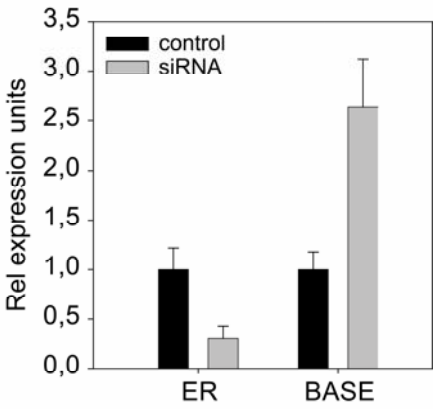


Figure R3. Knockdown of ERα leads to induction of the BASE gene.
MCF7 cells were transfected with siRNA against ERα for 24 hours. cDNA was analysed for BASE and ERα transcript levels. Expression levels were normalized to PPIA.

3. Cloning and testing of reporter constructs

To further investigate the regulation of BASE, luciferase reporter assays were chosen since this system would later allow rapid analyses of mutation effects. Based on the pGL3-basic vector (Promega) which lacks eukaryotic promoter and enhancer sequences, a reporter construct containing 2.4 kb of the BASE promoter upstream of luciferase gene was created (Fig. R4 A). In transient transfections in MCF7 cells, this reporter construct nicely recapitulates the regulation of the endogenous gene (Fig. R4 B) – strong repression with estradiol while ICI_{182780} slightly induces BASE expression.

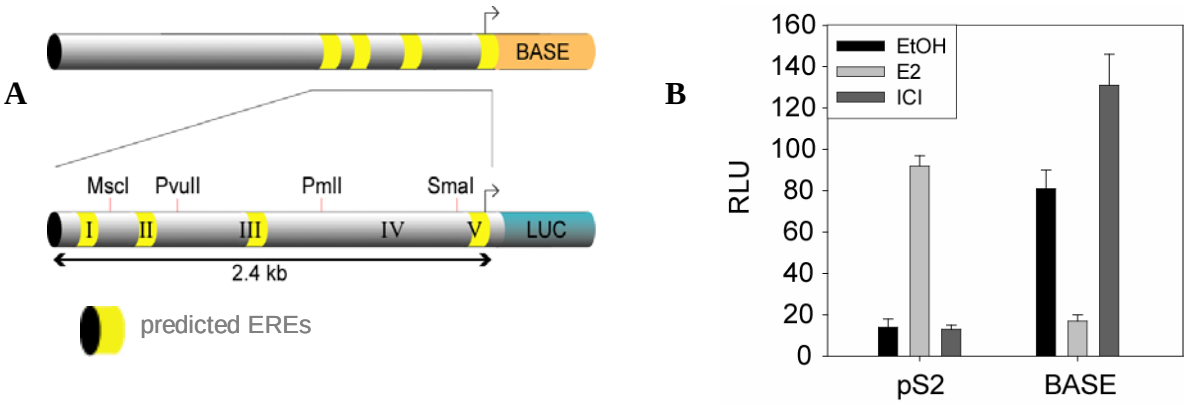


Figure R4. Regulation of BASE-luciferase reporter construct in MCF7 cells.
A. Schematic view of the BASE promoter with bioinformatically predicted EREs and the generated luciferase reporter construct below (wt). B. Regulation of pS2 and BASE reporter constructs after transient transfection in MCF7 cell and treatment with either vehicle (EtOH), E2 (10 nM), or ICI_{182780} (1 mM). The pS2 - luciferase reporter construct (-556 bp to +26 bp) has been generated in the group before. Relative luciferase units (RLU) were determined by normalization to Renilla luciferase.

4. BASE expression in different cell lines

In a first study, no expression of the BASE gene was detected in normal tissue apart from salivary glands (Egland *et al*., 2003). Although BASE mRNA was undetectable in mammary gland samples, high expression was detected in different breast cancer cell lines and few breast tumour samples.

To evaluate expression of BASE in cell lines used in our laboratory, cDNA was generated and analysed for the presence of BASE transcripts using semi-quantitative (end-point) PCR. To verify the quality of the cDNA templates, separate PCRs were performed for PPIA. Different breast cancer cell lines (ERα-positive: MCF7, ZR-75, T47D, ERα-negative: MDA-MB-231, SKBR3), the human cervical adenocarcinoma cell line HeLa, and the hepatocellular carcinoma cells line HepG2 (both ERα-negative) were tested. Furthermore, MDA-MB-231 stably expressing the full-length ERα66 (MDA66) or the short isoform ERα46 (MDA46) were also included. As predicted from the previous study, BASE was undetectable in HeLa and HepG2 cells, while BASE mRNA was detected in all breast cancer cell lines but MDA-MB-231. Surprising was the high expression of BASE in the ERα-negative cell line SKBR3 (Fig R5).

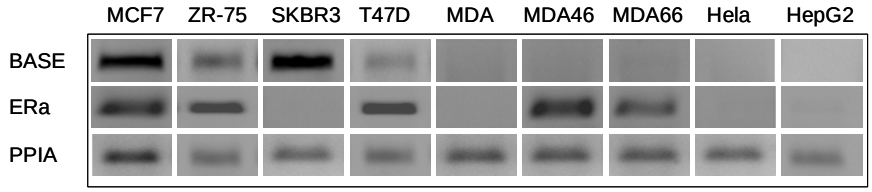


Figure R5. BASE expression in different cell lines
cDNA was prepared from different cell lines maintained in normal medium (for details see materials and methods). Expression of BASE, ERα, and PPIA was analysed with gene specific primers in end-point PCR.

After investigation of the endogenous gene expression levels, the 2.4 kb promoter fragment was assessed for its ability to drive expression in cell lines other than MCF7. In transient transfection assays using the luciferase reporter construct described before, the 2.4 kb proved to be sufficient for expression in the cell lines where endogenous BASE transcripts were detected (not all data shown). No luciferase activity was found in the MDA-MB-231, HeLa, and HepG2 cells (Fig. R6). Interestingly, although the BASE reporter construct was activated in ERα-negative SKBR3 cells, there was no regulation by either E2 or ICI_{182780}. This suggests a role for ERα in BASE regulation rather than basal expression. It is therefore not surprising that re-introduction of ERα isoforms (ERα46 and ERα66) in MDA-MB-231 cells did not stimulate BASE expression. The high pS2 promoter activity in HepG2 cells in the transfection assay, although E2-unresponsive, is in contrast to the absence of endogenous pS2 transcripts in these cells. In summary, ERα is not sufficient for BASE expression since BASE transcripts were detected in the ERα-positive and the ERα- negative cell line SKBR3.

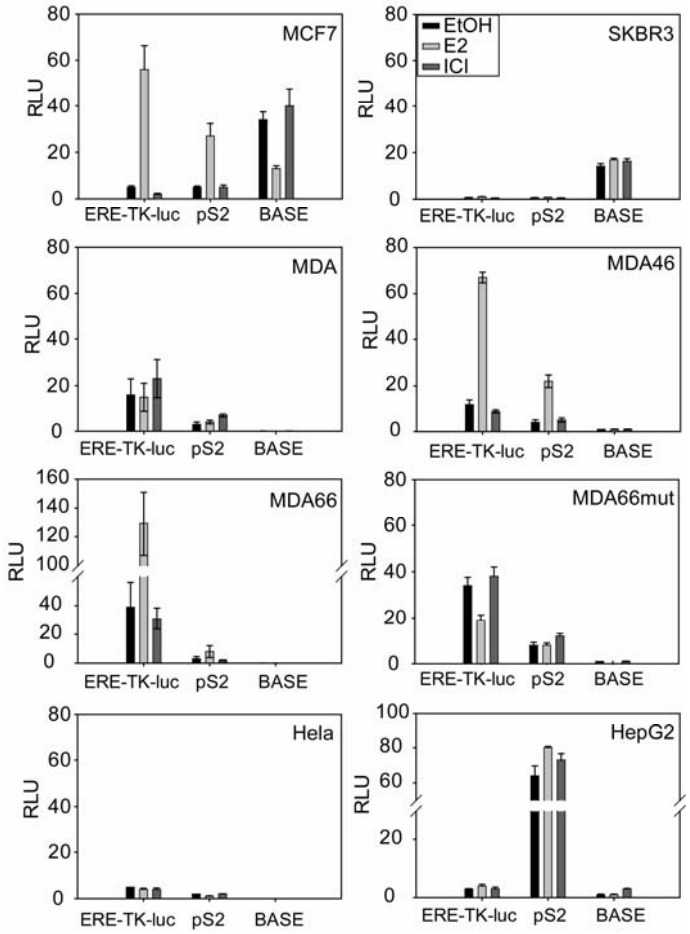


Figure R6. BASE promoter activity in different cell lines.

Cells were stripped for 3 days, transiently transfected with wt BASE reporter construct and treated with either vehicle (EtOH), 10^{-8} M E2 or 10^{-6} M ICI_{182780}. Luciferase activity was determined 24 hours later and normalized to Renilla luciferase activity.

5. BASE promoter analysis

5.1 A 600 bp regulatory region is essential for BASE expression

After establishing the luciferase reporter construct as a functional system resembling the regulation of the endogenous gene, a series of deletion mutations was generated (Fig. R7) to identify regions that have a role in BASE expression and regulation by estrogen or anti-estrogen ICI_{182780}. In this series, luciferase reporter constructs omitting different segments of the promoter were created from the 2.4 kb-basic construct and tested in transient transfection assays. Deletion of the most upstream region I (-2419/ -2353, ΔI) had no effect compared to the full length reporter construct. In strong contrast, deletion of the region between -2352 and -1689 (segment II, ΔII) reduced gene expression to almost undetectable levels. Absence of segment IV (-916 /-179, ΔIV) also reduced expression strongly, while deletion of segment III (-1688 /-917, ΔIII) resulted in slightly increased expression indicating that this region might harbour weak repressive features. All constructs that were expressed also showed repression by E2 and induction by ICI_{182780} (data not shown).

To summarize, promoter segment IV (-916/-179) contributes to BASE gene expression but is not necessary for regulation. The 600 bp region II is necessary for full expression of the BASE gene but whether in addition it plays a role in the repression by E2 could not be assessed with this approach due to the very low expression levels.

Contribution of different promoter regions to BASE expression and regulation

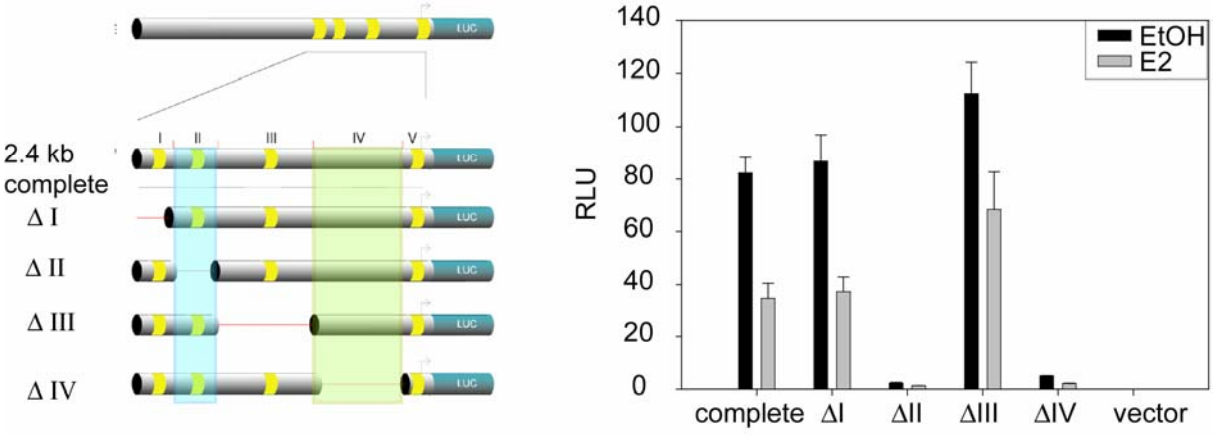


Figure R7. Identification of a 600 bp region essential for BASE expression that is located 2 kb upstream of the transcription start site.
MCF7 cells were stripped for 2 days, then treated with either EtOH or 10^{-8} M E2 and transfected with a series of luciferase reporter constructs omitting different segments of the BASE promoter. Firefly luciferase activity was determined 24 hours later and normalized to Renilla luciferase activity.

5.2 Regulatory potential of the enhancer and identification of the core promoter

To evaluate the role of region II in BASE regulation, promoter segments of increasing length have been subcloned into the pGL3-enhancer vector (Fig. R8). This vector is almost identical to the one used before but contains an additional SV40 enhancer which leads to increased basal expression and therefore allows to assess the regulation of constructs omitting the BASE enhancer region.

MCF7 cells were transiently transfected with sequential 5'-deletion constructs and constructs either omitting or exclusively containing the enhancer region (Fig. R8). The region up to -917 bp (segment IV) was identified as core promoter (E2 and E7). It imparts basal activity and has been shown to contribute to full promoter activity in previous experiments (compare Fig. R7), but not to the response to E2 or ICI_{182780} (ICI data not shown). Regulation and further increased expression was observed only when segment II, which can also be considered as an enhancer, was included. Interestingly, disruption of the ERE surrounding the transcription start site reduced E2-induced repression (E10). In summary, the enhancer (segment II) is necessary and (together with the BASE transcription start site) sufficient to mediate the response to E2 and ICI_{182780}. This is best illustrated by construct E9.

Identification of BASE core promoter

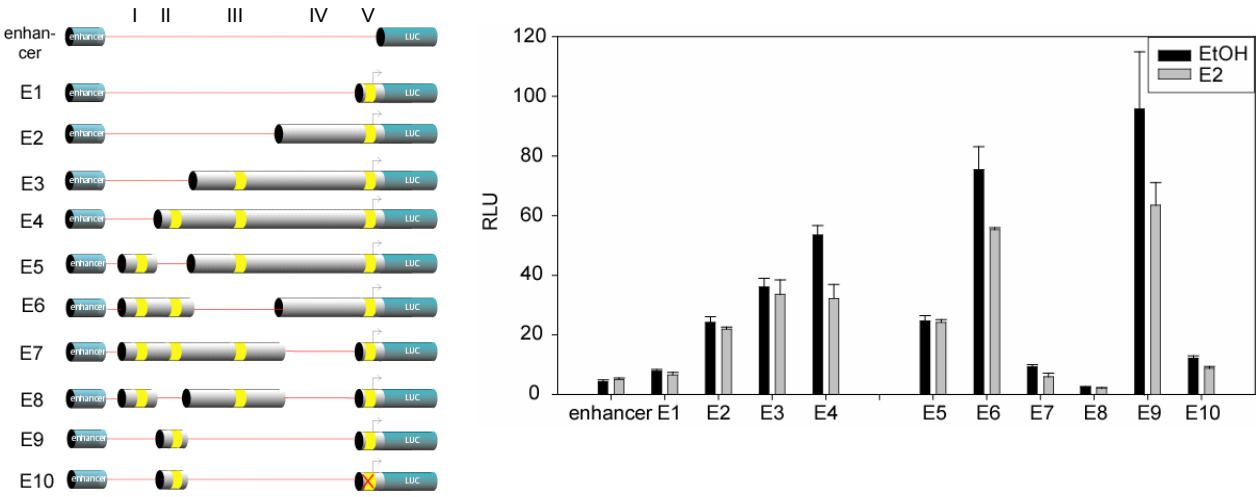


Figure R8. Core promoter analysis.

As series of promoter fragments of increasing length and omitting different promoter segments have been subcloned into the pGL3-enhancer construct (harboring an additional SV40 enhancer) and were tested for transcriptional activity in MCF7 cells 24 hours after transfection and treatment with either vehicle EtOH or 10^{-8} M E2. Values have been normalized to Renilla luciferase.

5.3 Impact of the transcription start site

So far the identified minimal hormone-responsive region consists of the 600 bp enhancer enhancer (segment II) and the transcription start site. The enhancer is essential for expression and regulation, but the role of the transcription start site in BASE regulation remained to be elucidated. To assess this question the –2419/-179 promoter region (segments I to IV) and sequential 3'-deletions were subcloned upstream of the SV40 promoter and analysed in transient transfection assays (Fig. R9). Consistent with the results obtained before, all constructs containing the 600bp enhancer (region II) showed hormone responsiveness (P1, P2, P3), indicating, that the transcription start site is not essential for BASE gene regulation by E2. However, the enhancer alone was not sufficient to mediate the hormone response (P6). Only inclusion of segment I (P3) immediately upstream caused hormone sensitivity. Taken together with previous experiments, the minimal sequence required for hormone response consists of the 600 bp enhancer (segment II) and either segment V (about 200 bp including the transcription start site) or segment I (the small region directly upstream of the enhancer region). Thus, the results point towards a two component regulation – involving an interaction between the enhancer (II) and either the transcription start site (V) or a small region immediately upstream of the enhancer (I).

The enhancer region is essential but not sufficient for E2-induced repression

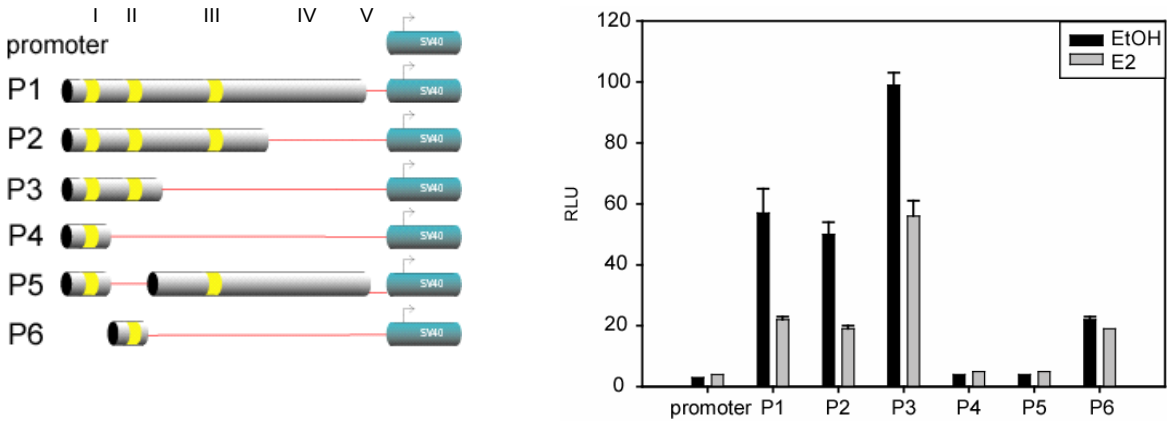


Figure R9. The enhancer is necessary but not sufficient for regulation.
BASE promoter segments of increasing length were subcloned upstream of the SV40 promoter and luciferase reporter. Transcriptional activity was analysed in MCF7 cells after transient transfections and treatment with either EtOH or 10^{-8} M E2 and normalized to Renilla activity.

5.4 Mutation of the EREs in enhancer and at transcription start site

All three regions potentially involved in mediating the hormone response contain bioinformatically predicted EREs. The most proximal ERE is located in segment V, directly flanking the transcription start site. A conceivable mechanism could be that ERα, by binding to this site, interferes with assembly of an activation complex and thereby represses gene expression. To test this hypothesis, the ERE was disrupted (in the whole promoter context (2.4 kb)) while the ATG of the TSS was preserved. These mutations reduced the gene expression to about 50 % compared to wild type while not altering the estrogen- and ICI_{182780}-dependent regulation. A similar result was obtained when the ERE in the enhancer region was mutated (Fig. R10 A to C). The complexity of promoters allows the combination of different signal pathways which makes assessment of single promoter features rather challenging. To minimize this problem further analysis was performed in a restricted background containing only the enhancer region (II) and the transcription start site (V). This combination is still able to confer the hormone response although to a slightly lower extent. In this background mutation of either one or both EREs also dropped expression while the regulation again was not dramatically affected (Fig. R10 D to F). This indicates that direct binding to the predicted EREs is not required for the repression.

Disruption of the EREs in the enhancer region and at the transcription start site (TSS)

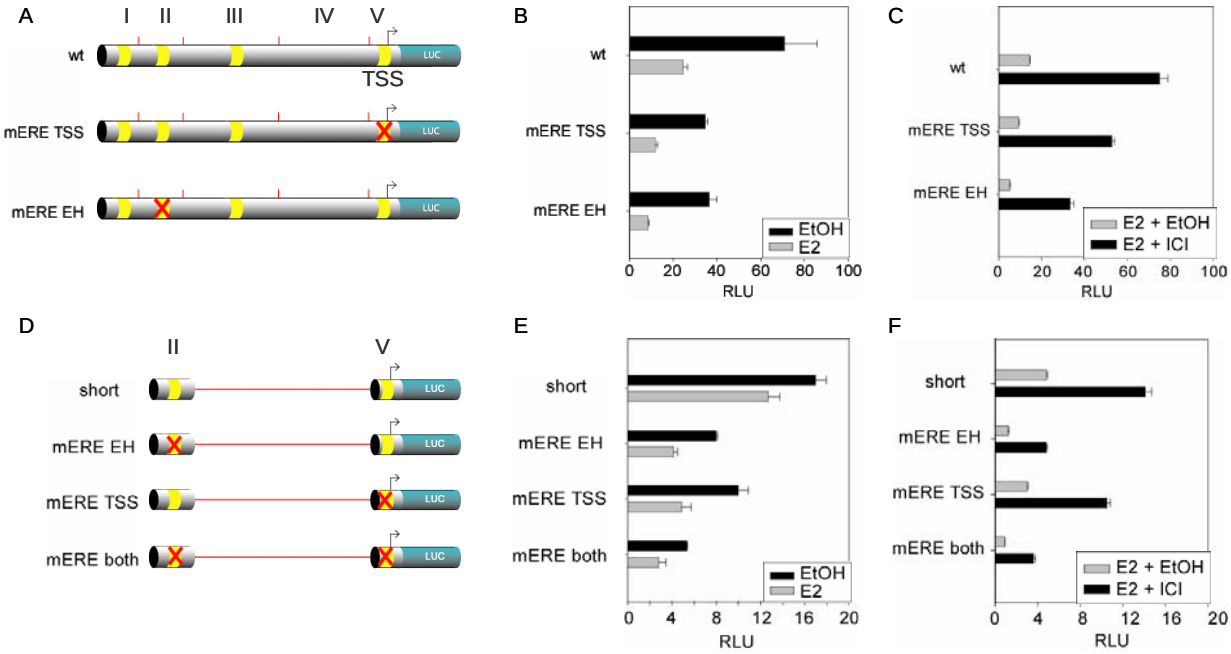
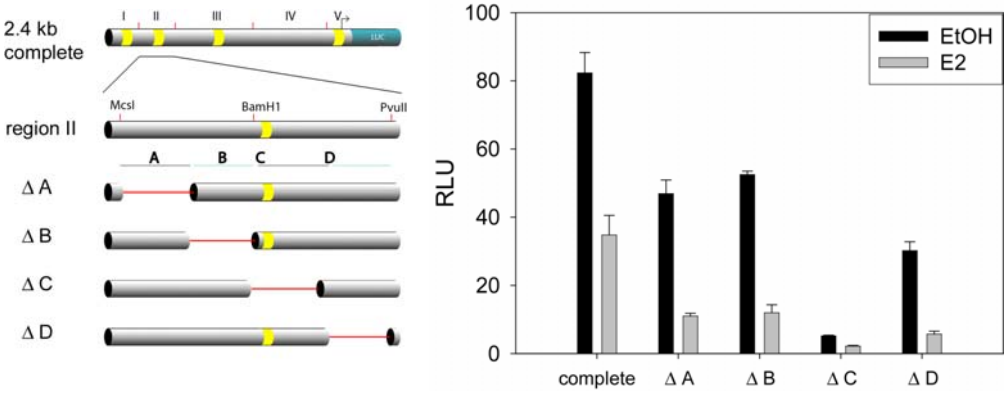


Figure R10. Mutation of EREs located in EH or at TSS affects expression levels but not regulation. A & D. Schematic display of reporter constructs. B & E. Luciferase assay. MCF7 cells have been cultured in stripped medium for 2 days before prior to treatment with either vehicle (EtOH) or 10^{-8} M E2 and transfection with reporter constructs containing the BASE promoter with disrupted EREs in the enhancer region or at the TSS. Firefly luciferase activity was determined 24 hours after transfection and normalized to Renilla activity. For C & F cells were maintained in normal medium containing E2 and treated with either EtOH or ICI_{182780}.

5.5 BASE expression and regulation can be separated

The identified enhancer region encompasses about 600 bp which are essential for regulation. To better define the important region, reporter constructs each omitting only about 150 bp were generated and tested for their activation potential and hormone sensitivity (Fig. R11 A). Deletion of the region between -1990 and -1835 (segment C) reduced the expression levels to the same extent as the deletion of the whole enhancer, indicating important binding motifs in this region. Absence of the segments A, B and D had less impact. In the subsequent step, segment C was divided into 3 subparts, each 50 bp long. Corresponding reporter constructs were generated and again tested in MCF7 cells (Fig. R11 B). The result surprisingly showed that omitting only 50 bp (C2) reduced transcriptional activity dramatically, while again not abolishing the regulation. Therefore, BASE gene expression and gene regulation in response to hormone can be separated. These 50 bp were further investigated regarding putative transcription sites located within this region.

A Dividing the 600 bp enhancer in 150 bp segments



B Omission of only 50 bp (C2) significantly reduces BASE expression

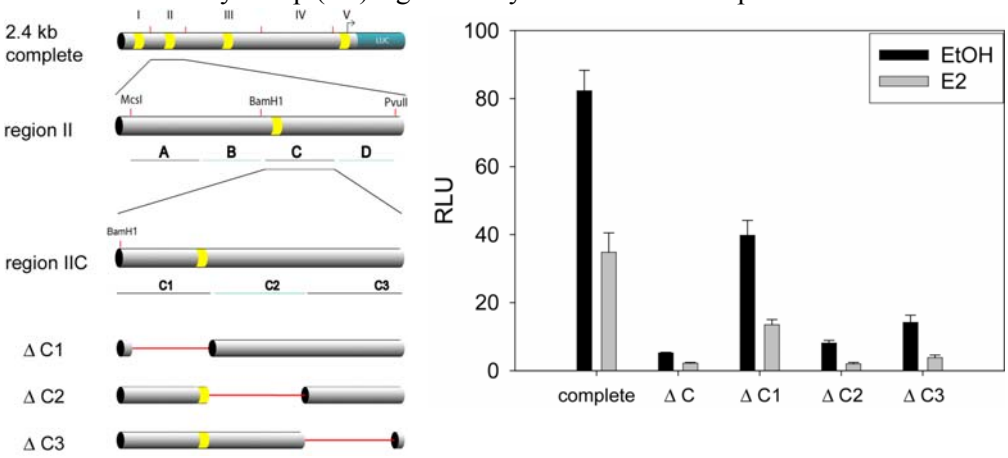


Figure R11. Promoter deletion analysis.
MCF7 cells were stripped for 2 days, then treated with either EtOH or E2 and transfected with a series of luciferase reporter constructs omitting different segments of the BASE promoter. Firefly luciferase activity was determined 24 hours later and normalized to Renilla luciferase activity. A. Deletion of 150 bp (C) is sufficient to reduce expression significantly. B. Deletion of an only 50bp segment (C2) reduced BASE expression to about 12 % of the complete construct.

6. ERα can bind the enhancer *in vitro* and *in vivo*

All three regions (segments I, II (enhancer) and V) involved in mediating the hormone response contain bioinformatically predicted EREs. Since the enhancer region is required for hormone response, the ERE in the 600 bp enhancer region was the most promising candidate. To test whether ERα can bind to this region, electromobility shift assays (EMSAs) were performed, using *in vitro* translated ERα and [32P] labeled 50 bp oligos containing either the C1 segment, C2 segment or pS2 promoter sequence (Fig. R12 B). Binding of ERα was observed for the 50 bp of the pS2 promoter containing the ERE which functioned as positive control. In the C1 region, which contains a predicted ERE, only very weak ERα binding could be detected. To confirm the observed weak binding of ERα to the C1 region a competition assay has been carried out. Oligos containing a perfect ERE were [32P] labeled and incubated with *in vitro* translated ERα while different cold oligos were used for competition (Fig. R12 C). Only the perfect ERE and the oligos covering region C1+C2 could compete for ERα binding, therefore confirming the ability of ERα to bind to the BASE promoter in the enhancer region.

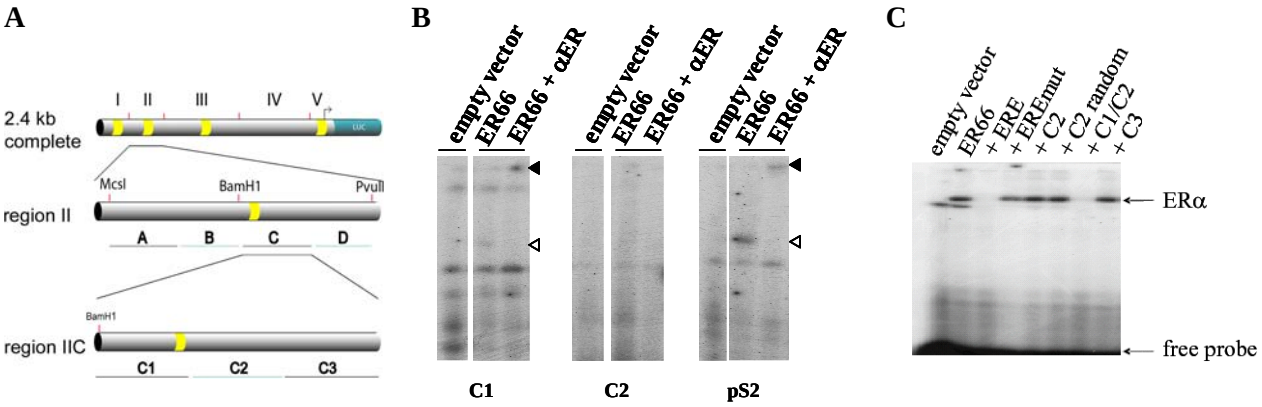


Figure R12. ERα can associate with BASE promoter *in vitro*.
EMSAs were conducted by incubating recombinant human ERα with [32P] labeled oligos and the corresponding antibody (◁ protein bound, ◀ corresponding supershift). A. ERα can bind to the pS2 promoter (positive control) and binds in the C1 region of the BASE promoter. B. Competition assay. Indicated oligos were tested for their ability to compete for ERα binding with the perfect ERE.

Association of ERα with the BASE enhancer was also confirmed *in vivo* by ChIP-on-Chip analysis (Fig. R13). Chromatin from MCF7 cells grown in normal media was applied to the ChIP procedure using an ERα-antibody. The analysis, including fluorescent labeled labelling with Cy3 (input) and Cy5 (immunoprecipitated material), and hybridization to custom made promoter array, was carried out by Agilent Technologies (Canada).

In the promoter region, ERα binding was observed in vicinity to the predicted ERα binding site in the identified enhancer region only. Although the enrichment is low (only about 2.7 fold) the extreme proximity of the probe with the highest enrichment and the predicted ERE strongly support the significance of this finding. Furthermore, preliminary conventional ChIP experiments also show ERα

association with the BASE promoter in this region (data not shown). However, whether ERα binds to the predicted ERE or whether it is recruited through protein-protein interaction remains to be determined. As a conclusion, ERα can bind to the BASE promoter in the enhancer region *in vitro* and *in vivo*.

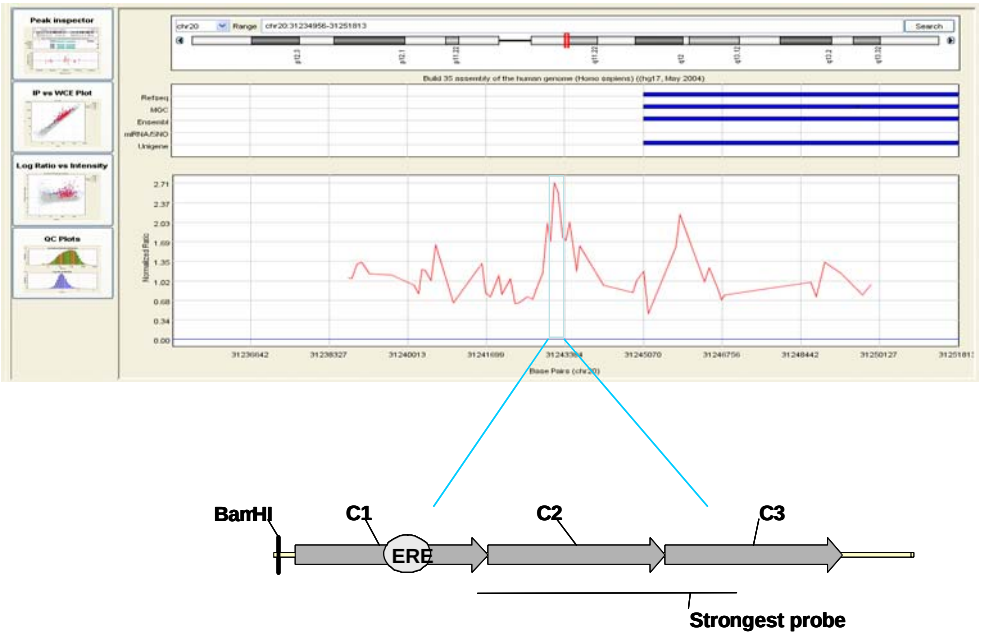


Figure R13. ERα can bind BASE promoter *in vivo* - ChIP-on-Chip analysis (in collaboration with Agilent, Canada).
MCF7 cells were crosslinked and submitted to the ChIP procedure using ERα antibody followed by hybridization on a custom made promoter Microarray. The fold enrichment of immunoprecipitated material vs input samples is displayed for the BASE promoter. The region of the most enriched probe matches the previously identified enhancer region.

7. Bioinformatic analysis of segment C2 & identification of FoxA1 sites important for expression

Sequence analysis of the enhancer using the transcription factor database TransFac (Matys *et al.*, 2003) indicated binding sites for FoxA1, Gata1 and the aryl hydrocarbon receptor (AhR) in the previously identified essential segment C2. To evaluate the potential of these binding sites we used site directed mutagenesis combined with luciferase reporter assays. Mutation of the AhR and the GATA1 site had mild effects on the expression but not on the estrogen-dependent regulation of BASE similar to the effect caused by disruption of the ERE. In contrast, disruption of the predicted FoxA1 binding site strongly reduced the expression of the BASE gene (Fig. R14). Thus, FoxA1 might have an essential role in BASE expression while the repressive role of estrogen was not affected.

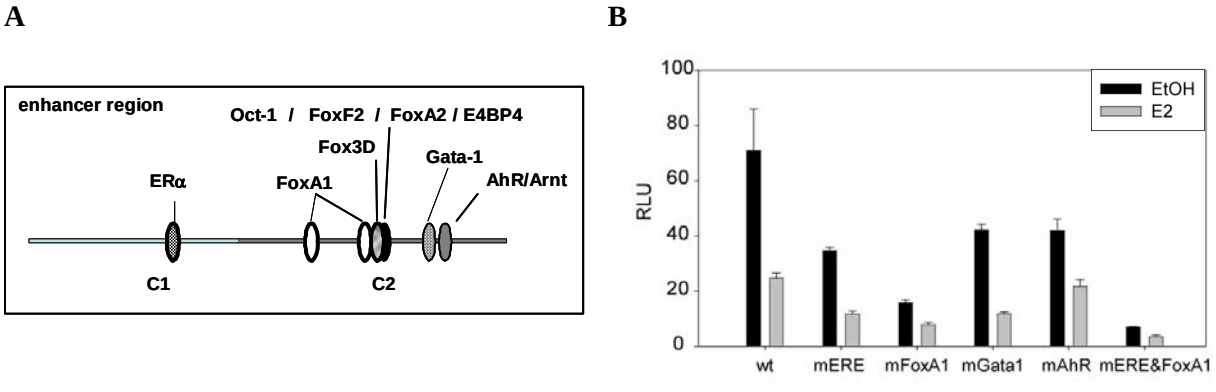


Figure R14. FoxA1 sites are important for BASE expression.
A. Schematic view of the promoter segments C1 and C2 of the enhancer region with putative transcription factor binding sites. B. Luciferase assay in MCF7. Luciferase reporter constructs containing mutations in different transcription factor binding sites were tested for their activity after treatment with either vehicle (EtOH) or 10^{-8} M E2.

8. FoxA1 is essential for BASE expression

ERE and FoxA1 binding sites located in close proximity are a common motif found in about 12% of direct ERα target gene promoters. For this subset, FoxA1 is required for recruitment of ERα (Laganiere *et al*., 2005). To evaluate the importance of FoxA1 in BASE expression several approaches have been undertaken.

To confirm the ability of FoxA1 to bind in the BASE enhancer regions, EMSAs were performed, using again [32P] labeled 50 bp oligos containing either the C1 segment, C2 segment or pS2 promoter sequence. In vitro translated FoxA1 was able to bind to the C2 oligo (containing the BASE FoxA1 binding sites) and could be supershifted with the corresponding antibody (Fig. R15 A). Preliminary data from ChIP experiments indicate association of FoxA1 with the BASE promoter *in vivo* (data not shown). These findings confirm the bioinformatic prediction of a FoxA1 binding site within C2.

If FoxA1 is important for BASE expression, then it should be present in cells lines that express BASE. Therefore, as a next step, expression of FoxA1 was tested in different cell lines at mRNA (Fig. R15 B) and protein level (Fig. R15 C). Using quantitative RT-PCR, high FoxA1 mRNA levels were detected in the ERα-positive breast cancer cell lines MCF7, ZR-75 and T47D while in the ERα-negative breast cancer cell lines SKBR3 and MDA-MB-321 transcript levels were lower or not detectable. Low levels were also detected in HeLa and HepG2 cells. Presence of the FoxA1 protein was confirmed for the ERα-positive cell lines as well as for HeLa and HepG2 cells. For SKBR3, detection of FoxA1 protein depended on the antibody used. Therefore, the FoxA1 expression pattern underlines the importance of FoxA1 as a key factor for BASE expression, since FoxA1 protein was present in all breast cancer cell lines where BASE is expressed. However, FoxA1 itself is not sufficient as HepG2 and HeLa do not express BASE. It implies the requirement of additional factors that are cell specific.

To further analyse the contribution of FoxA1 to BASE basal and hormone-dependent expression overexpression and siRNA experiments have been conducted. While overexpression of FoxA1 protein in the FoxA1-positive cell line MCF7 had no effect (data not shown), the knockdown using siRNA against FoxA1 dramatically reduced BASE expression (Fig. R15 D). FoxA1 siRNA reduced transcript levels of FoxA1 to about 40 %. mRNA levels of BASE were dramatically decreased (about 90 %). These results strongly support a role of FoxA1 in BASE gene expression at least in cell lines that express FoxA1 protein.

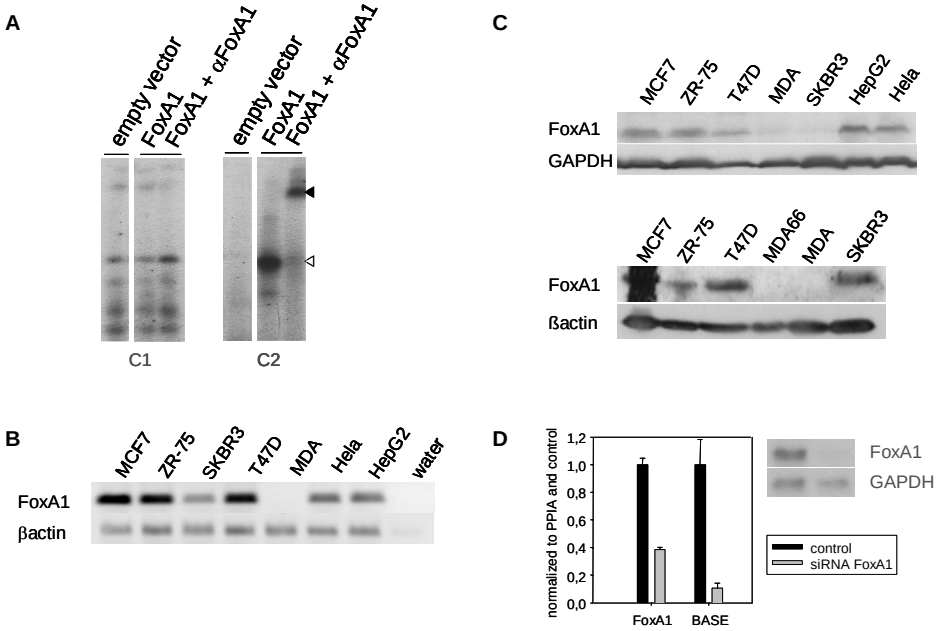


Figure R15. FoxA1 is important for BASE expression.
A. FoxA1 can bind to the C2 BASE promoter segment. EMSAs using [32P] labeled oligos and *in vitro* translated FoxA1 protein. The observed complex (◁) could be supershifted with an α-FoxA1 antibody (T-20, Santa Cruz) (◀). B. Detection of FoxA1 mRNA in different cell lines using qRT-PCR. ßactin levels are shown as loading control. C. FoxA1 protein levels detected by Western Blotting with FoxA1 antibody (upper part, M01, Abnova Corporation; lower part, T-20, Santa Cruz). D. Knockdown of FoxA1 using siRNA leads to reduction of BASE mRNA levels. MCF7 cells were reverse transfected with siRNA directed against FoxA1 (Dharmacon) and RNA isolation was performed 24 hours later. cDNA was generated and mRNA levels of FoxA1 and BASE were analysed with qPCR and normalized to PPIA transcript levels. Western blot analysis was used to test the efficiency of FoxA1 knockdown. GAPDH was used as loading control.

9. Bioinformatic analysis of the BASE promoter using MatInspector

The FoxA1 binding motif in the enhancer region plays a key role in BASE expression. However, although FoxA1 can bind to this motif, the data obtained so far are not conclusive whether FoxA1 is the key factor for high BASE expression. To assess, if other transcription factors could also bind at this site, the sequence of segment C was re-analysed using the further developed programme MatInspector (Cartharius *et al.*, 2005). This analysis revealed that the mutation of the previously identified FoxA1 site also affected putative binding sites of Oct-1 (octamer-binding transcription factor 1 (Pou2F1), NFIL3 (nuclear factor, interleukin 3-regulated, E4BP4), and IRF-3 (interferon regulatory factor 3).

Expression of Oct-1 and NFIL3 was analysed in different cell lines using RT-PCR (Fig. R16 A) and Western blot (Fig. R16 B). Ubiquitous expression was observed for Oct-1, although relative levels of mRNA did not correlate with protein levels. The breast cancer cell lines MCF7 and T47D showed the highest Oct-1 protein levels. NFIL3 mRNA was found only at very low levels. This is in agreement with the microarray data which also indicate low transcript levels. Interestingly, the NFIL3 protein seems to be modified in the MDA46, MDA66 and the non-breast cancer cells HeLa and HepG2 – all cell lines which do not express BASE. However, expression or levels of Oct-1 and NFIL3 did not correlate with BASE expression.

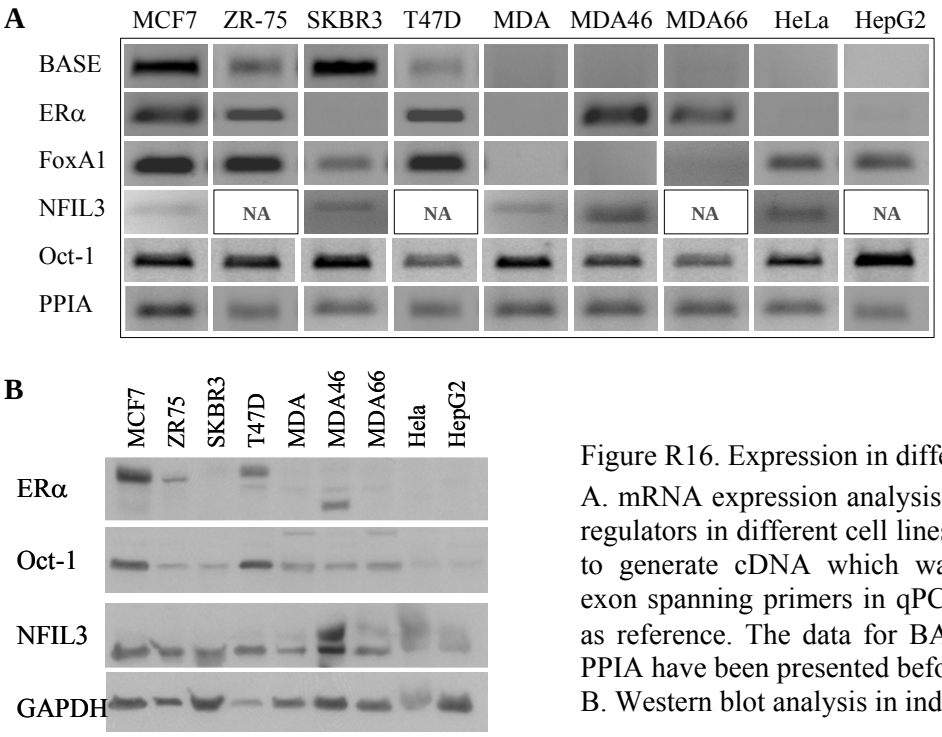


Figure R16. Expression in different cell lines.
A. mRNA expression analysis of BASE and putative regulators in different cell lines. Total RNA was used to generate cDNA which was then analysed with exon spanning primers in qPCR. PPIA was included as reference. The data for BASE, ERα, FoxA1 and PPIA have been presented before (Fig. R5, R15).
B. Western blot analysis in indicated cell lines.

10. Validation of key roles for FoxA1 and ERα in BASE expression and regulation

Based on the results presented distinct roles have been hypothesized for FoxA1 and ERα in BASE expression and hormone-dependent regulation. A possible role for Oct-1 was also suggested. To confirm these, siRNA experiments were performed in MCF7 cells in the presence and absence of hormone. The efficiency of the knockdown was controlled by Western blot, if possible, and RT-PCR. Application of siRNAs directed against FoxA1, ERα, and Oct-1, either alone or in combination significantly reduced the transcript and protein levels of their targets (no Western blot data for Oct-1 protein).

FoxA1 siRNA reduced FoxA1 transcripts to about 40 % and also affected Oct-1 and ERα levels. FoxA1 protein was significantly less abundant. The strongest impact at the transcript level was observed on BASE which expression dropped to almost undetectable levels. In contrast to the literature (Laganiere *et al*., 2005), pS2 gene expression and regulation by E2 was not affected.

As expected, ERα siRNA decreased ERα mRNA levels to about 16 % and also reduced pS2 transcript levels. Two findings were surprising: firstly, induction of pS2 by E2 was not affected, and secondly, Oct-1 mRNA levels were decreased to about 60 %. FoxA1 mRNA levels remained unchanged. As predicted by the hypothesis, that ERα is mediating BASE repression in presence of E2, lower levels of ERα led to release of BASE repression by E2. Remarkably, in the Western blot analysis ERα-antibody detected a protein at about 40 kDa only after treatment with E2. Whether the detected protein resembles an isoform or a degradation product of ERα remains to be analysed. Furthermore, it is unknown if this protein plays a role in BASE repression.

Knockdown of Oct-1 had no effect on FoxA1, ERα or pS2 transcript levels and regulation. However, BASE mRNA levels dropped to about 60 %.

Combination of FoxA1 siRNA with siRNA directed against ERα or Oct-1 did not reveal new insides.

In conclusion, knockdown of FoxA1 protein abolished BASE expression while knockdown of ERα did not alter expression of BASE but significantly reduced E2-induced repression. These results confirm that FoxA1 is essential for BASE expression while E2-mediated repression is dependent upon ERα.

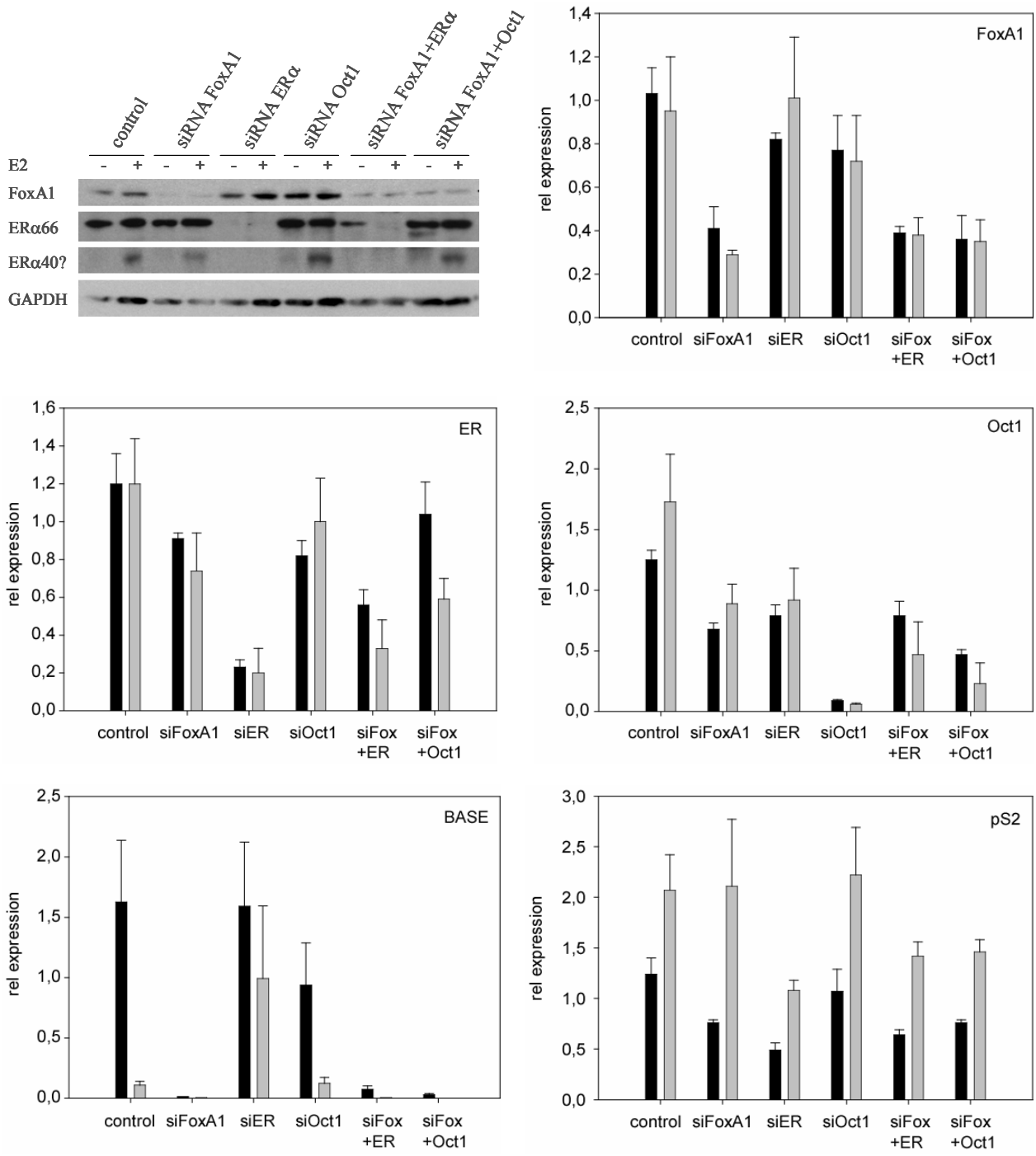


Figure R17. BASE expression depends on FoxA1 while repression in response to E2 is mediated by ERα. MCF7 cells were stripped for 36 hours and then reverse transfected with indicated siRNAs. Cells were then treated with either EtOH (■) or 10^{-8} M E2 (▭). Total RNA and cDNA were prepared 24 hours later. Gene expression was determined by qPCR and normalized to PPIA expression. Efficiency of knockdown was also analysed by Western blot.

11. Further putative factors involved in BASE regulation

After identification of regions I, II and V as crucial segments for BASE regulation in response to E2 and ICI_{182780}, these sequences were bioinformatically analysed for common motifs. Using MatInspector software only 3 transcription factor binding sites were found to be present in all three segments: INSM1 (Zinc finger protein insulinoma-associated 1, IA-1), ZNF219 (Zinc finger protein 219) and EF4 (Fig. R18).

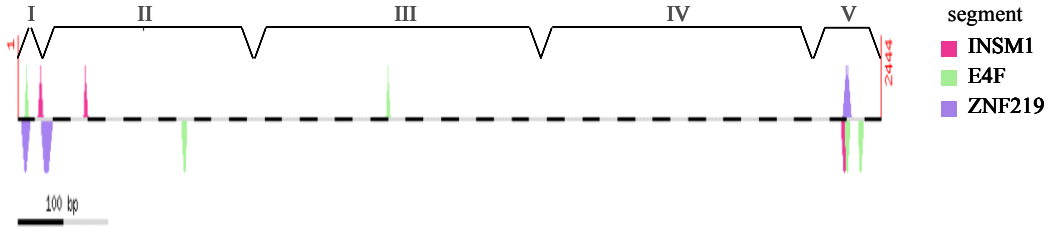


Figure R18. Schematic view of the BASE promoter (2.4 kb) with the putative binding sites of INSM1, E4F and ZNF219 predicted by MatInspector (Genomatix, Munich, Germany). These are the only three transcription factors that have predicted binding sites in region I, II and V. The different segments used for previous truncation studies are indicated (I – V).

E4F is an ubiquitinous expressed transcription factor which is E2-induced and ICI_{182780}-repressed in vascular smooth muscle cells (VSMC; Nakamura *et al*., 2004). Nevertheless, in microarray analysis performed in MCF7 cells in our laboratory E4F was not detectable and examination of mRNA levels using qPCR showed a slight increase only in one out of 3 biological replicates after 24 hour treatment with estradiol (Fig. R19). The Zinc finger protein 219 was slightly upregulated by E2 in the microarray analysis of MCF7 cells, but this could not be confirmed with RT-PCR (Fig. R19).

The INSM1 gene, also called Zinc finger protein insulinoma-associated 1 (IA-1), showed very low expression in the microarray and transcript levels were not modified in presence of hormone. However, a time course analysing INSM1 gene expression after hormone treatment a moderate increase of about 2.5 fold was observed between 6 and 12 hours (Fig. R19 A). To further investigate a possible role of INSM1 in BASE regulation, the expression of INSM1 at the transcript and protein level were tested (Fig. R19 A and B). mRNA abundance and protein levels did not correlate. While in ZR-75 high transcript levels were detected the protein level was lower than in T47D cells where the mRNA was less abundant. This discrepancy could be due to varying mRNA and protein half live or differences in translation efficiency. Notably, in SKBR3 in which BASE expression is not repressed after hormone treatment the INSM1 mRNA level was very low. And even more important the INSM1 protein was undetectable.

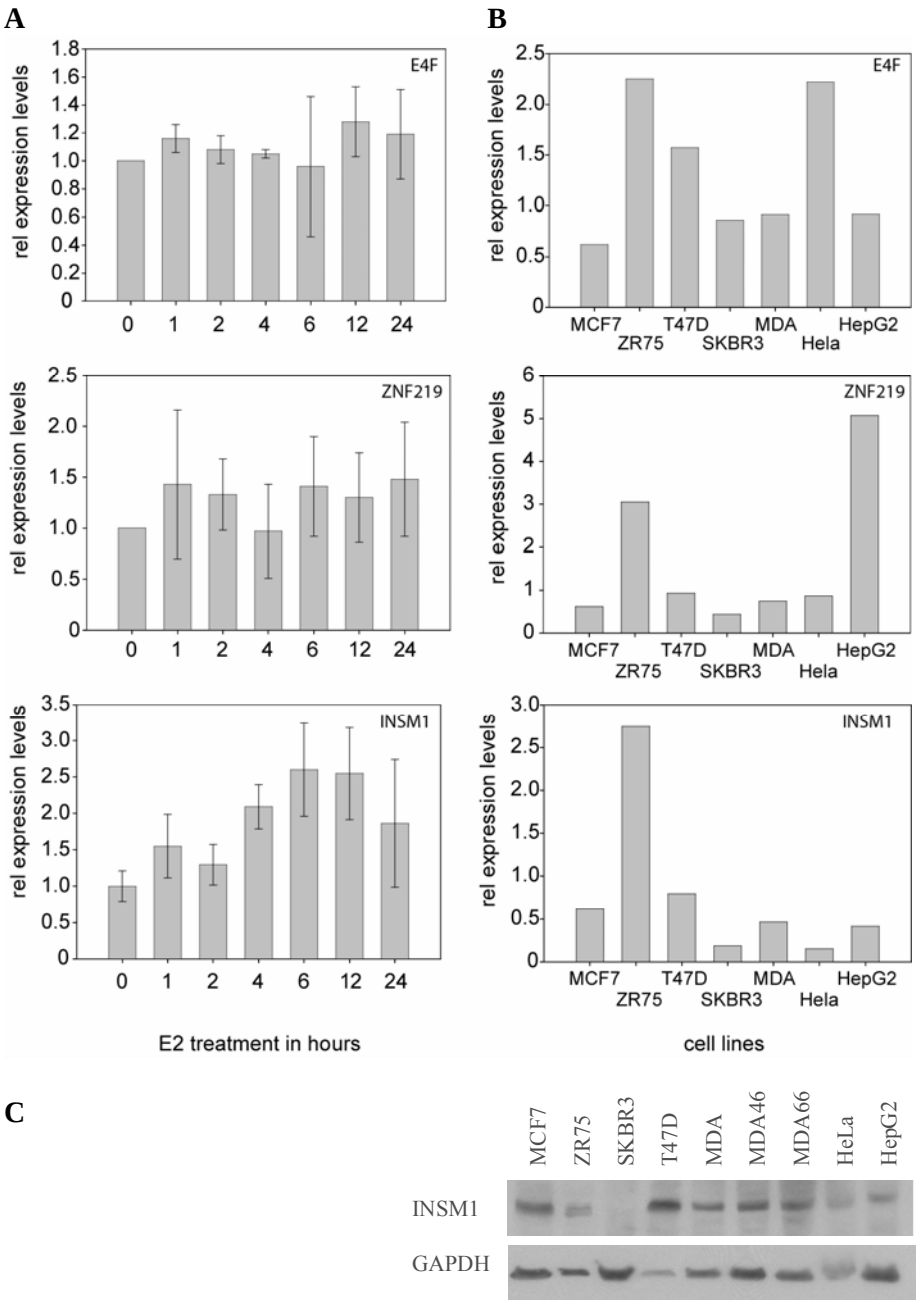


Figure R19. Expression analysis of putative BASE regulators.

A. Time course of gene expressions after E2 treatment in MCF7 cells for INSM1, ZNF219 and E4F. MCF7 cells were stripped for 3 days and then treated with E2 for indicated times. cDNA was prepared from 3 biological replicates and expression levels were determined and normalized to PPIA (left column). B. Gene expression levels were analysed in different cell lines and normalized to PPIA (single experiment, right column). C. Western Blot analysis for INSM1 protein in different cell lines.

12. Introduction of ERα in SKBR3 cells allows estrogen-induced regulation

The ERα-negative cell line SKBR3 expresses relatively high levels of BASE but in luciferase reporter assays treatment with neither E2 nor ICI_{182780} changes BASE promoter activity (Fig. R20). If ERα is a key factor for BASE regulation then reintroduction of the receptor into these cells should repress BASE promoter activity in presence of estrogen. Through usage of the different isoforms (ERα66, ERα46) and a DNA-binding mutant it should be possible to assess the receptor feature important for hormone induced repression. Therefore, serum-starved SKBR3 cells were transfected with BASE reporter constructs (complete (2.4 kb), short (II + V), and short dm (with mutated EREs)) and expression constructs for either ERα66, ERα46, or ERα66mut. Cells were treated with either EtOH, E2 or ICI_{182780}. Luciferase activity was determined 24 hours after transfection and normalized to Renilla luciferase activity (Fig. R20).

Co-transfection of full length and truncated ERα resulted in E2-dependent reduction and ICI_{182780} induced BASE promoter activity. Reduction of the promoter length and, even more important, mutation of the EREs reduced expression of BASE but it did not effect repression or induction after treatment. This indicates that direct binding of ERα to the BASE promoter is not necessary for hormone dependent regulation. Support for this hypothesis comes also from the ERα66 DNA binding mutant, which also has the ability to repress BASE in presence of E2. However, in the average of all experiments performed, the repressive potential of the DNA binding mutant is weaker than for the wild type. Also, ICI_{182780}-stimulated induction of BASE expression was not observed in all experiments with the DNA binding mutant. The reduced expression of the "short" and "short dm" reporter constructs is independent of ERα presence (empty vector), indicating that other factors might bind these sites (e.g INSM1) and contribute to BASE expression.

However, a significant difference was observed in the magnitude of regulation through ERα46 and ERα66. Both, E2-dependent repression and ICI_{182780}-induced stimulation are much more pronounced in presence of ERα46 (5 fold increase with ICI_{182780} for ERα46 compared to less then 2 fold for ERα66). Again, the treatment dependent alterations in expression were not dependent on promoter length or intact EREs. Similar transfection experiments were also performed for ERß and ERRγ. ERß had the ability to repress BASE promoter activity in response to E2 while presence of ERRγ had no effect under these conditions (data not shown).

ERα causes E2-induced BASE repression

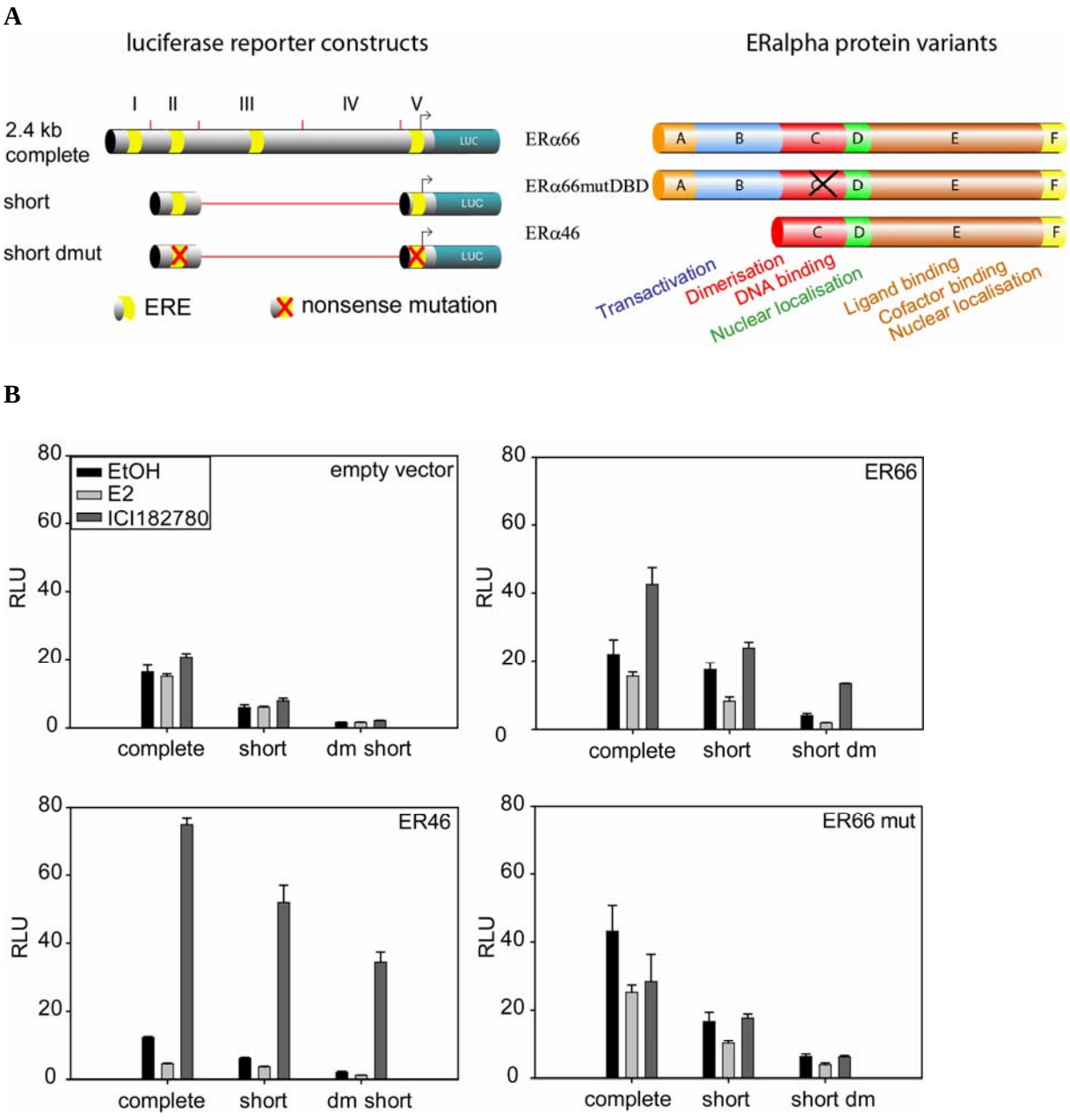


Figure R20. Reintroduction of ERα induces BASE regulation in response to E2 and ICI_{182780}.

BASE reporter constructs (A) were cotransfected with expression constructs for ERα66, ERα46, and ERα66mut into serum-starved SKBR3 cells which were then treated with either vehicle (EtOH), 10^{-8} M E2 or 10^{-6} M ICI_{182780} for 24 hours. Relative luciferase units were obtained by normalization to Renilla luciferase.

13. BASE main transcript is unlikely to be target of NMD

The BASE gene consists of 9 exons (Fig. R21 A). Comparison with the PLUNC gene family which are predicted to share the same gene structure indicated that the BASE gene might contain a point mutation in exon 6 introducing a putative premature stop codon (Bingle *et al.*, 2004b). Alignment of ESTs in the UCSC Genome, however, revealed that most ESTs end with exon 6, showing that the stop codon in exon 6 is not premature (Fig. R21 B). Thus, BASE mRNA is unlikely to be target of the nonsense-mediated decay (NMD) pathway. Since degradation of incorrect mRNAs depends on the translation, blockage of this process with cycloheximide would abolish the degradation leading to increasing transcript levels.

However, BASE exon 3 has two slicing acceptor sites that give rise to 2 splicing variants differing in only 17 bp. The longer variant leads to a frame shift, generating stop codons already in exon 3. This transcript will be degraded and not lead to a protein. Using primers spanning exon 2 and 3 the ratio between these splicing variants was determined and the 'nonsense' transcript was found to be much less abundant than the transcript without frame shift (Fig. R21 C). Therefore, the majority of BASE transcripts are unlikely to be degraded due to NMD.

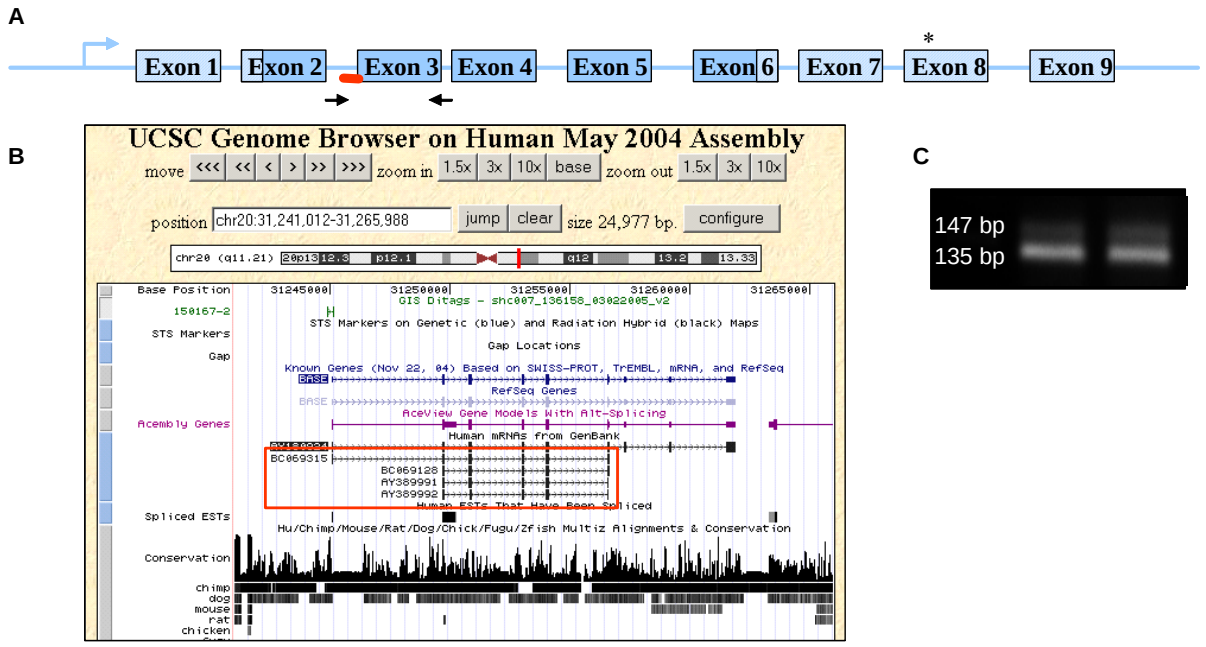


Figure R21. The BASE gene.

A Schematic view. ▭ UTR, ▭ CDS, ▭ splicing alternative, → RT primer, * stop codon in mouse. B. BASE transcripts displayed in the UCSC Genome Browser. Almost all ESTs end with exon 6. C. Alternative splice variants. cDNA from MCF7 cells was analysed with BASE RT primers spanning Exon 2 and Exon 3. Two splice variants of different lenght could be detected.

B. Patient studies

So far, the expression of BASE has only been examined using a commercially available tissue array and selected tumour samples (Egland *et al*., 2003). To test whether BASE expression is restricted to breast tumour tissue, thereby harbouring the potential to act as a breast cancer marker, an additional study comparing normal and tumour tissue was initiated in collaboration with the University College Hospital (Galway, Ireland). All experiments and statistical analysis were performed by David Coyle and Dr. Nicola Miller in the laboratory of Dr. Michael J. Kerin (Dept. Surgery, National University of Ireland, Galway, Ireland).

In total, 50 tumour samples, 4 benign and 4 normal samples were analysed by qPCR. BASE expression was detected in about 50 % of the samples tested (tumour: 26/50, benign 1/4, normal 2/4). The expression levels of BASE in normal tissue were 32 fold lower than in the tumour samples. Later on it turned out that the samples classified as “normal” were either benign tumour samples or were obtained as control biopsies from breast cancer patients and therefore do not represent good negative controls. Consequently, it is crucial that healthy breast tissue samples, e.g. from breast reduction surgery, will be tested. Nevertheless, BASE is expressed in the pathological samples. Through these findings, the potential of BASE as a putative marker for human breast cancer was confirmed and further studies are encouraged to increase the knowledge on the specificity of BASE expression.

C. The BASE protein – localization studies and antibody

A secondary interest of this project was the analysis of the potential of the BASE protein to function as breast cancer marker on the basis of its restricted expression and the prediction that the protein would be secreted (Egland *et al.*, 2003). To evaluate this possibility two peptide antibodies were generated (Eurogentec S.A., Belgium, Fig. R22). To obtain a positive control, the BASE coding sequence was cloned into different bacterial expression vectors (M80, M82, pET22b(+)) allowing expression and folding of the protein in different cell compartments. Since in the mature protein the predicted signal peptide would have been cleaved off, two variants of the BASE coding sequence were cloned – the full length and the short version missing the signal peptide. Production of the recombinant protein was accomplished by the Protein Expression and Purification Core Facility (Ario DeMarco) at the EMBL. The generated antibodies were tested on the recombinant bacterially expressed protein and in different cell lines. While both antibodies are able to detect the purified recombinant protein, specific endogenous BASE expression in cell lines could not be detected (Fig. R22). A commercially available antibody (CIM Antibody Core, Arizona State University) did not show a higher specificity (data not shown).

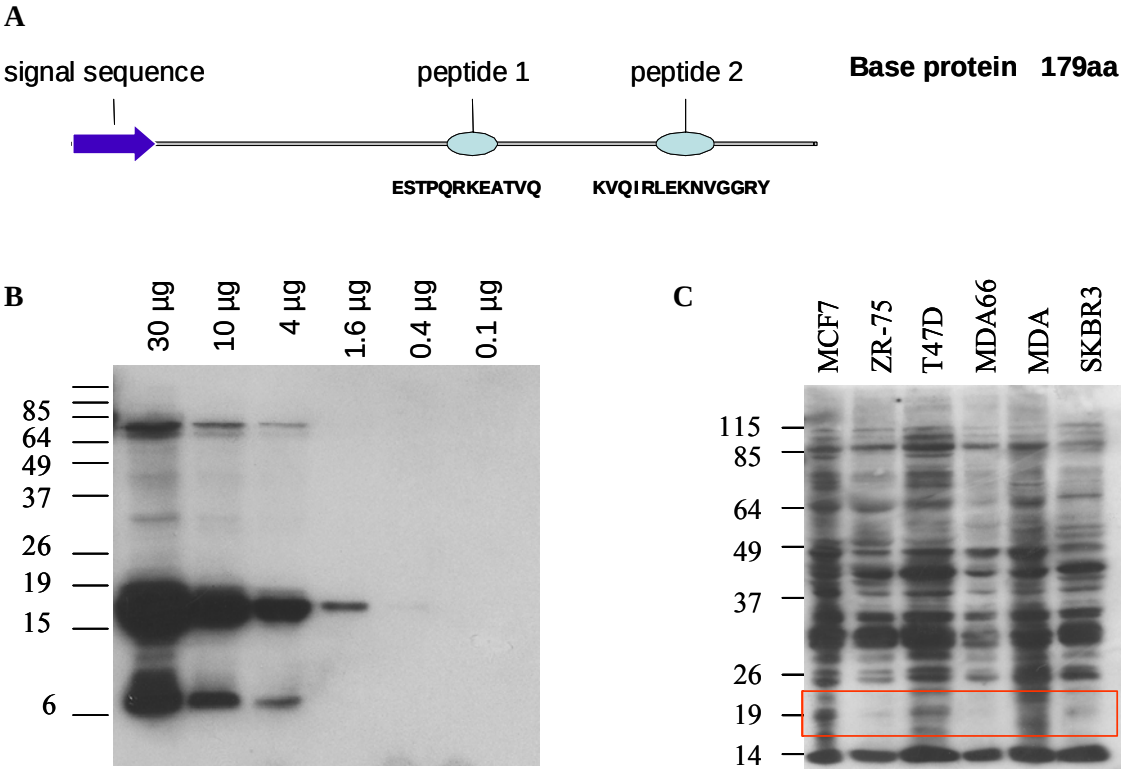


Figure R22. The BASE antibody.

A. Schematic display of the BASE protein and localization of the peptides used for antibody generation by Eurogentec. B Titration of recombinant BASE protein for detection with BASE-antibody (rabbit 3). C. Application of BASE antibody on cell extracts from different cell lines. Antibody from a second rabbit was less sensitive.

Since BASE protein was predicted to be secreted the cell culture medium was also tested for the presence of the protein. But again, no specific band could be detected (data not shown). It is possible that the protein levels are below the detection level of about 1 µg protein as determined with the recombinant protein (Fig. R22). Therefore it is concluded that the antibodies used for these studies are not sensitive enough to detect BASE protein in a complex protein extract.

To analyse the localization of the protein, BASE protein expression constructs for N- and C-terminal GFP fusion proteins were generated and transiently transfected into MCF7 cells. As expected, the C-terminal tagged protein could not be detected, indicating that the signal peptide is cleaved off. The N-terminal GFP-tagged protein could be detected. In MCF7, in co-transfections with RFP-tagged pSRß protein (kindly provided by J.Ellenberg), which localizes to the endoplasmatic reticulum, a co-localization was observed, supporting the theory that the protein might be secreted (Fig. R23). However, in western blot analysis of the culture medium the tagged BASE protein could not be detected (data not shown).

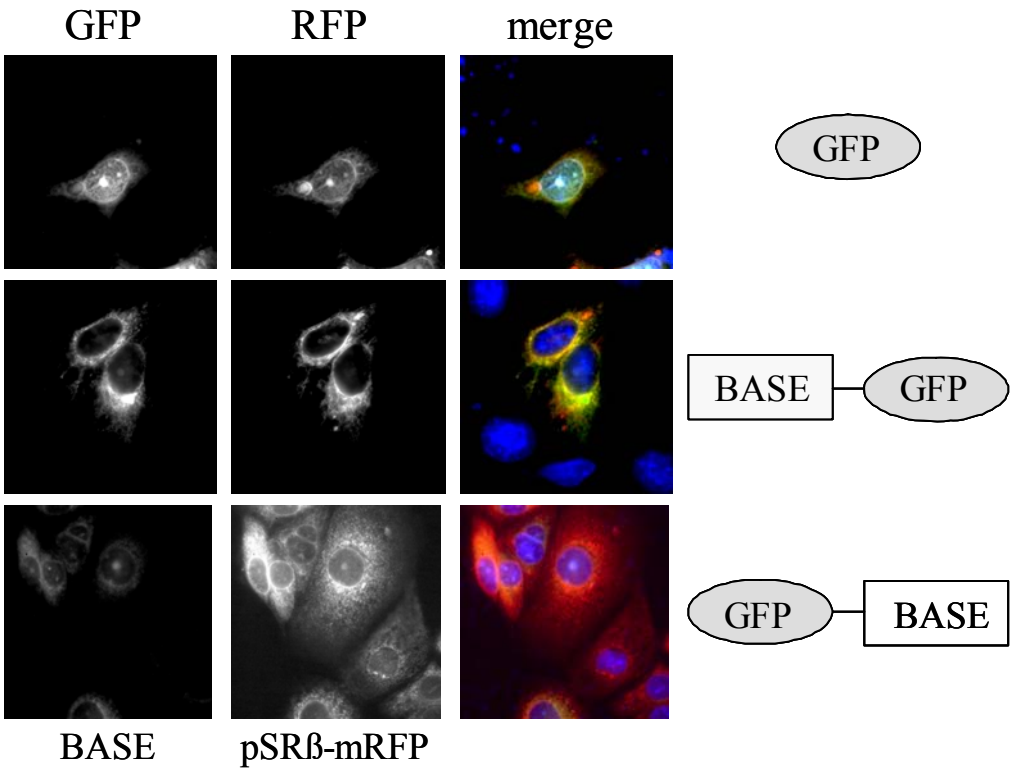


Figure R23. Localization studies of GFP-tagged BASE in MCF7 cells.
Cells were transiently transfected with expression constructs for GFP-tagged and pSRß-mRFP (localizing to the endoplasmatic reticulum).

D. Cathepsin D

1. Stimulation of CTSD expression upon E2 treatment

Cathepsin D is a well known estrogen responsive gene. Nevertheless, before conducting further experiments, the extent of CTSD induction by E2 was examined in the MCF7 cells used in our laboratory. E2 treatment after 3 day serum-starvation resulted in a steady increase of CTSD mRNA, reaching a 2.3 fold increase after 24 hours (Fig. R24). These results are in very good agreement with the microarray analysis performed in our group which shows 2.3 fold increase in transcript levels after estrogen treatment for 24 hours.

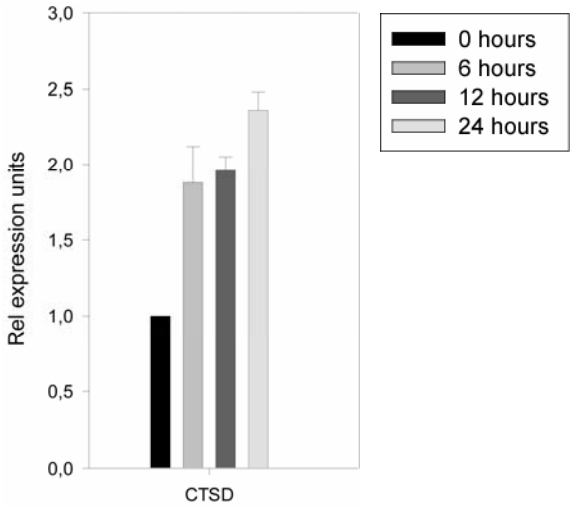


Figure R24. Time course of induction of CTSD expression by E2 in MCF7 cells. MCF7 cells stripped for 3 days were treated with hormone for indicated times. cDNA was prepared and transcript levels were determined by qPCR and normalized to ßactin expression.

2. Distal estrogen receptor binding sites in the CTSD promoter

Functional EREs have also been identified outside the proximal promoter, further upstream (about -9 kb and -33 kb; Bourdeau *et al.*, 2004; Carroll *et al.*, 2006). The ERE 9 kb upstream of the transcription start site differs from the consensus ERE in only 1 base pair (agGGTCAtggTG**g**CCcc). However, it is not known if binding of ERα to this almost perfect ERE can modulate CTSD gene expression. Therefore, luciferase-reporter constructs were generated (Fig. R25 A) containing either the distal ERE (-9446 to -8347, "distal"), the proximal promoter (-753 to +92, "proximal"), or both ("dis+prox"). Because these constructs were based on the pGL3-basic vector which does not contain a eukaryotic promoter, the expression of the "distal" construct was expected to be very low. To increase basal expression and allow better analysis of the regulatory potential, the "distal" sequence was subcloned into pGL3-promoter which contains the SV40 promoter upstream of the luciferase gene ("dis+SV40"). For further evaluation of the regulatory potential of the distal ERE this motif was also disrupted ("distal mut" and "distal mut+SV40").

In transient transfection experiments in MCF7 cells using the above described reporter constructs, the proximal promoter showed basal expression but only a slight induction after 24 hour E2 treatment. In contrast, the distal ERE displayed a high E2 responsiveness, both, without basal promoter or upstream of the SV40 promoter. Combination of distal and proximal regions resulted in increased basal expression and significant E2-dependent induction. This induction was completely abolished by disruption of the ERE motif.

In conclusion, the distal region, 9 kb upstream, confers significant higher estrogen responsiveness than the proximal promoter and is very likely to contribute to the estrogen induced upregulation of the CTSD gene.

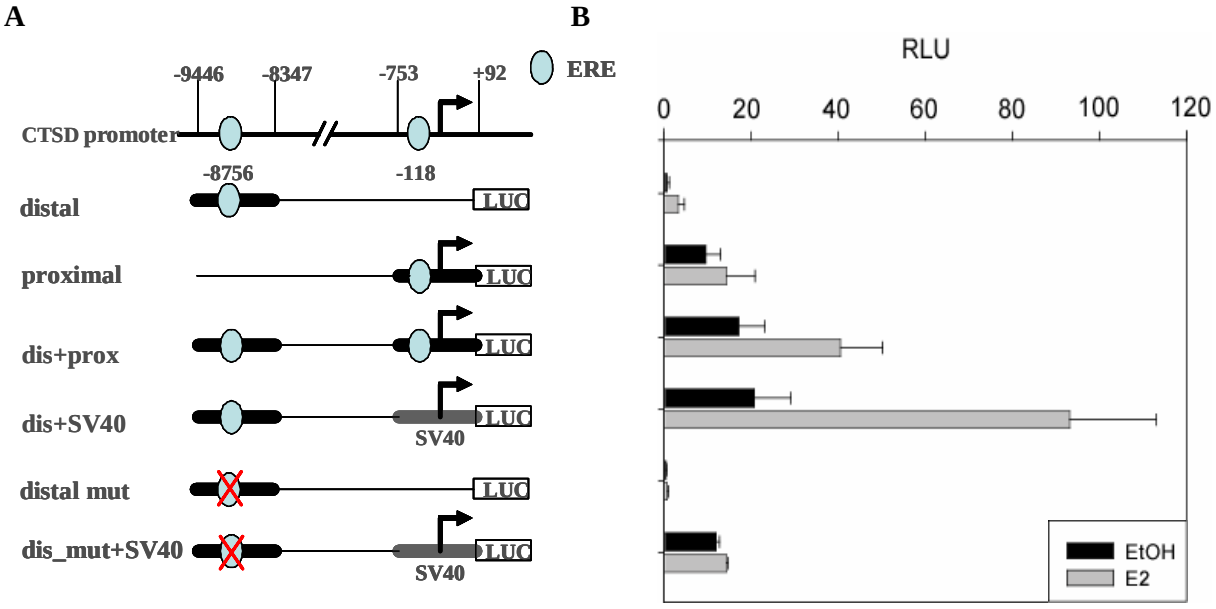


Figure R25. Promoter analysis of CTSD using luciferase reporter constructs. Schematic view of CTSD promoter and generated luciferase reporter constructs. MCF7 cells hormone deprived for 2 days were transfected with the indicated constructs and treated with either EtOH or E2. Luciferase activity was determined 24 hours later and normalized to Renilla luciferase.

3. ERα binds to the distal ERE in a cyclical manner

To test the association of ERα and RNA Polymerase II with the CTSD promoter, ChIP experiments were performed (Fig. R26 A). ERα and PolII are preferentially recruited to the CTSD proximal promoter in presence of E2. ERα binding was also observed at the distal ERE. Interestingly, in presence of E2, PolII was detected as well, indicating that the distal enhancer might be in close proximity to the transcription start site through loop formation. This hypothesis could be tested through Chromatin-Conformation-Capturing (3C).

In kinetic ChIP experiments, ERα cyclically associates with the distal ERE in the CTSD promoter with about 40 min periodicity (Fig. R26 B). The observed kinetics mirror the cycling times reported for the proximal promoter by Shang *et al*. (2000). Similar ERα cycling times have been observed at the pS2 promoter (Metivier *et al*., 2003).

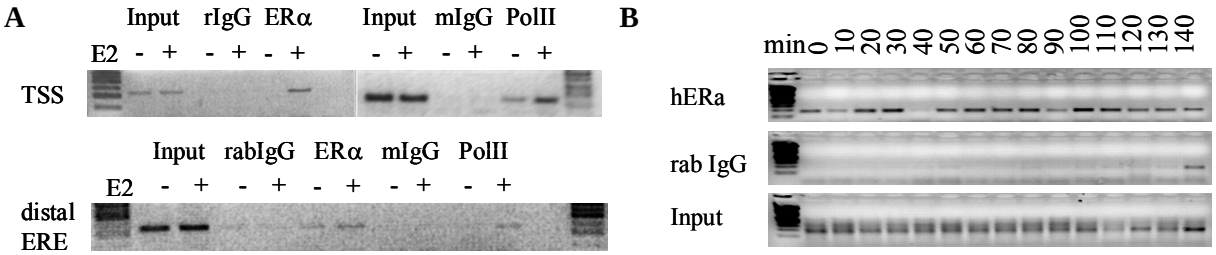


Figure R26. ERα can associate with the distal ERE *in vivo*.

A. Chromatin immunoprecipitation was performed for MCF7 cells (hormone deprived for 3 days and treated with either EtOH or E2 for 90 min). Chromatin was submitted to ChIP procedure using rabbit and mouse IgG, and antibodies against ERα and PolII. (TSS, transcription start site)

B. Cyclical recruitment of ERα to the distal ERE in MCF7 cells. Cells were hormone deprived for 3 days and then treated with E2. Samples were taken every 10 min from 0 to 140 min and then applied to ChIP procedure. Primers enclosing the distal ERE were used for PCR.

E. GREB1 & ELOVL2 – estrogen induced genes

1. Estrogen stimulates GREB1 and ELOVL2 gene expression

GREB1 (gene regulated in breast cancer 1) and ELOVL2 (elongation-of-very-long-chain-fatty-acids (family member 2)) are estrogen-induced genes. Stimulation by estrogen was also observed in our group in both, microarray analysis (performed by Dr. S. Denger) and RT-qPCR (Fig. R27).

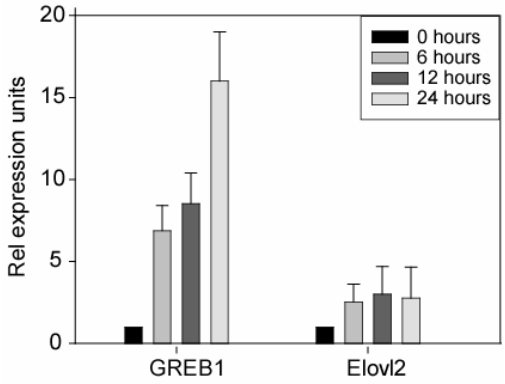


Figure R27. Estrogen induced GREB1 and ELOVL2 gene expression in MCF7 cells. MCF7 cells were hormone deprived for 3 days and treated with estrogen for indicated times. Total RNA was extracted, cDNA was generated and expression levels of GREB1 and ELVOL2 were determined and normalized to PPIA expression.

2. ERα associates with both promoters *in vivo*

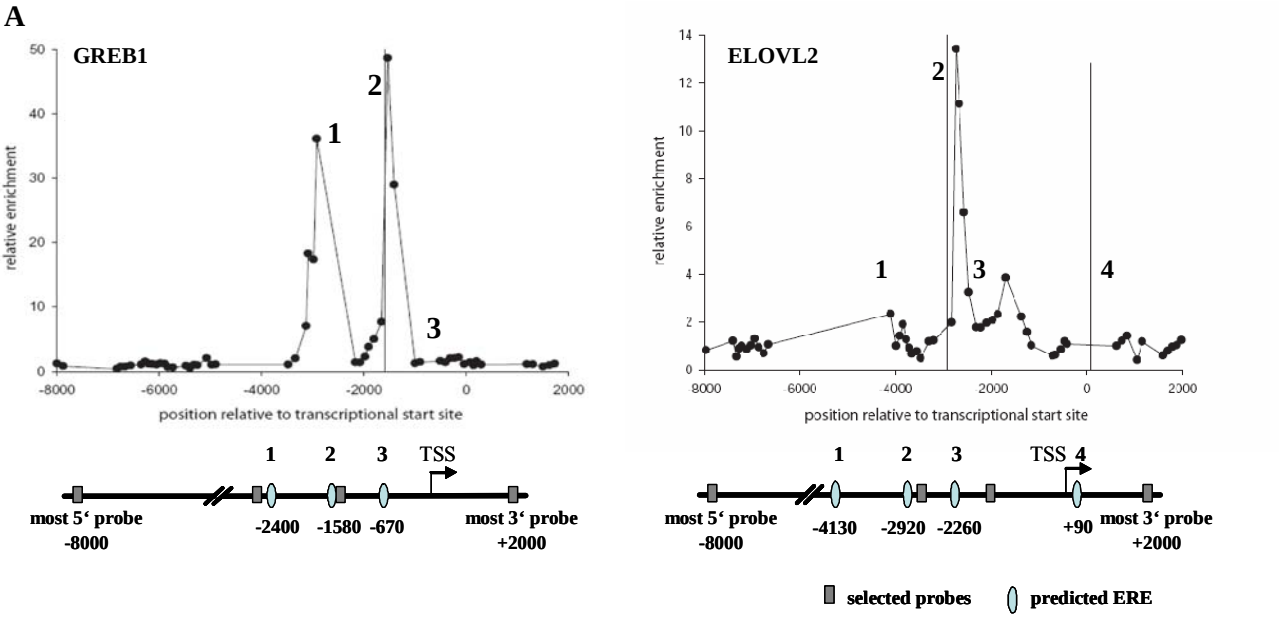
Customized promoter array (Agilent Technologies, Canada) covering the promoter region from -8 kb to 2 kb of selected genes, including GREB1 and ELOVL2, were used to identify estrogen receptor alpha binding sites. The outcome of this analysis is shown in figure R28 A. For the GREB1 promoter two of the identified ERα binding sites could correlate with predicted ERE 1 (-3400) and confirmed ERE 2 (-1560; Bourdeau *et al.*, 2004), respectively. A third predicted ERE (ERE3, -670) is located within a repetitive sequence and therefore not covered by the array.

Bioinformatic analysis of the ELOVL2 promoter identified 4 potential EREs within the 10 kb covered by the array (ERE1: -4130, ERE2: -2920, ERE3: -2260, ERE4: +90). However, significant enrichment was only observed for ERE 2 (14 fold over input) and a region at about -1.8 kb (about 4 fold). ERE1 and ERE4 are again not covered by the array due to their location in repetitive sequences. It is of note, that a large number of EREs are found in these sequences.

To confirm the results obtained by ChIP-on-Chip, conventional chromatin immunoprecipitation has been performed (Fig. R28 B). In agreement with the array, association of ERα with the GREB1 promoter was observed in the region including ERE2. For ERE1 only a very low enrichment was detected, which cannot explain the strong signal obtained by ChIP-on-Chip. This indicates that ERα is recruited to sequences other than the predicted ERE 1. Whether ERα is bound directly or is recruited through protein-protein interactions remains to be determined.

ChIP analysis also confirmed association of ERα with the ELOVL2 promoter in the region of ERE2, while no binding was detected for ERE1 and ERE3. Whether ERα is recruited to the predicted binding site at the transcription start site was not yet examined due to difficulties in primer design. FoxA1 was included in this analysis since ERE and FoxA1 sites are frequently found in close proximity and

important role has been assigned to FoxA1 in ERα-mediated gene regulation (e.g. at the pS2 promoter, (Laganiere *et al.*, 2005; Carroll *et al.*, 2005). While FoxA1 was not present at high levels in the tested regions of the GREB1 promoter, strong recruitment was detected in proximity of ERE2 in the ELOVL2 promoter. Nevertheless, FoxA1 could be recruited in other regions of the GREB1 promoter. Nevertheless, the results obtained so far indicates that GREB1 and ELVOL2 might belong to different subclasses of estrogen-responsive genes.



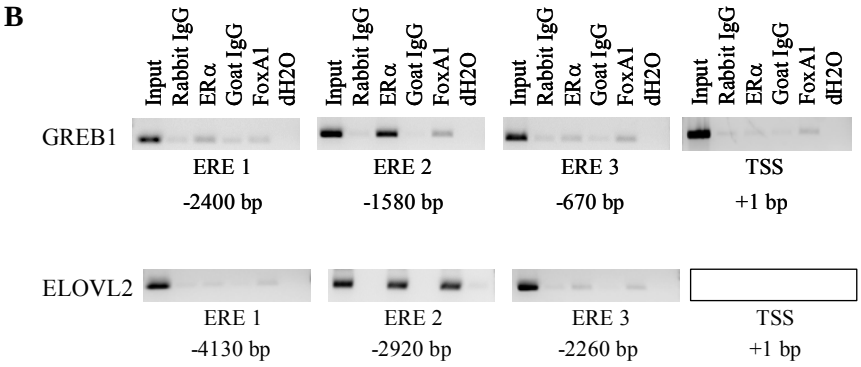


Figure R28. ERα is recruited to GREB1 and ELOVL2 promoter.
A. ERα association with the GREB1 and ELOVL2 promoters in MCF7 cells determined by ChIP-on-Chip analysis (Agilent Technologies, Canada). Predicted EREs are numbered. Vertical lines illustrate the localization of the predicted ERE in relation to the probes. B. Conventional ChIP analysis examining ERα presence at putative ERα binding sites in the promoters.

3. ERE2 is important for estrogen responsiveness of the ELOVL2 promoter

Although ERα is present at the ELOVL2 promoter it is still unclear whether the predicted EREs are the sites of association and if they are important for E2-mediated regulation of ELOVL2 expression. To analyse this, reporter constructs containing either 2.4 kb or 4 kb of the ELOVL2 promoter upstream of the luciferase gene were generated and tested in MCF7 cells (Fig. R29). Consistent with the ChIP and ChIP-on-ChIP results, the first 2.4 kb showed only a weak induction (+1.4 ± 0.3) after 24 hours E2 treatment and no repression with ICI_{182780} (1.1 ± 0.5). In contrast, E2 significantly induced luciferase activity from the 4.4 kb promoter fragment (4.5 ± 0.5) while ICI_{182780} repressed the activity (0.3 ± 0.1).

These results indicate that the first 2.4 kb contain the proximal promoter while the estrogen-responsive motifs are located between 2.4 kb and 4.4 kb. Thus, ELOVL2 is another example for an estrogen-induced gene for which estrogen response is mediated not by the proximal promoter but through regulatory region further upstream. Disruption of the EREs in the longer construct will reveal the contribution of ERE2 to estrogen-induced ELOVL2 expression.

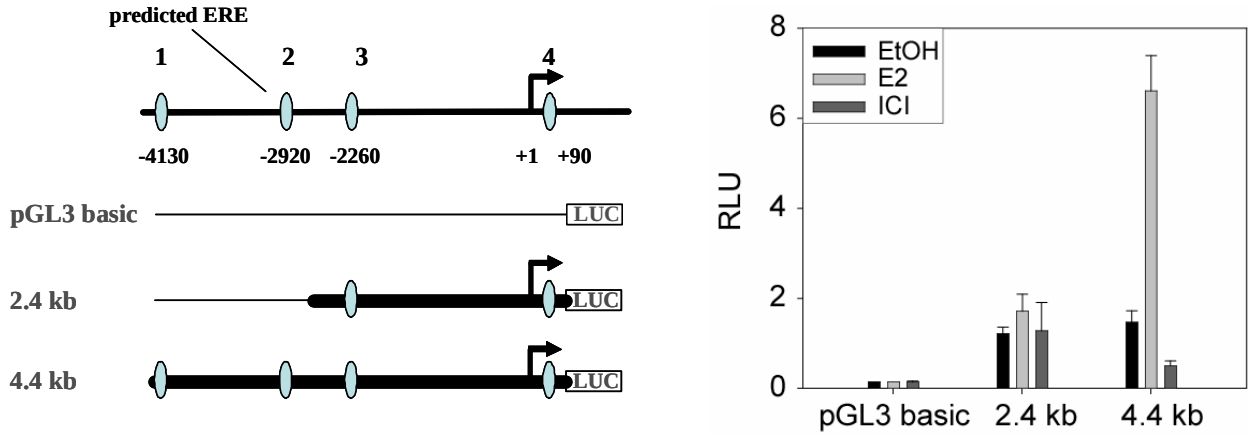


Figure R29. The major estrogen-response is conferred by motifs located between 2 and 4 kb upstream. Schematic display of luciferase reporter constructs containing either 2.4 kb or 4.4 kb of the ELOVL2 promoter. These constructs were tested in MCF7 cells (hormone deprived for 2 days) treated with either EtOH, 10^{-8}M E2, or 10^{-6} M ICI_{182780}. Luciferase activity was determined 24 hours later and normalized to Renilla activity.

DISCUSSION

In women, breast cancer is the most common cancer and accounts for most cancer deaths. The decline in cancer mortality in the last years is mainly due to improvements in breast cancer treatment and early detection. If the tumour is detected at an early stage, the 5-year survival rate increases dramatically. It rises from about 26 % for a distant-stage disease (metastasized in distant organs), to 81 % for regional disease (spread to surrounding tissue) and to 98 % for local-disease (confined to the breast) (Atlanta: American Cancer Society, 2005). Therefore, much attention has been devoted to identification of markers indicating malignant phenotype, metastatic potential and tumour growth. These markers could help to detect and classify tumours in very early stages and select the most promising therapy. Unfortunately, only few tumour markers, including the ERα, HER-2 and CTSD, have been identified so far (reviewed in Murphy *et al*., 2005).

A new potential tumour marker was identified recently - the Breast Cancer and Salivary Gland Expression gene (BASE). The gene, which encodes a putative secreted protein, shows a rather restricted expression to breast cancer cells and salivary gland, ideal for a breast cancer marker (Egland *et al*., 2003).

Our group found BASE as a highly estrogen-repressed gene, indicating that the ERα, a recognized tumour marker, could be involved in BASE expression. This study aimed to understand the mechanisms underlying BASE expression and regulation in breast cancer cells.

1. The BASE promoter

This study identified the core promoter of the BASE gene and a 600 bp regulatory region about 2 kb upstream of the transcription start site. These 600 bp were demonstrated to be necessary but not sufficient for estrogen-induced regulation (Fig. R9). However, fractionation of this area in 150 bp fragments (A to D) abolished the effect on estrogen-mediated repression (Fig. R 11A), indicating that most likely at least two sites in the regulatory region contribute to the hormone response. A strong decrease in expression was observed when only region C was deleted. It is unlikely that these effects are caused by shifts in positioning of the nucleosomes since deletions of 150 bp should not interfere unless a nucleosome positioning signal was affected. And furthermore, absence of the segments A, B and D had significant less impact. Moreover, a similar decrease was observed even if only a small fragment of 50 bp (C2) was omitted in the context of the whole 2.4 kb reporter construct (Fig. R11B). Further characterization of C2 revealed a key role for FoxA1 binding sites as they were essential for BASE gene expression. Binding of FoxA1 to these sites *in vitro* and requirement for FoxA1 for BASE expression was confirmed (Fig. R15A & R17).

2. Distinct roles for FoxA1 and ERα in BASE expression and regulation

Both, FoxA1 and ERα can bind to the BASE promoter in the regulatory region (Fig. R 12 & R15A). Whereas FoxA1 was essential for BASE expression it had no function in regulation in response to estrogen (Fig. R14 & R17). In contrast, presence of ERα was not required for BASE expression as shown by the ERα-negative but BASE-positive cell line SKBR3 (Fig. R5). However, ERα was crucial for estrogen-induced down-regulation (Fig. R17). As a conclusion, basal expression of the BASE gene and its regulation in response to hormone can be separated.

As stated above, ERα has been assigned a key role in estrogen-induced BASE repression. Absence of ERα, either due to gene silencing, treatment with anti-estrogen ICI_{182780}, or siRNA directed against ERα, abrogates repression in presence of hormone (Fig. R2, R3, R17). Binding of ERα to the regulatory region has been confirmed *in vitro* and *in vivo* (EMSA, ChIP, ChIP-on-ChIP, Fig. R12 & R13). However, ERα might not bind at the predicted ERE, as disruption of this motif did not alter repression in response to estrogen (Fig. R10). It is possible that ERα is recruited via protein-protein interaction to a nearby site. Furthermore, an indirect regulation through estrogen-regulated factors is conceivable. The very rapid repression of BASE in presence of estrogen (Fig. R2) argues against requirement of *de novo* protein synthesis. However, activation or inactivation through posttranslational modifications cannot be excluded. In this regard it is of note that estrogen can exert non-genomic activities which include rapid activation of signalling cascades like the MAPK pathway (reviewed in Song and Santen, 2006).

The promoter study and siRNA experiments identified FoxA1 as one factor essential for BASE expression. This was further supported by the presence of FoxA1 protein in all breast cancer cell lines that express BASE (Fig. R16). The only FoxA1-negative cell line tested, MBD-MB-231, is also negative for BASE. Furthermore, stable expression of ERα in this cell line (MDAERα66, MDAERα46) did not lead to BASE expression, showing again that ERα is not required for BASE expression in contrast to FoxA1. However, FoxA1 cannot be the only transcription factor required. Although FoxA1 is present in HeLa and HepG2, no expression of BASE was detected in these cell lines. This result points towards the involvement of another, most likely breast specific or even breast cancer specific factor. Contradictory reports exist regarding FoxA1 regulation by estrogen. Whereas some groups report estrogen-induced down-regulation of FoxA1 (e.g Frasor *et al*., 2003), Langanier *et al*., (2005) observed up-regulation of FoxA1 within 4 hours after estrogen treatment. In the MCF7 cells used in our group, FoxA1 mRNA levels did not change in response to exposure to estrogen (data not shown). Thus, reduction of FoxA1 levels is not the cause for down-regulation of BASE expression in presence of estrogen.

The clear separation of gene expression and hormone-dependent regulation was surprising. Although also expressed in ERα-negative tumours (Doane *et al*., 2006), FoxA1 expression is often associated with expression of ERα (Perou *et al*., 1999; 't Veer *et al*., 2002). Myles Browns group reported that about 50 % of ERα-binding sites are accompanied by Forkhead factor binding sites in close vicinity (Carroll *et al.*, 2005; Carroll *et al.*, 2006), and this applies for estrogen-stimulated and -repressed ERα target genes. Furthermore, in this subset of genes, FoxA1 was required for recruitment of ERα and gene expression in MCF7 cells. pS2 belongs to this reported subset. However, in contrast to the findings reported by Laganiere *et al*. (2005), in this study knockdown of FoxA1 in MCF7 cells had only a mild effect on pS2 expression (Fig. R17). This is in agreement with previous studies from our group where reintroduction of ERα into the ERα- and FoxA1-negative cell line MDA-MB-231 lead to expression and estrogen-dependent regulation of the pS2 gene, which is silenced otherwise (Metivier *et al*., 2004). This effect is not due to induction of FoxA1, which itself is an ERα target gene (Laganiere *et al*., 2005), as confirmed by RT-PCR and Western blot analysis performed on MDA-MB-231 and derived cell lines stably expressing ERα.

3. Other factors binding the FoxA1 motif

In addition to FoxA1 and ERα, several other factors might be involved in BASE expression and regulation. For example, the binding site of Oct-1 (octamer-binding transcription factor 1) overlaps with the FoxA1 motif and is affected by disruption of the FoxA1 motif in mutation studies. Oct-1 might cooperate or compete with FoxA1 for DNA binding and thereby modulate BASE expression.

Oct-1 has an important role in expression of the small breast epithelial mucin gene (SBEM). This gene shares several similarities with BASE including salivary and breast specific expression (Miksicek *et al*., 2002). Although Oct-1 is ubiquitously expressed it has been shown to play a role in regulation of tissue-specific genes (e.g. Dong and Zhao, 2007). Moreover, Oct-1 is involved in repression of the mouse gonadotropin-releasing hormone (GnRH) gene expression by glucocorticoids in cooperation with the glucocorticoid receptor GR (Chandran *et al*., 1999; Cheng *et al*., 2002). Although knockdown of Oct-1 reduced BASE expression (Fig. R17), the role of Oct-1 was not further evaluated due to time limitations. In addition, Oct-1 might not be the only relevant octamer-binding transcription factor involved in BASE expression. Jin *et al*. (1999) found Oct-1 (Pou2F1), Oct-2 (Pou2F2), Oct-3 (POU5F1) and Oct-11 (POU2F3) in the breast cancer cell line MCF7. The embryonic transcription factor Oct-3 was found to be expressed in breast cancer cell lines and tumours, while absent in normal breast tissue. Therefore, extended studies on recruitment of octamer-binding factors to the BASE promoter and there impact on BASE expression should also include Oct-3 and Oct-11.

Another factor which can bind in very close proximity to the FoxA1 motif is NFIL3 (nuclear factor, interleukin 3-regulated, E4BP4). It has been hypothesized that NFIL3 is involved in anti-inflammatory response, regulation of the circadian rhythm, as well as cell survival (Cowell, 2002). Furthermore, NFIL3 has been suggested to play a role in parathyroid hormone- and glucocorticoid induced gene repression (Wallace *et al*., 1997; Ozkurt and Tetradis, 2003). In contrast, at the human Interleukin-3 promoter in T-cells it functions as a transcriptional activator (Zhang *et al*., 1995). NFIL3 was present in all cell lines tested in this study (Fig. R17). However, in cell lines where BASE is not expressed, bands of higher molecular weight were detected. It could be hypothesized that these display posttranslational modifications, which might alter the activity or the DNA binding ability of NFIL3. A more speculative suggestion is that different posttranslational modifications might influence whether NFIL3 functions as activator or repressor of gene expression. NFIL3 can be induced by elevated Ca2+ levels (Nishimura and Tanaka, 2001; Priceman *et al*., 2006) and cAMP (Ozkurt *et al*., 2004). These secondary messengers can be triggered through non-genomic actions of estrogen. This could be a link between NFIL3 and estrogen-mediated repression. Furthermore, NFIL3 (E4BP4) is transcriptionally regulated by GATA-1 (Yu *et al*., 2005) whose transactivation ability can be inhibited by estrogen-activated ERα (Blobel and Orkin, 1996).

4. ERα partners in BASE regulation

Contradictory to the simple model that ERα binds the ERE and recruits corepressors, mutation of predicted and confirmed EREs did not affect the hormone-induced repression (Fig. R10). Therefore, ERα likely cooperates with other factors or even might regulate BASE only indirectly. Three regions in the promoter were important in hormone response – the essential enhancer (region II) and either a small region immediately upstream of the enhancer (region I) or an approximately 200 bp segment including the transcription start site (region V) (Fig. R8, R9, additional results). Although it is not necessary that factors mediating BASE regulation in these segments are identical it was worthwhile to bioinformatically analyse and compare these sequences for common motifs. Only for three transcription factors binding sites were predicted in all three segments: INSM1, E4F, and ZNF219 (Fig. R18).

A promising candidate is INSM1, also called Zinc finger protein insulinoma-associated 1 (IA-1). INSM1 protein was detected in all cell lines tested except for SKBR3 (Fig. R19). In this cell line, BASE is expressed but not repressed in presence of estrogen. This opens the possibility that if BASE is expressed, INSM1 could contribute to estrogen-mediated repression. In keep with this hypothesis, it is noted that INSM1 has been shown to repress the neuroD/beta2 gene in conjunction with cyclinD1, a well known ERα target, by recruiting HDACs (Liu *et al*., 2006). Furthermore, expression of INSM1 is associated with cancer. It is re-expressed in neuroendocrine tumours (Pedersen *et al*., 2006) and

(Taniwaki *et al*., 2006) showed that the expression is altered in small-cell lung cancer (SCLC). It is of note, that the predicted INSM1 binding site slightly overlaps with the confirmed ERE in the enhancer region of the BASE promoter. An overlap of binding sites also exists for INSM1 and ZNF219. The localization of these motifs opens the possibility for an "exchange/interactive" mechanism. A conceivable approach to evaluate whether INSM1 is involved in BASE regulation would be re-expression of INSM1 in SKBR3 cells and analysis of BASE expression in presence and absence of E2. Alternatively, since transient expression of ERα is sufficient to induce repression of BASE by estrogen, ERα could be expressed in SKBR3 cells and expression and regulation of both, INSM1 and BASE could be monitored.

Another factor of potential interest is E4F, an ubiquitously expressed transcription factor which is synthesized in two variants, the full-length 120 kDa protein (p120E4F) and a 50 kDA NH2-terminal fragment (p50E4F) created by proteolytic cleavage. Both variants recognize the same DNA motifs *in vitro*, but regulate gene expression differentially *in vivo* (Fernandes and Rooney, 1997). While p50E4F transactivates adenoviral E4 gene expression (Raychaudhuri *et al*., 1987; Raychaudhuri *et al*., 1989), p120E4F likely plays a role in mammalian cell cycle control (Fajas *et al*., 2001). Interestingly, E4F was identified as highly estrogen-induced and ICI_{182780}-repressed gene in vascular smooth muscle cells (VSMC; Nakamura *et al*., 2004). However, no response to hormone was observed in MCF7 cells in our laboratory.

Not much information is available for the Zinc finger protein 219, which was isolated and characterized by H. Maeda (Sakai *et al*., 2000). The same group identified the consensus binding site (CCCCC). Furthermore, they showed that ZNF219 functions as a transcriptional repressor of the HMGN1 promoter (Sakai *et al*., 2003). Thus, both factors could have the potential to mediate estrogen-induced repression. However, a possible role for E4F and ZNF219 in BASE regulation was not addressed in this study due to time limitations.

5. ERα features important for BASE regulation

Expression of BASE in the ERα-negative cell line SKBR3 presented an opportunity to dissect the ERα domains required for BASE repression. In absence of ERα and presence of E2 no repression was observed. Introduction of estrogen receptor alpha variants in the ERα-negative cell line SKBR3 confirmed the necessity of ERα for BASE repression. Interestingly, a significant difference was observed between the potential of the truncated ERα46 and the full-length receptor ERα66 in BASE regulation. Estrogen-stimulated repression as well as ICI_{182780}-mediated de-repression were more pronounced for ERα46. A possible explanation for these results is that ICI_{182780} renders both receptor isoforms inactive, but whereas hERα66 is rapidly degraded, ERα46 stability is only slightly decreased. Also, in presence of estrogen, levels of the full-length ERα decrease while levels of ERα46 even increase (our data, Valley *et al*., 2005). Therefore, when both isoforms are expressed, the ratio between the two isoforms is altered in response to estrogen or anti-estrogen. At least in absence of estrogen, ERα46 and ERα66 associate with distinct cofactors (Metivier *et al.*, 2004). Thus, in the case that BASE repression would be based on sequestering of required factors, the ratio between ERα46 and ERα66 may have an influence. However, reduction of ERα levels in absence of E2 had no effect on BASE expression level (Fig. R17), indicating that apo-ERα does not affect BASE expression. In concludion, BASE expression levels are only modulated by the estrogen-activated ERα while apo-ERα has no impact.

Compared to ERα66, ERα46 lacks the A/B domain which harbours the transactivation function 1 (AF-1). This domain is subject of frequent phosphorylation, which can activate the receptor in an estrogen-independent manner (reviewed in Lannigan, 2003). Thus, ERα46 can only be activated in a ligand-dependent manner. Furthermore, since ligand-activated ERα46 represses BASE this function is not dependent on AF-1, but seems to be exclusively based on AF-2. Metivier *et al*. (2002) proposed a model in which the A domain competes which helix 12 and the nuclear receptor corepressor (NCoR) for a hydrophobic cleft. This dynamic competition with the A domain is absent in ERα46 allowing unhampered interaction with cofactors. This might result in higher magnitudes of regulation.

Comparison of the repressive potential of ERα66 and a DNA binding mutant ERα66mut revealed that BASE repression does not require direct binding of ERα to DNA (Fig. R20). This result is in agreement with mutation studies where disruption of the EREs did not alter BASE regulation. Nevertheless, ERα is present at the BASE promoter (Fig. R13). This indicates either recruitment of ERα via protein-protein interactions or, in addition, indirect mechanisms such as regulation of a second gene or squelching of other factors. Repression by ERα without direct DNA binding has been reported for the TNFα gene (Cvoro *et al*., 2006). TNFα gene is induced by TNFα which results in

recruitment of AP-1 and NFκB. In a subsequent step, recruited unliganded ERα then potentiates the activation. In presence of estrogen, ERα was dismissed from the promoter, GRIP1 was recruited and TNFα expression was reduced.

In conclusion, apo-ERα does not contribute to BASE expression. And in presence of E2, BASE repression is mediated through AF-2 in a DNA binding-independent way.

6. Restricted expression of BASE to breast cancer and salivary gland?

BASE is a predicted member of the PLUNC gene family, which is expressed in the upper airways (Bingle *et al.*, 2004a). However, in a commercially available tissue array, no BASE expression was observed in lung. In fact, BASE expression was restricted to salivary gland and breast cancer cells (Egland *et al.*, 2003). The group also tested few breast cancer samples and found about 50 % of them BASE positive.

To complement this thesis a more comprehensive study was performed in collaboration with the group of Prof. Michael Kerin (Galway, Ireland). Within this study, 50 primary breast tumour samples were analysed for BASE expression so far. In agreement with the initial report by Egland *et al*. (2003), about 50 % (26/50) samples were positive for BASE expression in qPCR.

The as "normal" classified samples, of which 2 expressed BASE at very low levels, were obtained from either benign tumours or from control biopsies from breast cancer patients. They therefore do not represent valid controls and it is crucial that healthy tissue, e.g from breast reduction surgery, will be tested. In conclusion, BASE seems to be overexpressed in the pathological condition and could still function as breast cancer marker. However, BASE expression was not correlated with tumour stage, grade, ERα status, tumour size or histological subtype of breast cancer. Based on the results obtained in this thesis, it could be speculated that BASE expression might correlate with the FoxA1 status. Also, BASE was found to be more frequently expressed in ERα-positive tumours. This is in agreement with the positive correlation between ERα and FoxA1 expression (reviewed in Nakshatri and Badve, 2007). Nevertheless, an obvious question remaining is which changes occur during the transition from a normal to a malignant cell that induces BASE expression. A link between salivary gland, or more precise salivary gland tumour, and breast cancer has been reported. Female patients with a salivary gland tumour have a 2.5 times increased risk to develop breast cancer (In der Maur *et al.*, 2005). However, reports in the literature are contradictory, ranging from no increased risk to up to 8-fold increase (e.g. Berg *et al.*, 1968; Moertel and Elveback, 1969).

BASE is not the only gene with preferential expression in these tissues. The small breast epithelial mucin (SBEM) also shows a breast- and salivary gland-specific expression. However, in contrast to BASE, very low expression of SBEM was detected in some normal tissues including lung and breast (Miksicek *et al.*, 2002; Hube *et al*., 2004). Nevertheless, SBEM mRNA has been detected in more than 90% of breast tumours (e.g. Miksicek *et al.*, 2002; Colpitts *et al*., 2002). Also like BASE, SBEM has been detected in the well-differentiated breast cancer cell lines MCF7, ZR-75 and T47D but was undetectable in the poorly differentiated MDA-MB-231 cell line, HeLa and HepG2 cells (Miksicek *et al.*, 2002). The transcription factor Oct-1 was suggested to contribute to the strong expression of SBEM in breast tissue (Hube *et al*., 2006). Interestingly, an Oct-1/FoxA1 binding site is also essential for BASE expression. And furthermore, knockdown of Oct-1 also decreased BASE expression in MCF7 cells (Fig. R17). Therefore, it is possible that Oct-1, and in case of BASE also FoxA1, are essential for the breast-specific expression. In bioinformatic analyses of the first 3 kb of the SBEM promoter using MatInspector binding sites for ERα, estrogen-related receptors (ERRs) and NFIL3 (E4BP4) were predicted. All three transcription factors could also play a role in BASE expression. However, in contrast to BASE, SBEM is not regulated by estrogen or anti-estrogen ICI_{182780}. Both, SBEM and BASE are putative secreted proteins that have a salivary gland and breast specific expression pattern. As stated by (Miksicek *et al.*, 2002) for SBEM, the expression in salivary gland tissue does not undermine the potential use as breast cancer marker since salivary gland tumours can be easily distinguished. This is also underlined by the use of SBEM as breast cancer marker (Lacroix, 2006).

The potential diagnostic relevance of BASE is substantiated by the predicted secretion of the protein. BASE protein has not been detected yet, most likely due to the lack of specific antibodies. Although, expression in salivary gland has not been further investigated, it is possible that expression of BASE in breast cancer and salivary gland are linked. BASE was not found in a screen for proteins in human whole saliva (Vitorino *et al*., 2004). However, only 100 out of more than 200 protein spots on 2D-gel were tested and not all resulted in identifiable sequences (55%). Therefore, BASE could be present at low levels. Even more important, the analysed saliva sample was obtained from a healthy 25 year old man. The absence of BASE protein under these circumstances is expected. Therefore, this report does not undermine the potential of BASE determination in saliva as the previous study could not address this question. Furthermore, the possibility to detect breast cancer markers in saliva has been shown for HER2/neu (c-ERBB-2) whose levels in saliva strongly correlated with breast cancer in women (e.g. Bigler *et al*., 2002). Similar results have been reported for the Her2/neu protein in nipple aspirates (Kuerer *et al*., 2003). Detection of breast cancer markers in saliva or nipple aspirates opens the possibility of a non-invasive and inexpensive diagnostic tool for early detection and treatment response.

7. Conceivable mechanisms of BASE repression

A key finding of this thesis is the clear separation between basal expression of the BASE gene and the repression induced by estrogen. There is strong evidence that FoxA1 is a key factor in BASE expression while ERα is involved in the repression.

BASE basal expression

Exclusion of FoxA1 from the BASE promoter either by siRNA against FoxA1 or by mutation of the FoxA1 binding site greatly decreased transcription of the gene (Fig. R14 & R15). FoxA1 is a pioneer factor that can bind compact chromatin and initiate chromatin opening events (Cirillo *et al.*, 2002) which has been shown to support ERα-mediated transcription. Thus, FoxA1 might allow BASE expression by increasing the accessibility of the chromatin and thereby facilitate binding of other transcription factors including a breast and salivary gland specific factor (BSSF) (Fig. D1, I). Another possibility is that FoxA1 can displace a repressor binding the same DNA sequence (Fig. D1, II). A possible candidate is the transcriptional repressor NFIL3/E4BP4. This factor can bind in the same region as FoxA1. The model is supported by the fact that knockdown of Oct-1, another factor able to bind this site, has repressive effects on BASE expression (Fig. R17). However, it is also possible that FoxA1 and Oct-1 cooperate in BASE activation.

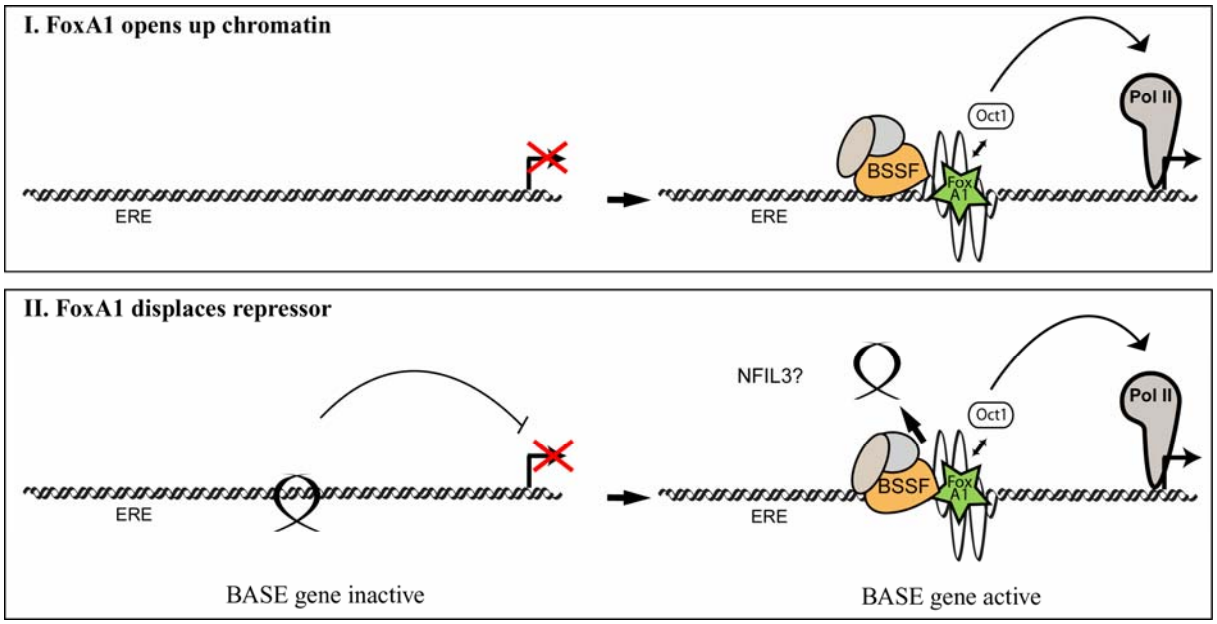


Figure D1. Schematic view of proposed mechanisms for activation of BASE gene basal transcription

BASE regulation

Repression of BASE in response to E2 requires ERα but no direct interaction with DNA. However, ERα can bind to the enhancer region *in vitro* and is present in the same region *in vivo* (Fig. R12 & R13). It is conceivable that ERα is recruited via protein-protein interactions, which are then stabilized by a direct binding to the half ERE. This hypothesis is able to explain the slight but reproducible difference between the repressive potential of the wild type and the DNA binding mutant of ERα. Nevertheless, whether presence of ERα is required for repression could not be conclusively defined.

Several mechanisms of repression have been outlined in the introduction (Fig.I6). The proposed models from the introduction will be discussed based on the results obtained in this study.

Model A has already been described for the repression of GATA-1 target genes. Ligand-activated ERα binds to GATA-1 preventing its binding to DNA and thereby activation of target gene promoters (Blobel *et al*., 1995; Blobel *et al.*, 1996). The model is compatible with the results obtained in this study. Direct binding of ERα to DNA is not required since the ERα DNA binding mutant can also repress BASE in transient transfection assays (Fig. R20). Furthermore, reduction of ERα through siRNA in presence of E2 increases BASE transcript levels (Fig. R3). Also the delayed induction of BASE expression after treatment with ICI_{182780} (Fig. R2), which inactivates ERα and accelerates its degradation, suggests a mechanism of de-repression rather than active induction. The only point undermining this model is the presence of ERα in the enhancer region of the BASE promoter (Fig. R13). However, whether this is required for repression remains unknown.

The second model suggested that ERα influences BASE expression by displacement of another transcription factor important for BASE activation from the promoter ("allosteric hindrance"). However, experiments with the ERα DNA binding mutant indicated that this is not the mechanism, since DNA binding is not required for repression (Fig. R20). Nevertheless, it is conceivable that ERα does not block a DNA sequence but rather a protein-protein interaction site.

Model C was based on the idea that liganded ERα would compete for coactivators, sequester them, and thereby decrease BASE expression. Such pronounced effects are unlikely caused by sequestering due to the redundancy in the transcription machinery and the shared usage of cofactors between transcription factors. In addition, ERα is present at the BASE promoter (Fig. R13). The opposite mechanism, in which apo-ERα sequesters repressive cofactors, is also not conceivable. If apo-ERα would titrate repressive factors away from the BASE promoter then knockdown of ERα by siRNA in absence of E2 would result in reduction of BASE expression, which was not observed (Fig. R17).

Model D proposed that apo-ERα would be absent from the active BASE promoter. In the presence of estrogen, ERα and subsequent repressors (e.g. HDACs) should be recruited, resulting in the inactivation of the BASE gene. This model is in agreement with the results if DNA binding is not the major requirement. It is possible that ERα is recruited through both, protein-protein interactions and direct DNA binding, and that these two modes act together to assure a stable association of ERα. In the absence of ERα, no repression would be observed. Furthermore, the observed difference in BASE activation by ICI_{182780} in the presence of ERα46 and ERα66 could be due to varying cofactor binding for the two isoforms. Whereas apo-ERα46 preferentially associates with corepressors, apo-ERα66 rather binds to coactivators (Metivier *et al.*, 2004). The A domain of ERα competes with helix H12 and corepressors for the same binding surface at the receptor (Metivier *et al.*, 2002). ERα46 lacks this domain and is more open for corepressor binding.

Model E was based on the theory that the sequence of the ERE induces changes in the ERα conformation which then determine whether binding sites for coactivators or corepressors are exposed. This scenario is highly unlikely since disruption of the ERE motif did not affect BASE repression in presence of E2 (Fig. R10) and even the DNA binding mutant could transmit the repression (Fig. R20).

Model F proposed that unliganded ERα contributes to BASE expression in the absence of hormone and is dismissed in presence of E2. In this case, knockdown of ERα with siRNA should reduce BASE expression levels. The contrary was observed, BASE levels in stripped medium were not affected, but the repression in presence of E2 was greatly diminished when ERα levels were reduced (Fig. R17). Therefore, the unliganded ERα is unlikely to contribute to basal BASE expression. This conclusion is further supported through experiments in the BASE expressing ERα-negative cell line SKBR3. Taken together, this model can also be neglected.

In another hypothesis, BASE repression was due to secondary effects. In this model, E2 would regulate expression of a second gene, whose product would then either repress or activate transcription of the BASE gene. A common method to distinguish between direct and indirect target genes of a transcription factor is the blockage of translation by cycloheximide. If the changes of expression in response to drugs are direct, meaning no additional protein synthesis is required, then the regulation should also be detectable in presence of cycloheximide, whereas secondary effects would be blocked. Unfortunately, two almost identical splicing variants of BASE exist. The less abundant form generates a nonsense-mRNA and is most likely target of NMD. Therefore, interference with translation led to increased levels of BASE transcripts even in presence of E2 (data not shown) since this second form was no longer degraded via the NMD pathway. However, down-regulation of BASE within 1 hour after exposure to E2 argues against this model (Fig. R2). Nevertheless, the rapid decrease in BASE transcripts could be due to blocked transcription and/or enhanced degradation of BASE mRNA. In

view of the weaker repression observed in luciferase reporter assays as compared to mRNA level, it is possible that mRNA destabilization might also contribute. A tempting hypothesis is that BASE mRNA is regulated through micro RNAs. In this context it is interesting that BASE is a predicted target for several micro RNAs (miRBASE, http://microrna.sanger.ac.uk). It is of note that this software only analyses the 3'UTR of the longest transcript which includes all 9 exons. However, analysis of the ESTs of BASE showed that most transcripts only include exons 2 to 6. Thus, the analysis does not include the majority of the transcripts.

In conclusion, the following models for BASE expression and regulation are proposed (Fig. D1). The pioneer factor FoxA1 binds to the inactive promoter and converts the chromatin structure into an active state (I) and/or replaces a repressor (II, e.g. NFIL3) which then allows recruitment of breast and salivary gland specifc factors and subsequent BASE expression. A cooperative role for Oct-1 is possible. However, which specific factors are recruited subsequently and whether FoxA1 is dismissed from the promoter in presence of hormone remains to be determined.

The level of expression of the active gene is then regulated by ERα in the presence of E2. In the proposed models (Fig. D2), ERα is only associated with the BASE promoter in presence of estrogen. In model III, ERα forms a complex with INSM1 and cyclin D1 and recruits co-repressors. A repressive mechanism involving INSM1, cyclin D1 and HDACs has been shown for the neuroD/beta2 gene (Liu *et al.*, 2006). The data obtained in this thesis support this model at several points. Firstly, BASE gene repression only occurs in presence of the estrogen-activated ERα (Fig. R17 & R20). Secondly, ERα can bind to the BASE promoter *in vitro* and *in vivo* (Fig. R12 & R13). Thirdly, INSM1 is expressed in cell lines where BASE is regulated by estrogen but not in SKBR3 cells where BASE is not regulated (Fig. R19). Fourthly, putative INSM1 binding sites have been identified in all three regions important for BASE regulation (Fig. R18). And finally, mutation of the ERE in the enhancer only slightly alters the INSM1 binding sites. All highly conserved bases are maintained and only non conserved bases are changed. This could explain the retention of regulation by E2 even if the ERE is disrupted.

There are further indications from the literature which might argue in favour of this model. Cyclin D1 is a well known estrogen-induced gene. Cyclin D1 can bind liganded and unliganded ERα and stimulates its transactivation ability (Neuman *et al.*, 1997; Lamb *et al.*, 2000). Zwijsen *et al.* (1997) reported a direct interaction between cyclin D1 and the LBD of ERα, and that this interaction is not affected by the pure-antiestrogen ICI_{164384}. In contrast, ICI_{182780} was reported to interfere with transactivation at ERE elements (Neuman *et al.*, 1997). However, interaction of ERα and cyclin D1 have not been studied in the context of estrogen-repressed promoters. Nevertheless it is of note, that cyclin D1 is overexpressed in about 50% of breast cancer tumours (Gillett *et al.*, 1996). Interestingly, BASE expression is also detected in about 50 % of tumours. It remains to be determined if a

correlation (positive or negative) between expression of cyclin D1 and BASE exists. An altered expression in some types of cancer has also been reported for INSM1. For example, it is re-expressed in neuroendocrine tumours (Pedersen *et al.*, 2006) and its expression is increased in small-cell lung cancer (SCLC, Taniwaki *et al.*, 2006). However, no link between INSM1 expression and breast cancer tumours has been reported so far.

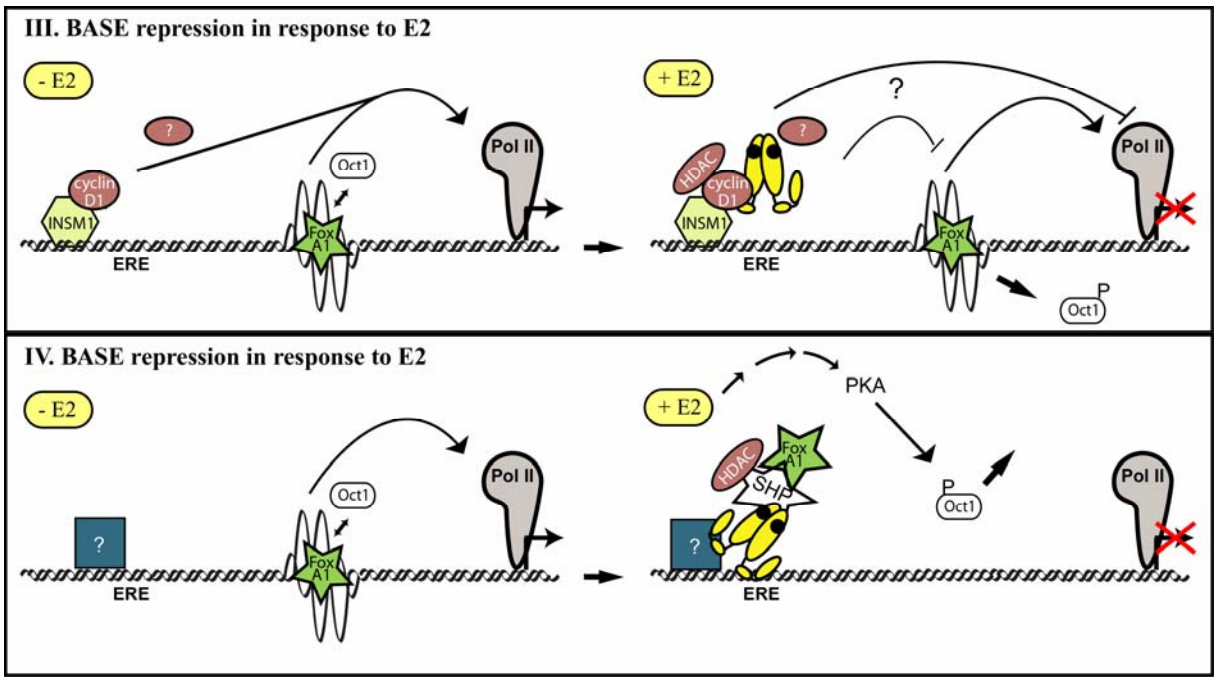


Figure D2. Schematic view of possible mechanisms of estrogen-induced repression of the BASE gene.

Model IV is also based on the requirement of FoxA1, and perhaps Oct-1, for BASE expression. The model proposes that the liganded ERα associates with the BASE promoter where it then interacts with a second factor (e.g SHP) which subsequently recruits co-repressors. The short heterodimer partner (SHP) binds via the LXXLL-related motif directly to estrogen-bound ERα (Johansson *et al.*, 2000). SHP has also been shown to directly interact with the DNA binding domain of FoxA1, thereby preventing its binding to DNA (Kim *et al.*, 2004). Moreover, SHP acts as a repressor by recruiting HDACs, in particular HDAC1 (Gobinet *et al.*, 2005).

For the second transcription factor involved, Oct-1, it is of note, that Oct-1 DNA binding is inhibited by phosphorylation through PKA (Segil *et al.*, 1991). Interestingly, PKA can indirectly be activated by E2 via the second messenger pathways (Doolan *et al.*, 2000).

To summarize, in presence of E2 both factors important for BASE expression would be prevented from binding to the promoter and therewith from activating the gene. As a result, BASE expression would be decreased.

8. Concluding remarks and future perspectives for BASE

This thesis investigated the regulation of the breast cancer and salivary expression gene (BASE) in breast cancer cell lines, predominantly in the well-differentiated estrogen receptor alpha positive cell line MCF7. BASE fulfilled the main criteria of a model gene for studies on repression of gene expression by E2: strong E2- dependent down-regulation and a compact promoter region that contains major elements contributing to expression and regulation of the gene. Additionally, the restricted expression pattern of the only recently discovered gene BASE implies biomedical relevance as BASE is potentially a new breast cancer marker.

One of the major findings of this study is the clear separation between gene expression and gene regulation in response to estrogen, with key roles for FoxA1 in expression and ERα in repression. Based on the data obtained, two models are favoured (Fig. D2). However, to distinguish between these and to refine the model, additional experiments would have to be conducted. These include chromatin immunoprecipitations to analyse presence of the pivotal factors (ERα, FoxA1, INSM1, cyclinD1, HDACs, SHP) at the BASE promoter in absence and presence of estrogen. Furthermore, transfection of SKBR3 cells with variants of ERα to induce regulation of BASE would be an excellent way to define the molecular basis of the control of the gene. If INSM1, which is not expressed in SKBR3, has a major role then it should be re-expressed under these conditions. Additionally, the application of HDAC inhibitors could clarify whether HDAC are involved in the process.

Primary studies of BASE expression in breast cancer samples conducted in collaboration with Prof. Michael Kerins group in Galway, Ireland, indicate that about 50 % of breast tumours are positive for BASE while normal breast samples were negative. This expression rate is higher than for the accepted breast cancer marker HER2, which is overexpressed in only 30 % of breast and ovarian cancers (reviewed in Nicolini *et al*., 2006). This promising result should be the basis for more comprehensive studies analysing BASE expression in normal human tissues, especially salivary gland, and different breast cancer types. To evaluate the potential of BASE as breast cancer marker, it is important to analyse whether its expression can be correlated with breast cancer subtypes, stage or prognosis.
Further studies should also focus on the BASE protein. The recombinant protein has been generated and an antibody will be raised against the full BASE protein. A BASE antibody would allow an immunohistochemical approach to investigate which in breast cell population expresses BASE. Furthermore, to address the function of BASE, the protein should be overexpressed and knocked down using siRNA. If the secreted protein could be detected, the value of BASE as a marker would even increase. Tests for BASE could then be performed in an inexpensive and non-invasive way by analysing the nipple aspirate fluid or maybe even saliva.

9. A distal enhancer of the cathepsin D gene

Cathepsin D (CTSD) is a lysosomal aspartyl protease that is overexpressed in aggressive human breast cancer cells where it is associated with poor clinical outcome. Whereas CTSD is constitutively expressed in ERα-negative cells, its expression is induced by estrogen in ERα-positive cells (Liaudet-Coopman *et al*., 2006). Transcription of CTSD is initiated at five sites located between -72 and -20 bp upstream from the initiation codon (Cavailles *et al*., 1993). Estrogen only induces transcription from the start site of at -20. This induction requires the promoter fragment -365 to -122. This region includes binding sites for ER (half EREs), and SP1 which cooperate in expression and the estrogen-dependent regulation of the CTSD gene (Krishnan *et al.*, 1994; Wang *et al.*, 1998; Wang *et al.*, 2001 and references therein). However, no canonical ERE has been identified. In contrast, the distal ERα binding site (GGTCAtggTG**G**CC), identified by S. Mader and co-workers (Bourdeau *et al*., 2004), contains only one mismatch compared to the consensus motif and has been shown by ChIP to bind ERα *in vivo*.

This study now shows the ability of the distal enhancer to induce estrogen-dependent activation in reporter gene assays. The distal enhancer caused significantly stronger estrogen stimulation than the proximal promoter (Fig. R25). Disruption of the ERE abolished this induction. Interestingly, the proximal promoter part showed only very weak estrogen-responsiveness. However, the proximal segment tested contained the core promoter and could drive transcription of the reporter gene, while only very low expression was seen with the enhancer alone. It is of note, that the proximal segment was slightly longer than the ones used in the previous studies that were mentioned. It therefore could include elements that attenuate the response to E2.

The general importance of distal enhancers has been accepted. Three general models of action to describe how distal elements can communicate with the proximal promoter have been discussed (reviewed in Bondarenko *et al*., 2003).

A. The looping model. Key transcription factors and coactivators or co-repressors assemble on the distal enhancer, which then contacts the transcription start site. The intervening chromatin loops out. This physical interaction leads to transcriptional activation or repression. Whether this is due to protein transfer between enhancer and the proximal promoter, or whether these factors only modify the promoter to set up a transcriptional permissive environment, is most likely dependent on the gene context. This mechanism has been reported for example for the monocyte chemoattractant protein 1 gene (MCP-1). There, upon stimulation by TNF, NFκB binds to a distal regulatory region and recruits factors with HAT activity. Subsequent direct interaction between the distal region and the promoter induced histone modifications that allow binding of other transcription factors and gene activation (Teferedegne *et al*., 2006).

B. The tracking model. In this model, again key factors assemble on the distal enhancer. From there, they track along the DNA to the proximal promoter. This mechanism has been shown for the prostate

specific antigen (PSA) promoter, where the introduction of an insulator between the enhancer and the transcription start site blocked tracking of PolII and thereby inhibited transcription (Wang *et al.*, 2005a).

C. The spreading-looping model suggests formation of a series of small DNA loops by polymerisation of a special protein through which the enhancer and promoter move in close vicinity. The protein-protein interaction is initialized at the enhancer and moves as a wave towards the promoter. Thereby the polymerizing protein can either interact with different factors bound to DNA or directly bind to multiple specific DNA binding sites between the enhancer and the promoter. This model is not suitable for transactivation over large distances.

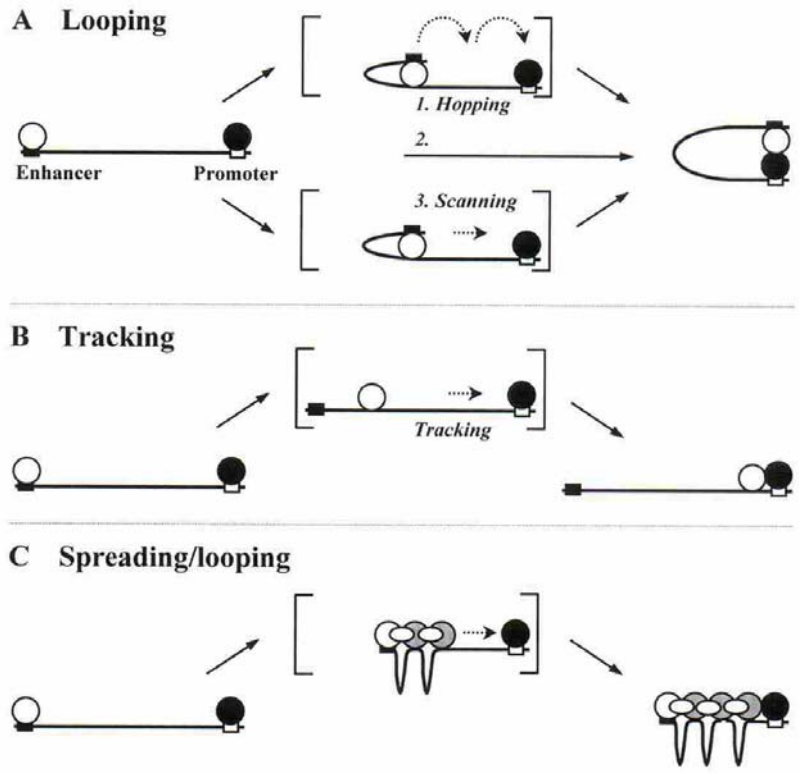


Figure D3.
Proposed mechanisms for communication between distal enhancer and promoter (from (Bondarenko *et al.*, 2003). For model description see text above.

It is very likely that different mechanisms and combinations apply depending on the promoter context. Enhancer motifs may be located tens or hundreds of kilobases upstream the gene (Kleinjan and van, V, 2005; Carroll *et al.*, 2006). In these cases, the tracking model and the spreading-looping model are rather implausible. In contrast, for enhancers that are only 4 kb upstream it is conceivable that PolII tracks along the DNA to the promoter.

For the CTSD, the enhancer is located 9 kb upstream. Therefore, the looping model is the more likely one (Fig. D4). Estrogen-dependent association of ERα and PolII with the enhancer has been shown in this study (Fig. R26), by Bourdeau *et al.* (2004) and very recently by Carroll *et al*. (2006). However, it remains to be investigated whether the looping model applies with the enhancer physically contacting the proximal promoter. Two approaches will be necessary to answer this question. The physical contact should be observed in a chromatin conformation capturing assay (3C) and using chromatin

immunoprecipitations PolII should be detected at the enhancer and the transcription start site but not on sequences in between. Furthermore, it has been shown that ERα associates with the proximal promoter in a cyclical manner (Shang *et al.*, 2000). Preliminary data in this thesis indicated that ERα also cycles on the distal enhancer. It would be very interesting to test, whether these dynamics are synchronized. These studies should also include another ERα binding site about 33 kb upstream of the transcription start site (Bourdeau *et al.*, 2004). The potential and importance of this binding site also remains to be analysed in further studies.

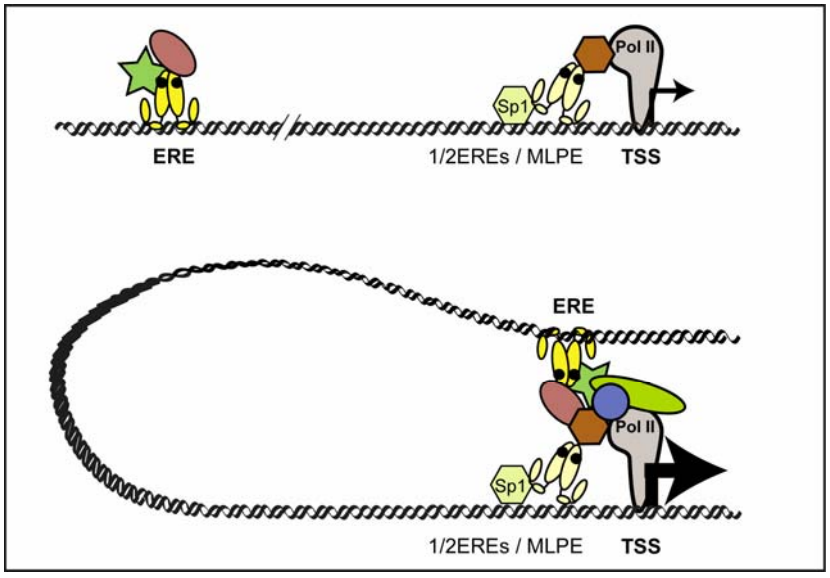


Figure D4. Looping model for induction of CTSD expression in response to estrogen

MATERIALS AND METHODS

1. Reagents and antibodies

α-amanitin, estradiol (E2), 4-hydroxytamoxifen (Tam), 12-O-Tetradecanoylphorbol 13-acetate (TPA), cycloheximide were purchased from Sigma (Taufkirchen, Germany). ICI_{182780} (ICI) was ordered from TOCRIS (Ellisville, MO). Protease inhibitor cocktail "complete (EDTA-free), T4 DNA ligase and selected restriction endonucleases were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Protein A Sepharose™ CL-4B (Ge Healthcare Bio-Sciences AB, Uppsala, Sweden). Selected restriction endonucleases were also obtained from New England Biolabs (Frankfurt am Main, Germany) and Fermentas GmbH (St.Leon-Rot, Germany). Primers were synthesized by Sigma-Aldrich Chemie GmbH (Munich, Germany).

Table M1. Antibodies used in this study. (WB – Western blot, ChIP – chromatin immunoprecipitation, IF – immunofluorescence, EMSA – electromobility shift assay)

Antigen	name	host	company	application
α-BASE (R3)	R3	rabbit	Eurogentec S.A., Belgium	WB
α-BASE (R4)	R4	rabbit	Eurogentec S.A., Belgium	WB
α-BASE	A0406	rabbit	CIM Antibody Core, Arizona State University	WB
α-E4BP4	H-300	rabbit	Santa Cruz, Heidelberg	ChIP, WB
α-ERα	HC-20	rabbit	Santa Cruz, Heidelberg	WB,ChIP, IF, EMSA
α-ERα	H-184	rabbit	Santa Cruz, Heidelberg	ChIP
α-FoxA1	T-20	goat	Santa Cruz, Heidelberg	ChIP, WB, EMSA
α-FoxA1	M01	mouse	Abnova Corporation, Heidelberg	WB
α-GAPDH	6C5	mouse	Santa Cruz, Heidelberg	WB
α-GFP	FL	rabiit	Santa Cruz, Heidelberg	WB, IF
α-INSM1	1800342133	rabbit	GenWay, San Diego,USA	ChIP, WB
α-Oct-1	C-21	rabbit	Santa Cruz, Heidelberg	ChIP
α-PolII	N-20	rabbit	Santa Cruz, Heidelberg	ChIP
α-ßactin	I-19	rabbit	Santa Cruz, Heidelberg	WB
α-goat-IgG-HRP			Santa Cruz, Heidelberg	WB
α-rabbit IgG HRP			GE Healthcare, UK	WB
α-mouse IgG HRP			Dianova, Hamburg.	WB
α-P-PolII	CTD4H8	mouse	Upstate, NY, USA	ChIP
normal mouse IgG	sc-2025	mouse	Santa Cruz, Heidelberg	ChIP
normal rabbit IgG	sc-2027	rabbit	Santa Cruz, Heidelberg	ChIP
normal goat IgG	sc-2028	goat	Santa Cruz, Heidelberg	ChIP

2. Cell lines, cell culture and analysis

ERα-positive cell lines used were mammary epithelial adenocarcinoma cell line MCF7, human mammary gland ductal carcinoma cell lines T47D and ZR75, ERα-negative cell lines were mammary adenocarcinoma cell lines SKBR3 and MDA-MB-231, human cervical adenocarcinoma cell line HeLa, hepatocellular carcinoma cells line HepG2. Most cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10 % fetal calf serum (FCS, Sigma), 2mM L-glutamine (Invitrogen), penicillin (100 units/ml, Invitrogen), and Streptomycin (100 µg/ml, Invitrogen) ("normal medium") at 37 °C under 5 % CO_2. ZR75 cells were cultured in RPMI 1640 medium containing the same supplements as DMEM. Cell lines stably expressing ERα (ERα46 or ERα66) derived from ERα-negative cell line MDA-MB231 were previously described (Reid *et al.*, 2003) and maintained in DMEM under hygromycin selection (0.8 mg/ml). When hormone deprivation was required, cells were cultured in phenol red free DMEM (Gibco) containing 2.5 % charcol-dextran stripped serum and antibiotics ("stripped medium") for 2 to 3 days before treatment. To evaluate direct effects on BASE regulation, cells were cultured in 6-well plates and treated with the protein synthesis inhibitor cycloheximide (1 µg/ml) alone and in conjunction with E2 and ICI_{182780} for 6 h, 4 h, 2 h, 1 h, and 0 h. Cells were harvested at the same time through lyses in 1 ml TriZol and subsequent storage at –80 °C until RNA was extracted.

3. General PCR protocol

In all PCR reactions performed FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) was used. For each primer pair PCR conditions were optimised by varying annealing temperature, extension time, $MgCl_2$ and DMSO concentration. A standard PCR reaction was setup as follows:

Table M2: Standard PCR reaction setup

component	volume used	final concentration (in 25 µl)
template	x µl	100 ng (plasmid) 2 µg (genomic DNA)
10X buffer w/ $MgCL_2$	2.50 µl	1x
dNTPs (25 mM)	0.25 µl	250 µM
forward Primer (10 µM)	1.00 µl	400 nM
reverse Primer (10 µM)	1.00 µl	400 nM
FastStart Taq DNA polymerase (10 U/µl)	0.20 µl	2 U
DMSO	1.25 µl	5 %
$MgCl_2$ (25 mM)	1.25 µl	1.25 mM
Nuclease-free water	Add up to 25µl	

4. Expression constructs

Expression constructs for human ERα66 (pcDNA3.1-hERα66, Metivier *et al.*, 2004), ERα46 (pcDNA3.1-hERα46, Metivier *et al.*, 2004), ERα66 with mutated DNA binding domain (pcDNA3.1-hERα66mut, unpublished) and ERß (pSG5-hERβ) had been previously generated in the laboratory. The FoxA1 expression construct, containing the FoxA1 coding sequence as BamH1 and Xho1 fragment in pcDNA3.1/hygro, was generated by Heike Brand. Expression constructs for INSM1 and E4BP4 (NFIL3) were kindly provided by Dr. Michael Lan (The Research Institute for Children, New Orleans, USA) and Dr. Sotiri Tetradis (UCLA School Dept Dentistry, Los Angeles, USA).

4.1 BASE protein expression constructs

The bacterial expression vectors M80, M82, and pET22b(+) were kindly provided by the Protein Expression and Purification Core Facility EMBL. The pcDNA3.1/hygro vector was obtained from Invitrogen Corporation (Carlsbad, CA) and the EGFP-N1 and EGFP-C1 vectors were purchased from Clontech Laboratories Inc. (Palo Alto, CA). The BASE coding sequence was obtained from the ENSEMBL genome browser and primers were designed accordingly. The BASE coding sequence was then amplified from MCF7 cDNA using FastSart DNA Polymerase (Roche). Different primer pairs (see table below) introducing various restriction sites were used to obtain suitable products for cloning into the expression vectors pEGFP-N1 (for C-terminal tagging) and pGFP-C1 (for N-terminal tagging), and the vectors M80, M82, p22b(+) for bacterial expression. Since the first 20 aa of BASE are predicted to be a signalling peptide, marking BASE for secretion, the sequence for the full length and the truncated protein, omitting the signal peptide, were generated. The GFP-tagged BASE was excised using Nhe1 and Not1 for the C-terminal GFP-tagging and Nhe1 and HindIII for the N-terminal tagging and subcloned into the pcDNA3.1hygro vector.

C-terminal tagging (pEGFP-N1, pcDNA3.1 hygro)
fwd2: gcg gctagc gcc *atg* ctg aat gtc tcc ggc (clamp Nhe1 Kozak *ATG*)

rev2: aattc agatct gtttgccacaacaaattttg (clamp BglII CDS BASE)

N-terminal tagging (pEGFP-C1, pcDNA3.1 hygro)
fwd3: at agatct *atg* ctg aat gtc tcc ggc (BglII- *ATG*)

rev3: ca aagctt *cta* tgtttgccacaacaaattttg (HindIII- *TAG*)

HA-tagging (pcDNA3.1+)
HA_fwd4: agcg gctagc gccacc *atg* ctg aat gtc tcc ggc (clamp Nhe1 Kozak *ATG*)
HA_rev4: gcca gaattc *cta* agcgtaatctggaacatcgtatgggta tccgcc tgtttgccacaacaaattttg
(clamp EcoR1-*TAG-HAGlyGlyCDS*)

Expression constructs (M80, M82, p22b(+))
fwd L-Nco1: cgcat cc*atg* gtg aat gtc tcc ggc (Nco1- *ATG*-whole protein)
fwd S-Nco1: cgcat cc*atg* gca cag gag gtc ctg gct (Nco1-*ATG*-w/o signal. peptide)
CDS rev: cgtcat ggt acc cta tgt ttg cca caa caa att ttg (Asp718- *TAG*)

5. In vitro transcription and translation

In vitro transcription and translation were mainly accomplished with the TNT T7 Quick Coupled Reticulocyte Lysate system from Promega Corp. (Madison, WI) following the manufacturer's protocol. In short, 40 µl master mix were combined with 1 µg DNA, 1 mM Methionine and 9 µl dH_2O containing 1 µg template DNA and incubated for 90 min at 30 °C. The expression vectors pcDNA3.1hygro-hERα66, pcDNA3.1hygro-hERα46 and pcDNA3.1hygro-FoxA1 were used as templates for transcription with T7 RNA polymerase followed by translation to generate human ERα66, ERα46 and FoxA1 proteins.

For non-radioactive EMSA experiments *in vitro* transcription/translation was also carried out using the RTS 100 Wheat Germ CECF Kit following the manufacturer's protocol. The reaction is set up on a microtiter plate consisting of feeding and reaction modules which are separated by a semi-permeable membrane. To obtain *in vitro* translated proteins 900 µl feeding mix, 80 µl amino acids and 20 ml methionine were combined in the feeding compartment. The reaction solution, containing 15 µl reaction mix, 4 µl amino acids, 1 µl methionine, 15 µl wheat germ lysate and 15 ml sterile water containing 2 µg plasmid, was placed into the reaction compartment. The modules were closed with adhesive film and incubated at 30 °C for 24 hours. After incubation the reaction mix was transferred to a fresh tube and stored at –80 °C until use. Production of protein was confirmed by Western blot.

6. Transient transfections and luciferase assays

Before transfection, cells were plated in 24-well plates and cultured over night in normal medium, or when hormone deprivation was required, maintained in stripped medium for 2 days. Transfections were carried out at 70 % confluency with Fugene6 Transfection Reagent (Roche) for MDA and derived cell lines and with ExGene^{TM}500 (Fermentas) for all other cell lines used. DNA mixture per well consisted of 1 µg reporter constructs, 100 ng phRL-TK Vector (Renilla luciferase, Promega), and for cotransfection experiments, 200 ng expression plasmids for either ERα66, ERα46, ERα66mut, FoxA1, Gata1, or ERRγ. Empty expression vector was used to adjust the final amount of DNA. Cells were treated immediately after transfection as indicated with either vehicle (EtOH), 10^{-8} M E2, 10^{-6} M ICI_{182780}, 10^{-7} M OH-Tam, 50 ng/ml TPA. After 24 h incubation, cells were harvested and cellular extracts were analysed for luciferase activity using the Dual-luciferase reporter system (Promega). The firefly luciferase reporter activities were normalized by Renilla luciferase activities and shown as relative light units (RLU). The data are the mean ± SD from a minimum of three independent experiments with triplicates for each experiment.

7. Generation of stable cell lines

MCF7 cells were seeded in 9cm plates and at 50 % confluency transfected with either carboxy-terminal GFP-tagged BASE (pcDNA3.1-BASE-GFP), N-terminal GFP-tagged BASE (pcDNA3.1-GFP-BASE), of C-terminal HA-tagged BASE (pcDNA3.1-BASE-HA). The pcDNA3.1 vector confers hygromycin resistance and drives expression of BASE under the control of a cytomegalovirus (CMV) promoter. 24 hours after transfection cells were set under hygromycin selection (0.8 mg/ml). Cell colonies were isolated and screened for tagged-BASE expression by Western blot analysis with GFP-antibody.

8. Identification of putative transcription factor binding sites

Identification of transcription factor binding sites was performed with Dragon ERE Finder (version 2) and MatInspector v.2.2 (Cartharius *et al*., 2005) software bases on the TransFac database (Matys *et al*., 2003). All three databases are available online (Dragon ERE Finder: sdmc.lit.org.sg/promoter, MatInspector: www.genomatix.de, TransFac: www.gene-regulation.com). All parameters were set as default except for matrix group (vertebrates).

9. Preparation of genomic DNA

Cells maintained in normal medium were washed twice with PBS, lysed Vo ml lysis buffer (3.6 M GTCI (Guanidine Thiocyanate/Isobutyl), 50 mM Tris-HCl (pH 8), 0.05 % SDS) and then transferred into a new tube (polypropylene). To extract the DNA Vo ml of phenol/chloroform/isoamyl alcohol (25:24:1; AppliChem GmbH, Darmstadt, Germany), mixed rigorously, and centrifuged at 2000 xg or 5 min. The aqueous phase was transferred to a fresh tube and the phenol/chloroform extraction was repeated until the aqueous phase was clear. After 2 washing steps using chloroform only, the DNA was precipitated by adding 1/10 of Vo 5 M NaCl and Vo of Isopropanol. DNA was pelleted through centrifugation at maximal speed for 10 min and then washed with 70 % EtOH. Genomic DNA was resuspended in 10 mM Tris (pH 8.0) and stored at 4 °C.

10. Measurement of DNA/RNA concentration

The light absorption-maximum of nucleic acids is at 260 nm (UV-light) while proteins absorb maximal at 280 nm. To determine the nucleic acid concentration and the impurity (contamination with proteins) the absorption was measured at both wavelengths and the ratio of A260/280 was calculated. dsDNA at the concentration of 50 µg/ml has an OD_{260} of 1. Ratios of A260/280 below 1.8 were considered impure and discarded or purified using EtOH-precipitation.

11. DNA purification/concentration through EtOH-precipitation

To purify or to concentrate the DNA it was precipitated by adding 1/10 volume of 3 M Na-acetate and 3 volumes of EtOH and incubation for at least 20 min @ -80 °C. DNA was pelleted by centrifugation at 14,000 rpm for 15 min (at 4 °C). After washing with 70 % EtOH and air drying for 10 min, DNA was resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA (pH 8.0)) or dH_2O.

12. Basic luciferase reporter constructs - cloning of the 5' upstream region

12.1 Basic 2.4 kb construct and first deletion series

2.4 kb of the 5' region upstream of the BASE gene were amplified from 2 µg genomic DNA from MCF7 cells using FastStart Polymerase (Roche). Primers were designed to introduce Kpn1 and Xho1 restriction sites at the end of the PCR product (2.4 kb fwd: gcg ggtacc tacatgactccaggctgtgg; start rev gcg ctcgag tgtgctgtcaagacactctgg). Using these restriction sites the BASE promoter region from -2418 to +23 was cloned into the multi cloning site of the pGL3-basic (Promega) reporter construct upstream of the luciferase reporter gene.

The ßactin promoter region (–1109 bp to +36 bp) was also amplified from 2 µg genomic DNA from MCF7 cells (forward primer: gcg ggtacc tgacaaggacagggtcttcc; reverse primer: gcg agatct caaaggcgaggctctgtg) and subcloned into the pGL3-basic vector using the restriction enzymes Kpn1 and BglII. The sequence was verified by DNA sequencing at the Genomics Core Facility using RV-primer3 (ctagcaaaataggctgtccc) and GL2 primer (ctttatgtttttggcgtcttcca). The Renilla-reporter vector (phRL-TK Vector) was purchased from (Promega) and the pS2-luciferase construct (containing the pS2 promoter from -556 to +26) was previously generated in the laboratory.

12.2 Modification of promoter region using digests

To identify the promoter regions important for expression and regulation of the BASE gene a series of deletion mutations (series 1) was created (constructs A to H). Construct 'A' was generated by PCR using the 2.4 kb construct as template and the following primers (2.2 fwd: gcg ggtacc ggagggagagggacacctac, start rev: gcg ctcgag tgtgctgtcaagacactctgg) introducing Kpn1 and Xho1 sites for cloning. The remaining constructs (B-H) were generated by digestion with different combinations of blunt cutting restriction enzymes (Tab. M3) and subsequent internal vector re-ligation.

Table M3: Information for generation of deletion constructs of BASE promoter

construct		restriction enzymes	sequence omitted
Δ I	A	PCR	-2419/ -2353
Δ II	B	Msc1, PvuII, 4+BSA	-2352 /-1689
Δ II/III*	C	Msc1, Pml1, 1+BSA	-2352 /-917
Δ II/III/IV*	D	Msc1, Sma1, 4+BSA	-2352 /-179
Δ III	E	PvuII, Pml1, 1+BSA	-1688 /-917
Δ IV	F	Pml1, Sma1, 4+BSA	-916 /-179
Δ III//IV*	G	PvuII, Sma1, 4+BSA	-1688 /-179
Δ /IV*	H	Msc1, PvuII, 4+BSA ; Pml1, Sma1, 4+BSA	-2352/-1689 and -916 /-179

* Constructs are not represented in results

12.3 Modification of the promoter region using site-directed mutagenesis

To evaluate the significance of specific transcription factor binding sites (EREs, FoxA1, Gata1, AhR, AP-1, Oct-1) point mutations were introduced using site-directed mutagenesis by overlap extension (Fig. M1). Two primer pairs were designed to amplify two overlapping DNA fragments (A & B) which harbour the mutation site in the overlapping region. The forward primer (primer 1) of set 1 contains a restriction site for subsequent subcloning while the reverse primer (mut primer 1) contains the mutation. In the second primer set the forward primer (mut primer 2) includes the mutation and the reverse primer (primer 2) does introduce an restriction site for cloning. If convenient restriction sites were present in the promoter the outside primers were not extended with additional restriction sites. All primers used are listed below. In the first step the fragments A & B were generated in separate amplifications using the primers described. The PCR products were analysed by electrophoresis and purified using the Qiagen Gel Purification kit. The two overlapping fragments function as templates in a second PCR reaction where they are first extended to form the full-length mutant DNA, and then amplified using only the outside primers (primer 1 and primer 2). The mutated DNA was also gel purified and subsequent digested with the appropriate restriction enzymes. The corresponding DNA fragment was excised from the original reporter construct (e.g. pGL3-BASE 2.4 kb) using the same enzymes. In the last step, the mutated sequence was ligated into the vector backbone substituting the wild type DNA sequence. For mutations close to the transcription start site, only one primer pair was used in which the reverse primer contained the mutation and a restriction site for cloning. A similar amplification and cloning strategy was applied for the generation for the deletion mutants omitting only 50 bp to 150 bp. Primers are listed below in tables M4 and M5.

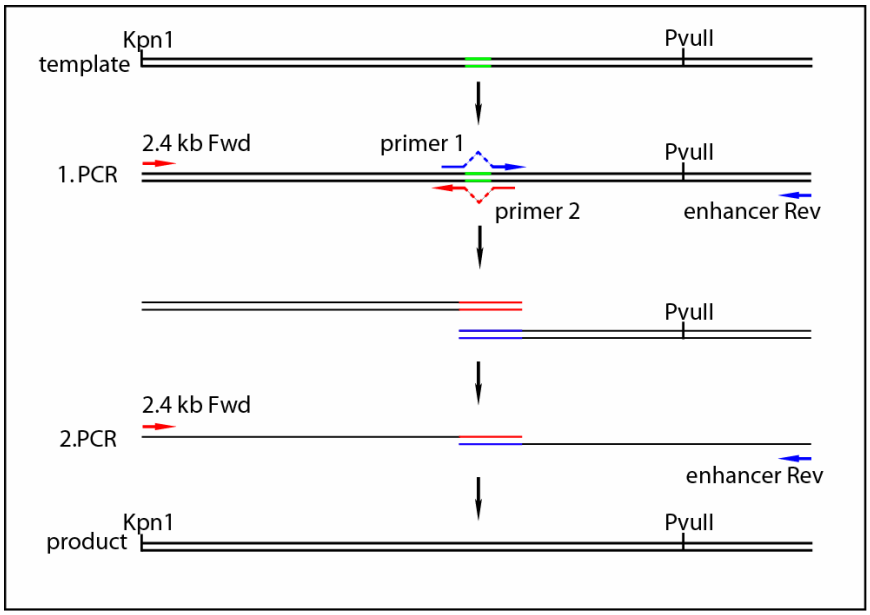


Figure M1. Schematic display of the mutation strategy.

Table M4: Primers used for further deletion mutations.

construct	primer	sequence	segment omitted
Δ A	fwd miss A	gacccactgaagactgatgt ctcaactctagtacaggctc	-2351 / -2099
	rev miss A	gagcctgtactagagttgag acatcagtcttcagtgggtc	
Δ B	fwd miss B	gttatccagagagtaaagca gataacatttcaggatcca	-2098 / -1984
	rev miss B	tggatcctgaaatgttatc tgctttactctctggataac	
Δ C	fwd miss C	ttcaggatccaaattgttaa atatttgctctgtgaatggg	-1983 / -1836
	rev miss C	cccattcacagagcaaatat ttaacaatttggatcctgaa	
Δ D	fwd miss D	gatggaaacctttattgtaa agctgggtgggaatac	-1835 / -1684
	rev miss D	gtattcccacccagct ttacaataaaggtttccatc	
Δ AB*	fwd miss AB	gacccactgaagactgatgt gataacatttcaggatcca	-2351 / -1984
	rev miss AB	tggatcctgaaatgttatca catcagtcttcagtgggtc	
Δ BC*	fwd miss BC	gttatccagagagtaaagca atatttgctctgtgaatggg	-2098 / -1836
	rev miss BC	cccattcacagagcaaatat tgctttactctctggataac	
Δ CD*	fwd miss CD	ttcaggatccaaattgttaa agctgggtgggaatac	-1983 / -1684
	rev miss CD	gtattcccacccagctttaa caatttggatcctgaa	
Δ C1	fwd miss C1	aggataacatttcaggatcc aatatttaccaaactggatc	-1984 / -1930
	rev miss C1	gatccagtttggtaaatatt ggatcctgaaatgttatcct	
Δ C2	fwd miss C2	accccaggaacacatcacga catatttcactacatctttg	-1930 / -1881
	rev miss C2	caaagatgtagtgaaatatg tcgtgatgtgttcctggggt	
Δ C3	fwd miss C3	cattacaatgatagcgtgta ttgctctgtgaatgggaata	-1880 / -1831
	rev miss C3	tattcccattcacagagcaa tacacgctatcattgtaatg	
	fwd 2.4 kb	gcg ggtacc tacatgactccaggctgtgg	
	rev enhancer	tggagaggcactgcttaggt	

With * marked constructs are not shown in results.

Table M5: Primers used for generation of point mutations. Mutated bases are shown in capital letters. Restriction enzymes used for cloning are shown in regular, while restriction sites introduced for screening are shown in italic.

primer	sequence	restriction enzyme
mutations at the transcription start site		
out fwd primer		
680 bp fwd1	gcg ggtacc ggaccttgggcaagatttgt	Kpn1
600 bp fwd2	gcg ggtacc ggagagacggagaccatgaa	Kpn1
mut rev primer		
mERE TSS rev1	gctctcgagac**T**ct**A**ct**A**g**T**ctctgtaggttga	Xho1, *Spe1*
mutations in the BASE enhancer region		
out fwd primer		
fwd 2.4 kb	gcg ggtacc tacatgactccaggctgtgg	Kpn1
out rev primer		
rev enhancer	tggagaggcactgcttaggt	
mut fwd & mut rev primer		
mERE EH fwd	aaattgttaactctatgcct t**AAC**ac**G**ttt**AAACG**ccccaggaacacatc	*Pme1*
mERE EH rev	gatgtgttcctgggg**CGTTT**aaa**C**gt**GTT**aaggcatagagttaacaattt	*Pme1*
mFoxA1 EH fwd	accccaggaacacatcacgaaa**A**a**GC**t**T**ccaaactggatctttc**ATGC**ta cattacaatg	*HindIII*
mFoxA1 EH rev	tacacgctatcattgtaatgta**GCAT**gaaagatccagtttgg**A**a**GC**t**T**tt tcgtgatgtg	*HindIII*
mGata1 EH fwd	tttctgtttacattaca**GAAT**t**C**gcgtgtacatatttcac	*EcoR1*
mGata1 EH rev	gtgaaatatgtacacgc**G**a**ATTC**tgtaatg taaacagaaa	*EcoR1*
mAhR EH fwd	tttctgtttacattacaatgatag**GCCT**tacatatttcac	*Stu1*
mAhR EH rev	gtgaaatatgta**AGGC**ctatcattgtaatgtaaacagaaa	*Stu1*
mERE EH perf fwd	ctctatgcctt**AACGGTCAC**tgtgaccccaggaacacatc	
mERE EH perf rev	gatgtgttcctggggtcaca**GTGACCGTT**a	
mOct1 EH perf fwd	ggatctttt**AC**gttta**G**attacaatgatagcgtgta	
mOct1 EH perf rev	tacacgctatcattgtaat**C**taaac**GT**aaagatcc	
mOct1 EH rev fwd	ggatctttt**ACATTTGTC**ttacaatgatagcgtgta	
mOct1 EH rev rev	tacacgctatcattgtaa**GACAAATGT**aaagatcc	
mFoxA1 EH 1 fwd	aa**A**a**GC**t**T**ccaaactggatctttctg	left site only
mFoxA1 EH 1 rev	gatccagtttgg**A**a**GC**t**T**tttcgtgatgtgttcctggggt	left site only

With * marked constructs are not shown in results.

12.4 Subclonings into pGL3-enhancer and pGL3-promoter constructs

To assess the role of the transcription start site and other promoter segments different promoter fragments were subcloned either in the pGL3-promoter vector or the pGL3-enhancer vector (both purchase from Promega). These vectors are almost identical to pGL3-basic but contain either an additional SV40 promoter or a SV40 enhancer. All constructs were generated by digestion and ligation using the original 2.4 kb reporter construct as template. The used restriction enzymes are listed in the table.

Table M6. Restriction enzymes used for subclonings into pGL3-promoter and pGL3-enhancer

construct name	internal number	template	enzymes
		pGL3- promoter	Kpn1/ Sma1
P1	1760	1236	Kpn1/ Sma1
P2	1764	1236	Kpn1/ Pml1
P3	1769	1236	Kpn1/ PvuII
P4	2555	1236	Kpn1/ Msc1
P5	1776	1282	Kpn1/ Sma1
P6	1828	1793	Kpn1/ PvuII
		pGL3- enhancer	Sma1/ Xho1
E1	1782	1236	Sma1/ Xho1
E2	1787	1236	Pml1/ Xho1
E3	1788	1236	PvuII/ Xho1
E4	1793	1236	Msc1/ Xho1
		pGL3- enhancer	Kpn1/ Xho1
E5	1797	1282	Kpn1/ Xho1
E6	1800	1291	Kpn1/ Xho1
E7	1805	1294	Kpn1/ Xho1
E8	1808	1322	Kpn1/ Xho1
E9	1834	1793	PvuII/Sma1
E10	1917	1793, 1327	Sma1/Xho1

13. Heat shock transformation and plasmid DNA preparation

Chemical competent DH5α cells were thawed and incubated with 5 µl ligation for 30 min on ice. Cells were then heat shocked for 45 s at 42 °C and kept on ice for 2 min before addition of 250 µl SOC medium (SOC medium: 0.5 % Yeast extract, 2.0 % tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose). After incubation for 1 h at 37 °C cells were plated on LB agar plates containing the appropriate antibiotic, which corresponds to the resistance gene of the introduced plasmid, and incubated at 37 °C overnight. Next day 2 ml LB medium aliquots containing the appropriate antibiotic were inoculated with single colonies and cultured for at least 8 hours at 37 °C. The cultures were then used to inoculate bigger cultures (maxiprep) or to isolate DNA (miniprep). For minipreps and maxipreps DNA was purified using Qiagen spin-miniprep kits or maxiprep kits according to manufacture instructions.

14. RT-PCR and quantitative real-time PCR

RNA was extracted from cells in 1 ml TriZol reagent (Invitrogen) followed by a phenol-chloroform phase extraction and isopropanol precipitation. cDNA reverse-transcription using poly-dT oligos (SigmaAldrich) was then performed on 3 µg total RNA using Expand Reverse Transcriptase (Roche) according to manufacturer's instructions.

2 µl cDNA were used in the subsequent real-time quantitative PCR containing 1x SYBR Green PCR Master Mix (Applied Biosystems) and 0.4 µM gene specific primers (synthesized by SigmaAldrich, listed below). PCR was performed on an ABIprism7500 (Applied Biosystems) with 40 cycles of two-step amplification (1x 2 min 50 °C, 1x 10 min 95 °C, 40 x 15 sec 95 °C, 1 min 60 °C measurement). The fold change of expression was calculated using either βactin or PPIA as an internal reference gene and the expression level was determined relative to the vehicle treated control.

Table M7: Primers used for RT-qPCR

Primer name	sequence	Product size
RT ßactin fwd	GTCTTCCCCTCCATCGTG	256
RT ßactin rev	GGGTACTTCAGGGTGAGGATG	
RT PPIA fwd	AGGGTTCCTGCTTTCACAGA	83
RT PPIA rev	CTTGCCACCAGTGCCATTAT	
RT pS2 fwd	CACCATGGAGAACAAGGTGA	134
RT pS2 rev	TGACACCAGGAAAACCACAA	
RT BASE (2/3) fwd	GGAGTTTCTTCCCAGCTCCT	135
RT BASE (2/3) rev	GATGTCCAGGAGGCCTGATA	
RT BASE (5/6) fwd	GGAGTTTCTTCCCAGCTCCT	176
RT BASE (5/6) rev	GATGTCCAGGAGGCCTGATA	
RT ERα fwd	CATGATCAGGTCCACCTTCT	170
RT ERα rev	AGCAGCATGTCGAAGATCTC	
RT ERRα fwd [1]	TCGCTGTCTGACCAGATGTC	502
RT ERRα rev [1]	CCTCGAGCATCTCCAAGAAC	
RT ERRß fwd [1]	CCCCTACCTGAGCTTACAGATTT	486
RT ERRß rev [1]	TACATTGAATCTGAGTTGGCAAG	
RT ERRγ fwd [1]	ACCATGAATGGCCATCAGAA	470
RT ERRγ rev [1]	ACCAGCTGAGGGTTCAGGTAT	
RT ERß fwd [1]	TGAAAAGGAAGGTTAGTGGGAACC	528
RT ERß rev [1]	TGGTCAGGGACATCATCATGG	
RT E4F fwd 2	CTGCTGGAGGTGGAGGAGTT	113
RT E4F rev 2	TCAGCTACCACGGACGAGAA	
RT INSM1 fwd 1	TTGGAACCCCCACTTTTACG	112
RT INSM1 rev 1	TTCCAACCACGAGACAAACG	
RT ZNF219 fwd 2	CTTTCCGCTCAGCACATCAC	82
RT ZNF219 rev 1	TAGTCGCAGTGCGGACACTT	
RT IRF3 fwd 1	CAAGAGGCTCGTGATGGTCA	142
RT IRF3 rev 2	GTCGGAGGTGAGGGAGAGTG	
RT FoxF2 fwd 1	CGCTGGAGCAGAGCTACTTG	
RT FoxF2 rev 1	CCCATTGAAGTTGAGGACGA	122
RT FoxF2 rev 2	CGCTAGCTGAGGGATGGAAA	150
RT ATF2 fwd 1	AACCGCCATGCAGAAGAAAT	123

[1] primer published by (Cheung *et al*., 2005)

Table M7: Primers used for RT-qPCR (continued)

Primer name	sequence	Product size
RT ATF2 rev 1	TTGGATGTGCTGACCGAACT	
RT FoxA1 fwd 1	GAAGATGGAAGGGCATGAAA	97
RT FoxA1 rev 1	GCCTGAGTTCATGTTGCTGA	
RT CTSD (5/6) fwd 1	CAGAAGCTGGTGGACCAGAAC	139
RT CTSD (5/6) rev 1	TGCGGGTGACATTCAGGTAG	
RT E4BP4 fwd 1	GCGCTCGGAACTGACCTACT	
RT E4BP4 rev 1	CCACATTGCTACTGGCATCA	117
RT E4BP4 rev 2	ACCATCATCTTGTCCACATTGC	130

15. siRNA experiments

siRNAs against FoxA1 and INSM1 were purchased from Dharmacon (SMARTpool siRNA) and Stealth™ siRNA against ERα, E4BP4, and Oct-1 were ordered from Invitrogen. MCF7 cells were transfected (in 6-well plates) with siRNA at a final concentration of 100 nM using reverse-transfection with Lipofectamine2000 (Invitrogen). The next day, cells were treated and 24 hours later harvested for RNA isolation or analysis by Western blot. When hormone deprivation was required, cells were set E2 free 24 hours prior transfection.

16. Antibody generation

BASE antibodies raised in two rabbits against two peptides (ESTPQRKEATVQ and KVQIRLEKNVGGRY) of the BASE protein were generated and purified by Eurogentec S.A. (Belgium). The antibodies were tested in Western Blot analysis using recombinant BASE protein produced by the Protein Expression and Purification Core Facility (Ario DeMarco) at the EMBL using the pET22b(+)-BASE short construct described above.

17. Western blotting

Cells were washed twice with phosphate buffered saline (PBS) and directly lysed in Lämmli buffer (4x: 250 mM Tris pH 6.8, 8 % SDS, 40 % glycerol, 150 mM DTT, bromphenol blue). Samples were sonicated to reduce the viscosity and proteins were resolved on a 10 % SDS-PAGE gel at 200 V in SDS/Page running buffer (5x: 25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS). Proteins were then transferred onto PVDF membranes (Millipore Corporation, Bedford, MA, USA) using wet transfer over night (50 V, 4 °C) (transfer buffer: 0.02 M Tris, 0.15 M glycine, 20 % MetOH).

.

Membranes were blocked for 1 h in 3 % milk + 0.05 % Tween®20 (Sigma-Aldrich,St. Louis, MO, USA), incubated 1 h with primary antibodies (Table M1), washed and incubated for another 1 h with corresponding horseradish peroxidase-conjugated secondary antibodies (1:10000), and developed

using ECL™ Western Blotting Detection reagents (PerkinElmer Life Sciences, Boston, MA, USA) and Kodak Biomax MR films. To reprobe the membranes these were stripped for 20 min in stripping buffer (0.1 M glycin (pH 2.8)), washed with PBS and reprobed with the desired primary antibody.
For protein analysis in the cell culture medium (for secreted proteins) samples were prepared through "Wessel-Flugge precipitation" (Wessel and Flugge, 1984). In short, 1 ml of medium was combined with 4 ml of MetOH and mixed well. After addition of 1 ml Chloroform, sample was vortexed again before 3 ml dH_2O were added to increase the volume. Following another vortexing step samples were centrifuged for 10 min at 14,000 xg at 4°C. The aqueous phase was removed and 3 ml MetOH were added. Proteins were collected by centrifugation at 4 °C for 30 min at 14,000 xg. The pellet was washed ones with 50 % MetOH, dried and resuspended in 1x Lämmli buffer.

Table M8: Western blot gel compositions

component	separation gel (10 %, 50 ml)	stacking gel (5 %, 10 ml)
30 % acrylamide	16.7 ml	1.7 ml
1.5 M Tris (pH 8.8)	12.5 ml	
1.0 M Tris (pH 6.8)		1.25 ml
10 %SDS	0.5 ml	0.1 ml
10 % ammonium persulfate	0.5 ml	0.1 ml
TEMED (Sigma)	0.02 ml	0.01 ml
dH2O	19.8 ml	6.8 ml

18. Immuostaining and Microscopy

For localization studies MCF7 cells were seeded in 6 cm plates containing sterile round cover slips and allowed to settle over night at 37 °C, 5 % CO_2. Using ExGene™500 cells were transiently transfected with either pcDNA3.1hygro-BASE-GFP, pcDNA3.1hygro-GFP-BASE, or pcDNA3.1hygro-GFP, and where indicated co-transfected with pSRß-mRFP, encoding red-fluorescent-protein (RFP) with a leading sequence which targets it to the endoplasmaic reticulum. The pSRß-mRFP plasmid was kindly provided by J. Ellenberg, EMBL, Heidelberg.
24-48 hours after transfection, cells were washed twice with PBS and fixed with ice cold MetOH for 5-10 min. Cell nuclei were stained with DAPI, washed, and cells were mounted in Moviol containing the radical scavenger DABCO and then examined using a Zeiss Axioscope fluorescent microscope (Carl Zeiss, Jena, Germany) using the x63 objective.

Moviol was prepared according to Osborn und Weber (1982).
2.4 g Mowiol 4-88 (Hoechst, Frankfurt), 6 g Glycerol and 6 ml ddH_2O were stirred for 3 h at room temperature. To dissolve the Moviol 12 ml of 0.2 M Tris-HCL (pH 8.5) were added followed by a 10 min incubation at 50 °C with occasional stirring. After centrifugation for 15 min at 5000 xg the supernatant was aliquoted and stored at –20 °C.

19. Electrophoretic mobility shift assay (EMSA) - radioactive and non-radioactive

Radioactive and non-radioactive EMSAs differ in 2 steps – the probe preparation and based on this the detection method.

Oligos (see table below) were ordered from SigmaAldrich either unmodified or for non-radioactive EMSAs with 5'-end biotinylation. The oligos were annealed by heating sense and antisense oligos in 1x annealing buffer (10x: 100 µl 1 M Tris (pH 8.0), 200 µl 5 M NaCl, 120 µl 0.5 M EDTA, 690 µl dH_2O) up to 100 °C and slow cooling to room temperature. Efficiency of annealing was analysed in a 12 % native gel. For radioactive EMSAs 2 µg dsoligos were end labelled in a 20 µl reaction in 1x PNK buffer with 1.5 µl T4 polynucleotide kinase (PNK, New England Biolabs) and 1 µl [γ-^{32}P]ATP (3,000 Ci/mM; PerkinElmer Life Sciences). After 90 min incubation at 37 °C probes were purified using Chromaspin columns (Clontech, USA). The columns were prepared by centrifugation at 2000 xg for 5 min. Probe volume was increased to 100 µl with TE buffer and applied to the columns. Probe was recovered by centrifugation for 5 min at 2000 xg. 1 µl was used for analysis in the scintillation counter.

In vitro translated proteins or extracts of MCF7 cells were pre-incubated in binding buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 3 mM $MgCl_2$, 100 µg/ml BSA, 12 % glycerol, 1 mM DTT, 100 mM KCl, if mentioned hormone) with 1 µg of poly (dI/dC) for 5 min at room temperature. The samples were then incubated for 30 min at room temperature with 1 ng of radioactive oligonucleotide probe (1-6*10^4 cpm) or non-radioactive Biotin labelled probes (SigmaAldrich). For competition assays 10 to 100x of unlabeled oligonucleotide was used. For supershift experiments, specific antibodies (1 mg/ml) were added 5 min after initial start of incubation. Protein-DNA complexes were then separated from free probe by non-denaturing electrophoresis on 4 % or 6 % polyacrylamide gels in 1x TBE (45 mM Tris/borate, 1 mM EDTA). The gels were pre-run at 4 °C for 30 min followed by electrophoresis for 2 h at 200 V.

For radioactive EMSAs gels were dried and subsequently exposed to Kodak Biomax film (Eastman Kodak Co., Rochester, NY). Non-radioactive gels applied to Western blot procedure using peroxidise-conjugated Streptavidin antibody (Jackson ImmunoResearch) to detect the Biotin-labeled oligos.

Table M9: Gel composition for EMSAs

	30 % acrylamide (BioRad)	10x TBE	10 % ammonium persulfate	TEMED (Sigma)	dH_20
4 %	6,7 ml	2,5 ml	0,5 ml	0,03 ml	40,3 ml
6 %	10 ml	2,5 ml	0,5 ml	0,03 ml	37 ml
12 %	20 ml	2,5 ml	0,5 ml	0,03 ml	27 ml

Table M10: Oligos used in EMSAs

Oligo name	Sequence
BASE_EH_I_sense	$^{-1994}$ttcaggatccaaattgttaactctatgccttggaacattttgTGACCcca^{-1944}
BASE_EH_II_sense	$^{-1954}$tgTGACCccaggaacacatcacgaaatatttaccaaactggatctttctg^{-1904}
BASE_EH_III_sense	$^{-1914}$gatctttctgtttacattacaatgatagcgtgtacatatttcactacatc^{-1864}
BASE_EH_IV_sense	$^{-1874}$tcactacatctttgccagcagatggaaacctttattgtaaatatttgctc^{-1824}
BASE C2_sense	$^{-1930}$aatatttaccaaactggatctttctgtttacattacaatgatagcgtgta^{-1880}
BASE C2_anti-sense	$^{-1880}$tacacgctatcattgtaatgtaaacagaaagatccagtttggtaaatatt^{-1930}
BASE C1*_sense	$^{-1970}$atgccttggaacattttgtgaccccaggaacacatcacgaaatatttacc^{-1920}
BASE C1*_anti-sense	$^{-1920}$ggtaaatatttcgtgatgtgttcctggggtcacaaaatgttccaaggcat^{-1970}
BASE random _sense	atcagtctagaactacaactaatctgtgttcatacaagttatagtttcta
BASE random_anti-sense	tagaaactataacttgtatgaacacagattagttgtagttctagactgat
BASE EH 100_sense	$^{-1970}$atgccttggaacattttgtgaccccaggaacacatcacgaaatatttaccaaactggatctttctg tttacattacaatgatagcgtgtacatatttcac^{-1870}
BASE EH 100_anti-sense	$^{-1870}$gtgaaatatgtacacgctatcattgtaatgtaaacagaaagatccagtttggtaaatatttcgtg atgtgttcctggggtcacaaaatgttccaaggcat^{-1970}
positiv controlle pS2_sense	$^{-414}$ccttcccttccccctgcaaggtcacggtggccaccccgtgagccactgtt^{-364}
ERE1_sense (control)	taatggaaGGTCAgtcTGACCtgagcacag
ERE1u_anti-sense (control)	ctgtgctcaGGTCAgacTGACCttccatta
EREmut_sense (control)	taatggaag**ca**cagtct**g**t**c**ctgagcacag
EREmutu_anti-sense(control)	ctgtgctcaggacagactgtgcttccatta

20. Chromatin immunoprecipitation (ChIP) assay

Cells were grown in 10 cm plates when necessary deprived of hormone for 3 days before stimulation with either vehicle (EtOH), 10^{-8} M E2 or 10^{-6}M $ICI_{182,780}$ for indicated times. Cells were crosslinked with 1 % formaldehyde for 15 min at room temperature. The reaction was stopped by addition of gylcin to the final concentration of 0.125 M. After two washes with cold PBS cells were scraped in 1 ml collection buffer (10 mM Tris-HCl, (pH 8.0), 150 mM NaCl, 1 mM EDTA) and collected for 5 min at 3000 xg. Cell pellet was resuspended in 400 µl lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 10 mM ß-glycerophosphate, 1 mM Na_3VO_4, proteinase inhibitors). Chromatin was sonicated to about 500 bp fragment size and cell debris was sedimented by centrifugation at 14,000 xg for 10 min.The supernatant was saved and diluted to 10 ml with ChIP dilution buffer (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 10 mM ß-glycerophosphate, 1 mM Na_3VO_4, proteinase inhibitors). Chromatin was either frozen at –80 °C for later use or processed immediately. 100 µl chromatin were incubated with 0.4 µg antibody or IgG (or nothing for input samples) for 4h or over night. To recover the protein-DNA-complexes 50 µl 50 % Protein A slurry (for goat antibodies Protein G was used) pre-absorbed with sheared salmon sperm DNA were added, and incubated for 2-3 h rotating at 4 °C. For the subsequent washing steps BioRad mini columns were prepared by adding 500 µl dilution solution. After transfer of the immunoprecipitations to the columns the tubes were rinsed with 500 µl dilution solution to ensure complete transfer of the beads. Subsequent washing steps were as follows: 2x with 1 ml TSEI (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl), 2x with 1 ml TSEII (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl), 2x with

TSEIII (1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1 % NP-40, 1 % sodium deoxycholate, 0.25 M LiCl), and 3x with 1 ml TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA).

For DNA recovering the bottom of the mini columns were capped and beads were resupended in 400 µl TE buffer and transferred to fresh tubes. Additional 500 µl of TE buffer were used to rinse the columns and transferred to the same tubes. After centrifugation at 3,000 xg for 2 min supernatant was aspirated and 100 µl of 10 % Chelex (in water) added. Beads were vortexed and incubated at 95 °C for 10 min to reverse the crosslinking. To release DNA and digest the proteins 2 µl Proteinase K solution (Invitrogen) was added and incubate at 55 °C for 30 min and then inactivated at 95 °C for 10 min. Supernatant was cleared by centrifugation for 2 min at full speed and transferred to a fresh tube. Remaining beads were washed with 100 µl water, sedimented again and supernatant was transferred to the same tube as in previous step. 2-5 µl were used as template in subsequent PCRs.

Table M11: Primers used for ChIP

Primer name	sequence	product size (bp)
BASE -575 rev	ccatctgctggcaaagatgtagtga	184
BASE w2 fwd	gatctttccaatgtttgcctga	
CTSD distal fwd	cctcctcaactgctcttgca	172
CTSD distal rev	gcggctgagatgctgagtca	
CTSD TSS fwd	accggtccgggtgcaaacacg	189
CTSD TSS rev	ctgaggcttcacctgacgagc	
CTSD unspecific fwd	cctcacaggtgcgtatctca	117
CTSD unspecific rev	agcaaggggtgaaagatggt	

21. ChIP-on-Chip

ChIP-on-ChIP experiments were performed by Agilent Technologies including Chromatin immunoprecipitation, fluorescent labelling with Cy3 (input) and Cy5 (immunoprecipitated material), hybridiziation to custom made promoter array provided by Agilent. Chromatin from MCF7 cells grown in normal media was provided by Heike Brand (EMBL, Gannon laboratorium). The array covered promoter regions of selected genes from -8 kb to +2 kb with 60 bp probes in 200 bp intervals. Repetitive sequences were omitted.

22. Expression analysis of BASE in human breast cancer tissues

(Department of Surgery, National University of Ireland, Galway, Ireland; D. Coyle, N. Miller, RE Mc Neill, MJ. Kerin)

Primary breast tumours were obtained from the Department of Surgery Biobank (Dept. of Surgery, University College Hospital, Galway) and divided into 6 groups of age-matched patients. The groups are summarized in table M12.

Table M12: Summary of patient groups

Patient Group	Progression of breast cancer at 5 year follow-up
Metastasis-free (n=11)	No metastasis of breast cancer detected
Bone Metastasis (n=8)	Bone metastasis only
Bone and Visceral Metastasis (n=9)	Metastasis to both bone and viscera
Visceral metastasis (n=2)	Metastasis to viscera only
Benign	Non-applicable, no follow-up required
Normal	Non-applicable, no follow-up required

Total RNA was isolated and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) according to manufacturers instruction. BASE expression was analysed in end-point PCRs using Amplitaq Gold® DNA polymerase (Applied Biosystems, Warrington, UK) or in quantitative RT-PCR using Taqman Universal Master mix (Applied Biosystems, Warrington, UK). BASE expression levels were normalized against the endogenous control PPIA gene expression levels by subtracting the average PPIA cycle threshold (Ct) from the average BASE Ct for each cDNA sample, yielding a level of mRNA expression for the target molecule relative to the endogenous RNA reference gene (ΔCT). The ΔCT for our calibrator sample, the breast cancer cell line T47D, was subtracted from the ΔCT values for all cDNA samples to yield mRNA expression relative to the calibrator sample (ΔΔCT). The relative quantity of gene expression for each sample was calculated using the formula $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001).

23. Generation of CTSD reporter constructs

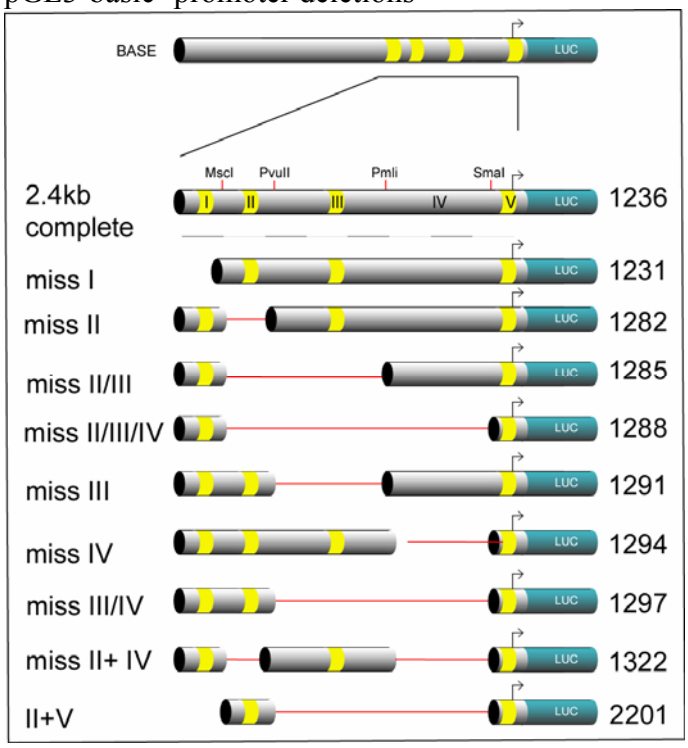
Two sections of the CTSD promoter were amplified using FastStart Taq Polymerase (Roche) and the primers listed below. The first primer pair (distal) flanks the -9698 to -8725 bp segment containing a putative enhancer while the second pair (proximal) amplifies the -753 to +96 segment containing the transcription start site and the proximal promoter. The PCR products were cloned into pGL3-basic vector both separate and combined using the restriction sites introduced by the primers.
To mutate the almost perfect ERE in the distal part the mut dERE primers, which introduce an EcoR1 site for easier screening, were used. All constructs were verified by sequencing.

Table M13: CTSD cloning and mutation primer. Introduced restriction sites for cloning are indicated in italic. Mutated bases are displayed as capital letters.

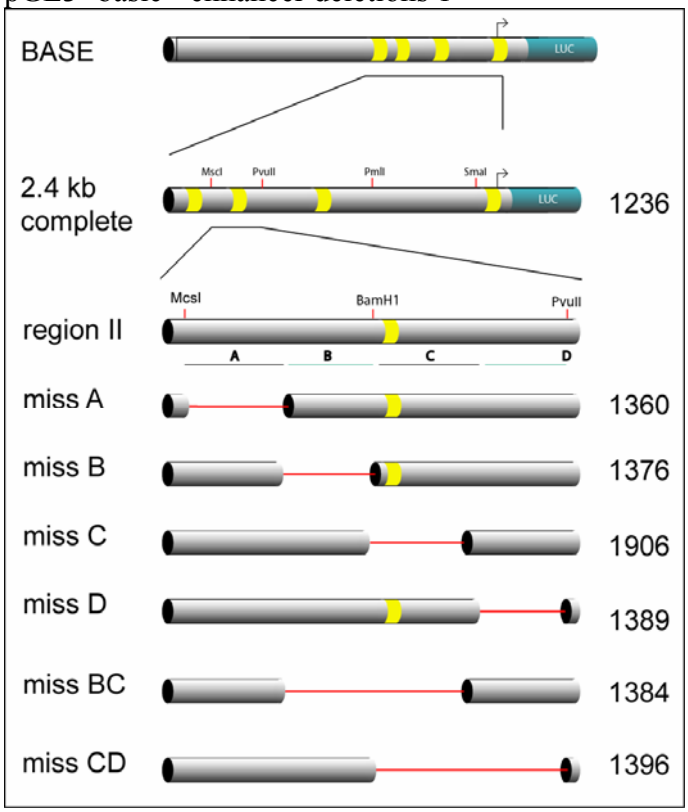
Primer name		sequence	product size
luc distal fwd	(*Nhe1*)	at *gctagc* atttgtgatcctggaaggtcaggt	973 bp
luc distal rev	(*BglII*)	gt *agatct* cctcctcttagggctgagtcactg	
luc prox fwd	(*BglII*)	gt *agatct* gagttgacgtgagtggacaaaagg	849 bp
luc prox rev	(*HindIII*)	ac *aagctt* gtgcgcttatagccgggatgac	
mut dERE fwd	(*EcoR1*)	tCCg *gAATTC*ggtTgccc cagctctgagagtg	
mut dERE rev	(*EcoR1*)	gggcAaccGAATTc cggaagagaaaggggggctgcg	

24. Summary of constructs

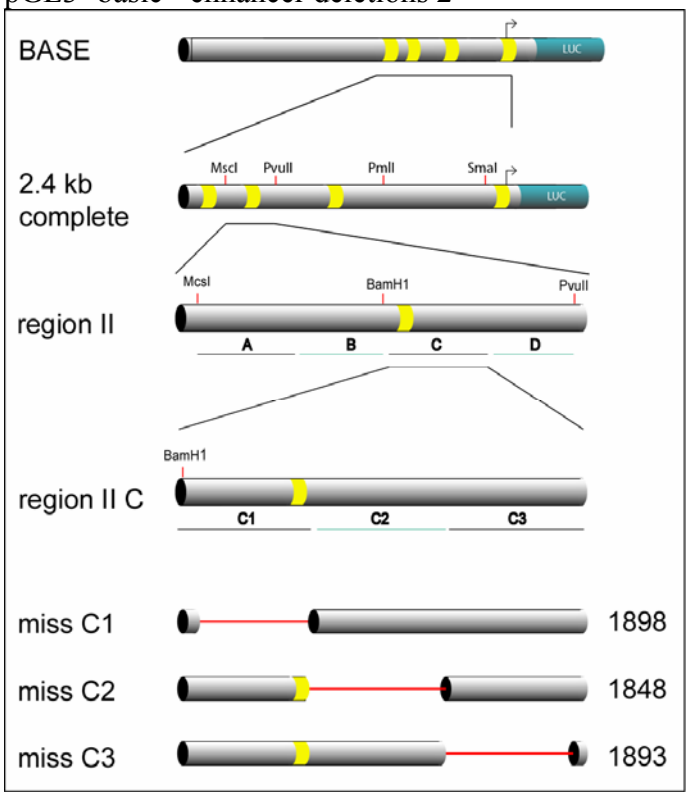
pGL3-basic- promoter deletions



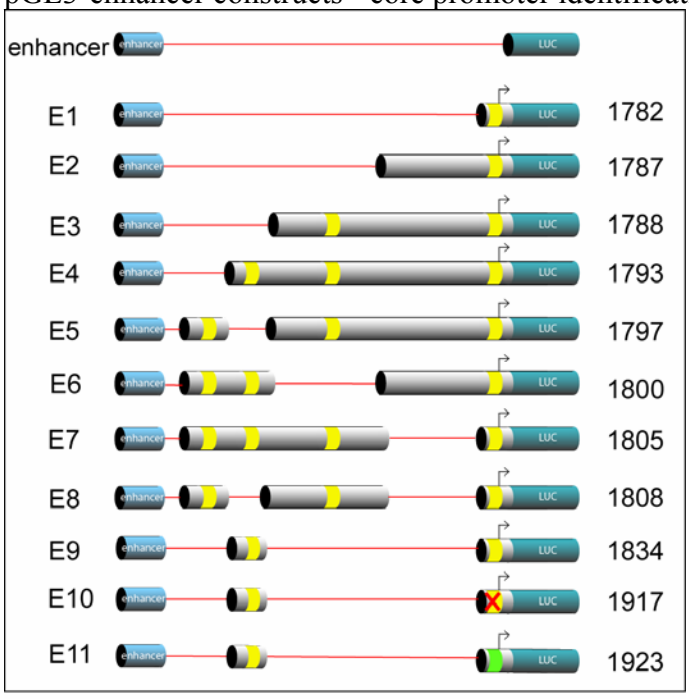
pGL3- basic - enhancer deletions 1



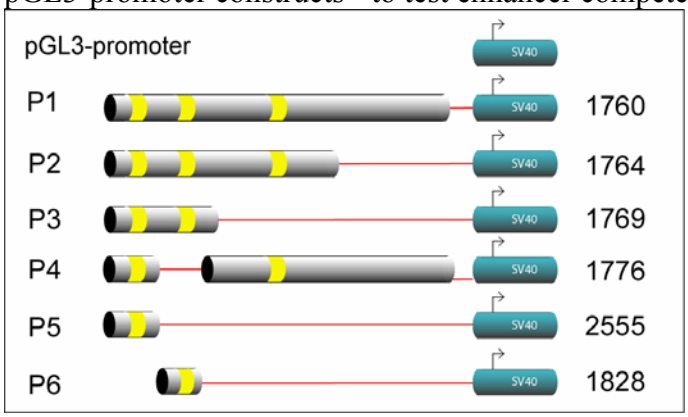
pGL3- basic - enhancer deletions 2



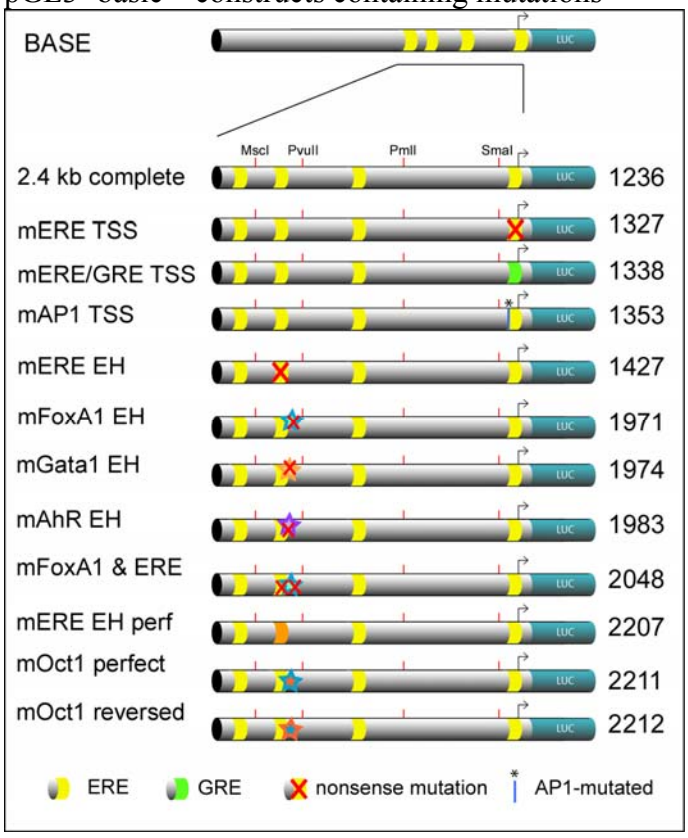
pGL3-enhancer constructs - core promoter identification



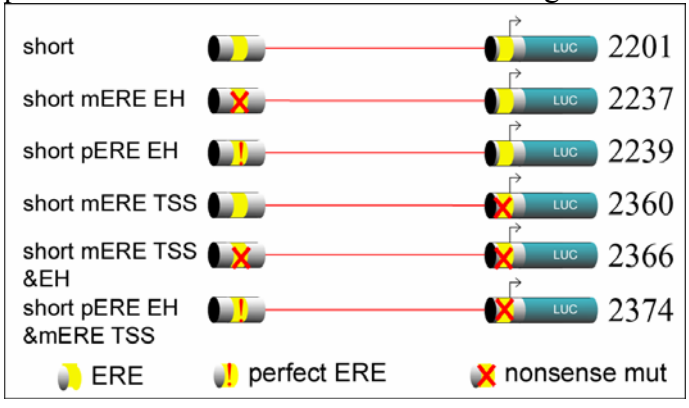
pGL3-promoter constructs - to test enhancer competence



pGL3- basic – constructs containing mutations



pGL3- basic - mutations in restricted background



ABBREVIATIONS

3C	chromatin-conformation-capturing
AF-1 / 2	activation function 1 / 2
AhR	aryl hydrocarbon receptor
AI	aromatase inhibitors
ATP	adenosine triphosphate
BASE	breast cancer and salivary gland expression gene
BPI	bacterial/permeability-increasing protein
BRCA1/2	breast cancer 1 gene/ breast cancer 2 gene
BSA	bovine serum albumin
cAMP	cyclical adenosine monophosphate
cDNA	complementary DNA
CDS	coding sequence
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
C-terminus	carboxy-terminus
CTSD	cathepsin D
DAPI	4,6-diamino-2-phenylindole
DBD	DNA binding domain
dNTP	deoxy nucleotide triphosphate
dsDNA	double-stranded DNA
E2	17ß-estradiol
E4BP4	E4 binding protein
EGFR	epidermal growth factor receptor (HER-1, Neu)
EH	enhancer
ELOVL2	elongation of very long chain fatty acids protein 2
EMSA	electromobility shift assays
ERα	estrogen receptor alpha
ERE	estrogen response element
ERR	estrogen-related receptor
ERß	estrogen receptor beta
EST	expressed sequence tag
EtOH	ethanol
FCS	fetal bovine serum
FoxA1	forkhead factor A1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gata-1	GATA-binding protein 1, erythroid transcription factor 1 (ERYF1)
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GREB1	gene regulated by estrogen in breast cancer protein
HAT	histone acetyl transferases
HDAC	histone deacetylase
HER-1	EGFR, Neu
HER-2	ErbB2 (((erythroblastic leukaemia viral oncogene homolog????????)))
HMT	histone methyl transferases
IgG	immunoglobulin G
IL-6	interleukin 6
INSM1	zinc finger protein insulinoma-associated 1, IA-1
IRF-3	interferon regulatory factor 3
kDa	kilo Dalton

LBD	ligand binding domain
LBP	ligand binding pocket
Luc	luciferase
Lys	lysin
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NCoR	nuclear receptor corepressor
NFIL3	nuclear factor, interleukin 3-regulated, E4BP4
NMD	nonsense-mediated mRNA decay
N-terminus	amino-terminus
NURD	nucleosome remodelling and deacetylating complex
Oct-1	octamer-binding transcription factor 1 (Pou2F1)
PCR	polymerase chain reaction
PI3K	phosphatidyl-inositol-3-kinase
PLUNC	palate, lung, and nasal epithelium carcinoma-associated protein
PPIA	peptidyl-prolyl isomerase
pS2	TFF1, trefoil factor 1
qPCR	quantitative PCR
RFP	red fluorescent protein
RLU	relative luciferase units
RT	reverse transcriptase
SBEM	small breast epithelial mucin
SCLC	small-cell lung cancer
Ser	serin
SERM	selective estrogen receptor modulator
SHP	short heterodimer partner
siRNA	small interference RNA
SUMO	small ubiquitin-like modifier
Tam	Tamoxifen
TERP1/2	truncated estrogen receptor product 1/2
TNF	tumor necrosis factor
TR	thyroid hormone
TSS	transcription start site
UTR	untranslated region
ZNF219	zinc finger protein 219

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