FULL PAPER

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ERRβ signalling through FST and BCAS2 inhibits cellular proliferation in breast cancer cells

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Background: The overexpression of oestrogen-related receptor-β (ERRβ) in breast cancer patients is correlated with improved prognosis and longer relapse-free survival, and the level of ERRβ mRNA is inversely correlated with the S-phase fraction of cells from breast cancer patients.

Methods: Chromatin immunoprecipitation (ChIP) cloning of ERRβ transcriptional targets and gel supershift assays identified *breast cancer amplified sequence 2* (*BCAS2*) and *Follistatin* (FST) as two important downstream genes that help to regulate tumourigenesis. Confocal microscopy, co-immunoprecipitation (CoIP), western blotting and quantitative real-time PCR confirmed the involvement of ERRβ in oestrogen signalling.

Results: Overexpressed ERRβ induced FST-mediated apoptosis in breast cancer cells, and E-cadherin expression was also enhanced through upregulation of FST. However, this anti-proliferative signalling function was challenged by ERRβ-mediated BCAS2 upregulation, which inhibited FST transcription through the downregulation of β-catenin/TCF4 recruitment to the FST promoter. Interestingly, ERRβ-mediated upregulation of BCAS2 downregulated the major G1-S transition marker cyclin D1, despite the predictable oncogenic properties of BCAS2.

Interpretation: Our study provides the first evidence that ERRβ, which is a coregulator of ERα also acts as a potential tumour-suppressor molecule in breast cancer. Our current report also provides novel insights into the entire cascade of ERRβ signalling events, which may lead to BCAS2-mediated blockage of the G1/S transition and inhibition of the epithelial to mesenchymal transition through FST-mediated regulation of E-cadherin. Importantly, matrix metalloprotease 7, which is a classical mediator of metastasis and E-cadherin cleavage, was also restricted as a result of ERRβ-mediated FST overexpression.

Oestrogen-related receptors (ERRs) are a group of nuclear receptors that are structurally and functionally related to oestrogen receptors (ERs) but do not bind oestrogen (Horard and Vanacker, 2003). Diethylstilbestrol (DES), which acts as an ERR ligand (Tremblay *et al*, 2001), inhibits the growth of ER-positive and tamoxifen-resistant ER-negative breast cancer cell lines (Lu *et al*, 2001).

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These actions suggest that ERR target genes should be investigated as potential therapeutic targets. Oestrogen-related receptor-β regulates tumourigenesis differently from ERRα and ERRγ; whereas ERRα and ERRγ expression levels are positively correlated with the development of steroid receptor-positive breast cancer (Riggins *et al*, 2010) and tamoxifen resistance (Riggins *et al*, 2008), *ERRβ* mRNA expression levels are inversely correlated with the S-phase fraction (Ariazi *et al*, 2002) of breast tumour cells, which suggests that cellular proliferation is inhibited by ERRβ.

Oestrogen-related receptor-β, like many other nuclear receptors, can be divided into five structural domains, including the A/B (activation function-1 (AF-1) or ligand-independent domain), C (DNA-binding domain), D (hinge region containing nuclear localisation signal), E (ligand-binding domain (LBD) or AF-2) and F domains (coregulator functions, dimerisation and localisation) (Nilsson *et al*, 2001). The C domain of ERRβ shares ∼73% homology with ERα (Zhou *et al*, 2006), and hence, as expected, ERRβ and ERα have common transcriptional targets, such as *pS2* (Lu *et al*, 2001), which is an important marker for cancer.

Oestrogen-related receptor-β binds as a homodimer to at least two types of response elements (Horard and Vanacker, 2003), including full oestrogen response elements (classical EREs: AGGTCAnnnTGACCT) and the SF-1 response element (SFRE/ERRE: TnAAGGTCA), which is an extended half ERE site. Oestrogen-related receptor-β also heterodimerises with other ERRs (Horard *et al*, 2004), and the F domain of ERRβ may heterodimerise with ERα to modulate ERα-mediated ERE-dependent gene transactivation (Bombail *et al*, 2010).

Oestrogen-related receptor-β precursor mRNA comprises 12 exons (Zhou *et al*, 2006), and it is spliced into 3 isoforms: *ERRβ2* (long form), *ERRβ2Δ10* (10th exon deleted) and *ERRβS* (short form with the F domain deleted). Only the short form of ERRβ is expressed in breast tissue (Zhou *et al*, 2006), and these isoforms also differ in tissue specificity, intracellular localisation and the modulation of ERE-dependent ERα transcriptional activity (Yu *et al*, 2008).

Oestrogen-related receptor-β may have anti-proliferative properties (Ariazi *et al*, 2002) in breast cancer cells, and this receptor may activate the $p21^{WAF1/CIP1}$ promoter (Yu *et al*, 2008) in prostate cancer cells, which is a universal inhibitor of cyclin-dependent kinases (CDKs; Xiong *et al*, 1993). P21 expression decreases with oestrogen treatment and the development of anti-oestrogen resistance (Mukherjee and Conrad, 2005), which supports the importance of ERRβ as a therapeutic agent in breast cancer.

Limited studies have reported the role of ERRβ in breast cancer cells (Lu *et al*, 2001; Ariazi *et al*, 2002; Bombail *et al*, 2010). One report found *ERRβ* to act as a proliferative gene (Lu *et al*, 2001), whereas a conflicting study raised the possibility of ERRβ acting as an anti-proliferative factor (Ariazi *et al*, 2002). Therefore, our study sought to unravel the functional significance of ERRβ in breast cancer cells and the deregulation of oestrogen signalling, as well as several key issues associated with breast tumourigenesis.

Our primary goal was to explore the association of ERRβ expression with the proliferative or anti-proliferative properties of the cells. Therefore, we performed chromatin immunoprecipitation (ChIP) cloning (Mishra *et al*, 2001) of ERRβ transcriptional targets and shortlisted two genes, *breast cancer amplified sequence 2* (*BCAS2*) and *Follistatin* (*FST*), on the basis of their opposing roles in proliferation. Subsequently, we identified the bona fide binding sites (i.e., the ERREs in the promoters of the *BCAS2* and *FST* genes) by confirming the binding of ERRβ to the identified sites. However, oestrogen signalling may also regulate *BCAS2* and *FST*, which we demonstrated by showing that ERα was recruited to the relevant ERREs. We also investigated the effect of ERs and ERRβ interactions on *BCAS2* and *FST*, and we successfully revealed the relationship between BCAS2 and FST signalling events. Moreover, Kaplan–Meier (KM) plotter survival analyses supported our conclusions (Gyorffy *et al*, 2010). Besides, our results suggest that regulation of the G1-S transition by β-catenin, of the epithelial to mesenchymal transition (EMT) by E-cadherin and of the induction of apoptosis in breast cancer cells is mediated through ERRβ transregulation of *BCAS2* and *FST*.

MATERIALS AND METHODS

Cell culture and treatments. The human breast carcinoma cell lines MCF-7 (ATCC HTB-22), MDA MB 231(ATCC HTB-26), ZR-75-1 (ATCC CRL-1500) and T47D (HTB-133) were purchased from National Centre for Cell Science, Pune, India. These cells were routinely subcultured in Dulbecco's modified Eagle's medium (DMEM with 4.5 g l^{-1} glucose, L-glutamine, sodium pyruvate, 3.7 g l^{-1} sodium bicarbonate; from Pan Biotech, GmbH, Aidenbach, Germany) containing 10% heat-inactivated foetal bovine serum (South American Origin, Aidenbach, Germany) and antibiotics (penicillin/streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C.

Cells were maintained in charcoal-stripped phenol red-free DMEM for at least 3 days before treatment. The cells were treated with 10 nM oestrogen (Sigma, St Louis, MO, USA), 10 nM DES (Sigma), 1 μM ICI182780 (Sigma) and 1 μM tamoxifen (Sigma) for the time periods indicated in the figures and text and processed for the extraction of whole-cell lysates for western blot.

Western blotting. Whole-cell lysates were prepared using RIPA buffer. Approximately 70 μg of cell lysates were loaded per lane. Transfer was performed onto PVDF membranes (Millipore, Temecula, CA, USA) overnight at 40 V. The transfer efficiency was monitored using Ponceau S staining, and the membranes were incubated in primary and secondary antibody according to the instructions on the antibody datasheets after blocking with non-fat dry milk (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membrane washes were performed in TBS-T, and the X-ray film (Kodak, Rochester, NY, USA) was developed after luminol (GE Healthcare, Buckinghamshire, UK) incubation over the membrane. Oestrogen-related receptor-β was developed, the membrane was stripped with stripping buffer (Pierce, Brebierers, France) and incubated with α-tubulin (Sigma)/FST (Santa Cruz Biotechnology, Inc.)/BCAS2 (Eurogentec, Seraing, Belgium)/ERRβ (Santa Cruz Biotechnology, Inc.)/ERβ (Epitomics, Burlingame, CA, USA) and ERα (Bethyl Laboratories, Inc., Montgomery, TX, USA)/E-cadherin (Epitomics)/β-catenin (Epitomics)/cyclin D1 (Santa Cruz Biotechnology, Inc.)/cleaved PARP (Cell Signaling Technology, Beverly, MA, USA)/GAPDH (Santa Cruz Biotechnology, Inc.) antibodies and developed following similar procedures.

ChIP assay/cloning. MCF-7 cells were grown to 90% confluence in DMEM containing 10% FBS. Cells were crosslinked with 1% (v/v) formaldehyde, and crosslinking was stopped with 0.125 M glycine. Immunoprecipitation was performed as per Farnham's ChIP protocol. The primers used in the ERRβ/ERα ChIP are mentioned in the Table 3.

For ChIP cloning, purified ChIP elutes were ligated to a pGEMT-Easy TA vector from Promega (Madison, WI, USA) and transformed in *E. coli* strain JM109. Plasmids were isolated from individual colonies and digested with *Eco*R1/*Not*I for the presence of inserts.

Plasmid and transfections. MCF-7 and MDA-MB 231 cells were subcultured 1 day before transfection to 70% confluence. Transfection was performed using GENECellin HTC (Larova, Jena, Germany) according to the manufacturer's protocol.

Real-time PCR. Total RNA was isolated from MCF-7 cells using the Trizol method. A total of 2 μg of total RNA was used for cDNA preparation using a first-strand cDNA synthesis kit

(Invitrogen, Carlsbad, CA, USA). This cDNA was used as the template for real-time PCR.

Flow cytometric analyses. Cells were stained using a PE Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol, and immediate acquisition was performed with a BD FACS Calibur using CellQuest Pro Software (San Jose, CA, USA). Using single positive controls in the setup mode, fluorescence compensation was adjusted to subtract the percentage of negative cells from positive cells and avoid spectral overlap. Approximately 10 000 cells were acquired and analysed using an FL3 filter for 7-AAD-positive cells and an FL2 filter for PE-annexin V-positive cells.

Confocal microscopy. Samples containing ERα-CFP, ERβ-CFP, ERRβ-YFP and propidium iodide (PI)/DRAQ5 were captured with a confocal microscope (Leica TCS SP5; Leica Microsystems CMS GmBH, Mannheim, Germany) using LAS AF (Leica Application Suite Advanced Fluorescence) 1.8.1 build 1390 software under an HCX PL APO lambda blue oil-immersion objective (63.0X/N.A.1.40) with a confocal pinhole set at Airy 1 and a resolution of 8 bits. CFP and YFP were excited at 458 nm, 514 nm (Argon laser 30% and AOTF 458 (40%) and YFP (35%)) sequentially with emission (CFP: 462–510 nm, YFP: 520–580 nm, DRAQ5: 650–700 nm; PI: ~617 nm) and a PMT gain of 1175 and 950 V, respectively. The offset was adjusted for a maximum range of fluorescence from 0 to 255 (50% green pixel). Propidium iodide was excited as follows: DPSS 561 nm laser, for DRAQ5- 633 nm laser (AOTF: 40%). Images were captured sequentially with CFP and YFP (emission at 600–630 nm and PMT gain 1050 V).

RNA interference. Breast cancer amplified sequence 2, FST and control siRNA against laminin were purchased from Eurogentec. β-Catenin and control shRNA were purchased from Addgene Cells (Cambridge, MA, USA) and transfected with siRNAs using the ICAFectin 442 (Eurogentec). Cells were subcultured 1 day before transfection to reach 60–70% confluency on the day of transfection. The growth medium was removed 30 min before transfection, and 500 μl fresh medium without serum was added. siRNA (100 nm) was used for transfection. Transfection was performed according to the manufacturer's protocol. Cells were harvested 24 h post-transfection.

Co-immunoprecipitation. Cells were washed with PBS pH 7.4 twice and lysed with NP40 buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP40). Lysates were precleared by the addition of 50 μl of agarose beads for 30 min. Total protein (600 μg) and 4 μg of antibody were used for each IP and rotated overnight in 4 °C. Beads (30 μg) were added to each IP and rotated for 2 h, followed by centrifugation at 1000 r.p.m. for 3 min. Supernatants were removed, and pellets were washed four times with NP40 buffer. Complexes were eluted in SDS lysis buffer.

EMSA. MCF-7 cells were lysed using cell lysis buffer (20 mM HEPES pH 7.9, 20% glycerol, 0.1% Triton X 100, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA and protease inhibitor cocktail), and nuclear lysates were prepared using 20 mM HEPES (pH 7.9, 20% glycerol, 420 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA and protease inhibitor cocktail). Oligos were labelled using γdATP. The EMSA reaction was performed using 5X binding buffer (20% glycerol, 100 mM HEPES pH 7.9, 300 mM KCl, 25 mM $MgCl_2$, 4 mM EDTA), 3 μg BSA, 1 μg poly dIdC at room temperature. The reaction was run on 6% nondenaturing PAGE gels.

KM plotter analyses. Relapse-free survival information was taken into account from GEO (Affymetrix HGU133A and HGU133 + 2 microarrays, Santa Clara, CA, USA), EGA and TCGA for the drawing of KM plots. Patient samples were divided into two groups by computing the median of the expression level of the proposed biomarker and selecting the best cut-off threshold using the percentiles between upper and lower quartiles to analyse the prognostic value of a particular gene. Optimal probe sets were selected for each gene using a scoring method established to assess specificity, coverage and degradation resistance. The two-patient cohorts of 2896 clinical samples were compared using KM survival plots, and the hazard ratio with 95% confidence intervals and log-rank *P*-values were calculated. Affymetrix gene ID: 207726_at, 203053_at and 204948_s_at were used to plot the survival curves of ERRβ, BCAS2 and FST, respectively.

3D structural modelling. The model of ERRβ *homo sapiens* protein (508AA) was built using I-TASSER, which generates 3D structural models using multiple threading alignments and iterative structural assembly simulations (Roy *et al*, 2010). The docking (ERRβ with ERα and ERRβ with ERβ) was performed using ZDOCK (Mintseris *et al*, 2007; Pierce *et al*, 2011), which uses a fast Fourier transformation to perform a 3D search of the spatial degrees of freedom between two molecules. The Z-dock can generate a 3D structural model of the interaction between two macromolecules. This study used docking without constraints on the protein structure. The best model was selected based on the Z-dock score and similarity with related protein complexes.

RESULTS

ERRβ overexpression is associated with apoptotic induction and improved prognosis in breast cancer cell lines and patients. No reports of relative ERRβ expression in breast cancer cell lines have been published. We therefore performed western blots to investigate ERRβ expression in breast cancer cell lines. Oestrogen-related receptor-β was overexpressed in immortalised normal MCF10A cells compared with ER-positive MCF-7 and T47D cells and ER-negative SKBR3, MDA MB 231 and MDA MB 453 cells (Figures 1A-I and II). We next analysed the morphological changes induced following ERRβ long-form (ERRβL) overexpression in the human breast cancer cell line MCF-7, based on the above-mentioned results and the reduced expression of ERRβ in cancer cell lines compared with immortalised cell lines (Yu *et al*, 2008). MCF-7 cells in which ERRβL was overexpressed showed distinct nuclear fragmentation (Figure 1B) according to PI staining, which is a morphological feature of apoptotic cells (Dini *et al*, 1996). Step-wise events associated with apoptosis (Wade *et al*, 2001), such as non-protruding crescents indicative of chromatin condensation and the formation of vessels containing condensed chromatin in the periphery of the nucleus, were also observed. We then overexpressed ERRβ in MCF-7 cells and performed an annexin V-PE apoptosis detection assay. In all 84.53% of the gated viable cells exhibited annexin-positive and 7AAD-negative staining; this result was indicative of early apoptotic events and was in contrast to the vector control, which demonstrated 2.32% positive staining (Figures 1C-I and II).

Oestrogen-related receptor-β overexpression in breast cancer cell lines was reduced compared with the immortalised normal breast cell line MCF-10A. Therefore, we investigated whether this reduced expression was associated with the development of malignancy. We isolated protein lysates from one benign (control) and two malignant tumour samples from breast tumour patients and performed western blotting. The benign sample was used as the control because normal breast tissue was not available, and the results indicated that ERRβ expression was reduced in the malignant samples (Figure 1D). This *in vivo* study suggested that decreased ERRβ expression was physiologically relevant in the development of malignancy.

We next performed KM plotter analysis ($n = 2978$) to obtain statistically sound data for the association of ERRβ with breast cancer patient survival. Kaplan–Meier plotter analyses

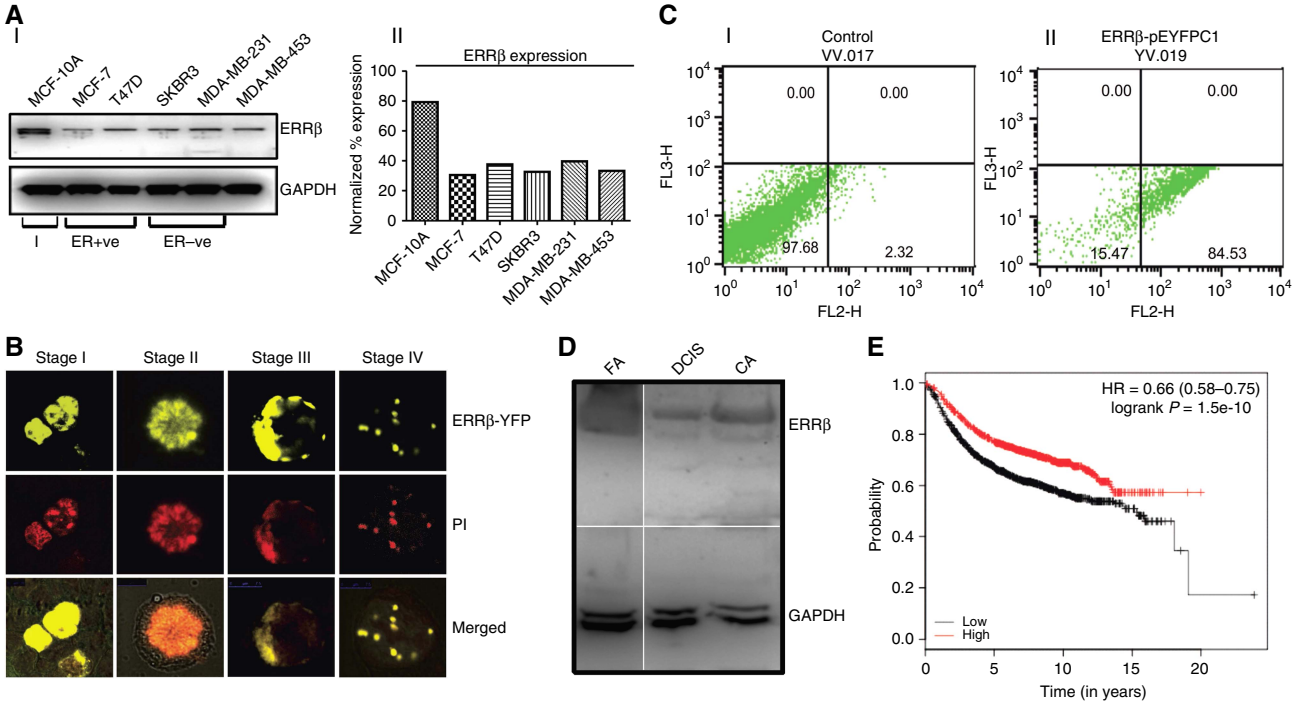

Figure 1. ERRβ as an anti-proliferative molecule. (**A**-I) Western blot showing ERRβ comparative expression in different breast cell lines. (II) Densitometric analyses denoting higher ERRβ expression in immortalised (I) breast cells (MCF-10A) compared to breast cancer cell lines. The histogram of % protein expression was calculated by normalising with the corresponding control. (**B**) Different stages apoptotic nuclear fragmentation as shown by confocal microscopy in MCF-7 cells. The emission maxima were 535 nm for ERR-YFP and 617 nm for PI. Propidium iodide staining was performed to monitor nuclear morphology post-RNase treatment. (**C**-I and II) Flow cytometric analyses showing apoptosis induction in ERRβ-pEYFPC1-overexpressing (II) MCF-7 cells in comparison with the vector control (I). Detection of apoptotic cells was performed using the FL2 channel of BD FACS Calibur and annexin V-PE as an apoptotic indicator. The dead cell population was gated out using 7-AAD as a vital stain and FL3 as the detection channel. (**D**) Western blot for human tumour samples showing reduced ERRβ expression in malignant tumours (DCIS and CA) compared with a benign tumour (FA). (**E**) Kaplan–Meier plotter analysis (for statistical significance) showing that high ERRβ expression was associated with better prognosis and longer relapse-free survival in breast cancer patients. Abbreviations: CA = carcinoma; DCIS = ductal carcinoma *in situ*; FA = fibroadenoma.

(Gyorffy *et al*, 2010) showed (Figure 1E) that high ERRβ expression was associated with improved prognosis and higher relapse-free survival rates compared with low ERRβ expression, which supports the anti-proliferative nature of ERRβ.

FST and BCAS2 are two important transcriptional targets of ERRβ that bind to ~6-kb and ~8-kb upstream of the BCAS2 and FST transcription start sites (TSSs), respectively, and upregulate their transcription. We performed ChIP cloning in MCF-7 breast carcinoma cells to identify the transcriptional targets of ERRβ (Supplementary Figures 1A, B, C, D, E and F). TA cloning of ERRβ target sequences isolated hundreds of colonies, which were sequenced to identify the important targets. The primers used for sequencing are listed in Table 1.

Table 1. Primer used for sequencing

Primer name	Sequence (5′–3′)
T7 forward primer	TAATACGACTCACTATAGGG

Nucleotide BLAST searches were performed in NCBI against human genomic DNA sequences, and probable target promoters were shortlisted (data not shown) based on the total score, ΔE value, query coverage and features flanking part of the subject sequence.

Chromatin immunoprecipitation cloning identified *BCAS2* and *FST* as significant target genes (Table I in Figure 2) of ERRβ. Breast cancer amplified sequence 2 is a co-activator of ERα (Qi *et al*, 2005) and a negative regulator of p53 (Kuo *et al*, 2009). Follistatin enhances the ability of R30C breast carcinoma cells to induce sub-G1 populations (Krneta *et al*, 2006) and inhibits multi-organ metastasis of small cell lung carcinoma in natural killer cell-deprived SCID mice (Talmadge, 2008).

The screening of up to 10-kb upstream from the *BCAS2* and *FST* TSSs revealed the presence of ERRE sites at −6077 to −6086 upstream from the *BCAS2* TSS (Supplementary Figure 1G) and −8678 to −8687 (distal site) and −5132 to −5141 (proximal site) upstream from the *FST* TSS (Supplementary Figure 1H). No full-length ERE was found up to 10-kb upstream from either TSS.

Electrophoretic mobility shift assays (EMSAs) were performed to confirm the specific recruitment of ERRβ to ERREs upstream of the *BCAS2* and *FST* promoters (Figures 2A and 3A). *Breast cancer amplified sequence 2* ERRE and distal ERRE (ERRE 2) of *FST* showed specific binding patterns (Vanacker *et al*, 1999) related to the co-recruitment of ERα and ERRβ. Supershift assays confirmed the *in vitro* ERRβ binding to the *FST* ERRE2. The *BCAS2* ERRE showed competition in the ERα band, which was less in the ERRβ band; however supershift for ERRβ was observed. The oligos used in the EMSA are listed in Table 2.

We next investigated the *in vivo* ERRβ binding to the *BCAS2* ERRE and *FST* ERRE2 under vehicle treatment and 24-h oestrogen treatment using the ChIP assay (Figures 2B and 3B). Oestrogen-related receptor-β binding to both ERREs was compromised under 24-h oestrogen treatment, which indicates that the oestrogen-dependent conformational change in ERRβ loosened ERRβ from the respective ERREs. The primers used in the ChIP assays are listed in Table 3.

Table I

S.no.	Accession no.	Reference gene name	No. of hits	Function
1	NT 007592.15	*Breast cancer amplified sequence 2*	4 times	Coactivator of ERα and negative regulator of p53
2	NM_013409.1	*Follistatin*	2 times	FSH inhibitor, inhibitor of multi-organ metastasis

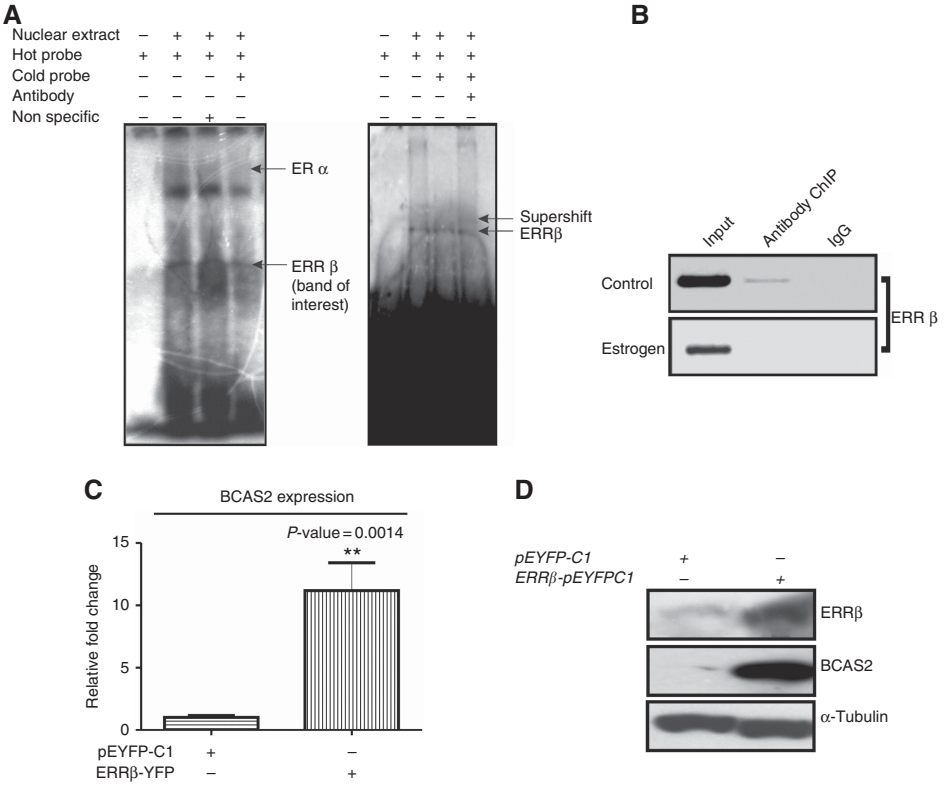

Figure 2. BCAS2 as a transcriptional target of ERR*β*. (**A**) Electrophoretic mobility shift assay showing the *in vitro* binding of transcription factors in the ERRE of BCAS2 promoters and supershift confirming ERR*β* binding. (**B**) Chromatin immunoprecipitation confirming the *in vivo* ERR*β* binding to BCAS2 ERRE in MCF-7 cells. (**C**) Real-time PCR data showing the upregulation of BCAS2 transcripts under ERR*β* overexpression. Asterisks (*) indicates significance (*t*-test compared with control; $P<0.05$). (**D**) Western blot confirming the upregulation of BCAS2 protein expression under ERR*β* overexpression. Abbreviation: ERRE = oestrogen related receptor response element.

We then investigated the effect of overexpressed ERR*β* on *BCAS2* and *FST* transcription using real-time PCR. Overexpressed ERR*β* upregulated *BCAS2* and *FST* mRNA levels (Figures 2C and 3C). The primers used for real-time PCR are listed in Table 4. Real-time figures of ERR*β* transfection are shown in Figure 2A. The upregulation of BCAS2 and FST proteins following ERR*β* overexpression was confirmed using western blotting (Figures 2D and 3D).

Involvement of ERs in ERR*β*-mediated BCAS2 and FST signalling. The western blotting, real-time PCR and ChIP results showed that the ERR*β* transcriptional targets *BCAS2* and *FST* were also regulated by ERα and that the ERR*β* antagonist DES reversed the effects of oestrogen. Therefore, we investigated the ERR*β* localisation under oestrogen and DES treatment (Figure 4A); no nuclear fragmentation was observed following either treatment; interestingly remarkable blockage in ERR*β* nuclear translocation was observed under DES treatment.

Confirmation of ERR*β* and ERα involvement in the regulation of BCAS2 and FST in MCF-7 cells. Real-time PCR data (Figure 4B) and western blots (Figures 4C-I and II) of 17*β*-oestradiol or ICI182780 in DES-treated MCF-7 cells confirmed the involvement of ERR*β* LBD and ERα in the regulation of BCAS2 and FST.

Follistatin was upregulated as a result of 10 nM oestrogen treatment, which was reversed with ICI182780 treatment. Breast cancer amplified sequence 2 was downregulated in the presence of oestradiol (E2), and ICI182780 reversed this effect in MCF-7 cells. These results revealed the involvement of ERα in BCAS2 and FST regulation.

However, DES treatment revealed rather complex regulation of BCAS2 and FST by ERR*β*. Diethylstilbestrol-mediated inhibition of ERR*β* nuclear translocation downregulated FST at the mRNA and protein levels, although BCAS2 expression was enhanced compared with the control. Therefore, the antagonistic activity of DES on endogenous ERR*β* transcriptional activity is questionable for BCAS2 expression. One explanation may lie in the functional domain of endogenous ERR*β*, which differentiates between oestrogen signalling and DES signalling. It seems, endogenous ERR*β* may not specifically upregulate BCAS2 upon overexpression; rather, it should actually downregulate BCAS2 due to which DES is able to upregulate its expression. We aligned the used sequence (full length) with the available full-length ERR*β* mRNA sequence through the NCBI and showed that approximately 78 amino acids of the exogenous sequence were deleted in the F domain (dimer interface; data not shown). Exogenous ERR*β* in MCF-7 cells alone seemed unable to translocate to the nucleus during DES treatment unless the ERs were also transfected exogenously. We next

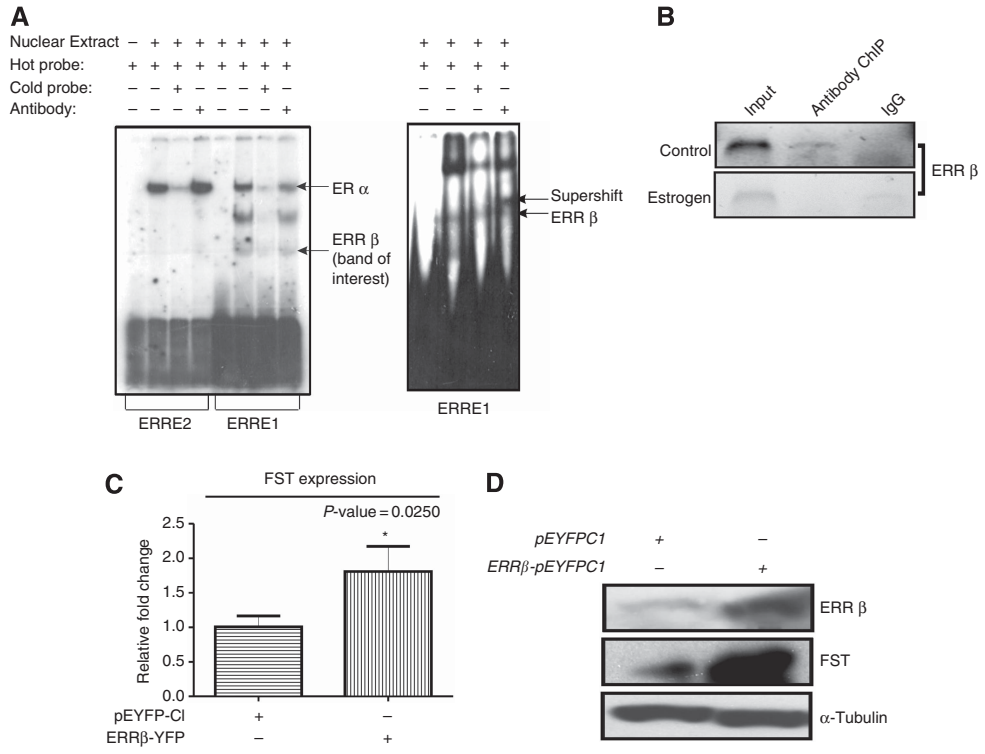

Figure 3. FST as a transcriptional target of ERR*β*. (**A**) Electrophoretic mobility shift assay showing the *in vitro* binding of transcription factors in ERREs of the FST promoter and supershift confirming ERR*β* binding in ERRE1. (**B**) Chromatin immunoprecipitation confirming the *in vivo* ERR*β* binding to FST ERRE1 in MCF-7 cells. (**C**) Real-time PCR data showing the upregulation of FST transcripts under ERR*β* overexpression. Asterisks (*) indicates significance (*t*-test compared with control; $P<0.05$). (**D**) Western blot confirming the upregulation of FST protein expression under ERR*β* overexpression.

Table 2. EMSA oligos

Primer name	Sequence (5′–3′)
FST ERRE1 F	GTGCTAAAGGTCACACACAG
FST ERRE1 R	CTGTGTGTGACCTTTAGCAC
FST ERRE2 F	TTACAGCTAAAGGTCACTTC
FST ERRE2 R	GAAGTGACCTTTAGCTGTAA
BCAS2 ERRE F	GAAAAGTGGAGTTAAAGGTCAGTTTCATTA
BCAS2 ERRE R	TAATGAAACTGACCTTTAACTCCACTTTTC

Abbreviations: BCAS2 = breast cancer amplified sequence 2; EMSA = electrophoretic mobility shift assay; FST = Follistatin.

Table 3. ChIP primers

Primer name	Sequence (5′–3′)
BCAS2 ERRE F	TTTTGTGGAGTTGGTAGGAC
BCAS2 ERRE R	CTCATGTTGGTGAAACTGTG
FST ERRE 1 F	TGCCTTCTCCTGGCTAACCCCT
FST ERRE 1 R	GCATCCACCAGAAGGGCTAGTGC

Abbreviations: BCAS2 = breast cancer amplified sequence 2; ChIP = chromatin immunoprecipitation; FST = Follistatin.

examined whether the release of ERR*β* from ERRE following E2 treatment occurred because of competition with ERα using ChIP (Figure 4D) since only ERα shared ERREs as common binding sites with ERRs. FST distal ERRE and BCAS2 ERRE showed ERα binding in vehicle-treated samples. Surprisingly, E2 treatment resulted in the release of ERα as well from ERREs indicating ERα AF-1 domain (ligand independent) is responsible for regulating FST and BCAS2 expressions at basal level in MCF-7 and convergence of the ERα and ERR*β* signaling pathways involving BCAS2 and FST.

The 3D structural model of the ERR*β* *homo sapiens* protein 508AA was built using I-TASSER. The docking of ERR*β* with ERα and ERR*β* with ER*β* was performed using Z-dock. Preliminary modelling showed that ERα binds to the D domain and partially the LBD of ERR*β*. The residues Arg179, 182, Lys184, Arg187, Ile201, Pro233, Glu372, Gln375, Asp376 and Glu234 of ERR*β* and Leu327, Tyr328, Arg352, Val355, His356, Ile358, Asn532, Pro535, Asp538 and Glu542 of ERα were found at the interface (Figure 5A). The residues of ERα that seemed to be part of the docking interface included a domain involved in the interaction with AKAP13 and the self-association and transactivation of the LBD region.

According to the ChIP assays, ERα and ERR*β* simultaneously regulate *BCAS2* and *FST* in an ERRE-dependent manner, and we therefore examined whether the transcription factor colocalises in MCF-7 cells using the overexpression of fluorescent constructs of ERR*β*L and ERα. Oestrogen-related receptor-*β* colocalised with ERα in the nucleus of MCF-7 cells (Figure 5B). ERα always colocalised with ERR*β* in co-transfection experiments in vehicle-, E2- and DES-treated cells.

The 3D structural modelling study and confocal microscopy suggested the occurrence of possible interactions between ERα and ERR*β*. *In vivo* CoIP experiments confirmed these results (Figure 5C), whereas oestrogen treatment compromised this interaction.

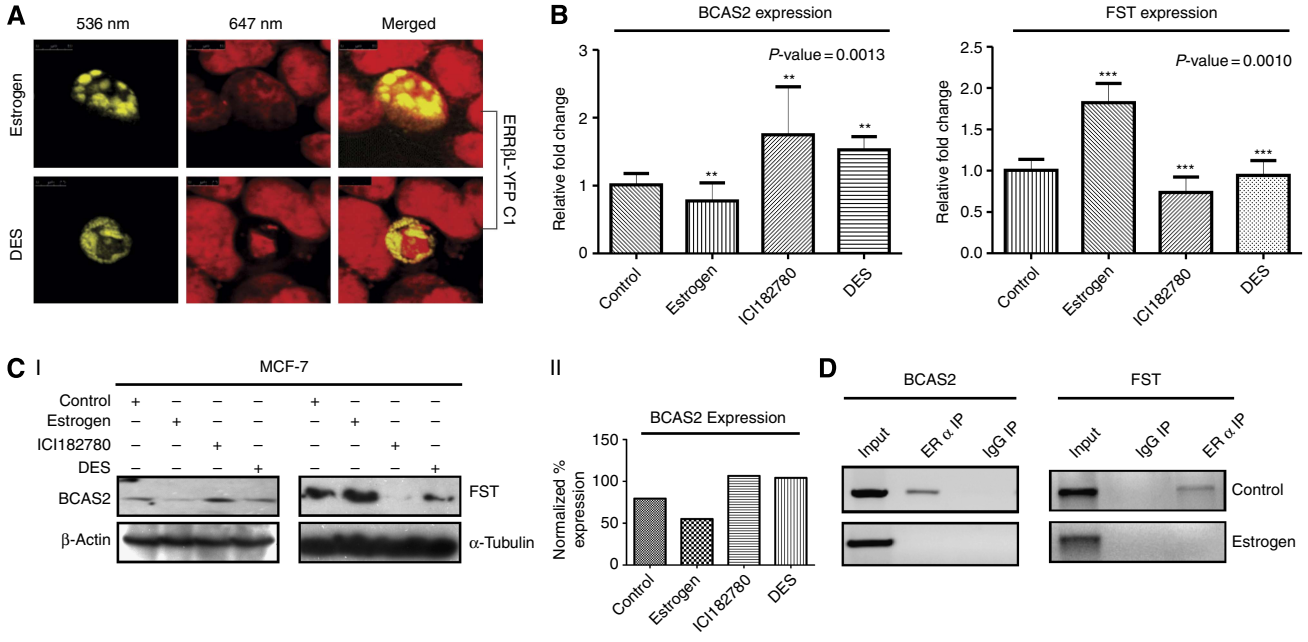

Figure 4. Involvement of ERs on the regulation of ERRβ transcriptional targets. (**A**) Effect of oestrogen and DES treatment on exogenously overexpressed ERRβ intracellular translocation as shown by confocal microscopy in MCF-7 cells. (**B**) Effect of 24-h treatment with E2, the pure ER antagonist ICI182780 and the ERRβ antagonist DES on BCAS2 and FST transcription using real-time PCR. Asterisks (*) indicates significance (one way ANOVA test compared with control; $P<0.05$). (**C**-I) Effect of 24-h treatment with E2, the pure ERα antagonist ICI182780 and the ERRβ antagonist DES on BCAS2 and FST protein expression using western blotting. (II) Densitometric analysis of the BCAS2 panel. (**D**) Chromatin immunoprecipitation PCR confirming the recruitment of ERα to the respective ERRE sites of BCAS2 and FST in MCF-7 cells.

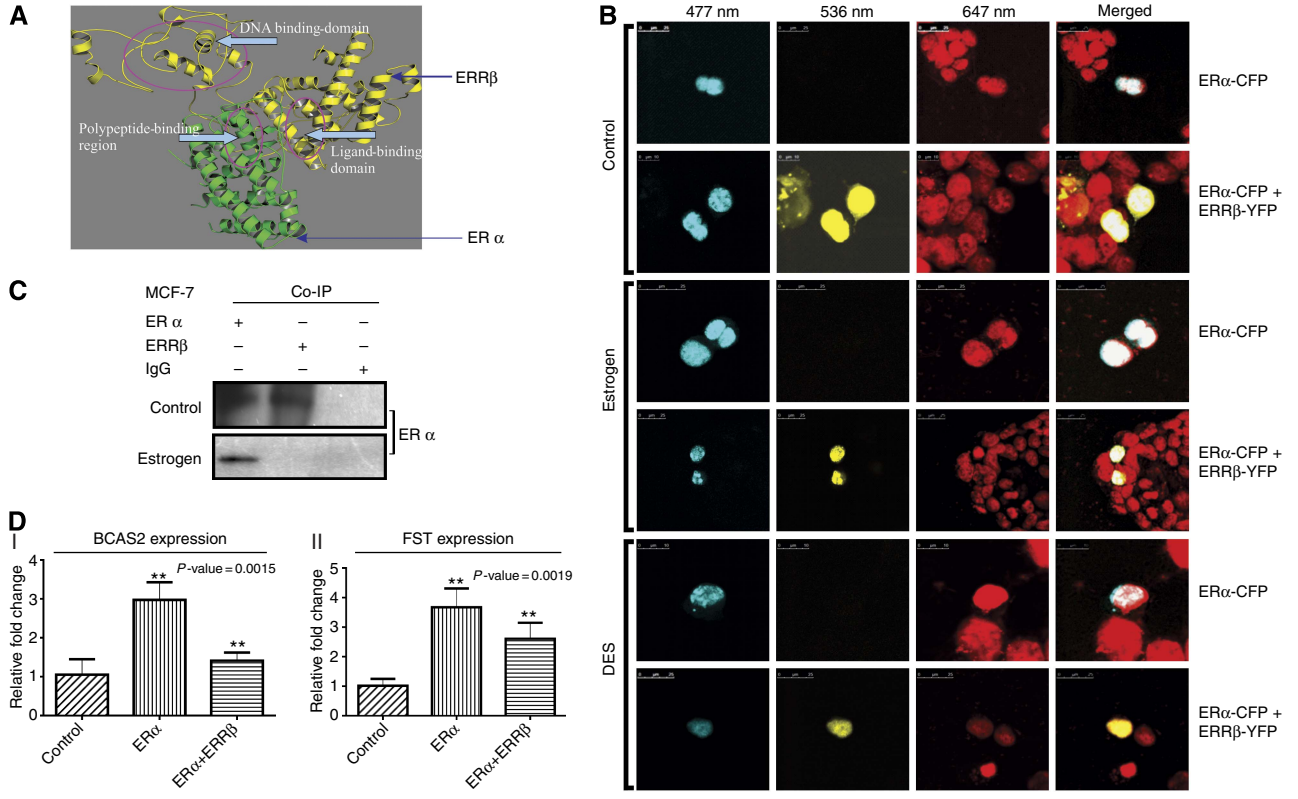

Figure 5. Modulation of ERα functional activity by ERRβ. (**A**) 3D structural model predicting the physical interaction of ERα (green) with ERRβ (yellow). (**B**) Intracellular localisation of exogenously overexpressed ERα alone and ERRβ under different treatment conditions in MCF-7 cells using confocal microscopy. (**C**) Co-immunoprecipitation assay showing the physical interactions between ERα and ERRβ in vehicle- and oestrogen-treated cells. (**D**) Real-time PCR data showing mRNA expression patterns of BCAS2 (I) and FST (II) under ERα overexpression alone and ERα with ERRβ in MCF-7 cells. Asterisks (*) indicates significance (one way ANOVA test compared with control; $P<0.05$).

The interactions of ERβ with ERα led us to perform real-time PCR to investigate the expression of the common targets BCAS2 and FST following co-transfection of ERα and ERβ. Oestrogen-related receptor-β significantly reduced the upregulation of BCAS2 and FST by ERα (Figures 5D-I and II). Real-time figures showing these transfections are shown in Supplementary Figures 2B-I, II and III. The primers used to examine the transfections are listed in Table 4.

Table 4. Real-time PCR primers

Primer name	Sequence (5′–3′)
BCAS2 RT primer F	GAAACGATATGAGCTTCCAG
BCAS2 RT primer R	CATTACATCCATGCTGTGACG
FST RT primer F	GGGAGAGGCCGGTGTTCCCT
FST RT primer R	TGGGGGAATACAGGGGAGCTGT
ERβ RT primer F	AAGCCATTGACCAAGATTGT
ERβ RT primer R	GGTCACAGAGAGTGGTCAGG
ERα RT primer F	AGCTCCTCCTCATCCTCTCC
ERα RT primer R	TCTCCAGCAGCAGGTCATAG
ERβ RT primer F	TTCCCAGCAATGTCACTAACTT
ERβ RT primer R	TTGAGGTTCCGCATACAGA
5′hMMP7 Ex2	TGGCCTACCTATAACTGGAA
3′hMMP7 Ex2	GTAGGTGACCACTTTGGAAG

Abbreviations: BCAS2 = breast cancer amplified sequence 2; ERβ = oestrogen receptor β; ERRβ = oestrogen-related receptor-β; FST = Follistatin; MMP7 = matrix metalloprotease.

The docking results of ERβ with ERRβ showed that ERβ binds to ERRβ at the LBD of ERRβ. The residues Arg400, Gln401, Ala404 Gln408, Tyr411 and Leu429 from ERRβ and Met379 and Ala456, Met460, Ser463, His467 and Lys471 from ERβ were found at the docking interface (Figure 6A). The ERβ residues at the docking interface include a domain that is involved in dimerisation interface/polypeptide binding. Based on our CoIP results, we extended our study towards colocalization pattern of ERβ and ERRβ with fluorescent constructs. ERβ demonstrated predominantly nuclear localisation under vehicle, E2 and DES treatment and with ERRβ co-transfection in MCF-7 cells (Figure 6B).

In contrast to MCF-7 cells, MDA MB 231 cells, which exhibit distinct ERβ expression but lack ERα expression (Li *et al*, 2010), restrict ERRβ functionality leading to the failure of exogenous ERα translocation to the nucleus. Therefore, we investigated whether the dependence of ERα nuclear translocation on ERRβ in the presence of oestrogen was based on a physical interaction using CoIP assays. We also performed CoIP of ERRβ with ERβ because of the structural similarities between ERα and ERβ (Nilsson *et al*, 2001). Interestingly, ERRβ was more interactive with ERβ *in vivo* compared with ERα in MCF-7 cells (Figures 5C and 6C). However, these interactions were compromised following oestrogen treatment. Oestrogen-related receptor-β also interacted with ERβ in MDA MB 231 cells, and this interaction was reduced as a result of oestrogen treatment.

Oestrogen receptor-β exhibited greater affinity for ERRβ than ERα. Therefore, we performed the same co-transfection

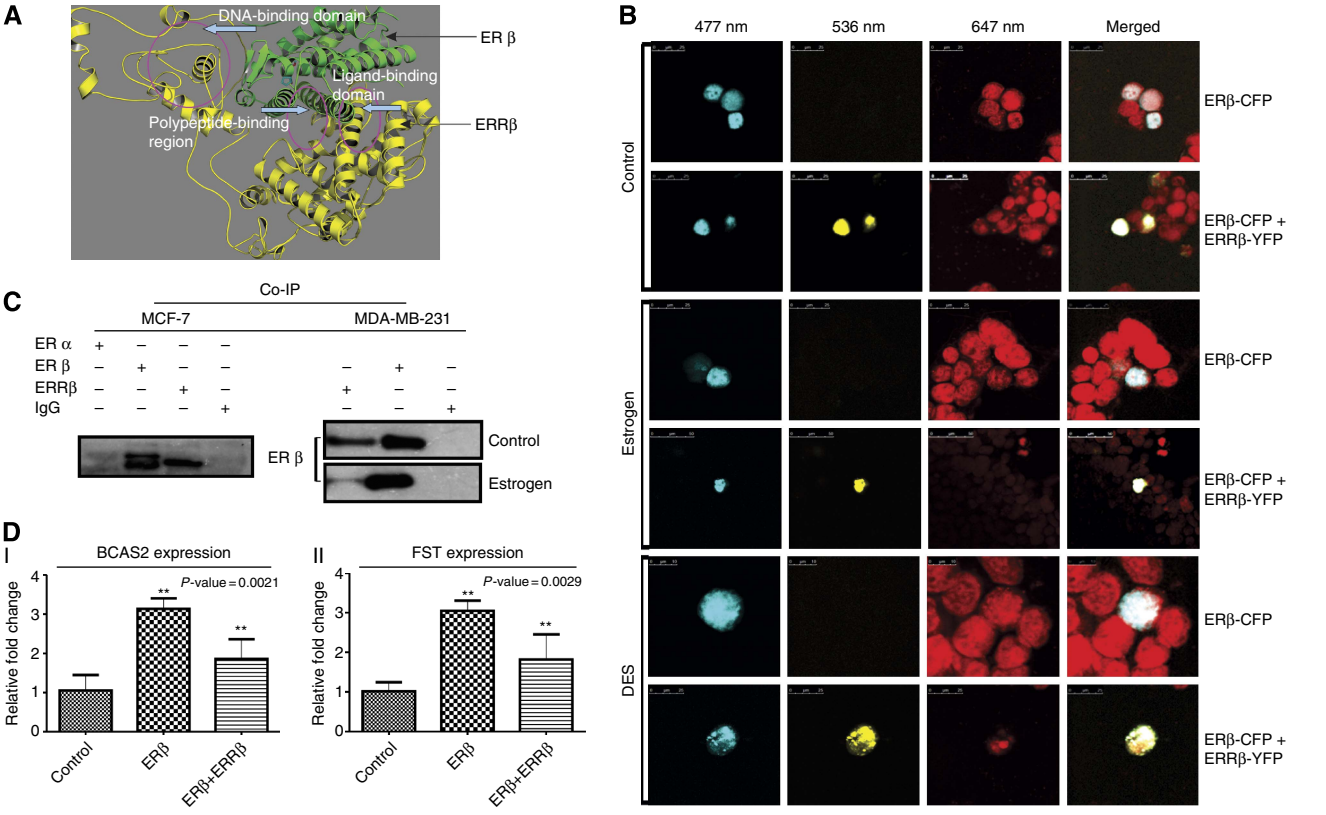

Figure 6. Modulation of ERβ functional activity by ERRβ. (**A**) 3D structural model predicting the physical interactions of ERβ (green) with ERRβ (yellow). (**B**) Intracellular localisation of exogenously overexpressed ERβ alone and with ERRβ under different treatment conditions in MCF-7 cell line using confocal microscopy. (**C**) Co-immunoprecipitation assay showing the physical interactions between ERβ and ERRβ in MCF-7 and in MDA-MB-231 (lacking ERα) cells following vehicle and oestrogen treatments. (**D**) Real-time PCR data showing mRNA expression patterns of BCAS2 (I) and FST (II) under ERα overexpression alone and with ERRβ in MCF-7 cells. Asterisks (*) indicates significance (one way ANOVA test compared with control; $P<0.05$).

experiment described above using ERβ. Oestrogen receptor-β-mediated activation of BCAS2 and FST expression was reduced following ERRβ co-transfection, similar to ERα (Figures 6D-I and II).

Triple-negative MDA MB 231 cells showing different patterns of FST and BCAS2 regulation depict the role of ERRβ as a coregulator of ERs for the regulation of ERRE-containing transcriptional target genes. MDA MB 231 cells, which are ERα null, uniquely regulate ERα target genes using ERβ because of its functional dissimilarity (Nilsson *et al*, 2001). We also observed different ERβ regulation patterns of our specified target genes, *BCAS2* and *FST*, in MDA MB 231 cells in western blots of treated samples (Supplementary Figure 3A). For example, BCAS2 was downregulated in oestrogen-treated cells, and ICI182780 did not reverse this downregulation, which confirmed ERβ involvement. A similar effect was observed for FST expression. Diethylstilbestrol also downregulated BCAS2 and FST, which indicated the agonistic activity of DES on ERβ and the inhibition of the transcriptional activity of ERRβ. This experiment also demonstrated that in the absence of oestrogen, ERRβ acted as a necessary coregulator of ERs to regulate the expression of ERRE-containing transcriptional target genes, such as BCAS2 and FST.

We next performed confocal experiments to explore the effect of ERRβL on exogenous ERα localisation in more aggressive MDA MB 231 cells (Supplementary Figure 3B). Surprisingly, exogenous ERα in cells receiving the vehicle control, oestrogen or DES treatment did not demonstrate nuclear localisation, and this cytoplasmic localisation did not change under conditions of ERRβL overexpression. Moreover, ERRβL overexpression did not affect ERβ cytoplasmic localisation following oestrogen treatment or DES treatment in MDA MB 231 cells, which indicated a major inhibitory mechanism in the nuclear translocation of ERs.

ERRβ, BCAS2 and FST show differential mRNA and protein expression patterns following time-dependent oestrogen treatment. Oestrogen-related receptor-β overexpression transactivated BCAS2 and FST which indicated that the effect on cellular metabolism because of their transactivation was directed in the same direction. However, the mechanism for this differential

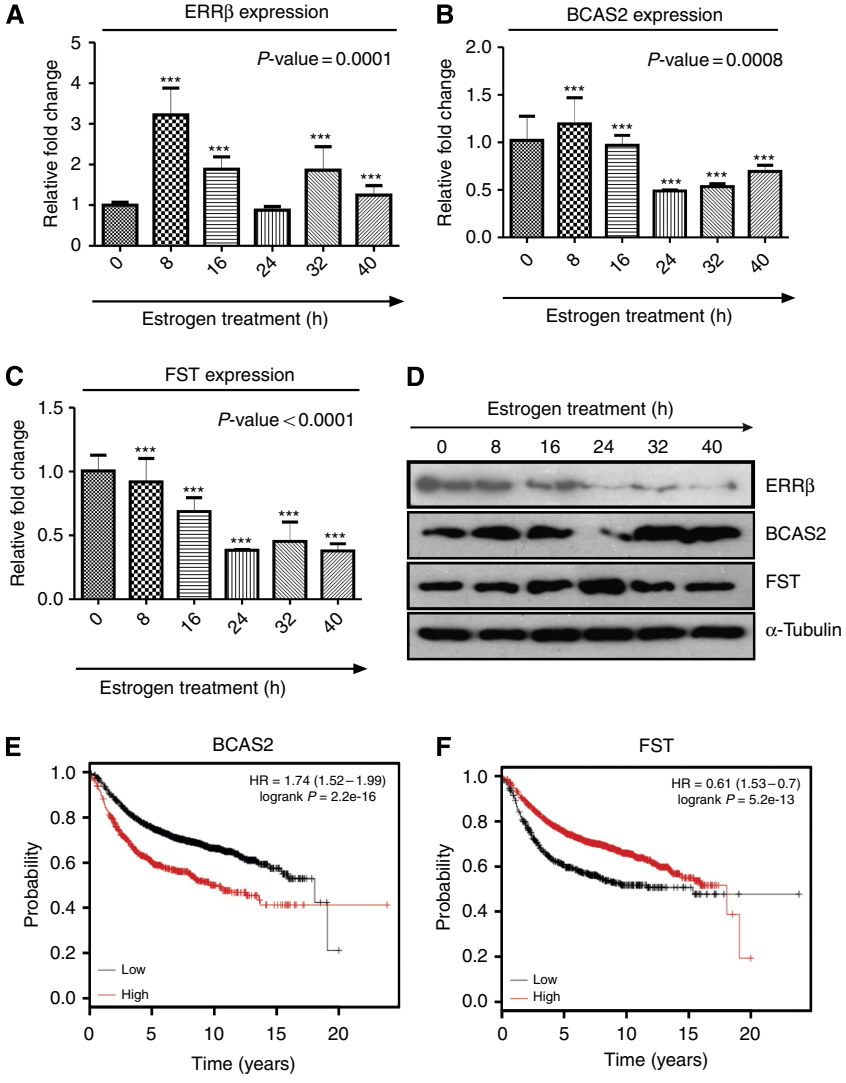

Figure 7. Effect of oestrogen on ERRβ transcriptional targets, their interconnected signalling events and patient prognosis. (**A**, **B** and **C**) Real-time PCR data showing time-dependent expression patterns of ERRβ, BCAS2 and FST, respectively. Asterisks (*) indicates significance (one way ANOVA test compared with control; $P<0.05$). (**D**) Western blot showing time-dependent protein expression patterns of ERRβ, BCAS2 and FST. (**E** and **F**) Kaplan–Meier plotter analyses showing the associations between low BCAS2 and high FST expression with better prognosis and longer relapse-free survival, respectively.

oestrogen-driven regulation of the above-mentioned genes is not known. Therefore, we investigated whether this regulation was altered in a time-dependent manner. Real-time PCR data (Figures 7A–C) showed similar expression patterns for BCAS2 and FST in response to oestrogen, whereas western blot data (Figure 7D) showed different expression patterns. Breast cancer amplified sequence 2 protein expression was stably upregulated after 32 h, but FST protein expression returned to basal levels after a transient increase at 16–24 h. Breast cancer amplified sequence 2 protein expression was at its lowest level at ~24 h, whereas FST protein expression was highest during this time. Moreover, the mRNA and protein expression of ERRβ was not necessarily correlated with BCAS2 or FST expression. For example, ERRβ protein expression was downregulated 24 h after E2 treatment. It seemed that oestrogen-related receptor-β was subjected to some type of protein degradation event during long-term E2 treatment, which also downregulated *BCAS2* and *FST* transcription. However, the stability of BCAS2 protein was also restored after long-term E2 treatment.

BCAS2 and FST exhibit opposite prognostic effects in breast cancer patients. Follistatin is a potential anti-proliferative molecule that is expressed at the highest level, whereas BCAS2 is completely downregulated, after 24-h E2 treatment. Therefore, we investigated the correlation between BCAS2 and FST expression with prognosis in clinical breast cancer patient samples ($n = 2978$) using KM plotter analysis. This analysis revealed improved relapse-free survival in FST-overexpressing patients compared with those with lower expression (Figure 7F). In contrast, lower BCAS2 expression was associated with higher relapse-free survival (Figure 7E).

BCAS2 downregulates FST expression through the inhibition of β-catenin/TCF4 signalling. Kaplan–Meier plotter analyses showed an inverse correlation between BCAS2 and FST. Therefore, we speculated that BCAS2, which is a known coregulator molecule by nature, may regulate FST. We performed real-time PCR for FST following BCAS2 knockdown in MCF-7 cells and observed significantly upregulated FST expression, which underscored the proliferative nature of BCAS2 (Figure 8A). However, knockdown of FST did not affect BCAS2 (Figure 8B), and this negative result confirmed that no downstream pathway of FST regulates BCAS2. Confirmation of siRNA transfection is shown in Supplementary Figures 4A-I and II. Sequences of the siRNAs used are listed in Table 5. Follistatin upregulation at the protein level following BCAS2 knockdown was also demonstrated using western blots (Figure 8C). In particular, we performed western blotting of FST after BCAS2 knockdown in the presence of tamoxifen (Figure 8C) and found that BCAS2-mediated regulation of FST remained intact, which supported the non-involvement of ERs.

ER coregulator BCAS2, as per our real time data acts as an inhibitor of FST. The E2 target gene (Pontes *et al*, 2010) β-catenin is a novel prognostic marker in breast cancer (Lin *et al*, 2000) and an upstream regulator of FST (Lin *et al*, 2000). Therefore, we investigated β-catenin expression following BCAS2 overexpression and observed that β-catenin expression was reduced following BCAS2 overexpression (Figure 8D). In addition, β-catenin overexpression upregulated FST (Figure 8E). Therefore, BCAS2 likely acts as a negative regulator of the β-catenin promoter, and downstream FST transcription is blocked as a result.

The downregulation of β-catenin by BCAS2 led us to further evaluate the regulation of downstream FST (Singh *et al*, 2009). However, knockdown of β-catenin reduced the luciferase activity of FST promoter fragments (2.9 kb) containing eight TCF-4E-binding sites located ~2870, 2329, 2298, 1823, 1803, 1387, 828 and 671-bp upstream of the TSS (Figure 8F). Follistatin upregulation also upregulated uncleaved E-cadherin (Figure 8E).

BCAS2 upregulated MMP7 and resulted in E-cadherin cleavage, which was counteracted by FST. We next investigated the E-cadherin protein expression level using western blotting to

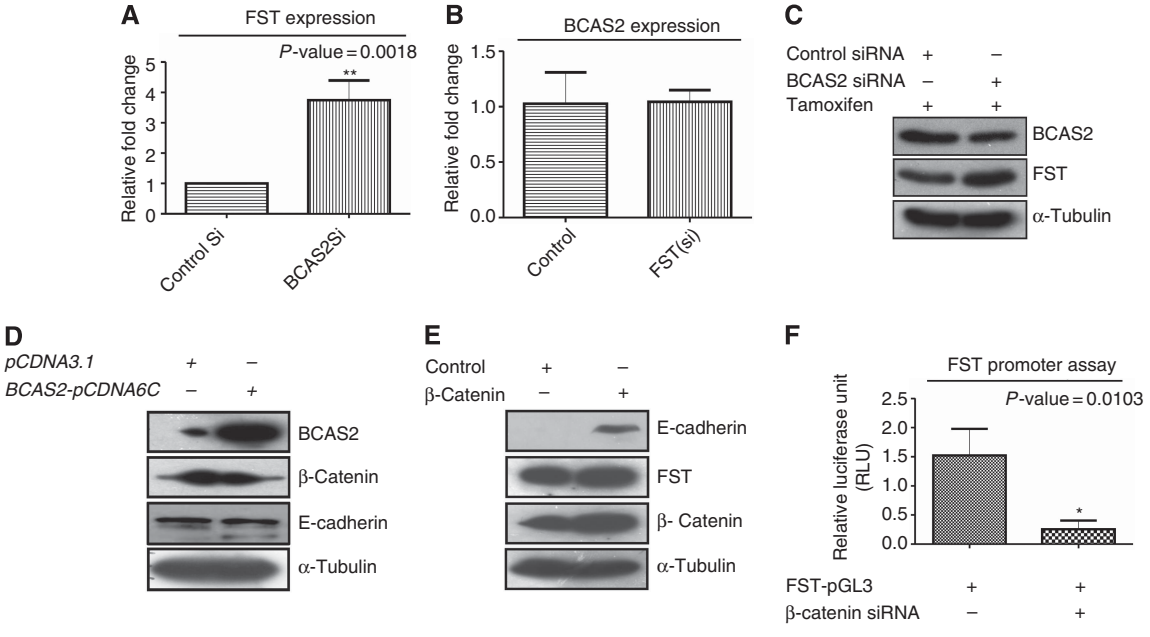

Figure 8. ER-independent regulation of FST by BCAS2 and the effect on E-cadherin. (**A** and **B**) Real-time PCR data showing FST and BCAS2 transcripts in BCAS2 and FST knockdown samples, respectively. Asterisks (*) indicates significance (*t*-test compared with control; $P < 0.05$). (**C**) Western blot confirming the regulation of FST by BCAS2 irrespective of the presence of the general ER antagonist tamoxifen. (**D**) Western blot showing the downregulation of β-catenin and cleavage of E-cadherin under BCAS2 overexpression. (**E**) Western blot showing FST and E-cadherin upregulation under β-catenin overexpression. (**F**) Dual luciferase assay data showing the downregulation of luciferase activity of the TCF4-binding site containing FST promoter fragment in control *vs* the β-catenin knockdown sample. Abbreviation: TCF4 = transcription factor 4.

determine the effect of BCAS2 overexpression on cell–cell adhesion mechanisms. An ~80 kDa cleavage product of E-cadherin was observed in the BCAS2 overexpression sample (Figure 8D). We searched for the molecules responsible for E-cadherin cleavage and identified matrix metalloprotease 7 (MMP7), a molecule downstream of FST. Breast cancer amplified sequence 2 upregulated MMP7 expression (Figure 9A), and FST downregulated MMP7 expression as expected (Figure 9B). However, ERRβ overexpression did not balance the opposite effects of FST and BCAS2 on MMP7 (Figure 9C). This redundant MMP7 activity also led to the cleavage of overexpressed E-cadherin (Figure 9D). The real-time PCR primers for MMP7 are shown in Table 4, and the transfection data are shown in Supplementary Figures 4B (I, II and III).

Table 5. siRNAs used

Primer name	Sequence (5′–3′)
BCAS2 siRNA F	GAAGGAACUUCAGAAGUUA55
BCAS2 siRNA R	UAACUUCUGAAGUUCCUUC55
FST siRNA F	GACCAAUAAUGCCUACUGU55
FST siRNA R	ACAGUAGGCAUUAUUGGUC55

Abbreviations: BCAS2 = breast cancer amplified sequence 2; FST = Follistatin; siRNA = small interfering RNA.

BCAS2 inhibition of β-catenin as a result of ERRβ overexpression resulted in the blockade of cyclin D1 expression. E-cadherin is a tumour-suppressor gene (Resnitzky *et al*, 1994; Wong and Gumbiner, 2003), and the mechanism of this suppressor function is mediated through the β-catenin-binding domain within its cytoplasmic tail. This binding inhibits β-catenin nuclear localisation and the subsequent transactivation of the cyclin D1 promoter (Lim and Lee, 2002), as cyclin D1 transactivation is required for the G1/S transition in the cell cycle (Resnitzky *et al*, 1994). As BCAS2 inhibited β-catenin expression, we hypothesised that cyclin D1 transactivation would also be blocked. Indeed, our western blot data (Figure 9E) supported this hypothesis and showed cyclin D1 downregulation following BCAS2 overexpression. Therefore, E-cadherin overexpression and β-catenin downregulation under ERRβ transfection supports the data of Ariazi *et al* (2002) by showing that ERRβ expression levels are inversely correlated to the S-phase fraction of cells (Ariazi *et al*, 2002).

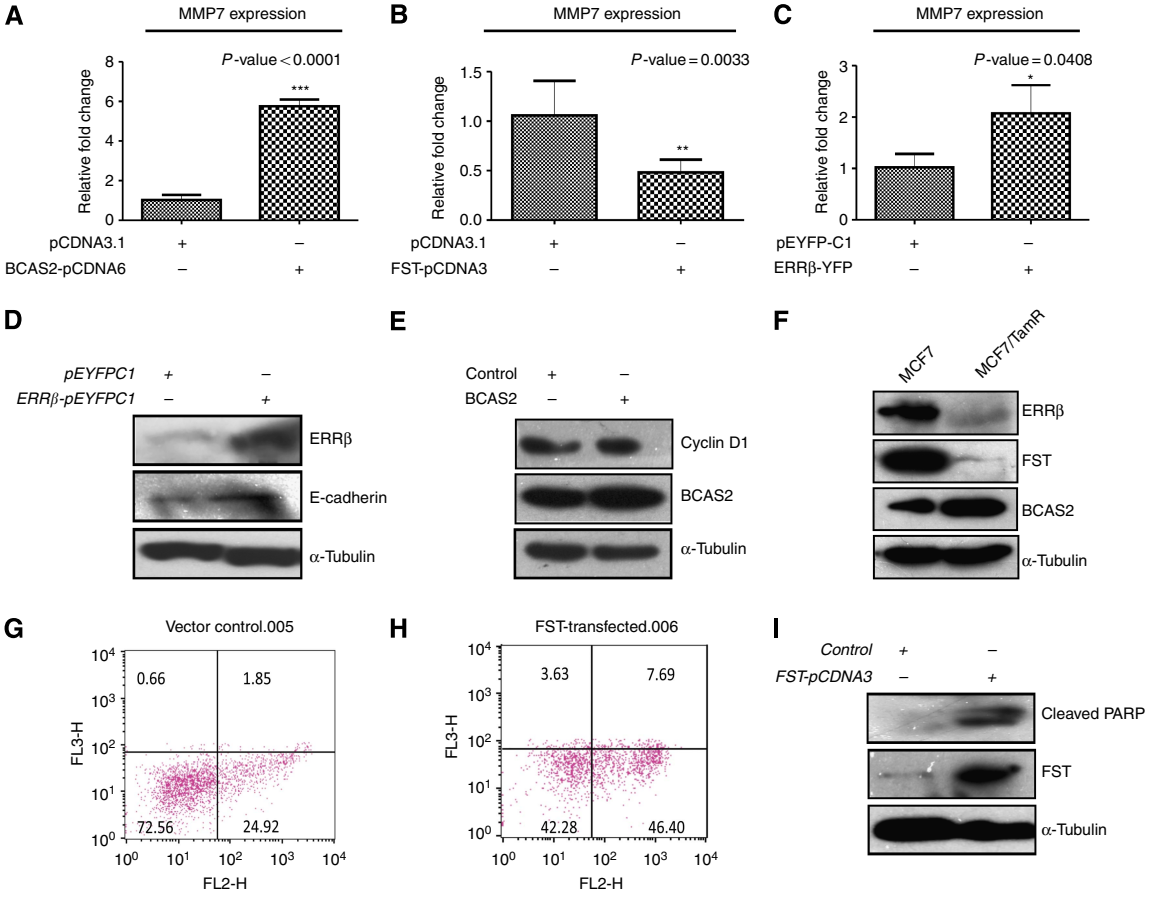

Figure 9. Role of ERRβ in controlling invasiveness, EMT and apoptosis in breast cancer cells. (**A**, **B** and **C**) Real-time PCR data showing MMP7 mRNA expression under BCAS2, FST and ERRβ overexpression, respectively. Asterisks (*) indicates significance (*t*-test compared with control; $P<0.05$). (**D**) Western blot showing the upregulation of uncleaved E-cadherin under ERRβ overexpression. (**E**) Western blot showing the downregulation of cyclin D1 under BCAS2 overexpression. (**F**) Western blot showing the overexpression of BCAS2 and the downregulation of ERRβ and FST in tamoxifen-resistant MCF-7 and MCF-7/ADR cells, respectively. (**G** and **H**) Annexin V-PE apoptosis detection assay data showing a significant induction of apoptosis in FST-overexpressing MCF-7 cells (**H**) compared with vector controls (**G**). (**I**) Western blot confirming apoptosis in FST-overexpressing MCF-7 cells via the detection of cleaved PARP. Abbreviations: ADR = adriamycin; PARP = poly ADP ribose polymerase; MMP-7 = matrix metalloprotease 7.

BCAS2 expression is upregulated, whereas ERRβ and FST expression is downregulated, in aggressive breast cancer cells. We next investigated BCAS2, FST and ERRβ expression in tamoxifen-resistant MCF-7 *vs* normal MCF-7 cells to evaluate the significance of the crosstalk between protein expression and prognosis in highly aggressive breast cancer cells. Oestrogen-related receptor-β and FST were downregulated and BCAS2 was upregulated in resistant MCF-7 cells compared with normal MCF-7 cells (Figure 9F).

FST overexpression induces apoptosis. Follistatin overexpression induces a sub-G1 population in mammary tumours and an R30C mammary tumour cell line (Krneta *et al*, 2006). Therefore, we overexpressed FST in MCF-7 cells to investigate its efficiency in apoptosis induction. Follistatin overexpression induced apoptosis in 54.09% of cells in contrast to 26.77% induction in the vector control (Figures 9G and H).

We next investigated the expression of cleaved PARP following FST overexpression in MCF-7 cells to further validate the FST-induced apoptotic signalling pathway. Western blot analyses showed the successful cleavage of PARP under FST overexpression but not in control cells (Figure 9I).

DISCUSSION

Breast cancer is the most common invasive cancer in women worldwide according to the World Cancer Report and estimates from the International Agency for Research on Cancer (Boyle *et al*, 2008). Previously, the expression status of certain receptors was adopted for the specific categorisation of invasive breast cancers for targeted therapy, although receptor status was subsequently combined with tumour grade to develop a new approach and improve diagnostics.

The most prevalent subtype of breast cancer is ER-positive tumours (Geyer *et al*, 2012). Oestrogen receptors and ERRs share some established transcriptional targets, such as pS2, osteopontin and lactoferin, which are used as breast cancer markers because of the structural and functional similarities (Lu *et al*, 2001; Zhou *et al*, 2006). Oestrogen-related receptor-β function differs from that of ERRα and ERRγ because of its anti-proliferative nature. Therefore, we studied two of its targets, a probable oncogenic molecule (BCAS2) and a tumour-suppressive molecule (FST), to unravel their differential regulation by ERRβ and characterise its precise role in tumourigenesis.

The relative expression of ERRβ was higher in an immortalised normal cell line, patients with longer relapse-free survival compared with breast cancer cell lines (ER-positive and ER-negative) and patients with shorter relapse-free survival. Reductions in ERRβ expression were observed *in vivo* in local breast tumour samples with developing malignancy, which indicates the physiological relevance of this receptor. Kaplan–Meier plotter analysis confirmed this difference statistically.

The overexpression of wild-type ERRβ significantly induced apoptosis in the MCF-7 breast cancer cell line (84.53% *vs* 2.32% in controls). One of the significant mediators of this apoptosis is the ERRβ transcriptional target, FST (46.40% early apoptotic induction compared with 24.92% in control cells). Follistatin inhibits tumourigenesis in R30C breast cancer cells and mouse mammary tumours (Krneta *et al*, 2006), although the downstream pathways in breast cancer, which are under FST regulation, are not well studied. However, FST is known to inhibit multi-organ metastasis in natural killer cell-deprived SCID mice. Moreover, MCF-7 cells are caspase 3-null (Janicke, 2009), and the 89 kDa cleavage product of PARP demonstrated the activation of alternative effector caspases under FST overexpression. In contrast, BCAS2 is amplified in breast cancer (Maass *et al*, 2002) which is indicative of its proliferative nature. Supportive evidences include its coactivator role for ERα and negative regulatory role for p53 transcriptional target p21. However, the downstream signalling events associated with BCAS2 remain poorly characterised.

We performed KM plotter analyses of BCAS2 and FST to investigate whether the opposite roles of BCAS2 and FST on cellular proliferation may reflect clinically significant differences in prognosis in breast cancer patients. We consistently observed a correlation between high BCAS2 and FST expression and poor and good prognoses, respectively.

Kaplan–Meier plotter analysis data further denoted a possible relationship between BCAS2 and FST signalling pathways. Therefore, we knocked down BCAS2 and performed real-time PCR for FST mRNA expression. Follistatin mRNA was highly upregulated in BCAS2 knockdown samples, which suggests that BCAS2 opposed the anti-proliferative effects of FST through the downregulation of FST mRNA transcription and acted as a negative regulator either directly or indirectly. However, the knockdown of FST did not alter BCAS2 protein levels, which showed that this signalling was not reversible and that FST was downstream of BCAS2. We next investigated whether the regulation of FST by BCAS2 involved ERα by treating MCF-7 cells with the ER antagonist tamoxifen (de Leeuw *et al*, 2011). Breast cancer amplified sequence 2 regulation of FST remained intact in the presence of tamoxifen, which demonstrated that the regulation was independent of ER. These results indicated that the downregulation of β-catenin/TCF-4 signalling on the FST promoter mediated the regulation of FST. In fact, loss of β-catenin expression is associated with bone metastasis in 82% of prostate cancer patients (Pontes *et al*, 2010), and this phenomenon may be assigned to the transcriptional blockade of downstream *FST*, which inhibits metastasis (Talmadge, 2008). Interestingly, ERRβ upregulates BCAS2, which results in the loss of β-catenin, but ERRβ also upregulates *FST*, which has anti-metastatic effects. The role of E-cadherin as a mediator of cell–cell adhesion also emerges as a potentially important mechanism (Onder *et al*, 2008) in this context. E-cadherin undergoes proteolysis and produces fragments that promote tumour growth and proliferation (David and Rajasekaran, 2012), and BCAS2 overexpression alone resulted in E-cadherin cleavage in our study. E-cadherin cleavage is also a signal of apoptosis induction (Steinhusen *et al*, 2001). However, the cleavage that occurred during apoptosis in mentioned study resulted in three fragments of molecular sizes 24, 29 and 84 kDa through two distinct proteolytic events because of caspase 3 and a metalloproteinase, likely secretase. Our findings revealed a single cleavage that resulted in a 135-kDa full-length band and an ~80 kDa fragment, which indicated the shredding of the extracellular domain (sE-cad) by α-secretase (David and Rajasekaran, 2012). The intracellular fragment containing the transmembrane segment and the cytoplasmic tail could not be examined for further cleavage events because the antibody used was raised against a synthetic peptide corresponding to the fifth cadherin domain of the extracellular segment of E-cadherin. Since, MCF-7 cell line is caspase 3-null (Janicke, 2009), only cleavage by γ-secretase is possible in the cytoplasmic domain, which remains to explored. The cytoplasmic segment contains a β-catenin-binding site, and β-catenin connects E-cadherin to the actin cytoskeleton via α-catenin (Ito *et al*, 1999). Therefore, the downstream β-catenin signalling events that result in cyclin D1 activation are dependent on the nuclear transport of free β-catenin, which is hindered by E-cadherin binding. But at least, dissociation of β-catenin from adherent junctions is confirmed through shredding of E-cadherin extracellular domain supporting positive role of BCAS2 in breast cancer metastasis.

However, ERRβ overexpression inhibited this shredding of the E-cadherin extracellular domain despite BCAS2 upregulation. Therefore, we further investigated the mechanism of E-cadherin cleavage inhibition, although the role of FST in E-cadherin regulation requires further investigation. Oestrogen-related receptor-β simultaneously upregulated BCAS2 and FST. Follistatin is an

inhibitor of activin, a TGFβ superfamily member that is responsible for the upregulation of MMP7 in oesophageal carcinoma cells (Yoshinaga *et al*, 2008). Matrix metalloprotease 7 is a protease responsible for the cleavage of E-cadherin in nontransformed epithelial cell lines, which results in loss of cell–cell contact, loss of epithelial cell polarisation and increased proliferation via Rho A activation (Lynch *et al*, 2010). Therefore, FST upregulation may lead to the downregulation of activin-mediated MMP7 upregulation, which inhibits E-cadherin cleavage.

Interestingly, BCAS2 was found to highly counteract this FST-mediated downregulation of MMP7. This counteractive activity surpasses the tumour-suppressive activity of ERRβ through FST by promoting the invasiveness induced by MMP7 in breast cancer cells, such as MCF-7 (Wang *et al*, 2006).

Our experimental findings also revealed E-cadherin upregulation in ERRβ-overexpressing samples. As the FST-target, activin, binds to type II activin receptors, which phosphorylate R-SMADs and SMAD2/SMAD3 (Chen *et al*, 2006), this complex may interact with coSMAD-SMAD4 and downregulate E-cadherin transcription (Vincent *et al*, 2009). Therefore, E-cadherin expression was increased when FST blocked the activin-mediated SMAD cascade.

In this study, we have tried to explore the effect of ERs and ERRβ on BCAS2 and FST transcription. Our findings indicate that ERRβ overexpression upregulates BCAS2 and FST expression at the mRNA and protein levels and reduces the ER-mediated transactivation of both genes, as demonstrated from our real-time PCR data. While, the FST protein level increases when ER and ERRβ interactions are abrogated as a result of 24-h E2 treatment, BCAS2 regulation is unique, as the maintenance of BCAS2 transcripts at the basal level requires the binding of ERs and ERRβ. Therefore, the interaction of ER with ERRβ was lost when oestrogen occupies the ER LBD (24-h E2 treatment), and the BCAS2 protein level decreased as a consequence of the lack of this physical interaction.

In light of these results, we also evaluated the interaction of ERs with ERRβ and the effect of the function of the ERRβ DNA-binding domain (DBD). Our 3D structural modelling data showed that ERα binding did not disturb the ERRβ DBD, whereas ERβ binding did. Therefore, ERRβ and ERα remain bound directly or indirectly to the DNA sequence flanking the concerned ERREs. In contrast, since oestrogen receptor-β binding to ERRβ may disturb the ERRβ DBD, therefore, co-recruitment of ERβ and ERRβ on same response element appears to be infeasible.

Overall, our study revealed the involvement of ERRβ in oestrogen signalling and provided sound mechanistic insights into ERRβ-regulated downstream pathways (Figure 10). Oestrogen-related receptor-β mediated induction of FST clearly supports its ability to induce apoptosis. However, BCAS2 upregulation, which is otherwise proliferative, is directed towards the downregulation of β-catenin, which results in the blockade of the G1 to S transition. The novelty of this finding is that a tumour suppressor was shown to convert the activity of an oncogene into a favourable outcome. Finally, the ERRβ-mediated upregulation of E-cadherin may serve

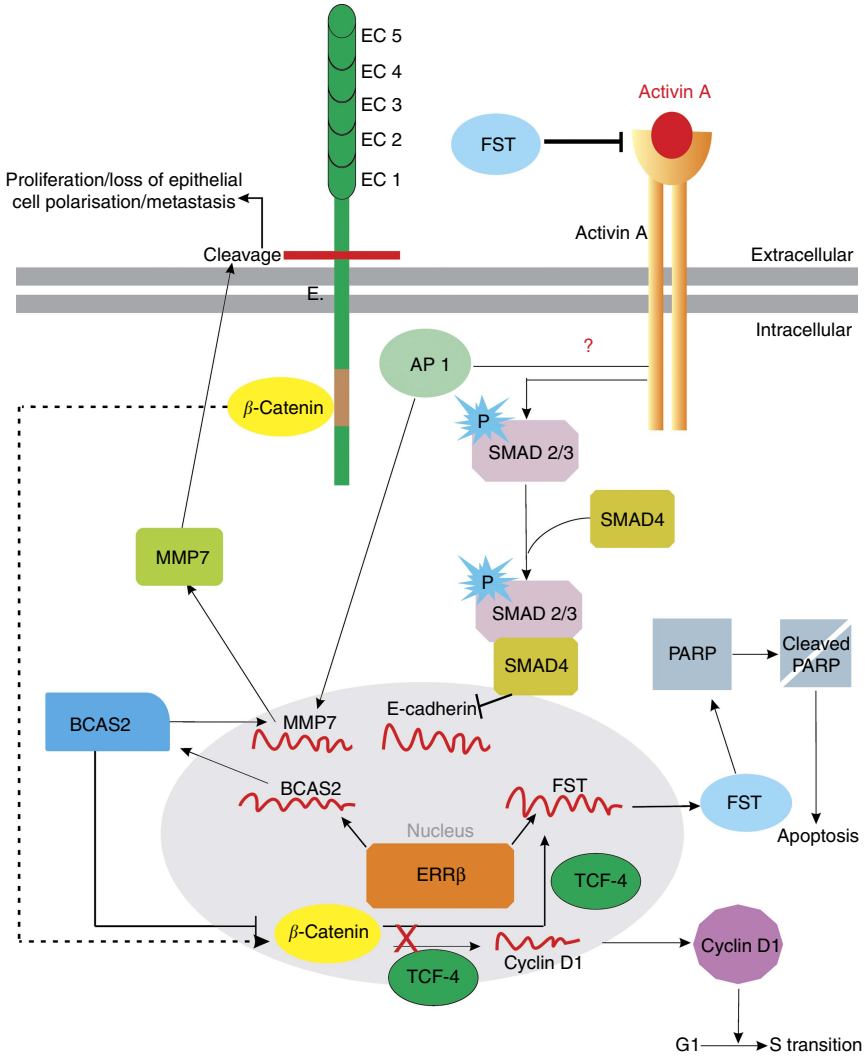

Figure 10. Diagrammatic representation showing different signalling pathways affected by ERRβ regulation through BCAS2 and FST.

as an indicator of the blockade of EMT transition, which is a primary event for metastasis (Benton *et al*, 2009). We strongly believe that the identification of molecules that create fluctuations in natural ERRβ activities should be pursued further. Future studies may identify more important transcriptional targets of ERRβ, which will provide in-depth knowledge of the functional significance of this molecule. Furthermore, more interacting partners and downstream targets of BCAS2 need to be identified to decipher the consequences of its amplification in breast cancer. Remarkably, FST is a candidate gene (Bhasin and Jasuja, 2009) for the development of selective androgen receptor modulators. New clinical trials of gene therapy using the overexpression of ERRβ and the simultaneous inhibition of MMP7 in metastatic breast cancer need to be conducted considering their capacity to induce apoptosis and inhibit G1-S transition, EMT and multi-organ metastasis of human breast cancer cells selectively.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DISCLAIMER

This is to certify that this work is not currently under consideration elsewhere, and all authors have seen and approved the submission of the manuscript for consideration for publication in the *British Journal of Cancer*.

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