



Published in final edited form as:

Breast Cancer Res Treat. 2010 January ; 119(1): 63–70. doi:10.1007/s10549-009-0330-4.

Regulation of α B-Crystallin Gene Expression by the Transcription Factor Ets1 in Breast Cancer

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Abstract

Recent studies indicate that the small heat shock protein α B-crystallin is expressed in poor prognosis basal-like breast tumors and likely contributes to their aggressive phenotype. However, the mechanisms underlying the deregulated expression of α B-crystallin in basal-like tumors are poorly understood. Using a bioinformatics approach, we identified a putative DNA binding motif in the human α B-crystallin promoter for the proto-oncogene Ets1, a member of the ETS transcription factor family that bind to DNA at palindromic ETS-binding sites (EBS). Here we demonstrate that ectopic expression of Ets1 activates the α B-crystallin promoter by an EBS-dependent mechanism and increases α B-crystallin protein levels, while silencing Ets1 reduces α B-crystallin promoter activity and protein levels. Chromatin immunoprecipitation analyses showed that endogenous Ets1 binds to the α B-crystallin promoter in basal-like breast cancer cells *in vivo*. Interrogation of publically available gene expression data revealed that Ets1 is expressed in human basal-like breast tumors and is associated with poor survival. Collectively, our results point to a previously unrecognized link between the oncogenic transcription factor Ets1 and α B-crystallin in basal-like breast cancer.

Keywords

α B-crystallin; Ets1; molecular chaperone; basal-like breast cancer; gene regulation

Introduction

The small heat shock protein α B-crystallin is a molecular chaperone that enhances survival in response to cellular stress by inhibiting protein aggregation and reducing intracellular reactive oxygen species levels [1–3]. In addition, α B-crystallin directly interacts with the cell death machinery to suppress apoptosis by inhibiting caspase-3 activation and preventing the mitochondrial translocation of proapoptotic Bcl-2 family members Bax and Bcl-x_s [4–6]. α B-crystallin is commonly expressed in many cancers, and its expression correlates with poor clinical outcomes in breast and head and neck carcinomas [7–9]. Consistent with its antiapoptotic function, α B-crystallin expression in breast cancer is associated with resistance to neoadjuvant chemotherapy [10]. Moreover, α B-crystallin is predominantly expressed in a subset of poor prognosis, triple (ER/PR/HER2) negative breast tumors with a basal epithelial gene expression profile (basal-like breast cancer) and likely contributes to the aggressive phenotype of these tumors [9–11].

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Despite its emerging pathogenic significance in cancer, very little is known about the regulation of α B-crystallin gene (CRYAB) expression in breast cancer or other malignancies. The mammalian α B-crystallin gene and the adjacent small heat shock protein HspB2/MKBP gene are arranged head-to-head and share a conserved intergenic promoter that is differentially transcribed in an orientation-specific manner [12,13]. Unlike in cancer, the transcriptional regulation of the murine α B-crystallin gene in normal tissue, such as muscle and lens, has been studied extensively. Multiple tissue-specific regulatory elements activated by distinct transcription factors, including MyoD, Pax-6 and HSF, have been identified [14–16].

In the present study, we used a bioinformatics approach to identify putative transcriptional regulators of the human α B-crystallin gene in breast cancer cells. One such candidate we identified was the oncogenic transcription factor Ets1. Ets1 is a cellular homologue of the avian erythroblastosis E26 viral oncogene that contains a conserved 85 amino acid ETS DNA binding domain that forms a winged helix-turn-helix motif [17,18]. ETS family members bind to palindromic ETS-binding sites (EBS) composed of a 5'-GGA(A/T)-3' consensus core sequence and regulate expression of genes involved in proliferation (Myc), invasion (matrix metalloproteinase (MMP)-1, MMP-3, MMP-9 and urokinase plasminogen activator), and angiogenesis (VEGF receptor 1). Here we report that Ets1 binds to the α B-crystallin promoter and regulates its expression by an EBS-dependent mechanism. We also show that overexpression of Ets1 in breast cancer cells increases α B-crystallin protein levels, while silencing Ets1 reduces α B-crystallin levels. In addition, we demonstrate that Ets1 is expressed in basal-like tumors from patients and is associated with poor survival. Taken together, our results point to a previously unrecognized and direct link between the proto-oncogene Ets1 and α B-crystallin in basal-like breast cancer.

Materials and Methods

Bioinformatics analysis of the shared human α B-crystallin/HspB2 promoter

The human α B-crystallin promoter, the 1111 base pair intergenic region between the ATG translational initiation sites of the adjacent α B-crystallin and HspB2 genes [12], was queried for DNA motifs within the TRANSFAC library using an internet-based MOTIF search (<http://motif.genome.jp/>). A cut-off score of 80 was used to identify putative transcription factor DNA binding elements.

Cell Culture

Human MCF-10A breast epithelial cells and MDA-MB-231 breast carcinoma cells were purchased from ATCC. MCF-10A cells were cultured in DMEM/F12 medium (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20 ng/ml of EGF (Sigma-Aldrich), 10 μ g/ml insulin (Sigma-Aldrich), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), and 1X Penicillin-Streptomycin-Glutamine (Invitrogen). MDA-MB-231 cells were grown in MEM medium with Earl's salts plus L-glutamine (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1X non-essential amino acids (Mediatech), 10 mM HEPES buffer (Mediatech) and 1X Penicillin-Streptomycin-Glutamine.

Reporter Assays

The full-length human α B-crystallin gene promoter (−1081/+30 relative to the transcription start site) and 5'-truncated promoter constructs were PCR amplified from genomic DNA isolated from human MCF-10A breast epithelial cells using KOD Hot Start DNA Polymerase (Novagen) according to manufacturer's protocol using a sense primer containing a *Sac*I restriction enzyme cleavage site (5'-CGAGCTCCATGGCTGCAGATGCAGC-3' (full-length promoter), 5'-CGAGCTCTGGTGTGACATGTTGACC-3' (−516 truncation) or 5'-CGAGCTCACTACGCCGGCTCCCATC-3' (−356 truncation)) and an antisense primer

containing a *Bgl*III site (5'-GGAAGATCTCATGGTGGCTAGGTGAGTGTGGGG-3'). PCR products were subcloned into the *Sac*I and *Bgl*III sites of the pGL3-Basic luciferase reporter plasmid (Promega). The putative EBS was mutated using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and the following primers: 5'-CCTGGGGCTCAGCCTAAAAAGATTTTAGTCCC-3' and 5'-GGGACTAAAATGTTTTTAGGCTGAGCCCCAGG-3'. All constructs were verified by DNA sequencing. For ectopic expression experiments, MCF-10A cells were transiently co-transfected with 700 ng of pcDNA3.1-Ets1 (kindly provided by Dr. M. Zhou, Emory University) or pcDNA3.1 vector, 100 ng of pGL3 firefly luciferase reporter, and 1 ng of control pRL-TK *Renilla* luciferase reporter using Lipofectamine 2000 (Invitrogen). A reporter pGL3-MMP9 (kindly provided by Dr. M. Sharon Stack, University of Missouri) was used as a positive control. Lysates were assayed for luciferase activity 48 h later with the Dual Luciferase Reporter Assay system (Promega) using a Clarity Luminescence Microplate Reader (Bio-Tek). Firefly luciferase activity was normalized to *Renilla* luciferase activity and expressed as fold induction relative to the activity in empty vector-transfected cells. For gene silencing experiments, MDA-MB-231 cells were transiently co-transfected with 300 ng of pGL3 reporter, 3 ng of pRL-TK reporter and 25 μ M of siRNA targeting Ets1 (Dharmacon ON-TARGETplus SMARTpool) or lamin A/C (control, Dharmacon) using Lipofectamine 2000. Luciferase activity was measured 72 h later as described above and expressed as fold induction relative to the activity in cells transfected with control lamin A/C siRNA.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using the LightShift Chemiluminescent EMSA kit (Pierce). The human α B-crystallin promoter region containing the putative EBS (WT: 5'-TCAGCCTAGGAAGATTCCAGTCCCTGC-3') or a mutant EBS (5'-TCAGCCTAAAAAGATTTTAGTCCCTGC-3') was duplexed with the corresponding antisense oligonucleotide and used as probes. Twenty fmol of biotinylated WT or mutant probe was added to 10 μ g of nuclear extract (Santa Cruz Biotechnology) for 20 minutes at RT. For competition experiments, 20 fmol of biotinylated WT probe was incubated in the presence of 4 pmol of unbiotinylated WT probe. For antibody abrogation experiments, 5 μ g of Ets1 antibody (SantaCruz Biotechnologies; sc-111) was added to the binding reaction for 20 minutes at RT before adding the WT probe; the reaction was then incubated for an additional 20 minutes at RT. Protein-DNA complexes were resolved by 5% native-PAGE using 0.5 \times Tris-Borate EDTA buffer, transferred to a Biorad B membrane (Pall Life Sciences) and UV cross-linked to the membrane. Biotin-labeled DNA was detected by chemiluminescence following the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the EZ ChIP kit (Upstate) according to the manufacturer's protocol. Briefly, $\sim 2 \times 10^7$ MDA-MB-231 cells were fixed with 1% formaldehyde for 10 min at RT. Crosslinked chromatin was sonicated using the Bioruptor 200 (Diagenode) for 30 min at full power with 30 second on/off cycles. Sheared chromatin was pre-cleared and incubated with 2 μ g of antibody (Ets1 or control rabbit IgG; SantaCruz Biotechnologies, catalogue no. sc-350 and sc-2027, respectively). Immunoprecipitated complexes were washed and eluted, crosslinks reversed, and samples RNase-treated according to the manufacturer's protocol. DNA was isolated using the QIAquick PCR Purification kit (QIAGEN) and PCR amplified with the following primers for the α B-crystallin promoter: 5'-AGATGGCTGGTGCTGACATGTTGA-3'; 5'-AATCAGGCCAGCAACTATCTTGGG-3. PCR products were resolved on a 2% agarose gel.

Creation of breast cancer cells stably overexpressing Ets1

Retroviral supernatants were generated by transfecting the Phoenix amphotrophic retrovirus packaging cell line (ATCC) with pBABE-Ets1 and pBABE vector as described previously [9]. MDA-MB-231 cells were retrovirally transduced as described [9] and puromycin-resistant pools were selected by growth in 2 µg/ml puromycin. The expression of Ets1 and αB-crystallin in puromycin-resistant pools was determined by immunoblotting as described using the following primary antibodies: αB-crystallin (Stressgen Biotechnologies; SPA-222), Ets1 (Abcam, ab10936) or actin (Sigma-Aldrich; # A4700).

Analysis of Ets1 gene expression data from human breast tumors

The expression of the human Ets1 gene in human breast tumors was determined from publically available gene profiling datasets by OncoPrint analysis (<http://www.oncoPrint.org/>) as described [19].

Statistical Analyses

The statistical significance of differences for reporter assays was determined by ANOVA with a Bonferroni posttest using Prism 4 software (GraphPad).

Results

Identification of a putative Ets1 binding site in the shared human αB-Crystallin/HspB2 promoter

We examined the shared human αB-crystallin/HspB2 promoter for putative transcription factor DNA binding elements using an internet-based MOTIF search. Our analysis identified previously published MyoD and heat shock elements [14,16], as well as a putative binding site for Ets1, a member of the ETS family of transcription factors (Fig. 1A). The putative palindromic EBS at -441 to -432 in the human αB-crystallin promoter (Fig. 1B, highlighted in gray) is absolutely conserved across many mammalian species.

Transcriptional regulation of the human αB-crystallin promoter by Ets1

To determine whether the putative EBS in the αB-crystallin promoter is activated by Ets1, we co-transfected human MCF-10A breast epithelial cells (which express low levels of Ets1 and αB-crystallin) with cDNAs encoding Ets1 and a luciferase reporter under the control of the αB-crystallin promoter (-1081/+30 for the full-length WT promoter) (Fig. 2A). Ectopic expression of Ets1 resulted in a ~2.5 fold increase in luciferase activity in cells co-transfected with the WT αB-crystallin promoter reporter (Fig. 2B). Importantly, the activation of the WT αB-crystallin promoter by Ets1 was similar in magnitude to the Ets1-induced activation of the MMP-9 promoter, a well-established Ets1 transcriptional target [20]. In contrast, the related ETS family member ESX did not significantly activate the WT αB-crystallin promoter (data not shown), indicating specificity within the ETS family. Moreover, Ets1 activated a truncated αB-crystallin promoter (-516/+30) that contains the putative EBS at -441 to -432, but not a truncated αB-crystallin promoter (-356/+30) that lacks the EBS. Mutation of both core elements within the putative EBS (**AAAAGATTTT**, nucleotide alterations in bold) in the αB-crystallin promoter abrogated Ets1 activation. These results indicate that the αB-crystallin promoter contains a functional EBS, which is required for its activation by Ets1.

We next examined whether silencing Ets1 inhibited αB-crystallin promoter activity. To this end, we co-transfected human MDA-MB-231 basal-like breast cancer cells (which express moderate levels of Ets1) with an Ets1 siRNA (or control lamin A/C siRNA) and each of the αB-crystallin promoter reporter constructs. The activity of the WT αB-crystallin promoter was inhibited in cells co-transfected with the Ets1 siRNA compared to cells co-transfected with a

control siRNA (Fig. 2C). Similarly, silencing Ets1 inhibited the activity of the α B-crystallin promoter truncated at -516 and the MMP-9 promoter, but did not inhibit activity of α B-crystallin promoter constructs lacking a functional EBS and shown to be unresponsive to Ets1 overexpression (Fig. 2B). Collectively, these findings demonstrate that the α B-crystallin promoter is regulated by the expression levels of Ets1, which activates transcription by an EBS-dependent mechanism.

Ets1 binds to the α B-crystallin promoter *in vitro* and *in vivo*

To determine whether Ets1 binds to the human α B-crystallin promoter, we performed electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) analyses. For EMSA experiments, nuclear extracts were incubated with a biotinylated probe containing the putative α B-crystallin promoter EBS (WT or mutant) in the absence or presence of excess unbiotinylated probe. Incubation of the nuclear extract with the WT probe led to a gel shift of the protein-DNA complex (Fig. 3A, lane 2) that was not observed when excess unbiotinylated probe was added (Fig. 3A, lane 3) or when a probe containing a mutant EBS was used (Fig. 3A, lane 4). Moreover, preincubating the nuclear extract with an Ets1 specific antibody (but not IgG control) prior to the addition of the biotinylated WT probe prevented the formation of the DNA-protein complex (Fig. 3A, lanes 5 and 6), confirming that Ets1 was responsible for the observed gel shift. To determine whether endogenous Ets1 binds to the human α B-crystallin promoter *in vivo*, we performed a ChIP assay using human MDA-MB-231 basal-like breast cancer cells. PCR amplification of Ets1-immunoprecipitated DNA with primers flanking the α B-crystallin promoter EBS revealed a band of the expected size that was also observed when input DNA was amplified (Fig. 3B). These results indicate that a putative EBS in the α B-crystallin promoter is both necessary and sufficient for Ets1 binding *in vitro* and that endogenous Ets1 binds to this EBS in the α B-crystallin promoter *in vivo*.

To determine whether Ets1 regulates α B-crystallin protein levels in breast cancer cells, we generated MDA-MB-231 pools stably expressing Ets1 by retroviral transduction. Stable overexpression of Ets1 resulted in a 2.4-fold increase in Ets1 protein levels and a 3.1-fold increase in α B-crystallin protein levels (Fig. 4A). Conversely, transfection of MDA-MB-231 cells with an Ets1 siRNA reduced Ets1 protein levels 2.3-fold and decreased α B-crystallin protein levels 6.0-fold compared to the levels in MDA-MB-231 cells transfected with a control lamin A/C siRNA (Fig. 4B). These latter findings indicate that the expression of α B-crystallin protein in these breast cancer cells is regulated by the endogenous levels of Ets1.

Ets1 is expressed in basal-like breast tumors and is associated with poor survival

Intriguingly, Ets1 mRNA and protein are expressed in the stroma, endothelial cells and/or epithelium of poor prognosis breast carcinomas and in basal-like breast cancer cell lines [21–25], suggesting that Ets1 and α B-crystallin may be co-expressed in human basal-like breast tumors. To determine whether Ets1 is differentially expressed in human breast tumor molecular subtypes, we interrogated publically available gene expression datasets by OncoPrint analysis [19]. The Ets1 gene was predominantly expressed in estrogen receptor (ER)-negative breast tumors (Fig. 5A) with a basal-like gene signature (Fig. 5B). Furthermore, Ets1 gene expression in human breast carcinomas was associated with poor survival at five years (Fig. 5C). Taken together, our results indicate that Ets1 is expressed in poor prognosis basal-like breast tumors, a distinctive gene expression pattern similar to that previously reported for α B-crystallin [9–11].

Discussion

We have described a previously unrecognized and direct link between the oncogenic transcription factor Ets1 and α B-crystallin in breast cancer: Ets1 is a novel transcriptional

activator of the α B-crystallin gene. Several lines of experimental evidence support this conclusion. First, ectopic expression of Ets1, but not the structurally related ETS family member ESX, activates the α B-crystallin promoter by an EBS-dependent mechanism. Conversely, silencing endogenous Ets1 reduces the activity of the α B-crystallin promoter. Second, Ets1 binds to the EBS in the α B-crystallin promoter *in vitro*. Third, endogenous Ets1 binds to the α B-crystallin promoter in basal-like breast cancer cells *in vivo*. Fourth, overexpression of Ets1 in breast cancer cells increases α B-crystallin protein levels, while silencing Ets1 reduces α B-crystallin levels. Although our results do not rule out the potential role of other ETS family members in regulating α B-crystallin gene expression, they point to a functionally important and specific role of Ets1 in this process. Such specificity may be conferred by the nucleotides flanking the conserved EBS or by coregulatory proteins that interact with ETS family members and cooperatively bind DNA [26,27]. Taken together, our results demonstrate unequivocally that endogenous Ets1 binds to the α B-crystallin promoter *in vivo*, directly regulating α B-crystallin gene and protein expression levels.

α B-crystallin, then, can be added to the growing network of cancer-related genes activated by Ets1 and related family members. Like Ets1, α B-crystallin has been implicated in angiogenesis, migration and invasion, and apoptosis-resistance [4,9,17,18,28–30]. For example, both Ets1 and α B-crystallin are selectively expressed in endothelial cells during developmental and tumor angiogenesis [28,29]. Recent studies indicate that α B-crystallin is required for endothelial cell survival during tube morphogenesis [29]. Moreover, the coordinated regulation of several MMPs (including 1, 3, and 9) and α B-crystallin by Ets1 likely promotes metastasis by initiating invasion and suppressing apoptosis. These findings suggest that α B-crystallin may be an important downstream target of Ets1 in promoting tumor progression, an hypothesis we will explore in future studies.

We have also shown that the Ets1 gene is expressed in clinically aggressive basal-like breast tumors, the same molecular subtype which expresses α B-crystallin [9–11]. Basal-like breast tumors are associated with a poor prognosis because they are highly proliferative and invasive, and they metastasize rapidly to the lungs and brain [31]. Given the well established role of ETS family members in promoting proliferation, invasion and angiogenesis [17,18], it is tempting to speculate that Ets1 may contribute to the aggressive phenotype of basal-like tumors. Consistent with our findings, Ets1 was one of ten proteins recently reported to define an immunohistochemistry phenotype capable of identifying basal-like breast cancer cell lines [24]. Although one clinical study suggested a correlation between Ets1 and HER2 expression [25], Oncomine analysis indicated that Ets1 was highly expressed in hereditary *Brcal*-associated breast tumors, which often have a basal-like gene expression profile [31], providing additional evidence linking Ets1 expression to basal-like tumors (data not shown). Intriguingly, the closely related ETS family member Ets2 has been shown to transcriptionally repress the *Brcal* gene [32]. Because reduced expression of BRCA1 and/or BRCA1 dysfunction is likely to play a key role in the pathogenesis of basal-like tumors [31], ETS family members may contribute to the molecular etiology of these tumors via their effects on multiple downstream targets. We also observed that Ets1 gene expression was associated with poor survival in breast cancer, consistent with the results of two earlier studies, one using RT-PCR to measure Ets1 levels, and the second using immunohistochemistry [22,25]. These findings from patient tumors are consistent with the preclinical data implicating Ets1 in tumor progression [33,34]. In future studies, it will be important to examine whether Ets1 and α B-crystallin are co-expressed in basal-like tumors and to evaluate their prognostic value in these tumors.

Acknowledgments

We thank Drs. M. Zhou and M. Sharon Stack for plasmids. This work was supported by NIH grants R01CA097198 (VLC), R21CA125181 (VLC) and T32GM08061 (JDB), and by the Breast Cancer Research Foundation (VLC).

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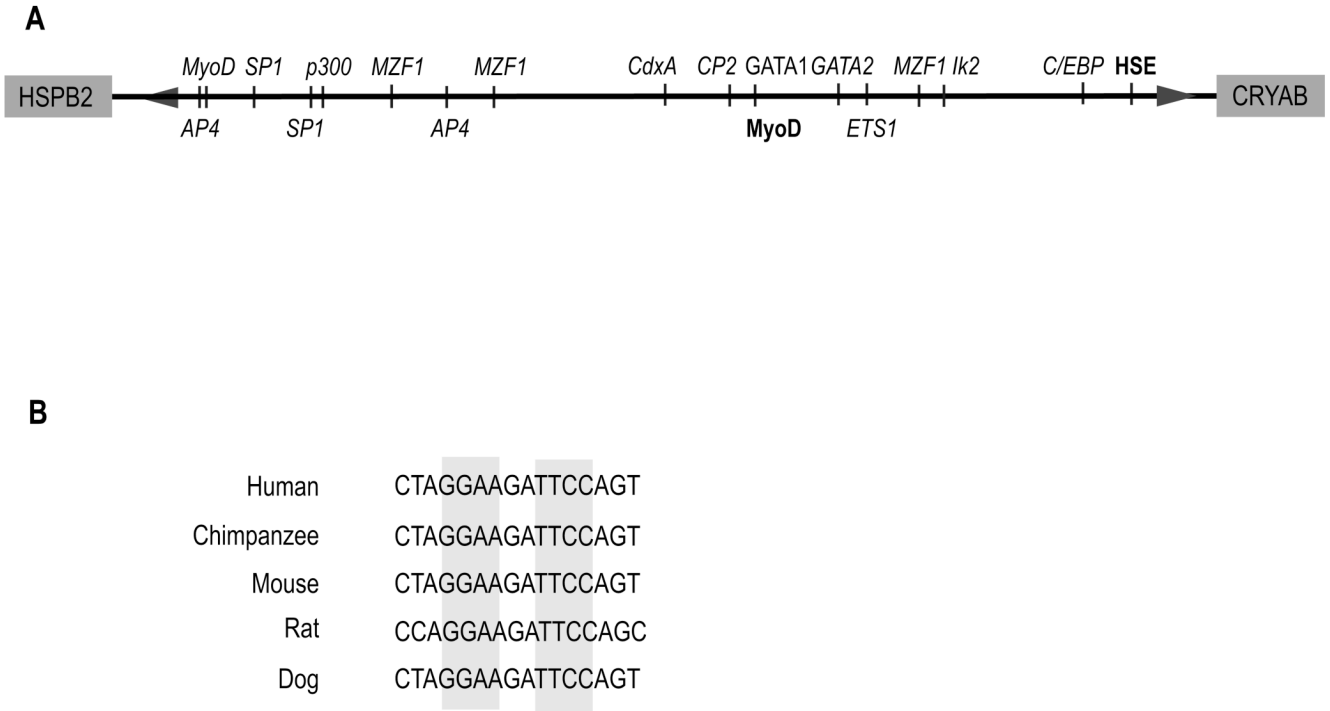


Figure 1. Schematic representation of the shared human α B-crystallin/HspB2 promoter and conservation of the putative ETS-binding site (EBS)

(A) Using the MOTIF-search platform, we performed a bioinformatics analysis of the shared human α B-crystallin/HspB2 promoter spanning the region between the start ATG for each gene. The search identified published (**bold**) transcriptional regulators of α B-crystallin, as well as additional putative (*italics*) regulators, including Ets1. (B) Alignment of the putative EBS in the human α B-crystallin promoter with the corresponding region from other species.

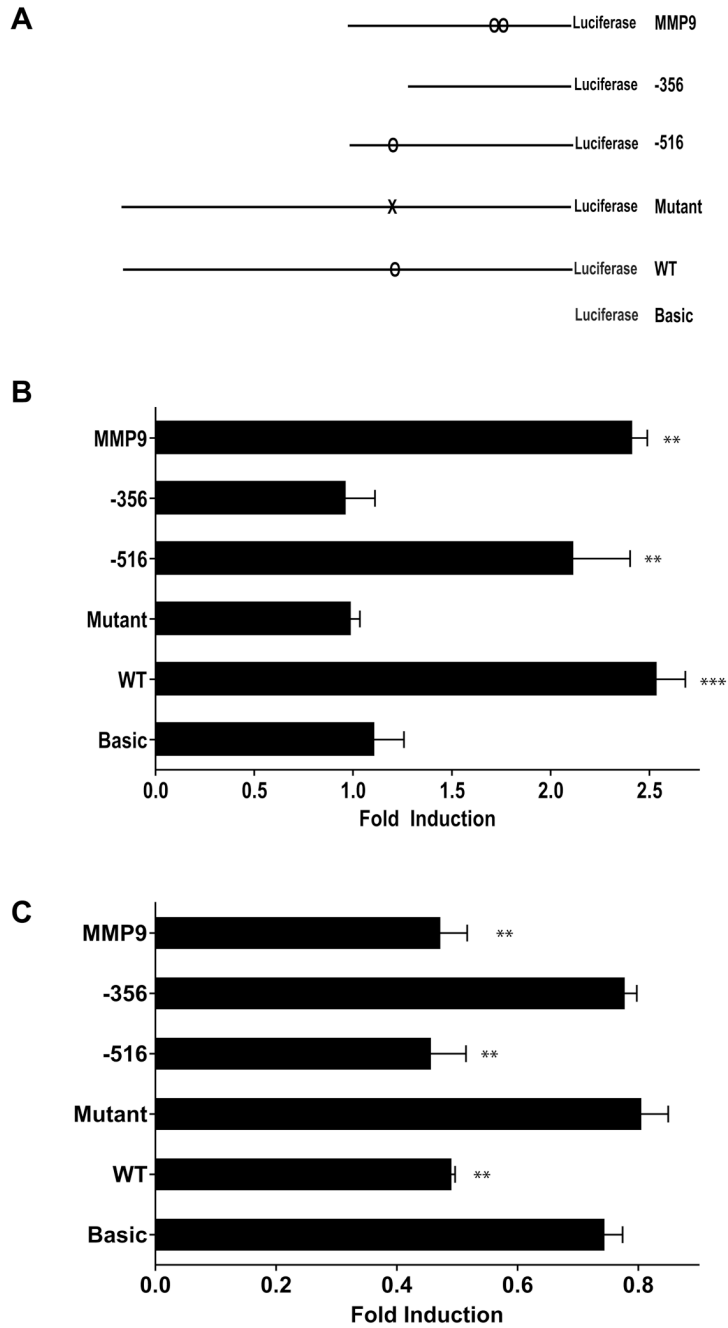


Figure 2. Transcriptional regulation of the human α B-crystallin promoter by Ets1
(A) Schematic representation of the reporter constructs used. The full-length WT human α B-crystallin promoter (–1081/+30) and 5' truncations (–516/+30, and –356/+30) were subcloned into the pGL3-Basic luciferase vector (Basic). The mutant EBS (AAAAGATTTT) was generated by site-directed mutagenesis. A pGL3-MMP9 reporter was used as a positive control.
(B) MCF-10A cells were transiently co-transfected with 700 ng of pcDNA3.1-Ets1 or vector, 100 ng of pGL3 firefly luciferase reporter, and 1 ng of control pRL-TK *Renilla* luciferase reporter. Firefly luciferase activity was normalized to *Renilla* luciferase activity and expressed as fold induction relative to the activity in empty vector-transfected cells. ** $p < 0.01$ *** $p < 0.001$ versus pGL3-Basic. **(C)** MDA-MB-231 cells were transiently co-transfected with 300

ng pGL3 reporter, 3 ng of pRL-TK reporter, and 25 μ M of siRNA targeting Ets1 or lamin A/C (control). Normalized firefly luciferase activity was expressed as fold induction relative to the activity in cells transfected with control lamin A/C siRNA. ** $p < 0.01$ versus pGL3-Basic.

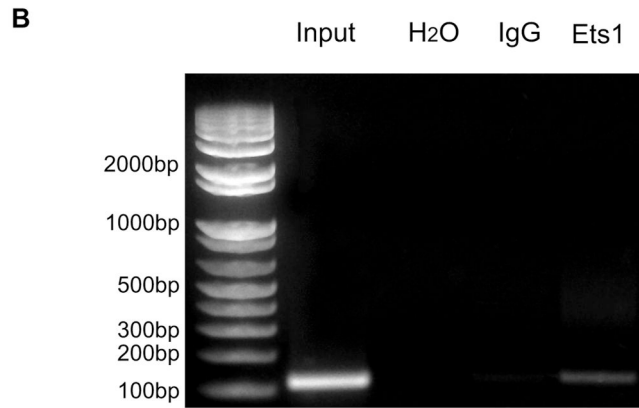
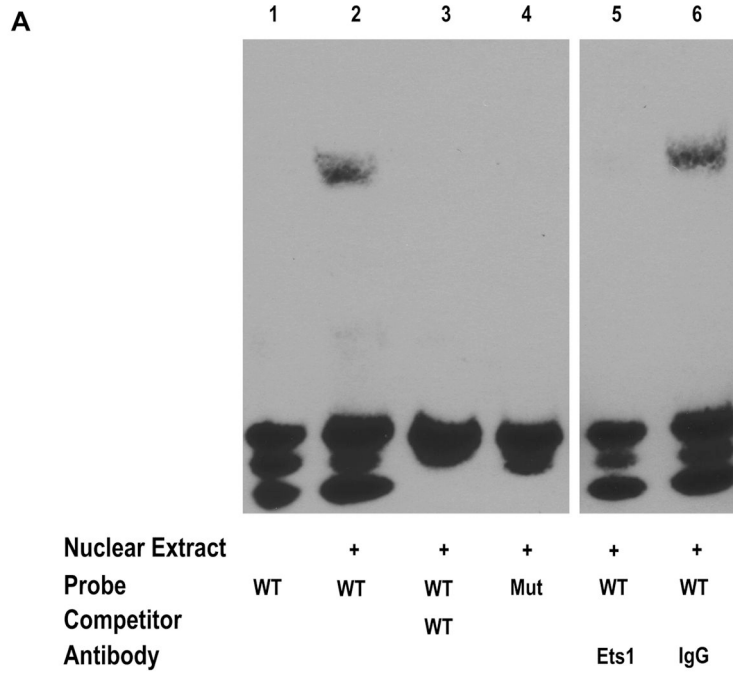
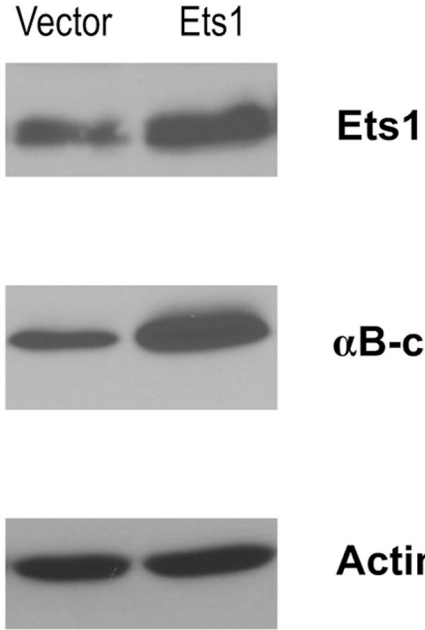


Figure 3. Ets1 binds to the α B-crystallin promoter *in vitro* and *in vivo*
(A) EMSA analysis of Ets1 binding to the putative EBS in the human α B-crystallin promoter *in vitro*. The human α B-crystallin promoter region containing the putative WT EBS or a mutant (Mut) EBS was duplexed with the corresponding antisense oligonucleotide and used as probes. Twenty fmol of biotinylated WT or mutant probe was added to nuclear extract in the absence or presence of 4 pmol of unbiotinylated WT probe. For antibody abrogation experiments, the binding reaction was preincubated with an Ets1 antibody or control IgG before adding the WT probe. Protein-DNA complexes were resolved by native-PAGE, transferred to a membrane, and detected by chemiluminescence. **(B)** ChIP analysis of endogenous Ets1 binding to the human α B-crystallin promoter *in vivo*. Input DNA-protein complexes or DNA-protein

complexes immunoprecipitated with water, IgG or Ets1 antibody were PCR amplified using primers flanking the α B-crystallin promoter EBS.

A

MDA-MB-231 Pools



B

siRNA

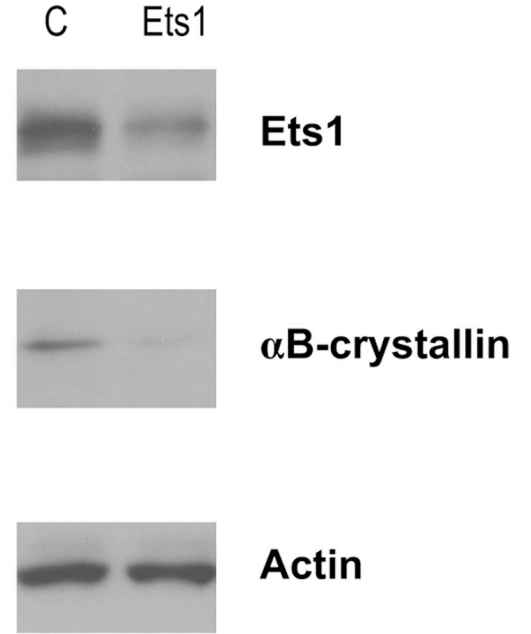


Figure 4. Regulation of endogenous α B-crystallin protein levels by Ets1 in basal-like breast cancer cells

(A) MDA-MB-231 pools stably expressing pBABE vector or pBABE-Ets1 were created by retroviral transduction. Ets1, α B-crystallin and actin levels were determined by immunoblotting. (B) MDA-MB-231 cells were transiently transfected with 25 μ M Ets1 siRNA or a control (C) lamin A/C siRNA. Ets1, α B-crystallin and actin levels were determined by immunoblotting 72 h later.

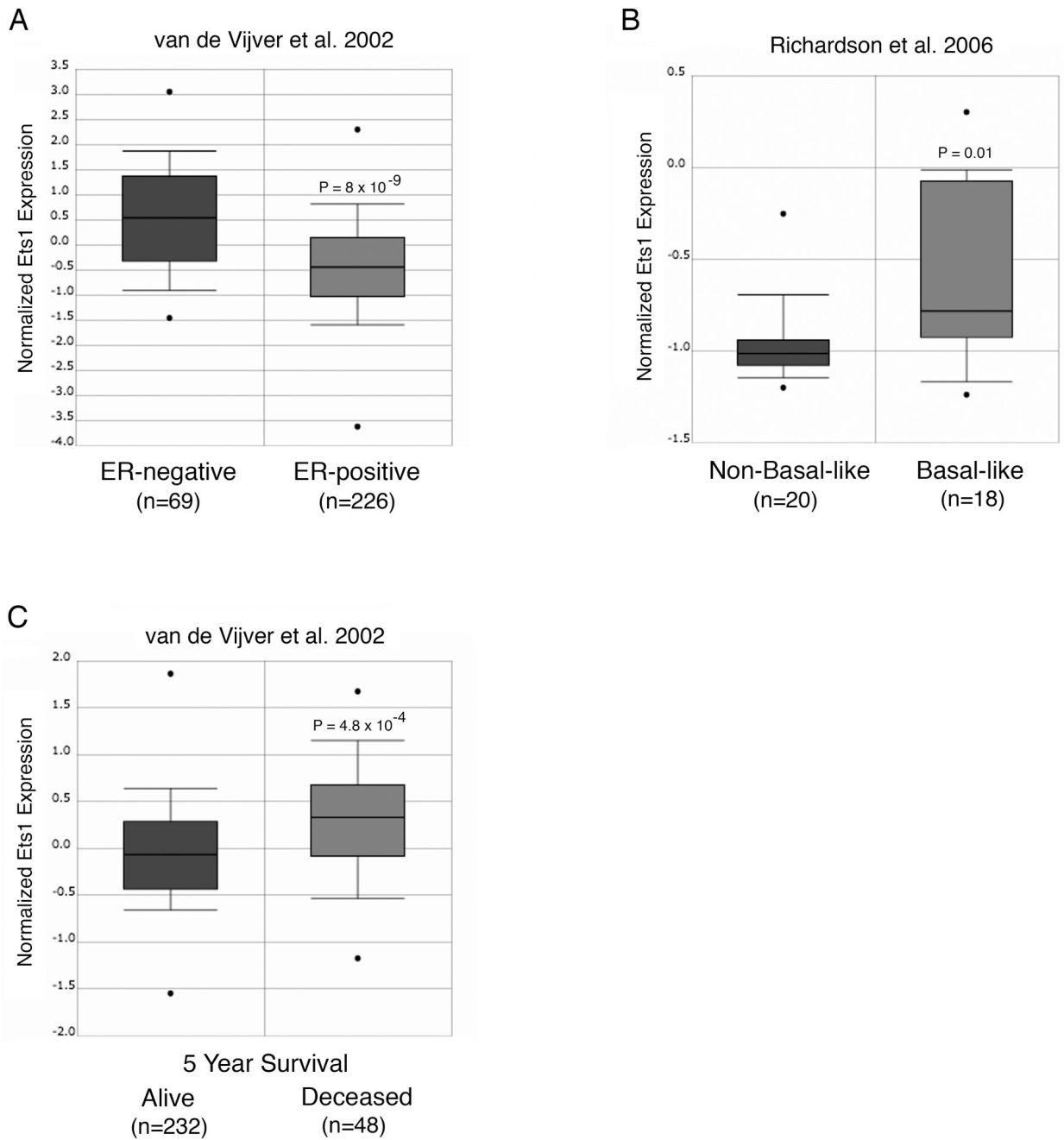


Figure 5. Ets1 is expressed in basal-like breast tumors and is associated with poor survival
Publically available gene expression datasets were interrogated by Oncomine analysis. **(A)** Ets1 expression as a function of ER-status in breast cancer [35]. **(B)** Ets1 expression in non-basal-like and basal-like breast tumors [36]. **(C)** Ets1 expression and five-year survival in breast cancer [35].