Breast Cancer Research

Proliferation-associated POU4F2/Brn-3b transcription factor expression is regulated by estrogen through ER-alpha and growth factors via MAPK pathway

Breast Cancer Research 2011, **13**:R5 doi:10.1186/bcr2809

Samir Ounzain (s.ounzain@ich.ucl.ac.uk)
Samantha Bowen (s.bowen@ich.ucl.ac.uk)
Chandrakant Patel (c.patel@ich.ucl.ac.uk)
Rieko Fujita (r.fujita@ich.ucl.ac.uk)
Richard J Heads (richard.heads@kcl.ca.uk)
Vishwanie S Budhram-Mahadeo (v.mahadeo@ich.ucl.ac.uk)

ISSN 1465-5411

Article type Research article

Submission date 9 July 2010

Acceptance date 17 January 2011

Publication date 17 January 2011

Article URL http://breast-cancer-research.com/content/13/1/R5

Proliferation-associated POU4F2/Brn-3b transcription factor expression is regulated by estrogen through ER-α and growth factors via MAPK pathway

Samir Ounzain[1], Samantha Bowen[1], Chandrakant Patel[1], Rieko Fujita[1], Richard J Heads[2], Vishwanie S Budhram-Mahadeo[1]*

[1]Medical Molecular Biology Unit, UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

[2]Cardiovascular Division, King's College London, Department of Cardiology, The Rayne Institute, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, UK

*Corresponding author, v.mahadeo@ich.ucl.ac.uk

Abstract

Introduction: In cancer cells, elevated Brn-3b transcription factor enhances proliferation *in-vitro* and increases tumour growth *in-vivo*, whilst conferring drug resistance and migratory potential, whereas reducing Brn-3b slows growth, both *in-vitro* and *in-vivo.* Brn-3b regulates distinct groups of key target genes that control cell growth and behaviour. Brn-3b is elevated in >65% of breast cancer biopsies, but mechanisms controlling its expression in these cells are not known.

Methods: Bioinformatics analysis was used to identify the regulatory promoter region and map transcription start site and transcription factors binding sites. Polymerase chain reaction (PCR) cloning was used to generate promoter constructs for reporter assays. Chromatin immunoprecipitation (ChIP) and site-directed mutagenesis were used to confirm transcription start site and auto-regulation. Breast cancer cell, MCF7 and Cos7 cells were used. Cells grown in culture were transfected with Brn-3b promoter and treated with growth factors or estradiol to test for effects on promoter activity. Quantitative reverse transcriptase PCR (q-RT-PCR) and immunoblotting were used to confirm changes in gene /protein expression.

Results: We cloned the Brn-3b promoter, mapped the transcription start site and showed stimulation by estradiol and growth factors, nerve growth factor (NGF) and epidermal growth factor (EGF), which are implicated in breast cancer initiation/progression. The effects of GFs are mediated through mitogen activated protein kinase (MAPK) pathway, whereas hormone effects act via the estrogen receptor, ERα. Brn-3b also auto-regulate its expression and co-operates with ERα to further enhance levels.

Conclusions: Key regulators of growth in cancer cells e.g. estrogens and growth factors can stimulate Brn-3b expression and auto-regulation also contributes to increasing Brn-3b in breast cancers. Since increasing Brn-3b profoundly enhances growth in these cells, then understanding how Brn-3b is increased in breast cancers will help to identify strategies for reducing its expression and thus effects on target genes, thereby reversing its effects in breast cancer cells.

Introduction

The POU4F2/Brn-3b transcription factor belongs to the class 4 POU (Pit-Oct-Unc) family of transcriptional regulators (hence POU4F2) and was named Brn-3b because of homology in the DNA binding domain to the related Brn-3a transcription factor. Brn-3b is highly expressed in a significant proportion (> 65%) of breast tumour biopsies analyzed [1]. Over-expression of Brn-3b in cancer cells is strongly associated with increased proliferation, in vitro, and enhanced tumour growth, in-vivo, whereas reducing Brn-3b (by antisense) decreases proliferation *in-vitro* and result in smaller, slower growing tumours *in-vivo* [2, 3]. Brn-3b also confers resistance to chemotherapeutic drugs that inhibit cell growth and increases migratory potential of cancer cells [2]. Recent studies also showed that Brn3b is increased in doxorubicin resistant breast cancer cells (unpublished data).

As a transcription factor, Brn-3b regulates the expression of critical genes that control different cellular processes. For example, increased proliferation by Brn-3b may be associated with its ability to transactivate the promoters of cyclin dependent kinase, CDK4, [4] and its regulatory partner cyclin D1[5], which are required for cell cycle progression whilst repressing BRCA1 [6], which is associated with cell cycle arrest in breast cancer cells. Invasiveness and drug resistance associated with Brn-3b in cancer cells are linked with its ability to transactivate genes such as the small heat shock protein, HSP27 [7] whilst repressing adhesion molecules e.g. γ-catenin/ plakoglobin [8].

However, whilst the effects of increased Brn-3b in cancer cells have been characterized and many of its target genes have been studied, we do not know what factors contribute to the elevated Brn-3b mRNA and protein levels observed in breast cancers. In this study, we have cloned and analyzed the regulatory region that controls Brn-3b expression in MCF7 breast cancer cells. Results presented herein, identifies a proximal promoter present in the 5' sequences upstream of Brn-3b gene which drives expression in MCF7 cells. This promoter is

transactivated by growth factors, NGF and EGF and the hormone, estradiol, all of which are known to promote proliferation/survival of breast cancer cells. NGF and EGF increase promoter activity by signalling through the MAPK pathway whereas the effects of estrogen are mediated via the estrogen receptor alpha (ERα) but not beta (ERβ). We also showed auto-regulation by Brn-3b to increase its own expression. These findings suggest that increased transcription of Brn-3b in breast cancer cells is stimulated by growth factors and hormones that enhance proliferation and propagated through auto-regulation.

Materials and methods

Materials

General laboratory reagents were purchased from Merck (UK) and Sigma (UK) unless otherwise stated. Primary antibodies were used at dilutions of 1: 1000-1500 and included Brn-3b-rabbit pAb (Abcam) or goat pAb (Santa Cruz Biotechnology Inc, USA); actin - goat pAb (I-19, Santa Cruz Biotechnology). HRP-conjugated secondary Ab from Dako (UK) was used for immunoblotting 1:2000). Estradiol (E4389), cyclic AMP and phorbol 12,13-dibutyrate (PDBu) and 4-hydroxytamoxifen (tamoxifen) were from Sigma; epidermal growth factor (EGF), transforming growth factor-β (TGF-β); insulin-like growth-1 (IGF 1) and nerve growth factor (NGF) were from Roche Diagnostics GmbH (Germany). Signalling pathways inhibitors Ro31-8220 (PKC), PD-98059 (MEK), SB-203580 kinase (p38), Genistein (tyrosine kinase), and Wortmannin (PI3-kinase) were from Calbiochem (Nottingham, UK). MCF7 breast cancer cell line was obtained from ATCC. Expression vectors, Brn-3b(l); Brn-3b(s); ER were previously described [9]. Dominant negative and constitutively active MEK expression vectors were kind gift from D.S. Latchman[10].

In-silico analysis of Brn-3b promoter

Homosapien chromosome 4 contig was analyzed using Blast to identify region containing Brn-3b gene. ~10 KB sequence (in BAC clone AC093887). Further analysis using Bioinformatics and Molecular Analysis Section (BIMAS) Promoter Scan identified putative promoter sequences in this region of DNA. VISTA Genome Browser[11] was used to generate homology plots whereas Genomatix Transfac analysis was used to identify binding sites for transcription factors in the putative promoter sequences.

Brn-3b reporter constructs

Brn-3b reporter constructs were generated so that the regulatory promoter region drives expression of firefly luciferase reporter gene, in pGL2 plasmid. The initial Brn-3b reporter construct was generated by amplifying ~1400 bp regions upstream of the gene sequence and incorporating part of exon 1 (Figure 1B). The resultant construct was designated BSX (BstX1/ Stu1 /Xho1) because it included sequences from the BstX1site (5'end) which extended to Xho1 site in exon 1 and included Stu1 site used for diagnostic digest. BSXEIE construct was subsequently generated by including the gene coding/genomic sequence (exon 1, intron and exon 2) upstream of luciferase reporter gene.

Chromatin Immunoprecipitation (ChIP) Assay

This technique was carried out as described by Lee et al. [7]. Anti-TBP Ab (Abcam) was used to immunoprecipitate regions of promoter bound by TBP in the transcription initiation complex and anti-goat Brn-3b Ab (Santa Cruz) was use to immunoprecipitate Brn-3b on chromatin in intact cells. Negative control ChIP was undertaken using anti-GAPDH (Abcam) or 2nd Ab (Dako) only. Shear size of DNA following ChIP and sonication was ~200-600 bp, as determined for agarose gel electrophoresis. PCR for transcriptional start site was performed on ChIP DNA using primers designed to amplify different regions of putative Brn-3b promoter detailed below.

(a) Upstream initiator, Fw-5'CTTGGGCCGCAACTTTATT3' and Rv-5'TACCTAAGGA CCAGCCTCCA3' (b) 278TATA Fw-5'CGGGGAGAGGGGAG TATAAC3' and Rv 5'GAT GCCTGACTCCGCTTG 3' (c) Intronic TA - Fw-5'TTGACAGCCCCCT TTATCTG3' and Rv-5'AGGCAACATCCCAGGTCATA3' and (d) negative control primers which amplified exon 2 sequence Fw-5'ACCATGAACCCCATGCAC3' and Rv-5'CTTGATGCCTCGCTGCTT3'. The distance between intronic (c) site and exonic sequences amplified was ~ 1 kb. Positive control primers amplified GAPDH promoter start site: Fw-5'TGAGCAGACCGG TGTCAC3' and Rv-5' AGGACTTTGGGAACGACTGA 3'. Primers to amplify promoter region containing Brn-3b site were Fw 5' GCCCCTTCTTCCTTTGATTG3' Rv 5' ACACACAC ACGCTCCTCTTG3'. Standard conditions for PCR included 2.5 mM $MgCl_2$ and cycling parameters: 1 cycle at 94^{o}C for 15 mins; 40 cycles of 95^{o}C for 30 sec; 58^{o}C for 30 sec and 72^{o}C for 30 sec; final cycle 72^{o}C for 5 mins. PCR products were resolved on a 2.5 % agarose/TBE gel.

Site-directed mutagenesis

Site directed mutagenesis of the Brn-3b or ERE sites in the Brn-3b promoter was carried out using QuickChange^{TM} site-directed mutagenesis kit (Stratagene) and used in accordance with the manufacturer's protocol. Primers to mutate Brn-3 site (-1324/-1312) were (Fw-5'CCCTTCTTCCTTTGATTGTGGCTAATGAAGAAGGATCCATCCAGGGGCAGGGTTT 3'Rv-5'AAACCCTGCCCCTGGATGGATCCTTCTTCATTAGCCACAATCAAAGGAAGA AGGG 3'). Primers to mutate ERE (-1263/-1255) were: (Fw-5' CATATGCGCTGTGTAATTT CTGGAATTCCCTCTCCCTGTCAGTTG 3', Rv-5' CAACTGACAGGGAGAGGGAATTC CAGAAATTACACAGCGCATATG 3'). Primers to alter upstream initiator (-1048/-1042) (Fw-5' CCAACGCTGGCTTGGGCCGCAACTCTAGATGGGAGTTTTCTTTTTC3',Rv-5' GAAA AAGAAACTCCCATCTAGAGTTGC GGCCCAAGCCAGCGTTGG3'). To mutate the proximal TATA (-278/-271) (Fw-5' GCGGGGAGAGGGGGGTACCCCTCGCCGGCCGCG 3', Rv-5'CGCGGCCGGCGAGGGGTACCCCCCTCTCCCCGC 3'). To mutate intronic TATA

(+293/+297) Fw-5' GTCTTCCAACCCCACCGGTGGGTACCCCTGCATAATCACCGCTTA AA 3', Rv-5'TTTAAGCGGTGATTATGCAGGGGTACCCACCGGTGGGGTTGGAAGAC3'. To alter upstream TATA +590/+604) Fw-5'-ACATTCCCAGATCGTTAAAATAATA ATAATAGGTACCCTGAAAACAGAGGTGAAGAGAAAAAGAGGG3', Rv-5'CCCTCTTT TTCTCTTCACCTCTGTTTTCAGGGTACCTATTATTATTATTTTAACGATCTGGGAATG T 3') motifs. Consecutive rounds of mutagenesis were used to generate double or triple mutants. Restriction analysis together with DNA sequencing confirmed the resulting mutation.

Western Blot Analysis

Total cellular proteins preparation and immunoblotting were undertaken as previously described [6] with 1 hour block in PBST, primary antibody incubation,1-3 hours, secondary antibody incubation for 45-60 min. Signals were developed using Enhanced Chemilumiescence reagent (ECL, Amersham, UK).

Cell Culture, transient transfections, reporter assays

MCF7 breast cancer cells were maintained in DMEM medium supplemented with 10% FCS; 1% NEAA and 1% penicillin/streptomycin. Cells were plated into 6 well plates ($5x10^4$ cells/well), 24 hours before transfection with reporter and expression vectors using Fugene™ (Roche; UK) or GeneJuice (Merck Biosciences). Transfections were undertaken according to manufacturer's protocol. To reduce activity endogenous ER levels, cells were grown in estrogen-depleted medium i.e. phenol-red less DMEM medium supplemented with charcoal stripped FCS [9] for up to 72 hours before transfection and subsequent analysis. 48 hours following transfection, promoter activity was measured using Dual-Luciferase reporter kit (Promega, UK) according to manufacturer's protocol using TD-20/20 luminometer (Turner Designs). Internal control, renilla luciferase reporter activity was used to control for variations in transfection efficiency and values were expressed as percentage of empty vector control.

Results

Identification of the Brn-3b promoter

Bioinformatics analysis of 5' sequences upstream of Brn-3b coding sequence using VISTA, revealed regions of high conservation across different species (Figure 1a). Such sequence homology often indicates key functions, [12] so in-silico analysis was undertaken for regulatory sequences in this non-coding region. Using BIMAS promoter prediction software, we identified putative transcription initiation sequences (e.g. TATA or initiator elements) within the proximal sequences (represented in Figure 1a) which can be indicative of promoters. Furthermore, Mat Inspector analysis of this sequence identified putative binding sites for TFs that are known to regulate growth of cancer cells e.g. estrogen response element (ERE), growth factor response element (EGRF) and serum response element (SRE). Because of the high conservation across species, we examined whether polymorphisms in these sequences might contribute to elevated Brn-3b expression in breast cancer biopsies by sequencing and comparing genomic DNA from 15 primary breast biopsies (including normal breast and breast cancer) and breast cancer cell lines, HB4A and MCF-7. No significant polymorphisms were observed except in microsatellite sequences (data not shown) suggesting that increased Brn-3b mRNA observed in breast tumours might result from activation of its promoter by upstream growth effectors/signalling pathways that stimulate gene transcription.

Cloning of promoter and mapping transcription start site

To identify factors that stimulate Brn-3b promoter activity and therefore gene expression in breast cancer cells, the BSX reporter construct, containing the putative Brn-3b promoter and regulatory sequences (cloned into pGL2 basic reporter vector, see Materials and methods, and Figure 1b) was used in transfection studies. Figure 1c shows high basal activity from Brn-3b promoter construct, compared with empty pGL control, thereby confirming that these sequences

were sufficient to drive gene expression. BSXEIE construct containing additional sequences including the intron region also give rise to similar results (not shown).

To identify site(s) from which transcription may be initiated on this promoter, in-vivo chromatin immunoprecipitation (ChIP) was undertaken using antibody to TBP component of the basal transcriptional complex [13]. Primers were designed to amplify regions that flanked putative transcription start sites shown in Figure 1d (position in reference to first ATG) i.e. upstream sequence (-1048-1042) or proximal TATA-like sequence (-278 to -272). Primers to amplify an intronic region with TA-like elements were also tested because this region was found to have an alternative promoter in the related Brn-3a gene, which has a similar genomic arrangement to Brn-3b. Primers to sequences in exon 2 were used as negative control.

Figure 1e shows the PCR products obtained following amplification of α-TBP ChIP DNA using primers to different putative start sites in the promoter. Panel B shows that primers flanking the putative TATA site at -278 (-278TATA) produced a strong band, not seen when primers were used to amplify control ChIP DNA (2^{nd} Ab). This was comparable with PCR products obtained using primers that amplified known start site in GAPDH gene (E), suggesting significant TBP binding to this proximal TATA containing region of the promoter. In contrast, amplification of sequences spanning the putative upstream initiator element (panel A) or intronic regions (panel C) gave rise to faint bands. This may either result from weak binding of TBP to these regions or from variability in shear size of ChIP DNA. No bands were seen with primers amplifying exon 2 (negative control) (D), indicating specificity of the assay. The data therefore suggested significant binding of TBP to proximal TATA and possibly weak binding to initiator elements and sequences within the intron.

To confirm which of these sites was required for transcription initiation, site-directed mutagenesis was used to alter bases at the proximal -278TATA; upstream site (-1042) or intronic TA-sequences either alone or in different combinations (Figure 2a). Mutated constructs

were used for co-transfection assays and results, shown in Figure 2b demonstrated that mutation of -278TATA alone (lane 3) resulted in significantly reduced promoter activity (by 70-80%) compared with WT promoter (lane1). Furthermore, when proximal (-278) TATA was mutated in any combination (with -1042 or intronic TA), similar loss of promoter activity was observed (lanes 7, 9). However, mutation of upstream initiator-like element (-1048 -1042) alone (lane 2) or intronic TATA-like elements alone or in combination (lanes 4-6) did not reduce promoter activity, if -278TATA was intact. These results suggest that the proximal TATA element (-278 from ATG) is essential for formation of basal promoter complex required to drive expression from Brn-3b promoter and hence, will mark the vicinity of the transcriptional start site. The intronic TA and distal initiator element did not appear to be sufficient or required for transcriptional initiation, independently of this proximal TATA, in breast cancer cells.

Since -278TATA is necessary for transcriptional activity, we next tested if altering this element affected Brn-3b expression in these cells. For the studies, we used the BSXEIE constructs, in which the wild type (WT) or mutant Brn-3b promoter (in which residues in -278 TATA are altered in the otherwise intact promoter) are cloned upstream of its own coding sequence (comprising exon-intron-exon, EIE) and therefore drives its own expression. Following transfections, protein extracts from cells transfected with WT or mutated -278TATA were used for immunoblotting to measure exogenous Brn-3b protein produced from transfected BSXEIE construct, compared with baseline expression (in empty vector transfected cells). Figure 2b (lane1) shows that levels of Brn-3b proteins were increased when WT promoter controlled gene expression compared with basal levels in untransfected control cells (lane 3). This was more evident for the longer Brn-3b(l) isoform because of the lower basal levels expressed in control cells compared with shorter Brn-3b(s). However, mutation of -278TATA resulted in loss of induction of Brn-3b protein (lane 2) since levels were similar to endogenous expression in control cells. From results of these different studies, we concluded that the

proximal TATA (-278 from ATG) marks the transcription start site for Brn-3b transcript breast cancer cells.

Brn-3b promoter is stimulated by growth factors, NGF & EGF via MAPK pathway

Since Brn-3b mRNA is increased in breast cancers, we next tested whether this promoter is regulated by growth factors that alter proliferation of these cancer cells. Therefore, MCF7 cells, transfected with BSX promoter, were treated with known growth regulators including cyclic AMP (cAMP); epidermal growth factor (EGF); nerve growth factor (NGF) and insulin-like growth factors (IGF-1)[14-17]. Transforming growth factor (TGFβ), which is an inhibitor of cell growth[18], was also tested. Figure 3a shows that NGF and EGF stimulated Brn-3b promoter whereas IGF-I, TGFβ and cyclic AMP had no effect on its activity in these cells.

Analysis of Brn-3b promoter, using Mat Inspector, identified multiple transcription factor binding sites for TFs stimulated by these GFs e.g. EGR/nerve growth factor induced protein C so we tested if this region of the promoter was necessary for stimulation by growth factors. Because of the presence of multiple sites in this region of the promoter, it was necessary to generate deletion constructs instead of mutating individual sites. Therefore, Sma1 restriction enzyme sites were used to delete a region of promoter containing 6 EGFR/SRE sites by restriction digest and re-ligation. The resultant deletion promoter construct, BS-SS (shown in Figure 3b) was used in similar co-transfection assays +/- NGF or EGF. Figure 3c shows that the BS-SS promoter was no longer stimulated by NGF or EGF, as seen in WT promoter. Although basal activity was slightly lower than WT promoter, this did not account for loss of inducibility by NGF and EGF, suggesting that key DNA binding sites present in this region are essential for increasing promoter activity in breast cancer cells.

NGF and EGF act as ligands, which when bound to specific receptors, activate signalling pathways that alter downstream TF which in turn modulates downstream gene

expression[16,19]. To identify pathway(s) that modify promoter activity, cells transfected with the Brn-3b reporter construct were treated with pharmacological inhibitors or activators of key signalling pathways. Figure 4a shows that PD98059, an inhibitor of MAPK (mitogen activated protein kinase) pathway, strongly and specifically repressed Brn-3b promoter activity whereas inhibitors of other pathways e.g. SB203580 (p38 kinase inhibitor), genestine (tyrosine kinase inhibitor), or Wortmannin (phosphoinositide 3-kinases (PI3Ks) inhibitor) had no effect on promoter activity. Furthermore, PD98059 also blocked activation by NGF and EGF, suggesting that these GFs stimulate Brn-3b promoter activity by signalling through MAPK pathway.

Interestingly, strong induction of promoter activity by phorbol 12,13-dibutyrate, (PDBu), a potent activator of protein kinase C, (Figure 4b) was also inhibited by PD98059, suggesting an important role for MAPK signalling pathway in controlling Brn-3b promoter activity in breast cancer cells through different upstream activators.

To confirm the requirement for MAPK pathway in stimulating this promoter, we over-expressed wild type (WT) MEK1 or dominant negative (dn)MEK1 with Brn-3b reporter construct, using co-transfection protocols. Figure 4c shows that over-expression of WT MEK1 increased promoter activity whereas dnMEK1 construct reduced promoter activity to levels seen with PD98059 treatment. Thus, Brn-3b promoter activity can be inhibited by blocking MAPK/ERK pathway either by using pharmacological inhibitors or dnMEK thereby placing MAPK/ERK pathway as a pivotal regulator of Brn-3b expression in breast cancer cells.

Activation of Brn-3b promoter by hormone, 17β-estradiol is via ERα but not ERβ

The hormone, estrogen, plays a critical role in initiation and progression of many breast cancers because breast epithelial cells are highly responsive to its proliferative effects. Therefore, we tested whether active estrogen (17β-estradiol) could stimulate Brn-3b promoter activity using MCF7 cells sensitized to estradiol, by growing in medium containing stripped serum [9]. Cells transfected with Brn-3b promoter construct were either untreated or treated

with different concentrations of 17β-estradiol. Figure 5a shows that 17β-estradiol significantly increased promoter activity compared with untreated cells suggesting that this hormone can stimulate Brn-3b transcription in breast cancer cells, thereby contributing to downstream estrogenic growth effects.

Estradiol can act through one of two receptors, estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ). Of these, increased ERα is implicated in etiology of breast cancers and often targeted for treatment. We therefore tested the effects of co-expressing either ERα or ERβ on Brn-3b promoter activity. Figure 5b shows that the promoter was strongly stimulated by ERα whereas ERβ did not alter its activity, suggesting that the effects of estrogen in breast cancer cells are likely to be mediated via ERα. However, ERα stimulated the Brn-3b promoter in the absence of estradiol in MCF7 cells grown in estrogen-depleted medium but there was further increases upon in the presence of this hormone, suggesting that in these cells, ERα mediates the effects of estrogens but can also act independently of this hormone. As expected, addition of the ER antagonist, tamoxifen, prevented activation of Brn-3b promoter by estrogen (Figure 5c), thus confirming that this receptor is required for stimulation of the Brn-3b promoter in MCF7 cells. This was further supported by studies carried out in ER-negative Cos7 cells, which showed that estradiol did not activate this promoter unless exogenous ER was expressed i.e. following transfection (Figure 5d). These results suggest that ERα is necessary to mediate the effects of estrogens in MCF7 breast cancer cells but can also act independently of estrogen to increase Brn-3b transcription.

Auto-regulation by Brn-3b and co-operation with ERα also increases promoter activity

Trans Fac analysis had revealed binding sites for Brn-3 proteins, suggesting that Brn-3b and/or related family member, Brn-3a, may also regulate promoter activity. A putative ERE site was identified within proximity to this site (Figure 6a) and since previous studies demonstrated

physical interaction between Brn-3b and ERα that can stimulate transcription of ERE containing target genes, we tested if Brn-3b could regulate its own expression and whether it cooperates with ERα to increase promoter activity.

Figure 6b shows that Brn-3b could weakly transactivate its own promoter whereas the related Brn-3a protein had no effect on promoter activity in these cells. Although ERα alone stimulated promoter activity, co-expression of this receptor with Brn-3b resulted in more significant increases suggesting that, as on other ERE target genes, Brn-3b cooperates with ERα to stimulate transcription from this promoter. ERβ did not affect promoter activity with or without Brn-3b, suggesting that a specific and unique cooperation occurs between ERα and Brn-3b to stimulate Brn-3b promoter activity in breast cancer cells.

We also tested whether increased promoter activation caused by co-expression of Brn-3b and ERα could also result in enhanced protein expression. For this study, we used the modified BSXE1E construct (Figure 6c), in which Brn-3b promoter (containing Brn-3 and ERE elements) drives expression of its own coding sequence. This BSXEIE construct was co-transfected with Brn-3b or ERα expression vectors, alone or together into MCF7 cells. Protein extracted from transfected cells after 48 hrs were used for immunoblotting to detect Brn-3b protein. Figure 6d shows that cells co-expressing exogenous Brn-3b and ERα produced higher levels of Brn-3b protein compared with basal levels in control cells (lane 1) or cells transfected with Brn-3b alone (lane 3), which represents exogenous and endogenous Brn-3b proteins. Thus, co-expression of Brn-3b with ERα at ratios of 1:1 and 1:2 (Brn-3b: ERα) resulted in increased Brn-3b protein but further increases in ERα (1:4 ratio) resulted in reduced protein levels, which is suggestive of squelching. To demonstrate this squelching effect, exposure times had to be reduced but this meant that changes in endogenous Brn-3b following transfection with ERα only was not evident here but is shown in Figure 6e.

Figure 6e (panel one) shows that transfection of increasing amounts of ER expression vector resulted in production of higher levels of ERα protein. Panel 2 shows that an increase in ERα also enhanced Brn-3b protein expression in MCF7. Thus, this receptor increases Brn-3b transcription but also cooperates with Brn-3b, when co-expressed, to further enhance expression. However, this cooperativity is influenced by the ratio of Brn-3b to ERα in cells.

Mutation of Brn-3 binding sites leads to loss of regulation by ERα

Analysis using BS-SS deletion construct, which lack the region of promoter containing Brn-3/ERE binding sites, showed loss of inducibility by Brn-3b and ERα (Figure 7a) suggesting that these sites were important for promoter transactivation. Therefore, to determine if these sites were necessary for promoter activation, we mutated the Brn-3 consensus sequence (Brn3 mut) and ERE (ERE mut), either alone or together (Brn3/ERE mut), using site directed mutagenesis. Effects of Brn-3b and ER were tested on mutant and WT promoter activity, following co-transfections. Figure 7b (black bars) shows the expected cooperation between Brn-3b and ERα on wild-type promoter whereas mutation of Brn-3 site (open bars) resulted in loss of induction by Brn-3b and also prevented activation by ERα or co-operative stimulation with Brn-3b. Mutation of the putative ERE did not affect promoter activity (Figure 7c-light grey bars) but loss of ERE and adjacent Brn-3 site, in double mutants (dark grey bars) abolished stimulation by ERα and cooperativity between Brn-3b and ER. These results suggested that ERα could stimulate this promoter by binding to Brn-3b, and thereby being recruited to the promoter. Therefore, ER mediated activation of this promoter is not solely dependent on the ERE site at this position.

Since the Brn-3 site was shown to be important for activation of this promoter, chromatin immunoprecipitation (ChIP) was utilized to show that Brn-3b does indeed bind to this site on the promoter, in-vivo, in intact cells. Figure 7d shows results of PCR using DNA

obtained after ChIP with Brn-3b antibody using MCF7 cells over expressing Brn-3b. PCR primers were used to amplify the promoter region containing the putative Brn-3b site. Input (lane 1) indicates amplification of chromatin from cells prior to IP, whereas ChIP DNA using Brn-3b Ab give rise to significant amplification products (Lane 3) which was not seen following PCR using ChIP DNA with control Ab (lane 2). These results therefore confirm that Brn-3b is indeed bound to this region of its own promoter in-vivo, in intact cells.

Discussion

The mechanisms underlying development and progression of breast cancer are not fully understood and this is particularly challenging because of the diverse etiology of these diseases [20]. However, it is clear that changes in gene expression are essential to drive different processes that occur during tumourigenesis [21]. Transcription factors control gene expression by binding to specific DNA sequences in gene promoters and often regulate multiple target genes. Because of this ability to control different target genes, de-regulation of transcription factors can drive events associated with initiation and progression of diseases such as cancers [22]. Previous studies have shown that the Brn-3b transcription factor is elevated in >60% of primary breast cancers [1] and when increased, it significantly enhances proliferation and anchorage independent growth in vitro and tumour growth, in-vivo [2, 3]. Elevated Brn-3b also confers resistance to growth inhibitory stimuli and increases migratory potential of cancer cells [2] suggesting that this transcription factor acts through complex mechanisms in cancer cells. More recent studies show increases in Brn-3b in drug-resistant, migratory breast cancer cells (unpublished data). Brn-3b transcription factor can give rise to such diverse effects because it regulates different subsets of target genes that control distinct aspects of cellular growth and behaviour e.g. Brn-3b might contribute to cellular proliferation by transactivating cell cycle regulators, CDK4 [4] and cyclinD1 [5] whilst repressing tumour suppressor, BRCA1 [6].

However, its effects on drug resistance and migration are likely to be associated with its ability to regulate other genes e.g. to transactivate Hsp-27 [7] whilst repressing adhesion molecules e.g. γ-catenin [8].

Interestingly, reducing Brn-3b was sufficient to change gene expression and reverse many growth effects[1]. Therefore, Brn-3b can act as a master regulator, whose expression profoundly alters growth of cancer cells. In this regard, Brn-3b might represent an important therapeutic target, whose reduction could alter expression of multiple downstream target genes and thereby reverse their effects on cancer cells. However, in order to identify strategies for reducing Brn-3b in these cells, we must understand the mechanisms that lead to its increased expression in breast cancer cells.

In this study, we utilized Bioinformatic analysis to identify the putative Brn-3b promoter and cloned this regulatory region into a reporter construct for further experimental analysis. By using chromatin immunoprecipitation and site directed mutagenesis, we identified a key TATA transcriptional start site located at ~278 from ATG, which is primarily associated with expression of Brn-3b mRNA in breast cancer cells. Although upstream initiation site and TA-like elements in the intronic sequence were weakly immunoprecipitated by TBP Ab, these do not appear to be candidates for transcriptional start site since mutation of any (or all) intronic TA sequences or upstream sequences did not reduce promoter activity if start site at -278 was intact. This is interesting because an intronic promoter is thought to be important to drive isoform specific expression of the related Brn-3a gene which has a similar genomic arrangement to Brn-3b. However, our results suggest that Brn-3b promoter activity in breast cancer cells is driven primarily from the proximal (-278) TATA, which is now used to define the transcription start site from this promoter.

Further analysis showed that the Brn-3b promoter can be stimulated specifically by growth factors, NGF and EGF but not IGF-I; cyclicAMP or TGFβ and these stimulatory effects

require a region of promoter that contains multiple EGFR and SRE sites. The ability of GFs such as NGF to increase transcription from the Brn-3b promoter by is significant because such GF are known to enhance growth of cancer cells. For example, NGF drives proliferation of breast cancer cells but not normal breast epithelial cells and blocking NGF was shown to inhibit tumour growth and metastasis [16, 23] suggesting a key role for NGF in controlling growth of cancer, but not normal cells. NGF is produced in an autocrine manner by breast cancer cells and its mitogenic effects in these cells are mediated through the mitogen activated protein kinase signalling pathway (MAPK) since it can be blocked by the pharmacological inhibitor PD98059, which targets ERK1 in this pathway [24]. Dominant negative MEK also blocks promoter activity, similar to ERK1 inhibitor, PD98059. It is therefore particularly interesting that stimulation of Brn-3b promoter by NGF is blocked by PD98059, suggesting that mitogenic effects of NGF in breast cancer cells may result, in part, from its ability to increase expression of regulators such as Brn-3b. The PKC analogue, PDBu, is also a potent activator of Brn-3b promoter and its effects are also blocked by PD98059 suggesting that this activator also converges on ERK1 pathway to stimulate Brn-3b promoter. Thus it would appear that the MAPK/ERK pathway is pivotal for activating Brn-3b promoter and hence expression in breast cancer cells.

In addition to stimulation by growth factors, the Brn-3b promoter is also strongly activated by the hormone, estradiol, which regulates growth/proliferation of normal breast epithelium and breast cancer cells and is important in the etiology of breast cancer [25]. Estrogens can regulate gene transcription by acting through one of two receptors i.e. ERα or ERβ. Our results showed that over-expression of ERα but not ERβ could strongly stimulate Brn-3b promoter. ERα is particularly relevant for development and progression of breast cancers because it is over-expressed in a significant proportion of breast cancers (>60%). Furthermore, ER-positive breast cancers are often treated using receptor antagonists e.g. tamoxifen as a first

line of therapy aimed at blocking ER mediated proliferative effects [26]. Therefore, the ability of ERα to stimulate Brn-3b suggests that proliferative effects of high ER levels may be associated with its ability to transactivate other regulators, such as Brn-3b, which in turn can modulate genes associated with growth in these cancer cells either alone or by co-operating with ERα.

The complexity underlying regulation of Brn-3b promoter is increased by auto-regulation, whereby Brn-3b can weakly stimulate its own expression by binding to recognition sequences present in its promoter but also co-operates with ERα to further enhance promoter activity. Such cooperation between Brn-3b and ERα to increase gene expression was previously observed on other ERE-containing target promoters e.g. HSP27, where Brn-3b stimulates expression directly by binding to specific sites in the promoter or indirectly by interacting and cooperating with ER to maximally activate this promoter [7]. This ability of Brn-3b to co-operate with ERα to enhance gene expression [9], including its own, is clearly relevant to breast cancer because ER expressing tumours that are responsive to estradiol will stimulate Brn-3b which can co-operate with ERα to further increase its own expression. Interestingly mutation of the putative ERE did not prevent ER-mediated promoter activity when co-expressed with Brn-3b but mutation of the nearby Brn-3 site abolished activation by ER and its cooperation with Brn-3b. This indicates that ERα could stimulate Brn-3b promoter even if it is not bound to ERE, possibly because interaction with Brn-3b allows recruitment of ER to the promoter. Auto-regulation of Brn-3b transcription, either alone or by cooperating with ER, is likely to increase its expression and subsequent target genes in these cells.

Although stimulation of Brn-3b promoter activity by the hormone, estrogen, can be independent of growth factor-mediated activation via MAPK signalling pathway, significant "cross talk" are known to exist between these pathways in breast cancer cells. Thus, estradiol primarily acts through its receptor, ERα in breast cancer cells but it can also indirectly stimulate

tyrosine kinase receptors (Kato 2001), which are also relevant to breast cancers. Similarly, transcription by estrogen receptor, ERα is also modulated by MAPK pathway stimulation [27]. Evidence for cross-talk between NGF or EGF and estradiol pathway have also been demonstrated [28] and the anti-estrogenic drug, tamoxifen can inhibit proliferation by EGF or NGF on MCF7 breast cancer cells [29].

Furthermore, increases in Brn-3b by either growth factor pathways (through MAPK activation) or estradiol (via ERα) will be propagated by auto-regulation, since Brn-3b co-operated with ERα to increase its own expression. Once elevated, Brn-3b will regulate expression of multiple downstream target genes, thereby affecting growth and behaviour in these cancer cells.

Conclusions

Elevated Brn-3b profoundly enhances growth and confers drug resistance in breast cancer cells, so it is important to identify what factors increase its expression in these cells. In these studies, we have cloned and analyse Brn-3b promoter and identified key pathways that converge to increase Brn-3b promoter activity and hence expression, in breast cancer cells. Thus, the hormone, estrogen and growth factors, NGF and EGF strongly stimulate activity of Brn-3b promoter increases mRNA and protein, suggesting that increased Brn-3b by such factors will be important in changing fate of these cells.Thus, Brn-3b it's a regulator whose expression is increased via different pathways e.g. NGF, EGF and estradiol, which are implicated in enhancing growth of breast cancer cells. These effects will be propagated by auto-regulation, thereby leading to changes in multiple Brn-3b target genes that control growth and behaviour of these cancer cells. By elucidating the mechanisms through which regulators, such as Brn-3b, are increased in cancer cells, we will increase understanding of how changes are brought about

during development and progression of this disease and may also be able to identify strategies to reduce its expression and reverse its effects in breast cancer cells.

Abbreviations

Brn-3b -transcription factor related Brn-3a regulator isolated from brain cDNA; BSX-Brn-3b promoter containing BstX1/Stu1Xho1 fragment; ChIP - chromatin immunoprecipitation; EGF-epidermal growth factor; ER- estrogen receptor; ERα-estrogen receptor alpha; ERβ-estrogen receptor beta; ERE-estrogen response element; GF-growth factors; MAPK-mitogen associated protein kinases; NGF-nerve growth factor; PI3K- phosphoinositide 3-kinases; POU4F2 - member of class 4 subgroup of Pit-Oct-Unc transcription factors; Q-RT-PCR- quantitative reverse transcriptase polymerase chain reaction; SRE-serum response element; TFs-Transcription factors; WT-wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SO mapped transcription start sites, used site directed mutagenesis and ChIP to identify key transcription factor binding sites and helped to identify signalling pathways associated with regulation of promoter activity. SB, CP and RF were involved in cloning and initial characterization of the promoter. RJH provided reagents required for aspects of the study. VBM is the principal investigator and was instrumental in designing, interpretation and processing of data and preparation of the manuscript for publication.

Acknowledgements

This work was supported by the Breast Cancer Campaign (BCC) UK and Association for International Cancer Research (AICR), UK. Dominant negative and constitutively active MEK expression vectors were kind gifts from DS Latchman.

References

1. Budhram-Mahadeo VS, Latchman DS: **Targeting Brn-3b in breast cancer therapy.** *Expert Opin Ther Targets* 2006, **10**:15-25.

2. Irshad S, Pedley RB, Anderson J, Latchman DS, Budhram-Mahadeo V: **The Brn-3b transcription factor regulates the growth, behavior, and invasiveness of human neuroblastoma cells in vitro and in vivo**. *J Biol Chem* 2004, **279**:21617-21627.

3. Dennis JH, Budhram-Mahadeo V, Latchman DS: **The Brn-3b POU family transcription factor regulates the cellular growth, proliferation, and anchorage dependence of MCF7 human breast cancer cells**. *Oncogene* 2001, **20**:4961-4971.

4. Samady L, Dennis J, Budhram-Mahadeo V, Latchman DS: **Activation of CDK4 Gene Expression in Human Breast Cancer Cells by the Brn-3b POU Family Transcription Factor**. *Cancer Biol Ther* 2004, **3**:317-323.

5. Budhram-Mahadeo VS, Irshad S, Bowen S, Lee SA, Samady L, Tonini GP, Latchman DS: **Proliferation-associated Brn-3b transcription factor can activate cyclin D1 expression in neuroblastoma and breast cancer cells**. *Oncogene* 2008, **27**:145-154.

6. Budhram-Mahadeo V, Ndisang D, Ward T, Weber BL, Latchman DS: **The Brn-3b POU family transcription factor represses expression of the BRCA-1 anti-oncogene in breast cancer cells**. *Oncogene* 1999, **18**:6684-6691.

7. Lee SA, Ndisang D, Patel C, Dennis JH, Faulkes DJ, D'Arrigo C, Samady L, Farooqui-Kabir S, Heads RJ, Latchman DS, Budhram-Mahadeo VS: **Expression of the Brn-3b transcription factor correlates with expression of HSP-27 in breast cancer biopsies and is required for maximal activation of the HSP-27 promoter**. *Cancer Res* 2005, **65**:3072-3080.

8. Samady L, Faulkes DJ, Budhram-Mahadeo V, Ndisang D, Potter E, Brabant G, Latchman DS: **The Brn-3b POU family transcription factor represses plakoglobin gene expression in human breast cancer cells**. *Int J Cancer* 2006, **118**:869-878.

9. Budhram-Mahadeo V, Parker M, Latchman DS: **POU transcription factors Brn-3a and Brn-3b interact with the estrogen receptor and differentially regulate transcriptional activity via an estrogen response element**. *Mol Cell Biol* 1998, **18**:1029-1041.

10. Liu YZ, Boxer LM, Latchman DS: **Activation of the Bcl-2 promoter by nerve growth factor is mediated by the p42/p44 MAPK cascade**. *Nucleic Acids Res* 1999, **27**:2086-2090.

11. Frazer KA, Pachter L, Poliakov A, Rubin EM, Dubchak I: **VISTA: computational tools for comparative genomics**. *Nucleic Acids Res* 2004, **32**:W273-W279.

12. Loots GG: **Genomic identification of regulatory elements by evolutionary sequence comparison and functional analysis**. *Adv Genet* 2008, **61**:269-293.

13. Burley SK, Roeder RG: **Biochemistry and structural biology of transcription factor IID (TFIID)**. *Annu Rev Biochem* 1996, **65**:769-799.

14. Lanzino M, Morelli C, Garofalo C, Panno ML, Mauro L, Ando S, Sisci D: **Interaction between estrogen receptor alpha and insulin/IGF signaling in breast cancer**. *Curr Cancer Drug Targets* 2008, **8**:597-610.

15. Lo HW, Hsu SC, Hung MC: **EGFR signaling pathway in breast cancers: from traditional signal transduction to direct nuclear translocalization**. *Breast Cancer Res Treat* 2006, **95**:211-218.

16. Descamps S, Toillon RA, Adriaenssens E, Pawlowski V, Cool SM, Nurcombe V, Le B, X, Boilly B, Peyrat JP, Hondermarck H: **Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways**. *J Biol Chem* 2001, **276**:17864-17870.

17. Descamps S, Pawlowski V, Revillion F, Hornez L, Hebbar M, Boilly B, Hondermarck H, Peyrat JP: **Expression of nerve growth factor receptors and their prognostic value in human breast cancer**. *Cancer Res* 2001, **61**:4337-4340.

18. Buck MB, Knabbe C: **TGF-beta signaling in breast cancer**. *Ann N Y Acad Sci* 2006, **1089**:119-126.

19. Tagliabue E, Castiglioni F, Ghirelli C, Modugno M, Asnaghi L, Somenzi G, Melani C, Menard S: **Nerve growth factor cooperates with p185(HER2) in activating growth of human breast carcinoma cells**. *J Biol Chem* 2000, **275**:5388-5394.

20. Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G: **Breast cancer**. *Lancet* 2005, **365**:1727-1741.

21. Cleator S, Ashworth A: **Molecular profiling of breast cancer: clinical implications**. *Br J Cancer* 2004, **90**:1120-1124.

22. Liu S, Dontu G, Wicha MS: **Mammary stem cells, self-renewal pathways, and carcinogenesis**. *Breast Cancer Res* 2005, **7**:86-95.

23. Descamps S, Lebourhis X, Delehedde M, Boilly B, Hondermarck H: **Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells**. *J Biol Chem* 1998, **273**:16659-16662.

24. Melck D, Rueda D, Galve-Roperh I, De PL, Guzman M, Di M, V: **Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-**

proliferative effects of anandamide in human breast cancer cells. *FEBS Lett* 1999, **463**:235-240.

25. Sommer S, Fuqua SA: **Estrogen receptor and breast cancer**. *Semin Cancer Biol* 2001, **11**:339-352.

26. Iwase H: **Molecular action of the estrogen receptor and hormone dependency in breast cancer**. *Breast Cancer* 2003, **10**:89-96.

27. Hart LL, Davie JR: **The estrogen receptor: more than the average transcription factor**. *Biochem Cell Biol* 2002, **80**:335-341.

28. Kato S: **Estrogen receptor-mediated cross-talk with growth factor signaling pathways**. *Breast Cancer* 2001, **8**:3-9.

29. Chiarenza A, Lazarovici P, Lempereur L, Cantarella G, Bianchi A, Bernardini R: **Tamoxifen inhibits nerve growth factor-induced proliferation of the human breast cancerous cell line MCF-7**. *Cancer Res* 2001, **61**:3002-3008.

Figure legends

Figure 1. Identification, cloning and identification of transcription start site in Brn-3b promoter. **(a)** Homology plots (VISTA Genome browser) showing regions of similarity in Brn-3b gene and 5' upstream sequences between human (top) and dog, horse and mouse genomic sequences. Regions of homology are indicated by peaks and grey shading and positions of Brn-3b exons and intronic sequences are shown. **(b)** Schematic diagram to show cloned BSX construct containing putative Brn-3b promoter and regulatory sequences or BSXEIE expression construct also containing the coding sequences, used for subsequent studies. Promoter/regulatory sequences are shown in striped grey; 5' non-coding sequences at the beginning of exon 1 are indicated by black bar whereas the white stripe at the start of exon 2 represent unique sequences that are only present in Brn-3b(s) transcripts but not in Brn-3b(l). Position of restriction sites Bstx1 (B), Sac1 (S) and Xho1 (X), used for cloning are also shown. **(c)** Luciferase activity of Brn-3b reporter constructs following transfection into MCF7 cells was compared with baseline luciferase activity of the empty reporter vector. Values were equalized with renilla internal control. **(d)** Schematic diagram showing positions of putative start sites identified by in-silico analysis. Initiator element and proximal TATA sequences are shown

relative to ATG in exon1 and putative intronic TATA sequences are indicated. Arrows show relative positions of primers used for PCR following chromatin immunoprecipitation (ChIP) with α-TBP antibody to analyse TBP binding to the different sites. **(e)** PCR products obtained when using ChIP DNA (obtained with α-TBP Ab or 2nd control Ab) for amplification with primers flanking either the upstream initiator elements (-1048); -278TATA or intronic TA sequences. Primers to sequences within exon 2 (>1kb from ATG) was used for amplification in the negative control. Positive control represent PCR products using primers that amplified known start site of GAPDH gene using α-TBP or control ChIP DNA. Input represents amplification using 1/10 of DNA isolated before ChIP). Lane 3 shows the products obtained following amplification of control ChIP antibody (α-rabbit secondary Ab) using indicated primers.

Figure 2. Analyzing transcription start sites of Brn-3b promoter. (a) Analysis of Brn-3b promoter (BSX) activity following mutation of key sites to identify the transcriptional start site. Wild type (WT) promoter in lane 1 is represented as 100% and mutations are expressed as % of WT promoter. Schematic diagram shows positions of mutations at key sites (indicated by x) e.g. upstream initiator alone (lane 2) or in combination with other sites (lanes 7-9). Proximal (278) TATA mutation is shown in (lane 3) or in combination (lane 7 and 9). Effects of mutation of different intronic TA alone (lanes 4-6) or in combination (lanes 8-10) are also shown. Values were equalized to internal control (renilla luciferase) and results represent data from 3 independent experiments. **(b)** Western blot analysis of Brn-3b protein expression in cellular extract prepared from cells transfected with BSXE1E expression constructs in which Brn-3b promoter drives expression of the Brn-3b gene. Wild type promoter activity is shown in lane 1. Lane 2 shows reduction in Brn-3b protein from expression constructs containing mutation within the proximal (278) TATA promoter of an otherwise intact construct. Lane 3

shows untransfected control cells with no reporter and therefore represents levels of endogenous Brn-3b protein, in these cells.

Figure 3. Analysing effect of growth factors on Brn-3b promoter activity. **(a)** Brn-3b promoter (BSX) activity was measured following transfection into MCF7 cells and treatment with different growth factors. Values were equalized with internal control, renilla luciferase activity and expressed as percentage of promoter only (set at 100%). The data shown represent mean +/- SD from at least three independent experiments. **(b)** Schematic diagram to show position of EGRF or SRE sites in the Brn-3b promoter. The location of two Sma1 sites used to generate the deletion construct is shown in relation to the DNA binding sites. The resultant truncated promoter is represented schematically, below. **(c)** Luciferase activity of WT or Sma1 deletion promoters is shown following transfection into MCF 7 cells. Grey bars represent WT promoter activity either alone or following treatment with NGF or EGF whereas stippled bars show activity of Sma1 deletion construct +/- growth factors. Values represent mean +/- SE of three independent experiments.

Figure 4. Brn-3b promoter is activated via MAPK/ERK pathway in breast cancer cells. **(a)** Brn-3b promoter activity is shown following treatment of transfected cells with inhibitors of different signalling pathways (as indicated) in absence or presence of NGF or EGF. Values have been adjusted using renilla luciferase activity as internal control and represent mean +/- SD in at least three independent experiments. **(b)** Increase in Brn-3b promoter activity following treatment of transfected cells with PKC analogue, PDBU in absence or presence of ERK inhibitor, PD98059. Values represent relative luciferase activity after adjusting for renilla internal control. **(c)** Brn-3b promoter activity is shown following co-transfection with either dominant negative MEK (dnMEK) or wild type MEK compared with activity in presence of

ERK inhibitor, PD98059. Values represent relative luciferase activity after adjusting for renilla internal control.

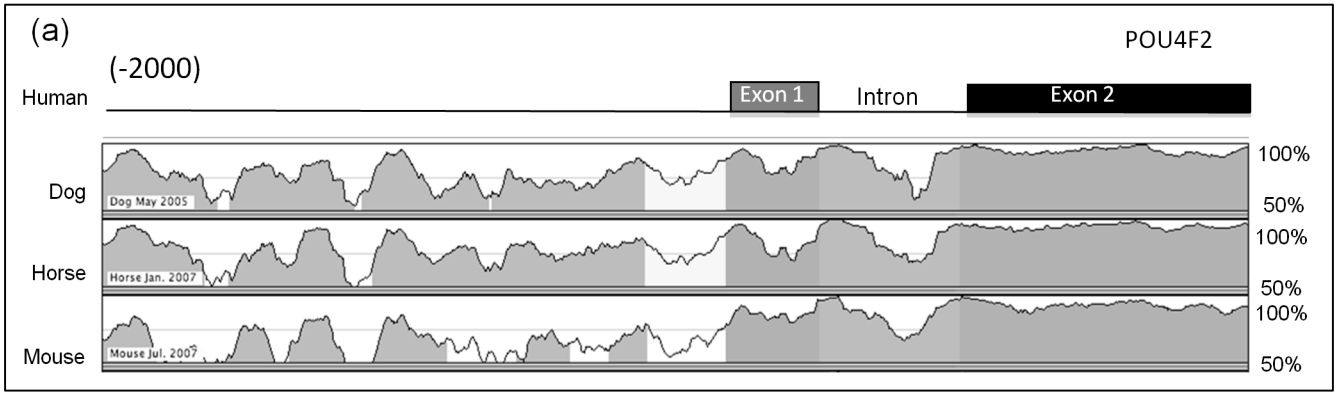
Figure 5. Brn-3b promoter activity is strongly stimulated by active estrogen,17β-estradiol via activation of estrogen receptor–α (ERα). **(a)** Brn-3b promoter activity following treatment of transfected MCF7 cells with different concentration of 17-β-estradiol. Values have been adjusted with internal control, renilla luciferase and represent mean +/- SD of three independent experiments. **(b)** Effect of different estrogen receptors, ERα or ERβ on Brn-3b promoter activity are shown after co-transfection into sensitized MCF7 cells (grown in stripped serum medium for 48 hrs). Promoter activity is adjusted to internal renilla control and expressed as percentage of levels seen with empty vector only (set at 100%). Values represent data seen in 3 independent experiments. **(c)** Brn-3b promoter activation by ERα can be blocked by receptor antagonist, tamoxifen. Luciferase activity is shown after co-transfection of Brn-3b promoter with ERα into sensitized MCF7 cells (grown in estrogen-depleted medium for 48 hrs) and treatment with 1 μmol 5-HT (tamoxifen). Promoter activity is adjusted to internal renilla control and expressed as percentage of levels seen with untreated control (set at 100%). **(d)** Activity of Brn-3b promoter in ER negative Cos7 cells treated with estradiol in the absence and presence of the receptor, and from ERα. Luciferase activity was measured following transfection of Brn-3b promoter and treatment with either vehicle (-E2) or 10 nmol estradiol (+ E2) in the absence or presence of ERα.

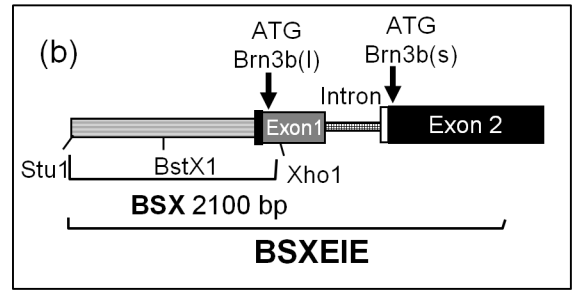
Figure 6. Co-operation between Brn-3b and ER can stimulate promoter activity. **(a)** Schematic diagram to show position of Brn-3 consensus binding sites or ERE site relative to the proximal (-278)TATA, now designated +1. **(b)** Promoter activity following co-transfection of BSX reporter construct with Brn-3b, Brn-3a, ERα or ERβ alone or in different combinations

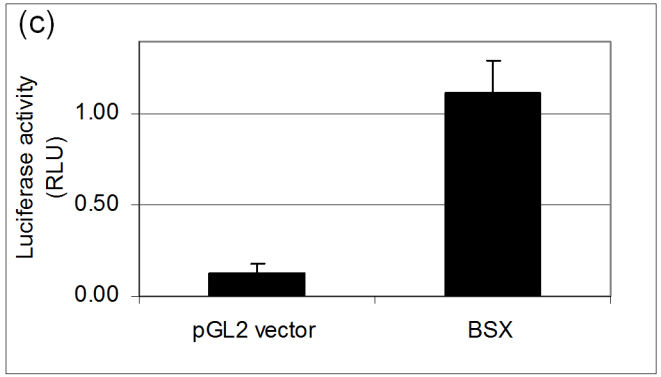
into MCF7 cells. Values have been equalized with internal renilla control and represent mean +/- SD of three independent experiments. **(c)** Brn-3b promoter activity with Brn-3b and ERα, alone or together, in cells depleted of estrogens and endogenous ER expression (by growing in estrogen-depleted medium) and either left untreated or treat with estradiol. Grey bars show inducibility in the absence of estradiol whereas black bars demonstrate effects of adding estradiol after transfection with the constructs described. **(d)** Schematic diagram to show the modified expression construct, BSXE1E, in which Brn-3b promoter drives expression of Brn-3b gene upstream of luciferase reporter (L). **(e)** Immunoblotting was carried out to show changes in Brn-3b protein produced from BSXE1E expression construct in the presence of Brn-3b and ERα either alone or when co-expressed together at different ratios (Brn-3b: ER – 1:1 to 1:4). Lower exposure times are shown to demonstrate the squelching effect observed when ER alpha is increased relative to Brn-3b. At this exposure, Brn-3b expression is not evident when ER only is expressed. Actin immunoblot indicates variation in protein loading. **(f)** Immunoblotting to show increases in ER protein following transfection with expression construct (panel 1). Panel 2 shows changes in Brn-3b protein from BSXE1E expression construct in the presence of Brn-3b or ERα either alone or co-expressed together at different ratios. Panel 3 shows an actin immunoblot to indicate variation in protein loading.

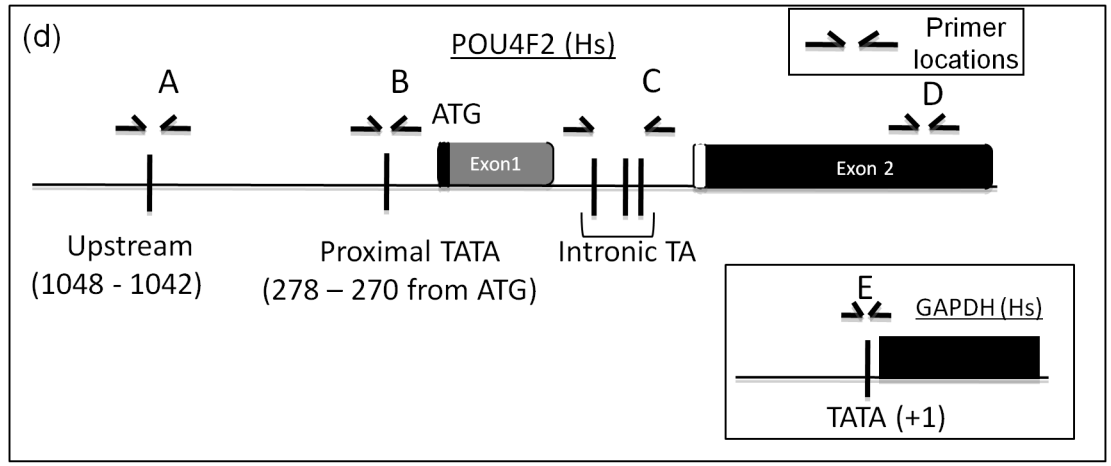
Figure 7. Mutation of Brn-3 site reduces inducibility of promoter by ERα and also abolishes cooperativity. **(a)** Testing effects of ER or ER and Brn-3b on activity of BS-SS promoter, which lack putative binding site for these TFs. Reporter gene activity was adjusted using renilla luciferase and shown relative to activity in control cells containing mutated promoter and compared with activation on wild-type promoter. **(b)** Reporter gene activity following co-transfection of Brn-3b or ER (alone or together) with WT or mutant Brn-3b reporter construct, in which Brn-3b site is mutated. Values have been adjusted for internal

renilla luciferase control and are expressed as percentage of activity in empty vector transfected cells (set at 100%) for respective promoter. Results represent mean +/- SD from three independent experiments. **(c)** Similar reporter assays were undertaken using Brn-3b promoter containing mutation of ERE alone (ERE mutant) or double mutant lacking both sites (Brn-3/ERE mutant). Results represented data from 3 independent experiments +/- SD. **(d)** Representative PCR product obtained using ChIP DNA (immunoprecipitated with Brn-3b antibody) and primers to amplify and promoter region containing the putative Brn-3b binding site. Input represents PCR amplification using 1/10 of isolated DNA before ChIP; lane 2 shows product following ChIP with –ve control (α-rabbit Ab only); lane 3 shows product resulting from ChIP using Brn-3b Ab.


(a)
POU4F2
(-2000)
Human
Exon 1
Intron
Exon 2
Dog
Dog May 2005
Horse
Horse Jan. 2007
Mouse
Mouse Jul. 2007
100%
50%
100%
50%
100%
50%


(b)
ATG
Brn3b(l)
ATG
Brn3b(s)
Intron
Exon1
Exon 2
Stu1
BstX1
Xho1
BSX 2100 bp
BSXEIE


(c)
Luciferase activity (RLU)
1.00
0.50
0.00
pGL2 vector
BSX


(d)
POU4F2 (Hs)
Primer locations
A
B
C
D
ATG
Exon1
Exon 2
Upstream
(1048 - 1042)
Proximal TATA
(278 – 270 from ATG)
Intronic TA
E
GAPDH (Hs)
TATA (+1)

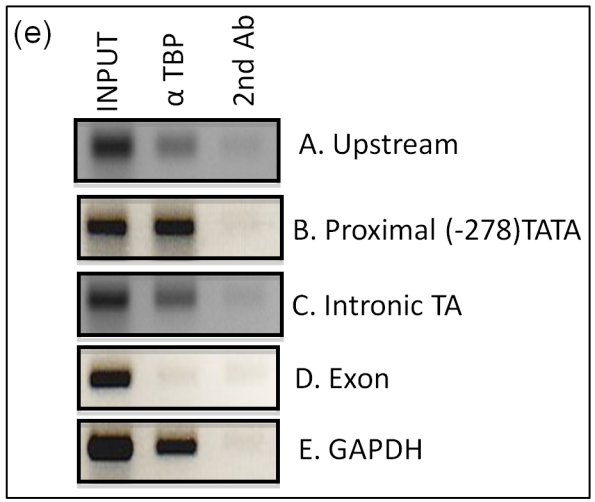

(e)
INPUT
α TBP
2nd Ab
A. Upstream
B. Proximal (-278)TATA
C. Intronic TA
D. Exon
E. GAPDH

Figure 1

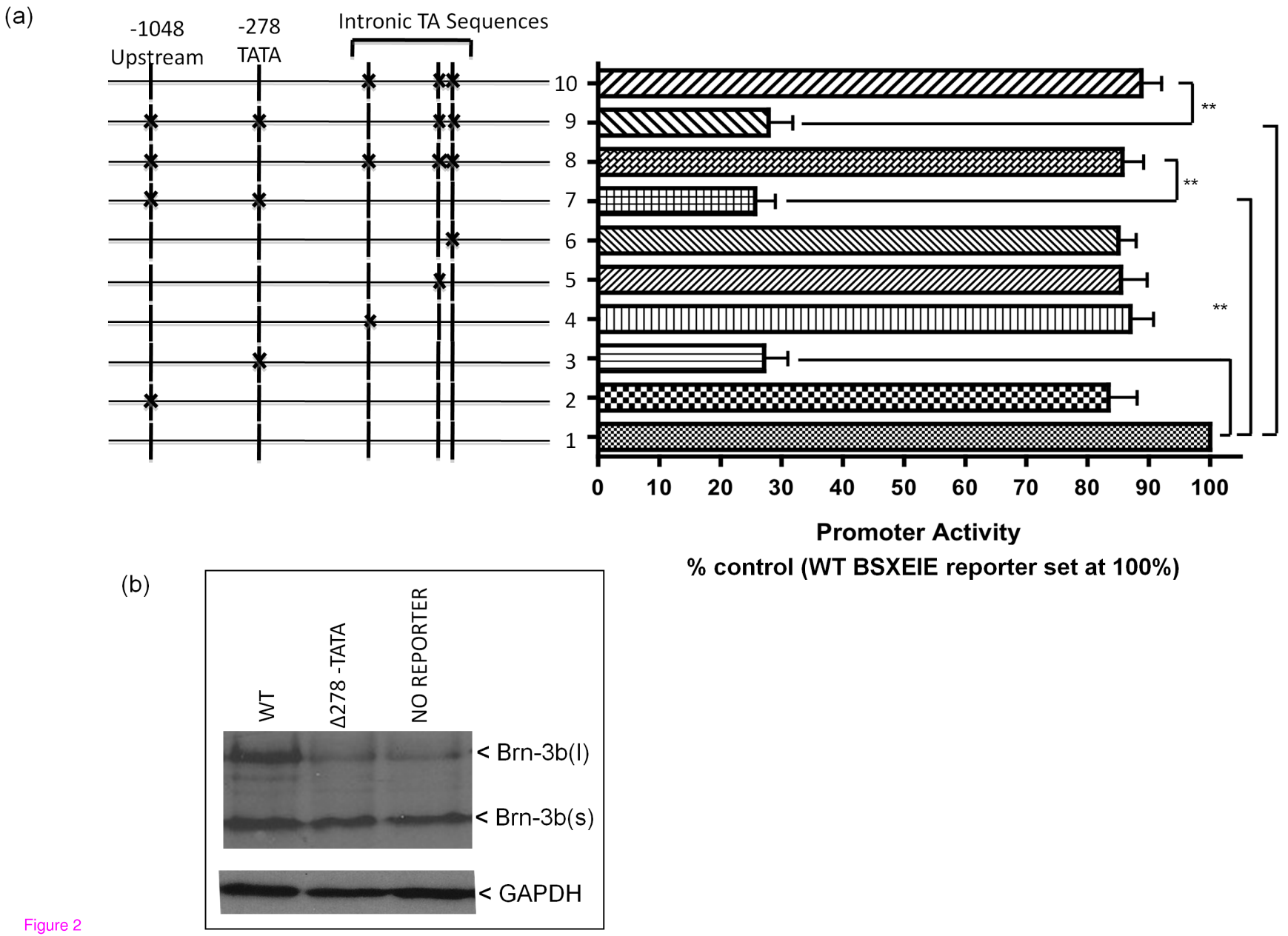
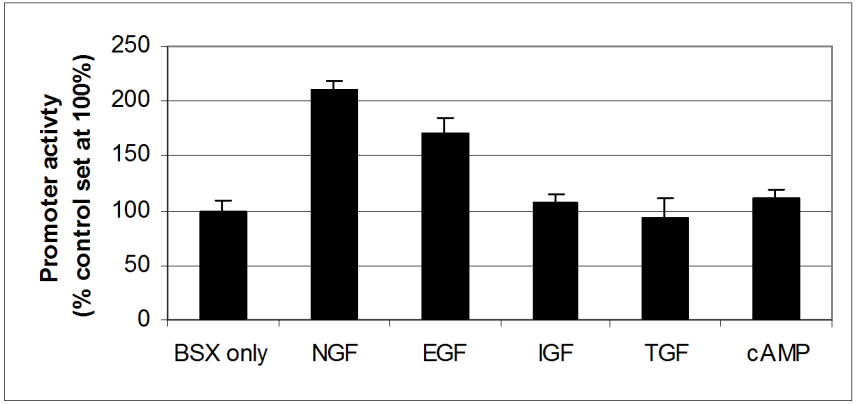
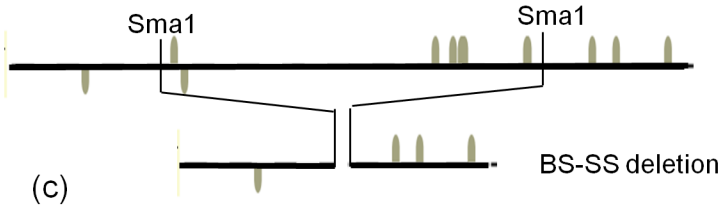

(a)
-1048
Upstream
-278
TATA
Intronic TA Sequences
10
9
8
7
6
5
4
3
2
1
**
**
**
0
10
20
30
40
50
60
70
80
90
100
Promoter Activity
% control (WT BSXEIE reporter set at 100%)
(b)
WT
Δ278 -TATA
NO REPORTER
< Brn-3b(l)
< Brn-3b(s)
< GAPDH

Figure 2

(a)


Promoter activty
(% control set at 100%)
250
200
150
100
50
0
BSX only
NGF
EGF
IGF
TGF
cAMP

(b)


Sma1
Sma1
BS-SS deletion

(c)

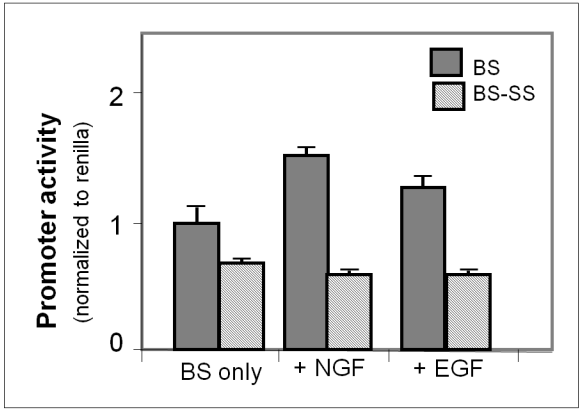
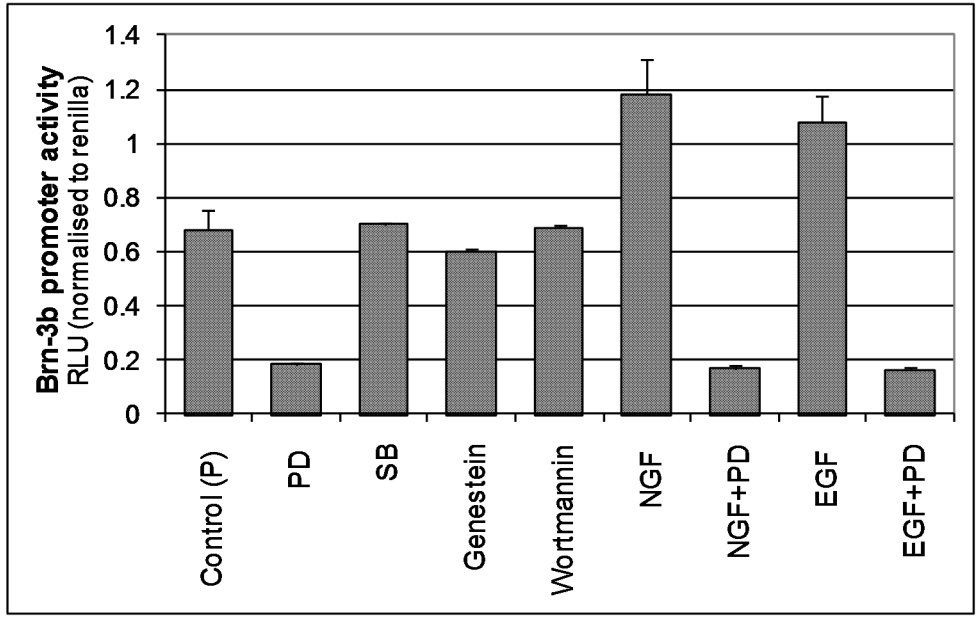
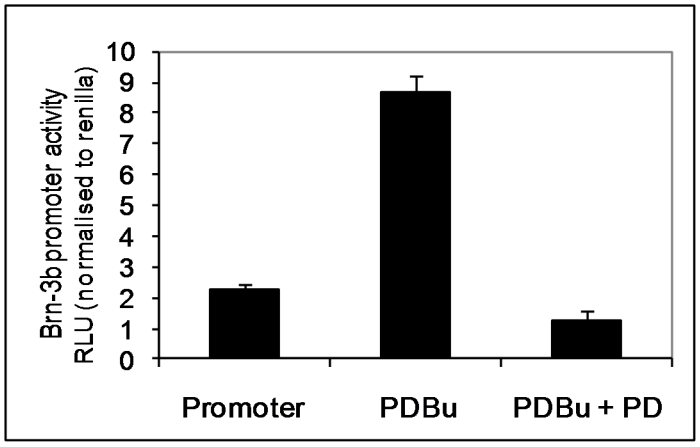

Promoter activity
(normalized to renilla)
2
1
0
BS
BS-SS
BS only
+ NGF
+ EGF

Figure 3

(a)


Brn-3b promoter activity
RLU (normalised to renilla)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control (P)
PD
SB
Genestein
Wortmannin
NGF
NGF+PD
EGF
EGF+PD

(b)


Brn-3b promoter activity
RLU (normalised to renilla)
10
9
8
7
6
5
4
3
2
1
0
Promoter
PDBu
PDBu + PD

(c)

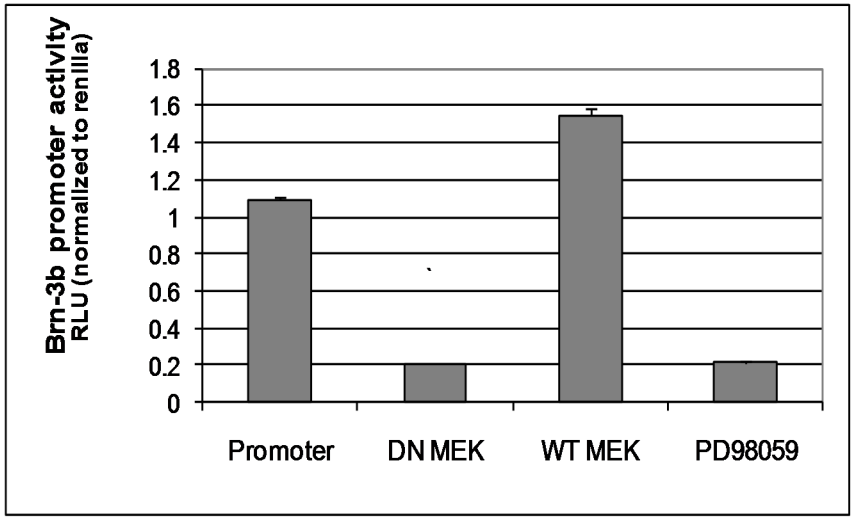
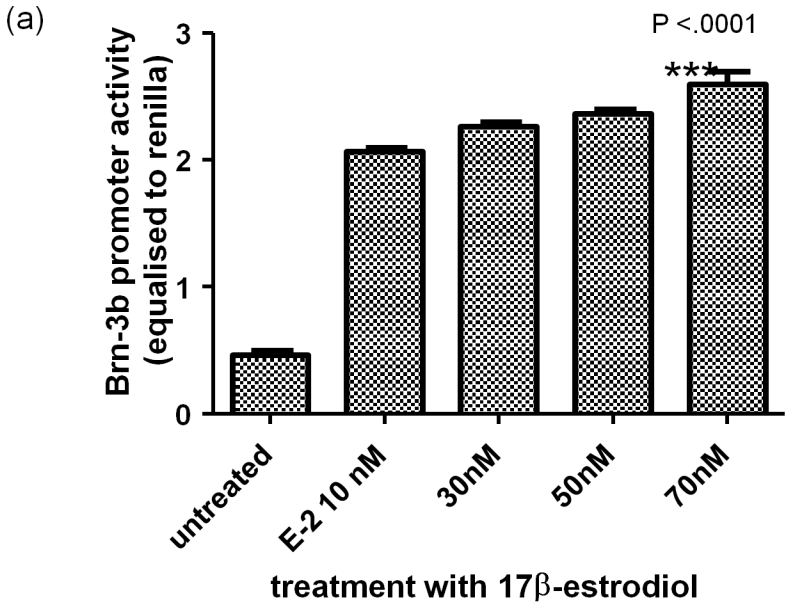
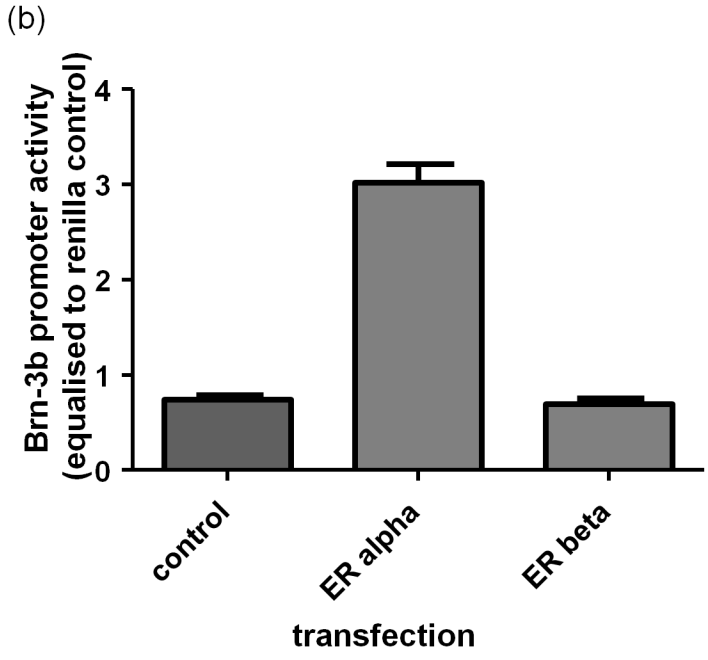
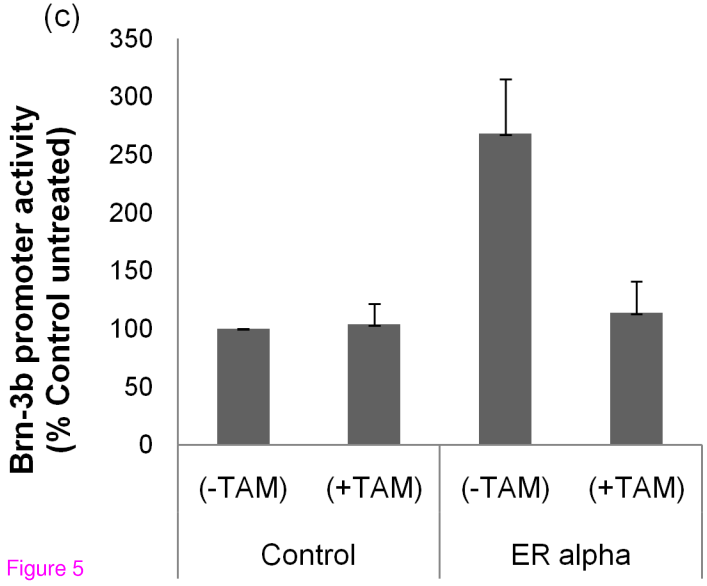

Brn-3b promoter activity
RLU (normallzed to renllla)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Promoter
DN MEK
WT MEK
PD98059

Figure 4


(a)
P <.0001
***
Brn-3b promoter activity
(equalised to renilla)
3
2
1
0
untreated
E-2 10 nM
30nM
50nM
70nM
treatment with 17β-estrodiol


(b)
Brn-3b promoter activity
(equalised to renilla control)
4
3
2
1
0
control
ER alpha
ER beta
transfection


(c)
Brn-3b promoter activity
(% Control untreated)
350
300
250
200
150
100
50
0
(-TAM)
(+TAM)
(-TAM)
(+TAM)
Control
ER alpha

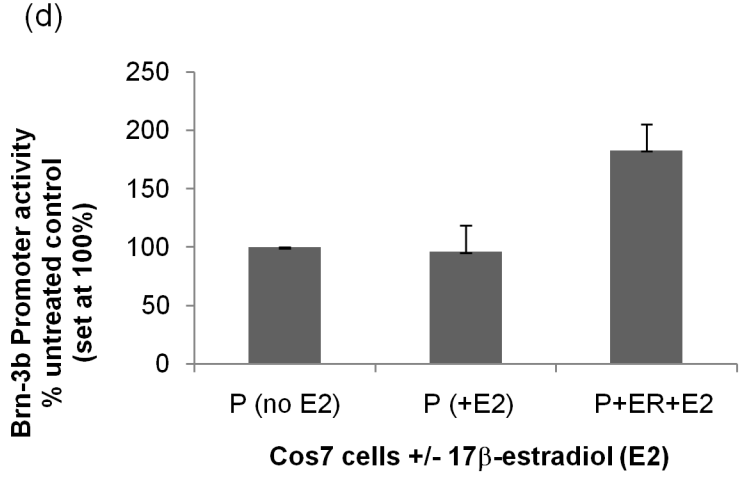
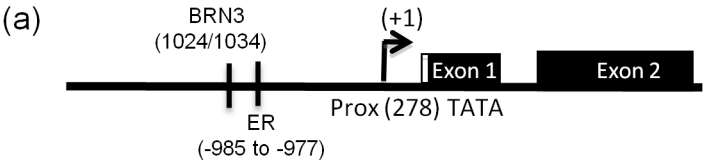
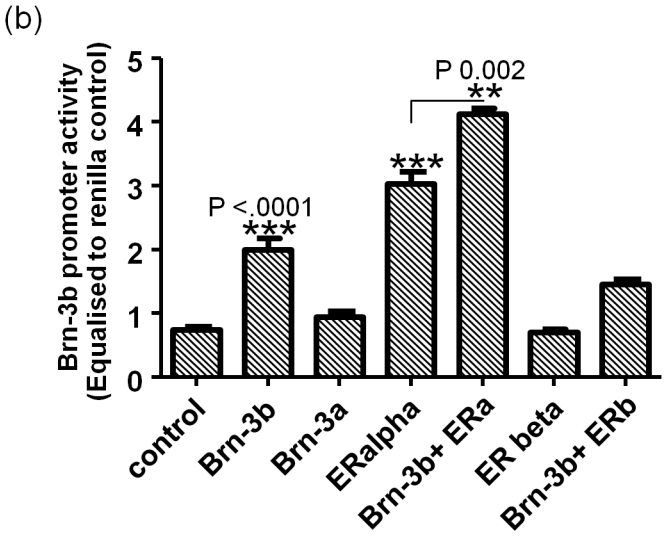
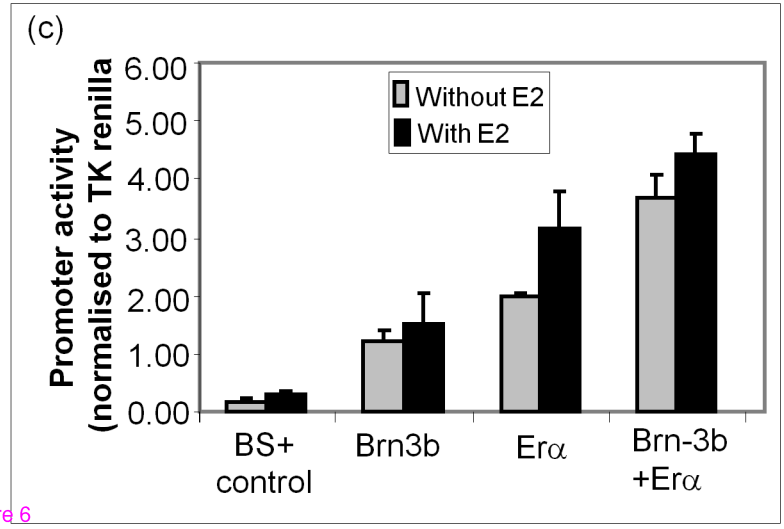
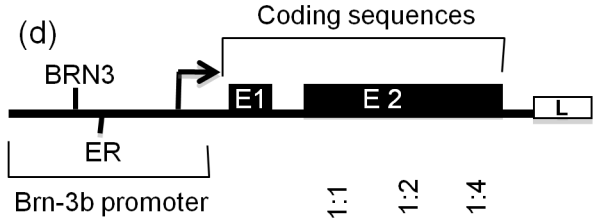

(d)
Brn-3b Promoter activity
% untreated control
(set at 100%)
250
200
150
100
50
0
P (no E2)
P (+E2)
P+ER+E2
Cos7 cells +/- 17β-estradiol (E2)

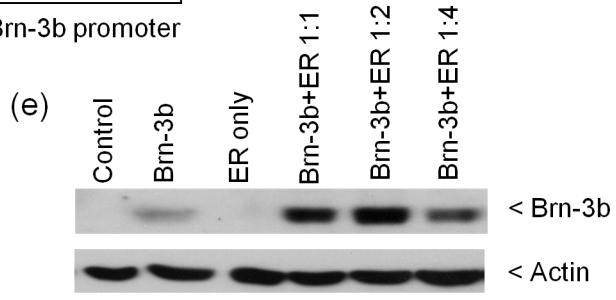
Figure 5


(a)
BRN3
(1024/1034)
(+1)
Exon 1
Exon 2
Prox (278) TATA
ER
(-985 to -977)


(b)
Brn-3b promoter activity
(Equalised to renilla control)
P <.0001
***
P 0.002
**
***
control
Brn-3b
Brn-3a
ERalpha
Brn-3b+ ERa
ER beta
Brn-3b+ ERb


(c)
Promoter activity
(normalised to TK renilla
Without E2
With E2
BS+
control
Brn3b
Erα
Brn-3b
+Erα


(d)
Coding sequences
BRN3
E1
E 2
L
ER
Brn-3b promoter


(e)
Control
Brn-3b
ER only
Brn-3b+ER 1:1
Brn-3b+ER 1:2
Brn-3b+ER 1:4
< Brn-3b
< Actin

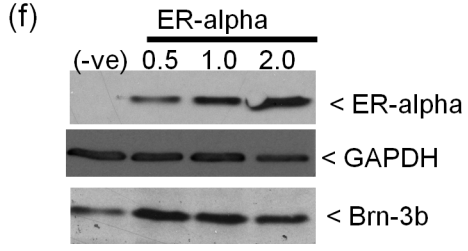
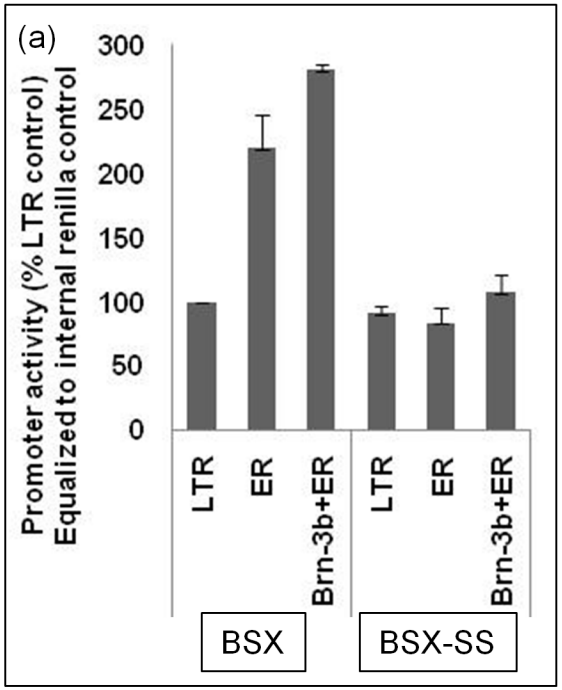
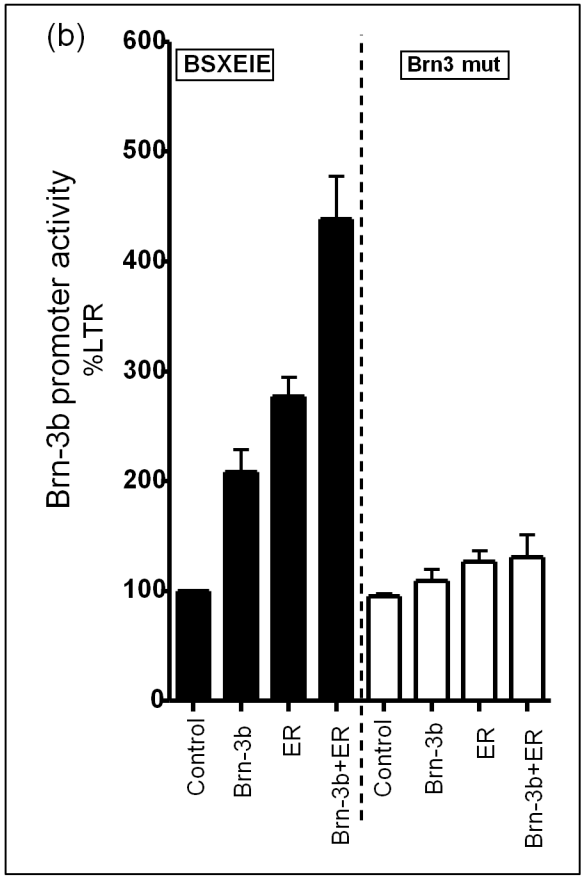
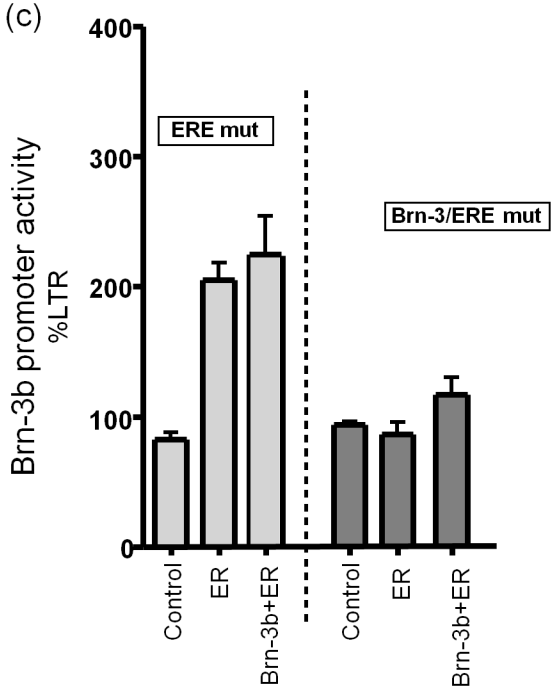

(f)
ER-alpha
(-ve) 0.5 1.0 2.0
< ER-alpha
< GAPDH
< Brn-3b

Figure 6


(a)
Promoter activity (% LTR control)
Equalized to internal renilla control
300
250
200
150
100
50
0
LTR
ER
Brn-3b+ER
LTR
ER
Brn-3b+ER
BSX
BSX-SS


(b)
Brn-3b promoter activity
%LTR
600
500
400
300
200
100
0
BSXEIE
Brn3 mut
Control
Brn-3b
ER
Brn-3b+ER
Control
Brn-3b
ER
Brn-3b+ER


(c)
Brn-3b promoter activity
%LTR
400
300
200
100
0
ERE mut
Brn-3/ERE mut
Control
ER
Brn-3b+ER
Control
ER
Brn-3b+ER

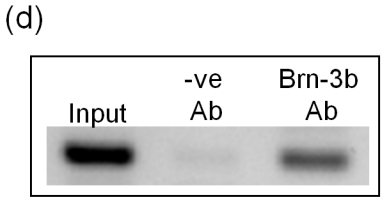

(d)
Input
-ve
Ab
Brn-3b
Ab

Figure 7