

## Regulation of p130<sup>Cas</sup>/BCAR1 Expression in Tamoxifen-Sensitive and Tamoxifen-Resistant Breast Cancer Cells by EGR1 and NAB2<sup>1</sup>

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### Abstract

Elevated levels of p130<sup>Cas</sup>/BCAR1 (Crk-associated substrate/breast cancer antiestrogen resistance 1) are found in aggressive breast tumors and are associated with tamoxifen resistance of mammary cancers. p130<sup>Cas</sup> promotes the integration of protein complexes involved in multiple signaling pathways frequently deregulated in breast cancer. To elucidate mechanisms leading to p130<sup>Cas</sup> up-regulation in mammary carcinomas and during acquired tamoxifen resistance, the regulation of p130<sup>Cas</sup>/BCAR1 was studied. Because multiple putative binding motifs for the inducible transcription factor EGR1 were identified in the 5' region of BCAR1, the p130<sup>Cas</sup>/BCAR1 regulation by EGR1 and its coregulator NAB2 was investigated. Overexpression or short interfering RNA (siRNA)-mediated down-regulation of EGR1 or NAB2, and chromatin immunoprecipitations indicated that EGR1 and NAB2 act in concert to positively regulate p130<sup>Cas</sup>/BCAR1 expression in breast cancer cells. p130<sup>Cas</sup> depletion using siRNA showed that, in tamoxifen-sensitive MCF-7 cells, p130<sup>Cas</sup> regulates EGR1 and NAB2 expression, whereas in the derivative tamoxifen-resistant TAM-R cells, only NAB2 levels were influenced. BCAR1 messenger RNA and p130<sup>Cas</sup> protein were upregulated by phorbol esters following the kinetics of late response genes in MCF-7 but not in TAM-R cells. Thus, in MCF-7 cells, we identified a positive feedback loop where p130<sup>Cas</sup> positively regulates EGR1 and NAB2, which in turn induce p130<sup>Cas</sup> expression. Importantly, compared with MCF-7, enhanced NAB2 expression and increased EGR1 binding to the BCAR1 5' region observed in TAM-R may lead to the constitutively increased p130<sup>Cas</sup>/BCAR1 levels in TAM-R cells. The uncovered differences in this EGR1/NAB2/p130<sup>Cas</sup> network in MCF-7 versus TAM-R cells may also contribute to p130<sup>Cas</sup> up-regulation during acquired tamoxifen resistance.

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### Introduction

p130 Crk-associated substrate (p130<sup>Cas</sup>) is an adapter protein that was first isolated from v-Src- and v-Crk-transformed rat fibroblasts [1]. Its gene was independently identified as breast cancer antiestrogen resistance 1 (BCAR1) [2,3]. Here, we refer to the gene and messenger RNA (mRNA) as BCAR1 and to the protein as p130<sup>Cas</sup>. p130<sup>Cas</sup> is the founding member of the Cas family, which includes HEF1 (NEDD9), Efs/Sin, and HEF1-Efs-p130<sup>Cas</sup>-Like (HEPL) [4–6]. Proteins of this family function as scaffolds integrating large multiprotein complexes in response to stimuli, such as growth factor and hormone release, and integrin engagement [5,7–9]. Evidence shows that signaling pathways activated by several of these stimuli are frequently enhanced in breast cancer, for example, by overexpression of receptor tyrosine kinases (RTKs) of the ErbB family (ErbB1 [EGF receptor] and ErbB2 [Her2/neu]) [10,11], integrin receptors ( $\alpha_3\beta_1$  and  $\alpha_v\beta_3$  integrins) [8,12], and estrogen [13].

Abbreviations: BCAR1, breast cancer antiestrogen resistance 1; ChIP, chromatin immunoprecipitation; p130<sup>Cas</sup>, Crk-associated substrate; dn, dominant negative; EGR, early growth response; ev, empty vector; ER, estrogen receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF, hypoxia-inducible factor; NAB2, NGFI-A binding protein 2; PPIA, peptidylprolyl isomerase A; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription–polymerase chain reaction; RPLP0, ribosomal protein, large, P0; RNV18S1, RNA 18S ribosomal 1; siRNA, short interfering RNA; TF, transcription factor; TSS, transcription start site; WB, Western blot analysis; WCE, whole cell extract; wt, wild-type

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In breast cancer patients, elevated p130<sup>Cas</sup> levels are associated with an increased rate of relapse and aggressiveness of the disease [14]. p130<sup>Cas</sup> expression is higher in pleural effusions of breast cancer patients compared with primary tumors [15]. Also, in feline and canine models of breast cancer, p130<sup>Cas</sup> levels positively correlate with advanced breast carcinomas [16]. Increased p130<sup>Cas</sup> protein expression has been implicated in mediating resistance to Adriamycin and tamoxifen [17,18]. Recent *in vitro* studies have shown elevated p130<sup>Cas</sup> levels in tamoxifen-resistant breast cancer cells (TAM-R) that were derived from tamoxifen-sensitive MCF-7 cells [19]. Resistance to antiestrogens such as tamoxifen and fulvestrant has also been associated with enhanced growth factor signaling involving the up-regulation of RTKs of the ErbB family and augmented Akt pathway activation [19–21]. Disruption of the p130<sup>Cas</sup> signaling node leads to attenuation of the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt survival pathways, reduced migration, and resensitization of TAM-R cells to tamoxifen [19].

Members of the early growth response (EGR) transcription factor (TF) family have been implicated in breast cancer progression and antiestrogen resistance [22,23]. The zinc finger factors EGR1 (NGFI-A), EGR2 (Krox-20), and EGR3 are immediate early response genes that are important for the regulation of differentiation, proliferation, and cell death in response to environmental stimuli [24,25]. Extracellular signals such as cytokines, growth factors, and hormones that lead to the activation/phosphorylation of p130<sup>Cas</sup> also rapidly and transiently induce the expression of the EGR family members [26,27]. EGR target genes comprise, for example, *vascular endothelial growth factor (VEGF)* [28], *PDGF* [29], as well as *cyclin-dependent kinase 1 inhibitor<sup>p21Waf1/Cip1</sup>* [22]. The activity of the EGR TFs is modulated in part through the gene product of its own target, the delayed early response gene *NGFI-A binding protein 2 (NAB2)* [30–32].

Given the number of studies that link p130<sup>Cas</sup> levels to breast cancer progression and the fact that tamoxifen is still a commonly used drug in hormonal adjuvant therapy, understanding the p130<sup>Cas</sup> gene regulation in mammary carcinomas is of great importance. However, the regulatory mechanisms that upregulate p130<sup>Cas</sup> expression in mammary carcinomas and during acquired tamoxifen resistance in human breast tumors are elusive. Here, we demonstrate the presence of two *BCAR1* mRNAs, using alternative first exons, in normal human mammary gland tissue and in breast tumors. The results suggest that, in breast cancer cells, p130<sup>Cas</sup> expression/signaling upregulates the TF EGR1 and its coregulator NAB2, which, in turn, are involved in the induction of

p130<sup>Cas</sup> expression. This regulatory network was altered in tamoxifen-resistant breast cancer cells, which exhibited higher NAB2 levels. Therefore, our studies identified a transcriptional circuit that controls p130<sup>Cas</sup> expression in breast cancer cells and a p130<sup>Cas</sup>-controlled regulatory network that may contribute to acquired tamoxifen resistance.

## Materials and Methods

### Cell Lines and Culture Conditions

Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) or obtained as indicated. The following cell lines were used: BT-20 (Sam Lee, Massachusetts General Hospital; human breast carcinoma, estrogen receptor [ER] negative); ZR-75-1, MCF-7, and the MCF-7-derivative TAM-R (Robert Nicholson, Cardiff, United Kingdom; human breast carcinoma, ER positive). MCF-7 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. TAM-R cells were cultured in phenol red-free RPMI-1640 and 10% charcoal-stripped FBS, supplemented with 4 mM glutamine and antibiotics/sodium pyruvate as previously mentioned. BT-20 and ZR-75-1 cells were cultured in Dulbecco modified Eagle medium, 10% FBS, and supplemented as previously mentioned. Cells were maintained at 37°C and 5% CO<sub>2</sub> and tested for *Mycoplasma* contamination.

For phorbol myristate acetate (PMA) treatment, 2 × 10<sup>5</sup> cells were seeded in six-well plates and, after 24 hours of plating, serum starved (0.5% serum) for 16 hours before stimulation by the addition of PMA (final concentration 10 ng/ml; Sigma, St Louis, MO) for indicated times.

### Generation of Expression Constructs

Human wild-type (wt) and dominant negative (dn) NAB2 complementary DNAs (cDNA) [32,33] were subcloned into pCX<sub>bsr</sub> digested with *Bam*HI. Human EGR1 cDNA was obtained by reverse transcription (RT) of total RNA prepared from MCF-7 cells stimulated for 2 hours with PMA using the EGR1-specific reverse primer *EGR1-NoI*, including a *NoI* site (Table 1, for primer sequences). The obtained EGR1 cDNA was amplified by polymerase chain reaction (PCR) using *Pfu* polymerase with the *EGR1-EcoRI* forward primer and the *EGR1-NoI* reverse primer and subcloned into the *EcoRI/NoI*-digested pCX<sub>bsr</sub> vector. Constructs were validated by sequencing. QIAGEN Plasmid Maxi Kit (Qiagen, Valencia, CA) was used for DNA preparation.

**Table 1.** Primer Sequences.

Name	Forward (5'–3')	Name	Reverse (5'–3')
<i>EGR1-EcoRI</i>	ATAGAATTCATGGCCGCGGCCAAGGCCG	<i>EGR1-NoI</i>	ATAGCGGCCGCTTAGCAAATTTCAATTGTCCTGG
<i>BCAR1</i> total	TGGAGCAGGACACGCAGGG	<i>BCAR1-rev</i>	GGTGGGCACCACCCTTTG
<i>BCAR1-1</i>	CGGACACCATGAACCACCTGAA		
<i>BCAR1-1'</i>	CCAAGATGTCCGTGCCTAAC	<i>BCAR1q-rev</i>	TCATACATGCCCAACCAAGAT
<i>BCAR1-1q</i>	GACACCATGAACCACCTGAA		
<i>BCAR1-1'q</i>	CGGCCAAGATGTCCGTGC		
<i>BCAR1</i> totalq-f	GTCACCTACAGGGGAAGGA	<i>BCAR1</i> totalq-rev	CGTGACACCTCCTGTTCCAG
<i>GAPDHq-f</i>	TGCACCACCAACTGCTTAGC	<i>GAPDHq-r</i>	GGCATGGACTGTGGTCATGAG
<i>PPIAq-f</i>	TTGGGCCAATAAGAAGTTGTGAA	<i>PPIAq-r</i>	AACCGACTGGCTAAAATCCAC
<i>RPLP0q-f</i>	GCTGCTGCCCGTGTGGTG	<i>RPLP0q-r</i>	TGGTGCCCTGGAGATTTAGTGG
<i>RN18S1q-f</i>	CGGTACCACATCCAAGGAA	<i>RN18S1q-r</i>	TTTTTCGTCACCTACCTCCCG
<i>BCAR1-1 -226</i>	CTCGGGCAGGGCCCTGGAG	<i>BCAR1-1 -58</i>	AGCCCGATCCCAGCATGC
<i>BCAR1_1' -360</i>	TCCTCTTCCCTGGGGTCTC	<i>BCAR1-1' -162</i>	TCTGGGTGCGCCCAACCAAGTC
control-f	ATGGTTGCCACTGGGGATCT	control-r	TGCCAAAGCCTAGGGGAAGA

### In Silico Analysis

Analyses of *BCAR1* and the exon 1 mRNA and protein variants were conducted using database resources and software from AceView [34], the EMBL–European Bioinformatics Institute, Genomatix [35], and the National Center for Biotechnology Information. *In silico* analysis of putative promoter regions was performed using the Genomatix suite of programs.

### Retroviral Transduction

Retroviral transduction was carried out as described [19,36]. Briefly, transient expression of EGR1, wt or dn NAB2, and/or empty vector (ev) control in MCF-7, TAM-R, and BT-20 cells was achieved by transduction with equal volumes of supernatant containing amphotropic CX<sub>bsr</sub> virus particles that carry either the cDNAs for EGR1, wt or dn NAB2, or ev. Equal amounts of ev control supernatant were added to the single-gene transductions to control for DNA amounts/virus titer. To enrich for cells expressing the introduced cDNAs, cells were selected with 10 µg/ml Blasticidin (Invitrogen, Grand Island, NY) for 3 days before harvesting.

### RNA Interference

Transfection of p130<sup>Cas</sup> short-interfering RNAs (siRNAs) was performed as previously described [19]. Depletion of human EGR1 was achieved by transfection of pooled siRNA (50 nM) in 12-well plates after 38 hours using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. ON-TARGETplus SMART pool siRNA targeting *EGR1* (catalog no. L-006526-00-0005) and negative control (catalog no. D-001810-01-05) were purchased from Dharmacon (Chicago, IL). Sequences of siRNA-specific for *EGR1* were as follows: 5'-GAUGAACGCAAGAGGCAUA-3'; 5'-CGACAGCA-GUCCCAUUUAC-3'; 5'-GGACAUGACAGCAACCUUU-3'; 5'-GACCUGAAGGCCCUCAAUA-3'. Down-regulation of human NAB2 was achieved by transfection of pooled siRNAs (50 nM) in 12-well plates after 72 hours using Lipofectamine 2000. siRNA sequences targeting *NAB2* (catalog no. sc-36014; Santa Cruz Biotechnology, Santa Cruz, CA) were as follows: 5'-CCAACCUC-CUUUCCUACUAAtt-3'; 5'-CAACCUCUUUCCUACUAUAtt-3'; 5'-CCUACUUGUCCUCCUUGAAtt-3'.

### Protein Analysis

Whole cell extracts (WCEs) were prepared and analyzed by Western blot analysis (WB) as described [37]. Briefly, cells were incubated in HNTG lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA) supplemented with protease inhibitor cocktail (Roche, Nutley, NJ) and phosphatase inhibitors (1 mM sodium vanadate and 1 mM sodium fluoride) for 30 minutes on ice followed by centrifugation at 16,000g and 4°C for 30 minutes. Equal amounts of protein were analyzed by SDS-PAGE. Antibodies (Abs) used were mAbs against β-actin (Sigma), NAB2 [38], and p130<sup>Cas</sup> (clone 21; BD Transduction Laboratories, Sparks, MD). Polyclonal rabbit Abs against EGR1 (588; sc-110) were purchased from Santa Cruz Biotechnology.

### Reverse Transcription–PCR

Cells were washed twice with cold PBS and RNA was extracted using TRIzol reagent (Invitrogen). Total RNA (2 µg) was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers (Invitrogen) according to the manufacturer's recommendations. cDNAs reverse transcribed using oligo-dT

primer from human breast tissue mRNA were purchased from Biochain Institute (Hayward, CA). PCR was performed using 0.5 µl of undiluted cDNA in a 20-µl PCR with the use of *Taq* DNA polymerase and reaction buffer from Biochain Institute. To check for linear amplification, each PCR was performed at different cycle numbers. Exon overlapping PCR primers were applied to exclude amplification of genomic DNA and cDNA generated from unspliced precursor mRNA. Primer sequences are found in Table 1. Reverse primer *BCAR1-rev* was used for *BCAR1* total, *BCAR1-1*, and *BCAR1-1'* amplifications. Forward primers and amplicon sizes were as follows: *BCAR1 total*, 551 bp; *BCAR1-1*, 657 bp; *BCAR1-1'*, 653 bp. Reaction conditions (annealing temperature and cycle number) were as follows: *BCAR1 total*, 62°C and 25; *BCAR1-1*, 64°C and 27; and *BCAR1-1'*, 64°C and 27. The *BCAR1* total reaction amplifies parts of exon 2 and exon 3, which are present in both exon 1 variants. To control for the integrity and uniformity of the sample preparations, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* mRNA was amplified as described previously [33]. Products were separated by electrophoresis through 1.2% agarose gels and validated by sequencing.

### Quantification of Gel Bands

Densitometric analysis of gel bands was performed using Quantity One software (Bio-Rad). Equal adjustments of contrast and brightness of gel pictures and scanned films were applied to all parts of the image using Adobe Photoshop CS2 version 9.0. *Vertical lines* in Figures 1D, 3, 4A and B, and 8A indicate that results from different WB are shown.

### Real-time PCR

Real-time PCR was performed in 20-µl reactions in triplicate using ABI PRISM 7300 Sequence Detection System Version 1.4.0 (Applied Biosystems, Carlsbad, CA) and 2× FastStart Universal SYBR Green PCR Master (ROX; Roche Applied Science, Indianapolis, IN) as recommended by the manufacturer using 2 ng of cDNA, reverse transcribed as described previously. Comparative *C<sub>t</sub>* method was applied for relative quantification of each gene expression between samples [39] and normalized to *GAPDH*, *peptidylprolyl isomerase A (PPIA)*, *ribosomal protein, large, P0 (RPLP0)*, and *RNA 18S ribosomal 1 (RN18S1)* levels, as indicated. PCR specificity and amplicon size were validated by electrophoresis through a 2% agarose gel and subsequent sequencing. Efficiency of each PCR was more than 98% as determined by performing the PCRs for each primer pair with serial DNA dilutions. Primer sequences are presented in Table 1. Reverse primer *BCAR1q-rev* was used for *BCAR1-1* and *BCAR1-1'* amplifications. Forward primers and amplicon sizes were as follows: *BCAR1-1q*, 209 bp; *BCAR1-1'q*, 211 bp. *BCAR1* total levels were detected by amplification of a region from exon 5 to exon 7, which is present in both exon 1 variants, using the primers *BCAR1 totalq-f* and *BCAR1 totalq-rev*. Primers used in *GAPDH*, *PPIA*, *RPLP0*, and *RN18S1* PCRs are shown in Table 1. Primers for *EGR1* and *NAB2* were described previously [33].

### Chromatin Immunoprecipitation Real-time PCR

Briefly, 2.5 × 10<sup>6</sup> (MCF-7) or 3 × 10<sup>6</sup> (TAM-R) cells were seeded in 10-cm dishes and after 1 day of plating serum starved (0.5% serum) for 16 hours before harvesting. Subsequently, chromatin immunoprecipitation (ChIP) was performed using EZ-ChIP kit (Millipore, Billerica, MA) following the manufacturer's protocol using 4 µg of antibody. Abs used were as follows: rabbit anti-EGR1 (588, sc-110; Santa Cruz

Biotechnology) and normal rabbit IgG control (sc-2027; Santa Cruz Biotechnology). ChIP real-time PCRs were performed as described previously using 3  $\mu$ l of purified DNA. For amplification of the *BCAR1-1* and *BCAR1-1'* 5' regions, 4  $\mu$ l of 5 M betaine (Sigma) was added to the PCRs. Primers (for sequences, see Table 1) and amplicon sizes were as follows: *BCAR1-1* –226 and *BCAR1-1* –58, 169 bp; *BCAR1-1'* –360 and *BCAR1-1'* –162, 199 bp. As control for chromatin bound nonspecifically, a human negative control primer set (**control-f** and **control-r**; amplicon size, 174 bp; Active Motif, Carlsbad, CA) was used. Specificity of the PCRs was validated by electrophoresis through 1.6% agarose gels and subsequent sequencing. The enrichment of EGR1 to the genomic *BCAR1* 5' or negative control regions was calculated as followed. The percentage of the input DNA (1% of the nuclear extract used) that was recovered was calculated for each sample, and the EGR1 values were related to the percentage computed for the rabbit IgG control. Subsequently, the resulting fold of IgG values obtained by the EGR1-

ChIP for the *BCAR1* 5' regions were divided by the fold of IgG retrieved by the EGR1-ChIP for the genomic negative control region to account for possible unspecific precipitation of genomic DNA by the EGR1 antibody in different samples/cell lines.

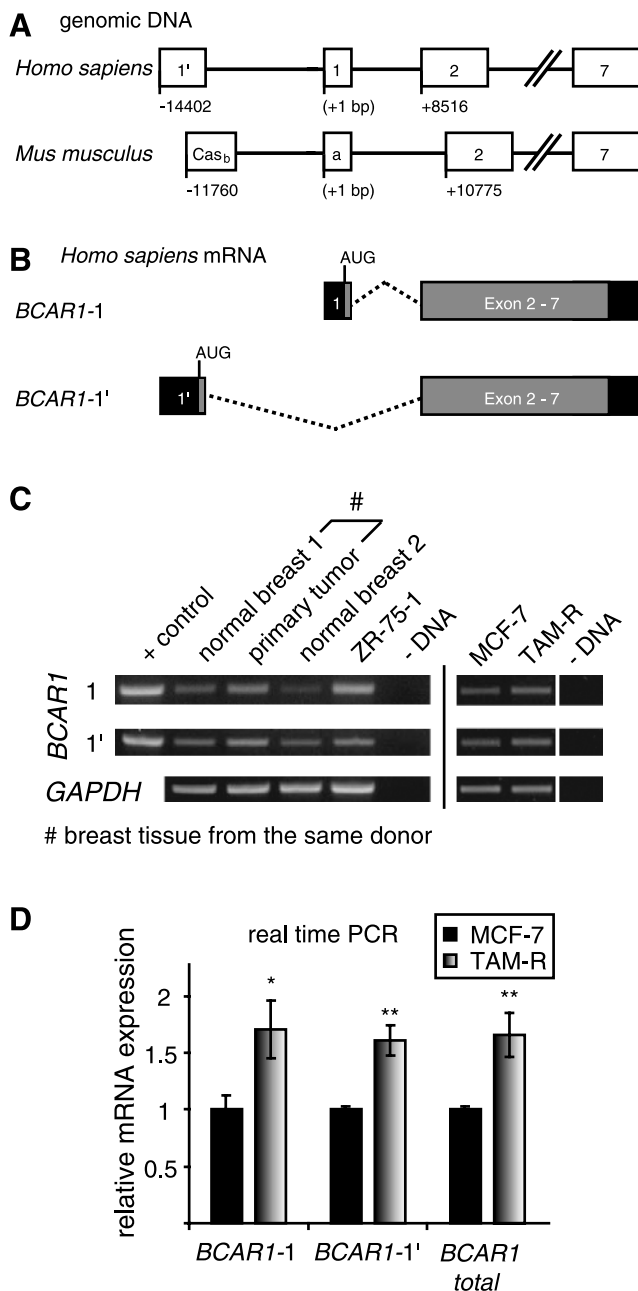
## Results

### BCAR1—Alternative First Exon Usage in Normal and Cancerous Breast Tissues

Recent studies have shown elevated p130<sup>Cas</sup> protein levels in tamoxifen-resistant breast cancer cells [19] and, more importantly, in antiestrogen-resistant breast cancers in patients [40]. Since to date the regulation of p130<sup>Cas</sup>/BCAR1 expression remains enigmatic, a comprehensive analysis of the *BCAR1* gene was performed to decipher mechanisms that may contribute to the observed up-regulation of p130<sup>Cas</sup> expression.

Database searches and gene structure analysis revealed that the human *BCAR1* gene uses an alternative first exon (*BCAR1-1'*, NM\_001170718) located 14,402 bp upstream of the first described exon 1 (*BCAR1-1*, RefSeq NM\_014567.3) (Figure 1, A and B). Comparative genomics indicated that *BCAR1-1* and *BCAR1-1'* reflect the p130<sup>Cas</sup> isoforms Cas<sub>a</sub> and Cas<sub>b</sub> described in mice, respectively [41]. Both first exons encode for only three distinct amino acids (aa) (NHL [*BCAR1-1*] and SVP [*BCAR1-1'*]) and the resulting isoforms are conserved in mammals. The genomic distance between the two first exons points to the use of alternative promoters.

To study the regulation of *BCAR1* in mammary carcinomas, we examined the expression of *BCAR1* exon 1 variant mRNA in two normal



**Figure 1.** *BCAR1* uses alternative first exons in normal and cancerous breast tissues. (A) Comparison of the human and mouse *BCAR1* genes. The human *BCAR1-1* and *BCAR1-1'* reflect the murine p130<sup>Cas</sup> isoforms Cas<sub>a</sub> and Cas<sub>b</sub>, respectively. Boxes represent exons. // indicates that sequences of exons 3 through 6 were omitted. Numbering indicates the TSS of variant 1' relative to exon 1 TSS (+1). (B) Schematic of the *BCAR1* Exon 1 mRNA variants. Relative position of the translation start site (AUG) for each variant is indicated. Black boxes indicate untranslated regions; gray boxes, translated regions; dashed lines, spliced-out intron region. (C) Levels of *BCAR1-1*, *BCAR1-1'*, and *GAPDH* (control) mRNA in human breast tissues and ZR-75-1, MCF-7 (tamoxifen-sensitive), and TAM-R (tamoxifen-resistant) mammary carcinoma cell lines were determined by RT-PCR as described previously. + control, positive control for PCR; - DNA, negative control for PCR. Results are from one representative experiment of two performed. (D) Expression of *BCAR1* mRNAs is increased in tamoxifen-resistant TAM-R cells. Tamoxifen-sensitive MCF-7 and its derivative tamoxifen-resistant TAM-R cells were cultured similarly for 2 weeks and seeded at  $3 \times 10^5$  cells in six-well plates. After 3 days at a confluence of 70% to 90%, cells were harvested and mRNA was prepared and reverse transcribed as described previously. Levels of *BCAR1-1*, *BCAR1-1'*, total *BCAR1*, and the internal controls *GAPDH*, *PPIA*, and *RPLP0* mRNA in MCF-7 cells and TAM-R were determined by real-time PCR as described previously. Shown are the results after normalization to *GAPDH*. Similar results were obtained after normalization to *PPIA* and *RPLP0*. Average levels relative to the expression in MCF-7 cells (black bar, set to 1) and SD from triplicate were determined as described previously. *P* values were calculated using *t* test. \**P* < .05. \*\**P* < .01. Results are from one representative experiment of two performed.

human breast tissues, a human primary tumor, and in the human breast cancer cell line ZR-75-1 (Figure 1C). Furthermore, to elucidate differences in acquired tamoxifen resistance, the tamoxifen-sensitive MCF-7 breast cancer cells and its resistant derivative, TAM-R, expressing higher p130<sup>Cas</sup> protein levels [19], were used as a model system and therefore also included. In all samples, both *BCAR1* variants were detected. Compared with normal breast tissue 1, the levels of *BCAR1-1* as well as of *BCAR1-1'* were increased in the primary tumor sample, which was derived from the same donor. Higher levels of *BCAR1-1'* over *BCAR1-1* or vice versa were found in normal breast tissue 2 and ZR-75-1 cells, respectively, suggesting a distinct regulation of each *BCAR1*/p130<sup>Cas</sup> variant. In MCF-7 and TAM-R cells, a similar expression of both variants was detected. However, a slight increase in both *BCAR1* variants was observed in TAM-R versus MCF-7 cells.

To further investigate whether the enhanced protein levels in TAM-R cells correlate with higher mRNA expression, *BCAR1-1*, *BCAR1-1'*, and total *BCAR1* (determined by amplification of a region spanning exons 5 to 7) mRNA expression levels were analyzed by real-time PCR in MCF-7 and TAM-R cells (Figure 1D). Significant increases in *BCAR1-1* (1.71-fold,  $P < .05$ ), *BCAR1-1'* (1.61-fold,  $P < .01$ ), and total *BCAR1* (1.66-fold,  $P < .01$ ) were observed in TAM-R compared to MCF-7 cells.

Taken together, these results show that *BCAR1-1* and *BCAR1-1'* are coexpressed in human breast tissues and breast cancer cell lines, and both variants seem to be differentially regulated in some circumstances. Furthermore, tamoxifen-resistant cells have elevated mRNA levels compared to tamoxifen sensitive.

### ***In Silico Analysis of the Flanking Regions of the BCAR1 Exon 1 Variants Reveals Multiple Clusters of Binding Sites for Transcription Factors of the EGR Family***

To gain insight into the mechanisms regulating the p130<sup>Cas</sup> variant expression, a comprehensive phylogenetic analysis of the 5' and 3' flanking regions of the alternative first exons of *BCAR1* was performed (Figure 2).

Putative promoters colocalized with CpG islands were identified in the regions flanking human exons 1 and 1'. A CAAT box was only found at bp -92 of exon 1 and no TATA box or initiator element is present in both regions. The lack of these core promoter elements points to the involvement of specific TFs in the regulation of *BCAR1* expression. Owing to the elevated p130<sup>Cas</sup> levels in breast cancer putative binding sites for TFs including members of the EGR, NF- $\kappa$ B, and hypoxia-inducible factor (HIF) family, p53, AP2, estrogen response elements (ERE), and cAMP-responsive elements binding proteins (CREB), involved in tumor development and progression, were explored. The region bp -800 to +400 relative to the transcription start site (TSS, +1) of exon 1, contains 14 putative binding sites for the EGR family, most of which overlap with nine SP1 motifs, seven p53, two AP2 sites, and a single HIF and CREB motif. Of note is a conserved cluster of overlapping binding sites (bp -196 to -125) including six EGR and five SP1 motifs. Several EGR/Sp1 motifs within bp -500 to +150 and two p53 binding sites near the CAAT box are conserved among the different species (Figure 2).

In the 5' region of exon 1', an evolutionary conserved cluster of four EGR and three overlapping SP1 sites at bp -29 to +2, one EGR1/SP1 site, and two ERE at bp +150 to +180 were predicted. In addition, nine EGR, five SP1, four NF- $\kappa$ B, two HIF sites, and one ERE were identified (bp -800 to +400).

These results point to a complex regulation of the expression of the *BCAR1-1* and *BCAR1-1'* variants involving members of the EGR family and other TFs, such as NF- $\kappa$ B, and p53.

### ***EGR1 and Its Coregulator NAB2 Are Involved in the Regulation of p130<sup>Cas</sup> Expression in Human Breast Cancer Cells***

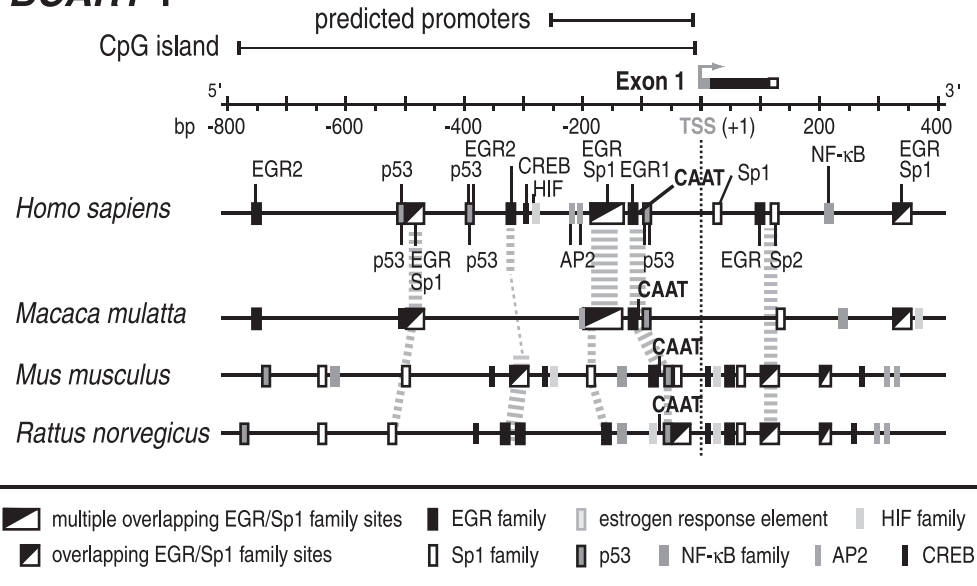
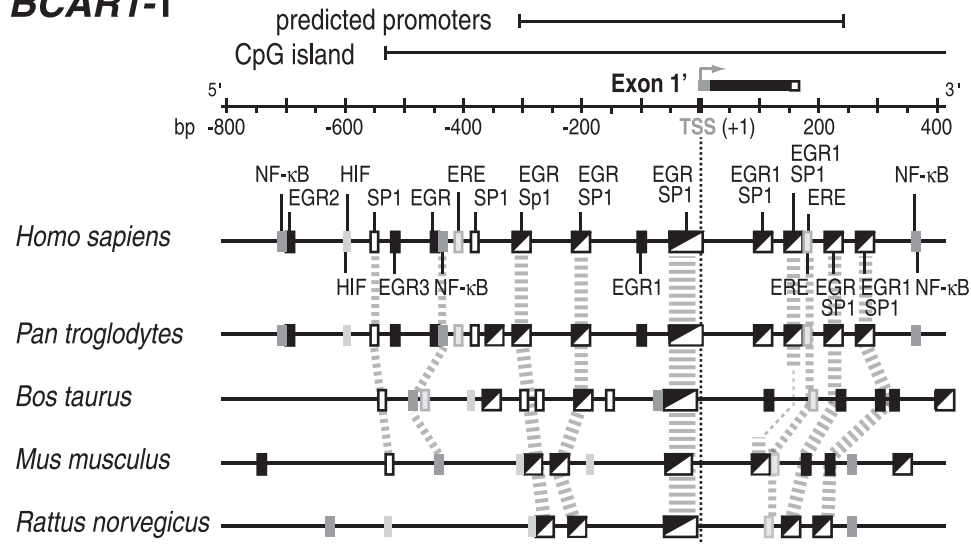
To elucidate a potential role of EGR1 and the influence of its coregulator NAB2 on p130<sup>Cas</sup> expression, MCF-7 and TAM-R cells were transduced with EGR1, NAB2 (wt or dn), and ev control (Figure 3). *NAB2* is a target gene of EGR1 and, in turn, can act as corepressor [31,32] and coactivator [30] of EGR1-mediated transcription. dnNAB2 contains an aa exchange that prevents the binding to EGR1. dnNAB2 is still able to form complexes with endogenous NAB2, thereby greatly abolishing the binding of also endogenous NAB2 to EGR1 [42]. Protein levels were analyzed by WB after 5 days, which included 3 days of selection with blasticidin to enrich for cells expressing the introduced cDNAs. Cells transduced with EGR1 had profoundly higher amounts of p130<sup>Cas</sup> and NAB2. The influence of NAB2 on EGR1-mediated p130<sup>Cas</sup> expression was examined by cotransduction of EGR1 and NAB2. p130<sup>Cas</sup> was not altered by coexpression of EGR1 and NAB2 compared to EGR1 single transduction, suggesting that NAB2 does not inhibit EGR1-mediated p130<sup>Cas</sup> expression. Cells transduced with NAB2 alone showed also elevated p130<sup>Cas</sup> levels. Importantly, ectopic expression of dnNAB2 did not result in higher p130<sup>Cas</sup> expression in MCF-7 and TAM-R cells (Figure 3B). These experiments suggest that EGR1 and NAB2 complex formation is required to increase p130<sup>Cas</sup> levels in breast cancer cell lines.

### ***EGR1 and NAB2 Depletion Attenuates p130<sup>Cas</sup>/BCAR1 Expression at the mRNA and Protein Levels***

Subsequently, the importance of endogenous EGR1 and NAB2 in the regulation of p130<sup>Cas</sup> expression was further investigated using siRNAs. MCF-7, TAM-R, or BT-20 breast cancer cells were transfected with siRNAs directed against *EGR1*, *NAB2*, or a negative control siRNA, and the effects on p130<sup>Cas</sup>, EGR1, and NAB2 were assessed by WB (Figure 4, A and B). Results in Figure 4A are presented for TAM-R and BT-20 cells because no adequate depletion of EGR1 levels was achieved in MCF-7 cells. Transfection of *EGR1* siRNA resulted not only in a reduction of EGR1 levels by 70% and 41% in TAM-R and BT-20 cells, respectively, but also of p130<sup>Cas</sup> expression by 41% ( $P < .01$ ) and 50% ( $P < .01$ ). NAB2 levels were also significantly ( $P < .01$ ) reduced by 73% (TAM-R) and 44% (BT-20).

Depletion of NAB2 by siRNA targeting *NAB2* by 63% (MCF-7) and 74% (TAM-R) also led to a reduction of p130<sup>Cas</sup> levels ( $P < .001$ ) by 30% and 40% in MCF-7 and TAM-R cells, respectively (Figure 4B), underscoring the importance of NAB2 in regulating p130<sup>Cas</sup> expression. Furthermore, EGR1 expression was reduced in TAM-R cells by 47% ( $P < .001$ ), whereas in MCF-7 cells, a reduction of only 20% ( $P < .05$ ) was observed.

To investigate whether endogenous EGR1 and NAB2 are involved also in the transcriptional regulation of p130<sup>Cas</sup> expression, the *BCAR1* mRNA levels were analyzed after depletion of NAB2 by siRNA (Figure 4C). After *NAB2* siRNA transfection, the mRNA levels of *BCAR1-1*, *BCAR1-1'*, total *BCAR1*, *NAB2*, and *EGR1* were determined in MCF-7 and TAM-R cells by real-time PCR. Transfection of *NAB2* siRNA resulted in a reduction of *NAB2* levels by 49% and 88% in MCF-7 and TAM-R cells, respectively, correlating with

**BCAR1-1****BCAR1-1'**

**Figure 2.** Schematic representation of putative regulatory elements in the flanking regions of the alternative first exons of *BCAR1* in mammalian species, as indicated. Comparative genomics were performed using database resources from AceView and the Genomatix suite of programs. Different species were used for the analysis of the *BCAR1-1* and *BCAR1-1'* regions owing to a restricted availability and gaps in the genomic sequence. Predicted promoter regions, CpG islands, and TF binding sites are indicated for *BCAR1-1* and *BCAR1-1'*. Conserved *cis* regulatory elements are emphasized by gray dashed lines/blocks. Numbering is relative to TSS (+1, gray boxes); exon 1: black boxes, untranslated regions; small white boxes, translated regions.

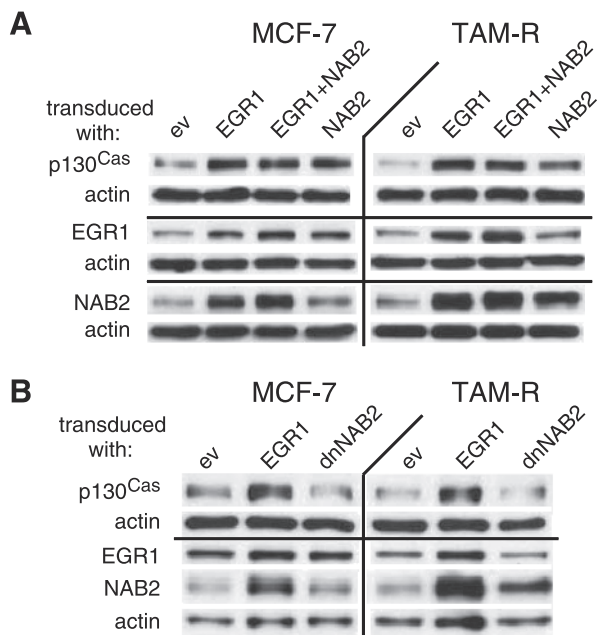
reductions in total *BCAR1* expression by 56% ( $P < .01$ ) and 65% ( $P < .001$ ). Corresponding to the minor effects of NAB2 depletion on EGR1 protein levels in MCF-7 (Figure 4B), *EGR1* mRNA expression was only significantly reduced in TAM-R cells (by 78%,  $P < .001$ ). Interestingly, *BCAR1-1'* levels were substantially depleted ( $P < .001$ ) by 49% (MCF-7) and 68% (TAM-R) in both cell lines, whereas *BCAR1-1* expression was reduced to a much greater extent in TAM-R by 67% ( $P < .001$ ) than in MCF-7 cells by 14% ( $P < .05$ ).

Taken together, these results indicate that the TF EGR1 and its coregulator NAB2 are positive regulators of p130<sup>Cas</sup>/*BCAR1* expression in human breast cancer cells. Importantly, NAB2 seems to be involved in the regulation of *BCAR1-1* and *BCAR1-1'* expression in the tamoxifen-

resistant TAM-R cells, whereas NAB2 influences mostly *BCAR1-1'* levels in the tamoxifen-sensitive MCF-7 cells.

#### *Increased Binding of EGR1 to the BCAR1 5' Regions in Tamoxifen-Resistant TAM-R Compared to Tamoxifen-Sensitive MCF-7 Cells*

Because RNA interference experiments suggested that EGR1 regulates *BCAR1* expression, EGR1 was tested for its ability to directly bind to the potential *BCAR1* promoter regions. ChIP assays were performed on isolated chromatin from MCF-7 and TAM-R cells and evaluated by real-time PCR for the genomic DNA 5' regions of *BCAR1-1* and *BCAR1-1'* that contain multiple putative EGR1 binding



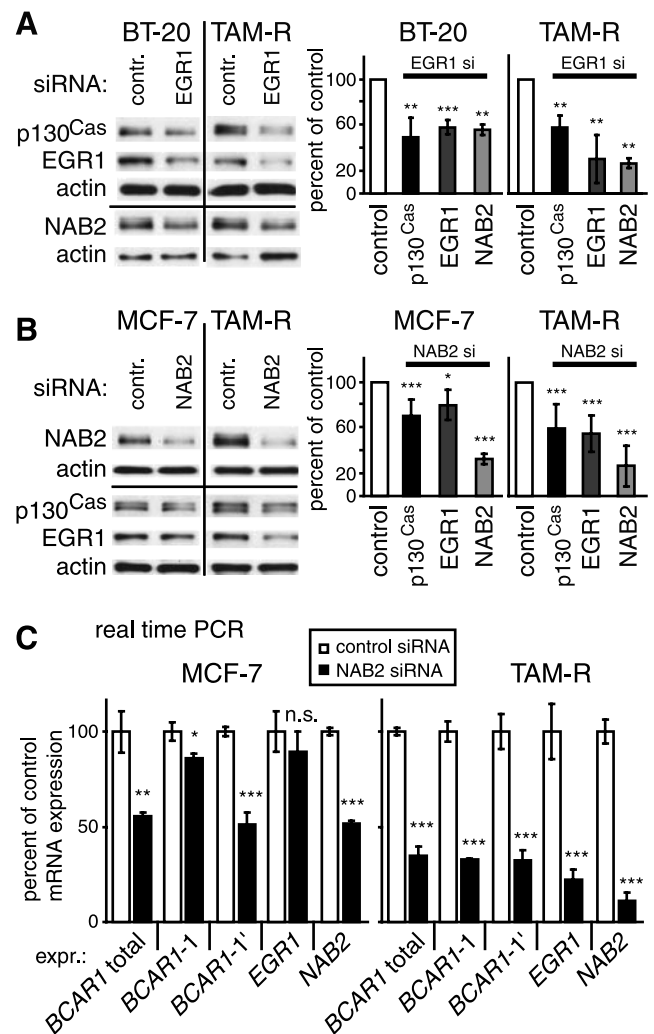
**Figure 3.** EGR1 and NAB2 are involved in the regulation of p130<sup>Cas</sup> expression. (A and B) MCF-7 and TAM-R cells were transfected with the indicated cDNAs for 2 days and selected for 3 days with blasticidin as described previously. WCEs (20  $\mu$ g) were separated by SDS-PAGE, subjected to WB, and probed with the indicated antibodies. Data are presented from one experiment of at least three performed with similar results.

sites (Figure 5A). Binding to these specific regions was investigated because they are located in the predicted promoter regions, and similar clusters of EGR/Sp1 sites in a comparable distance from the TSS were identified as crucial in multiple other promoters [28,29,32]. To account for nonspecific binding of EGR1, the recovery of DNA by IgG control serum and enrichment to a genomic negative control region were included in the calculation as described in the Materials and Methods. In MCF-7 cells, EGR1 binding was found located within the 5' region from bp -226 to -58 of *BCAR1-1* (2.9-fold enrichment,  $P < .001$ ) containing a cluster of multiple overlapping EGR/Sp1 and a single EGR1 site, whereas no binding was detectable to the 5' region of *BCAR1-1'* from bp -360 to -162 (Figure 5B). This region similarly contains two clusters of overlapping EGR/Sp1 motifs. In contrast, in TAM-R cells, binding of EGR1 to *BCAR1-1* (7.96-fold enrichment,  $P < .001$ ) and *BCAR1-1'* (2.53-fold enrichment,  $P < .05$ ) was observed. The binding to *BCAR1-1* in TAM-R cells was significantly higher ( $P < .001$ ) than in MCF-7 cells.

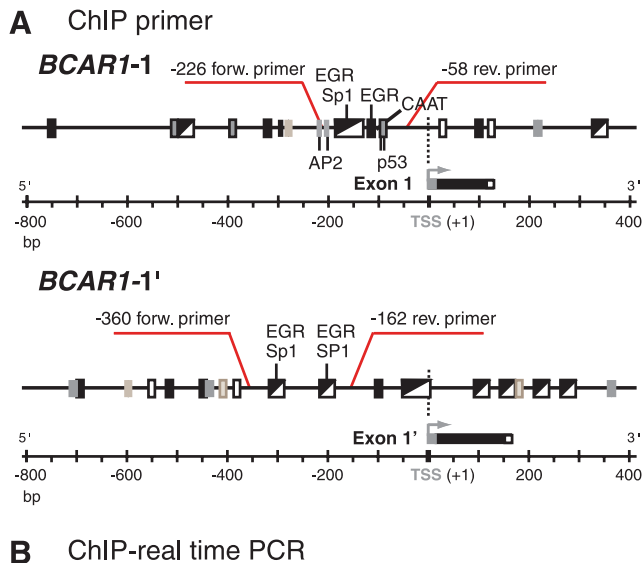
These results suggest differential regulation of the p130<sup>Cas</sup> variant expression in MCF-7 versus TAM-R cells by EGR1.

#### Aggressive and Late-Stage Breast Cancers Display Elevated NAB2 Levels

As more significant effects by depletion of the EGR1 coregulator NAB2 were observed in TAM-R versus MCF-7 cells, NAB2 expression in both cell lines was compared. MCF-7 and TAM-R cells were cultured similarly for 2 weeks to eliminate differences in the basal expression of EGR1 and NAB2 induced by varied culture conditions. WB of basal p130<sup>Cas</sup>, EGR1, and NAB2 protein levels (Figure 6A) showed similar EGR1 levels in both cell lines but profoundly



**Figure 4.** Endogenous EGR1 and NAB2 are important regulators of p130<sup>Cas</sup> expression. (A) BT-20 and TAM-R cells were transfected with siRNAs for *EGR1* or control siRNA. WCEs (20  $\mu$ g) were analyzed for p130<sup>Cas</sup>, EGR1, NAB2, and actin (loading control) expression by WB 38 hours after treatment (left panel). Right panel: Densitometric analysis of protein expression. (B) MCF-7 and TAM-R cells were transfected with siRNAs for *NAB2* or control siRNA. WCEs (20  $\mu$ g) were analyzed for p130<sup>Cas</sup>, EGR1, NAB2, and actin (loading control) expression by WB 72 hours after treatment. Right panel: Densitometric analysis of protein expression. (A and B) Average expression levels relative to negative control (black bar, set to 100%) and SD from three representative experiments performed were determined as described previously. (C) MCF-7 and TAM-R cells were transfected with siRNAs for *NAB2* or control siRNA. After 72 hours, mRNA levels of *BCAR1-1*, *BCAR1-1'*, total *BCAR1*, *NAB2*, *EGR1*, and the internal controls *RPLP0*, *PPIA*, and *RN18S1* in MCF-7 and TAM-R cells were determined by real-time PCR as described previously. Shown are the results after normalization to *RPLP0*. Similar results were obtained after normalization to *PPIA* and *RN18S1*. Average levels relative to the expression in MCF-7 cells (black bar, set to 100%) and SD from triplicate were determined as described previously. Results are from one representative experiment of two performed. (A-C)  $P$  values were calculated using  $t$  test. \* $P < .05$ . \*\* $P < .01$ . \*\*\* $P < .001$ . n.s. indicates not significant.



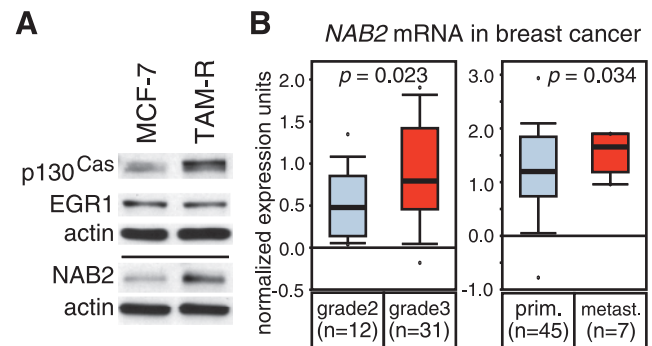
**Figure 5.** Distinct binding of EGR1 to the *BCAR1-1* and *BCAR1-1'* 5' regions in MCF-7 and TAM-R cells. ChIP real-time PCR was used to investigate EGR1 binding to the *BCAR1* 5' regions as described in the Materials and Methods. (A) Schematic of the flanking regions of the alternative first exons of *BCAR1*. Positions of primers used for ChIP analyses and putative TF binding sites within the amplified regions are indicated. (B) Immunoprecipitated *BCAR1-1*, *BCAR1-1'*, and negative control regions were amplified by real-time PCR as described previously. Shown is the average relative fold EGR1 enrichment at the *BCAR1* 5' regions in relation to the EGR1 binding at the negative control DNA region (control region, set to 1) and SD calculated from triplicate as explained in detail in the Materials and Methods. Control region: PCR to control for chromatin bound non-specifically. Results are from one representative experiment of at least three performed. *P* values of negative control region *versus BCAR1* 5' regions, or as indicated, were calculated using *t* test. \**P* < .05. \*\*\**P* < .001. n.s. indicates not significant.

elevated NAB2 levels in tamoxifen-resistant TAM-R cells. Commonly, breast cancers with acquired tamoxifen resistance represent more advanced tumors that are difficult to treat. Therefore, we investigated whether increased *NAB2* levels correlate with mammary carcinoma grade and/or malignancy by analyzing microarray gene expression data sets

available at [www.oncomine.org](http://www.oncomine.org). For microarray studies to be included, we required a minimum of five samples for each group and a similar trend in expression differences if multiple reporters were used in these studies. The *NAB2* mRNA levels significantly increased from grade 2 to grade 3 specimens of human invasive ductal breast cancers in the Ginestier study (*P* = .023, reporter number 216017\_s\_at) [43] (Figure 6B, left panel). A similar increase was observed in the study of Zhao et al. [44] (*P* = .033, reporter IMAGE:1926749; not shown), whereas no significant changes from grade 2 to grade 3 were detected in the Radvanyi study (reporter number U48361) [45] (not shown). Thus, the analyses were extended to compare *NAB2* levels in primary sites and metastases of breast cancer patients. The levels of *NAB2* mRNA were significantly higher in metastasis versus primary site in the Radvanyi study (*P* = .023, reporter number U48361) [45] (Figure 6B, right panel). Similar results were found in two other microarray studies (not shown) that fulfilled our requirements, supporting these findings. These data suggest that advanced human breast cancers express higher levels of NAB2.

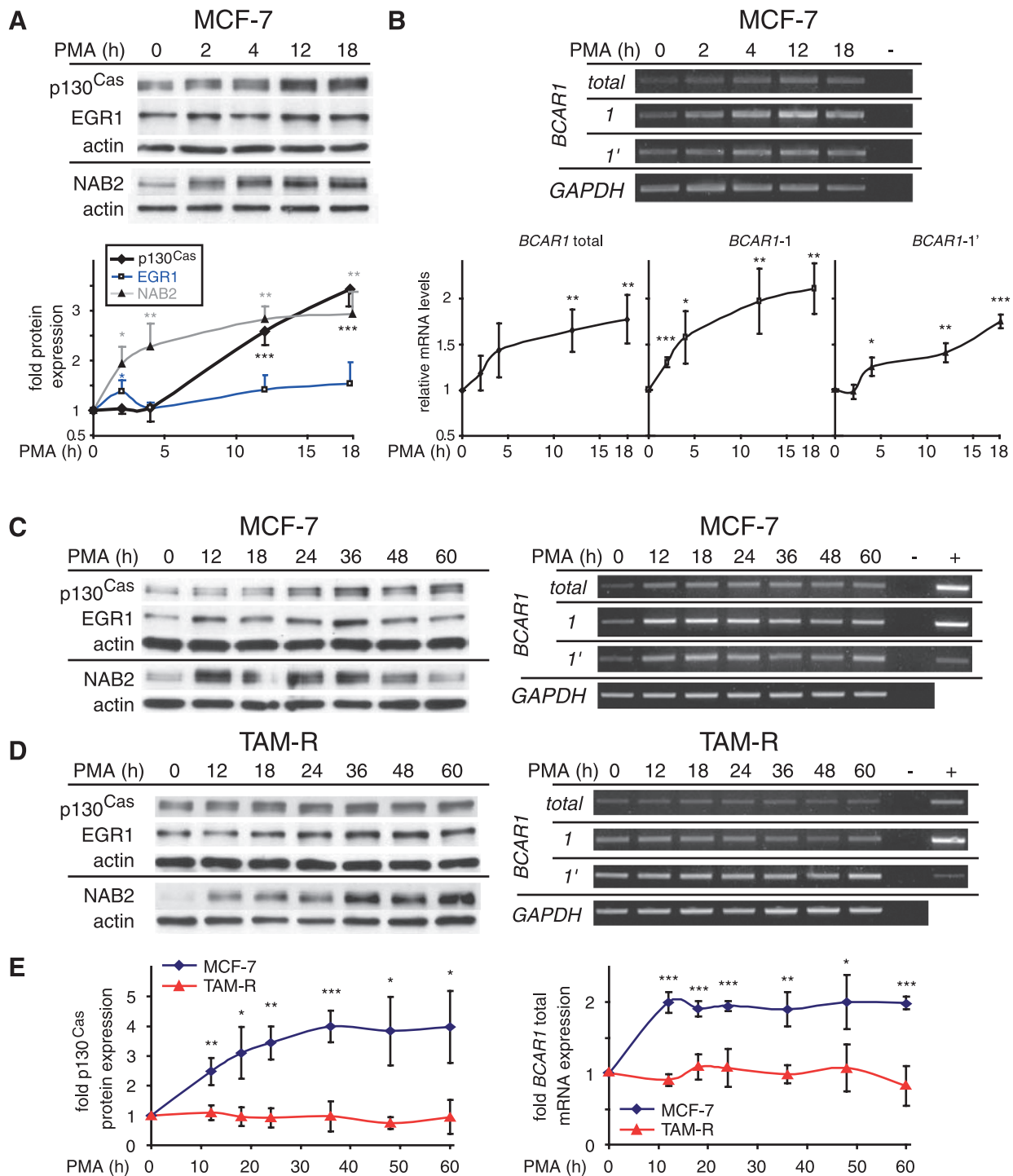
### *p130<sup>Cas</sup>/BCAR1 Expression Is Induced by Phorbol Esters Following the Kinetics of a Late Response Gene in Human Breast Cancer Cells*

The immediate early gene *EGR1* and its coregulator, the delayed early response gene *NAB2*, are highly inducible by PMA [24,31,38], which is an activator of protein kinase C, thereby mimicking growth factor signaling. Accordingly, depletion of growth factors leads to down-regulation of *EGR1* and *NAB2*, which can vary in time and strength in different cell types. To elucidate whether *BCAR1/p130<sup>Cas</sup>* expression



**Figure 6.** NAB2 expression is elevated in aggressive and late-stage breast cancers. (A) MCF-7 and TAM-R cells were cultured similarly for 2 weeks and seeded at  $3 \times 10^5$  cells in six-well plates. After 3 days at a confluence of 70% to 90%, cells were harvested and WCEs (20  $\mu$ g) were subjected to WB to compare the basal p130<sup>Cas</sup>, EGR1, and NAB2 protein levels. Actin was used as a loading control. Results are presented from one representative experiment of three performed. (B) Box plots of data from breast carcinoma microarray data sets using the Oncomine Cancer Profiling Database ([www.oncomine.org](http://www.oncomine.org)) plotted on a log scale. The *t* test was performed directly through the Oncomine 4.4 software. *n* indicates the number of samples in each group. Left panel: a box plot of data from the Ginestier study (reporter number 216017\_s\_at) [43] including grade 2 and grade 3 human invasive ductal breast cancer samples. The difference in *NAB2* mRNA expression between the two groups was significant (*P* = .023). Right panel: data from the Radvanyi study (reporter number U48361) [45] including samples of human breast cancer obtained from the primary site (prim.) or metastasis (metast.). The difference in *NAB2* expression between the two groups was significant (*P* = .034).





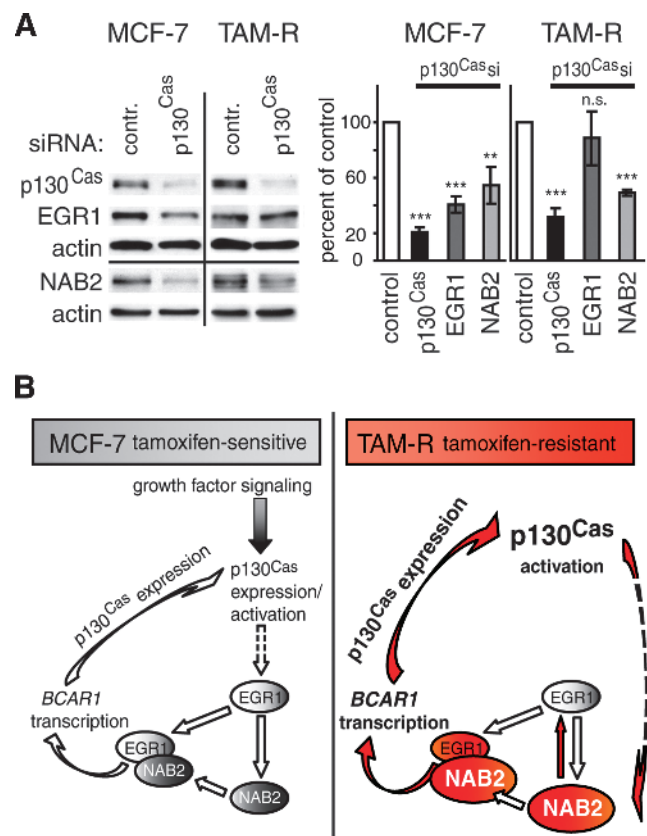
**Figure 7.** p130<sup>Cas</sup> mRNA and protein expression are inducible in response to phorbol esters. (A-D) MCF-7 and TAM-R cells, as indicated, were serum starved for 16 hours followed by stimulation with PMA for indicated times up to 60 hours or left untreated (0 h). (A and B, upper panel, and C and D) Results are presented from one experiment of three performed with similar results. (A) Upper panel: WCEs (20  $\mu$ g) were analyzed for p130<sup>Cas</sup>, EGR1, and NAB2 protein expression by WB. Lower panel: Densitometric analysis of protein expression. (B) Upper panel: expression of total *BCAR1* mRNA and of *BCAR1-1*, *BCAR1-1'*, and *GAPDH* (control) mRNAs was determined by RT-PCR as described previously. - DNA indicates the negative control for PCR. Lower panel: Densitometric analysis of total *BCAR1* mRNA, *BCAR1-1*, and *BCAR1-1'* expression. (C and D) Left panels: WCEs (20  $\mu$ g) were analyzed for p130<sup>Cas</sup>, EGR1, and NAB2 protein expression by WB. Right panels: Expression of total *BCAR1* mRNA and of *BCAR1-1*, *BCAR1-1'*, and *GAPDH* (control) mRNAs was determined by RT-PCR as described previously. + control indicates positive control for PCR; - DNA, negative control for PCR. (E) Densitometric analysis of p130<sup>Cas</sup> protein (left panel) and total *BCAR1* mRNA (right panel) expression from MCF-7 and TAM-R cells. (A and B, lower panel, and E) Average expression levels in relation to untreated cells (set to 1) and SD from three experiments performed were determined. Statistically significant changes compared with untreated cells are indicated: \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001. *P* values were calculated using *t* test.

levels can be influenced by environmental signals, an analysis of the expression kinetics of p130<sup>Cas</sup> in response to PMA was performed in MCF-7 and TAM-R cells. Breast cancer cells were serum starved for 16 hours followed by PMA treatment for indicated times and the p130<sup>Cas</sup>, EGR1, and NAB2 protein expression and total BCAR1, BCAR1-1, and BCAR1-1' mRNA levels were monitored by WB and RT-PCR, respectively (Figure 7). Initial treatment of MCF-7 cells for up to 18 hours (Figure 7, A and B) showed a weak induction of total BCAR1 (1.18-fold, not significant) and BCAR1-1 (1.3-fold,  $P < .001$ ) mRNA levels at 2 hours continuously increasing until 18 hours (1.77-fold [ $P < .01$ ] and 2.11-fold [ $P < .01$ ], respectively). A delayed up-regulation of BCAR1-1' was observed at 4 hours (1.25-fold,  $P < .05$ ) further increasing until 18 hours (1.74-fold,  $P < .001$ ). Accordingly, major p130<sup>Cas</sup> protein induction occurred at 12 hours, which was stable for up to 18 hours. Coinciding with the initial up-regulation of total BCAR1 and BCAR1-1, a transient induction of EGR1 at 2 hours and increasing levels of NAB2 protein were detected. EGR1 returned to basal levels at 4 hours and was reinduced at 12 and 18 hours, whereas NAB2 expression constantly increased until 18 hours. Because a strong up-regulation of p130<sup>Cas</sup> levels was still present, at 18 hours, the time of the PMA treatment was extended to 60 hours to elucidate the late expression kinetics. This study included also the tamoxifen-resistant TAM-R (Figure 7, C-E) and ER-negative BT-20 breast cancer (not shown) cells. In MCF-7 cells, the total BCAR1 mRNA induction remained similar for up to 60 hours, whereas a continuous increase in protein levels was observed, reaching its maximum at 36 hours (4-fold), which was stable for up to 60 hours (Figure 7C). Whereas the induction of BCAR1-1' mRNA was relatively stable for 60 hours, BCAR1-1 levels slightly declined from 24 to 60 hours. The late p130<sup>Cas</sup> protein and mRNA expression patterns also correlated with the protein levels of EGR1 and NAB2 in MCF-7 cells. Similar results were obtained in BT-20 cells (not shown).

In TAM-R cells, no major changes of total p130<sup>Cas</sup> protein and BCAR1 mRNA levels were observed (Figure 7D). Increased EGR1 expression was observed from 24 to 60 hours, whereas NAB2 levels were induced earlier further increasing until reaching a plateau at ~36 hours. These results indicate that p130<sup>Cas</sup> protein and mRNA expression is inducible in response to PMA treatment in tamoxifen-sensitive MCF-7 cells, following the kinetics of a late response gene, but not in tamoxifen-resistant TAM-R cells (as compared in Figure 7E).

#### Differential p130<sup>Cas</sup> Regulation of EGR1 and NAB2 Expression in Tamoxifen-Sensitive MCF-7 and Tamoxifen-Resistant TAM-R Cells

In response to numerous stimuli, p130<sup>Cas</sup> mediates the activation of multiple signaling pathways including the Ras/mitogen-activated protein kinase and PI3K/Akt cascades [5,19]. Because EGR1 expression is regulated through mitogen-activated protein kinase and PI3K-dependent pathways [46], the influence of p130<sup>Cas</sup> on endogenous EGR1 levels was investigated. MCF-7 and TAM-R cells were transiently transfected with siRNAs specific for BCAR1 or a control siRNA (Figure 8A). In MCF-7 cells depletion of p130<sup>Cas</sup> levels by 79% led to a reduction of EGR1 and NAB2 expression of 60% ( $P < .001$ ) and 45% ( $P < .01$ ), respectively. Interestingly, a comparable down-regulation of p130<sup>Cas</sup> in TAM-R cells did not significantly reduce EGR1 levels but decreased NAB2 expression by 50% ( $P < .001$ ). This difference was also indirectly observed in MCF-7 and TAM-R cells as similar EGR1 levels were found in both cell lines but profoundly elevated NAB2 levels in TAM-R cells (Figure 6A). This suggests that the



**Figure 8.** p130<sup>Cas</sup> is involved in the regulation of EGR1 and NAB2 expression. (A) MCF-7 and TAM-R cells were transfected with BCAR1-specific siRNAs or control siRNA. Left panel: WCEs (20  $\mu$ g) were analyzed for p130<sup>Cas</sup>, EGR1, NAB2, and actin (control) expression by WB 48 hours after treatment. Results are presented from one representative experiment of three performed. Right panel: Densitometric analysis of p130<sup>Cas</sup>, EGR1, and NAB2 protein expression in siRNA-treated cells. Average expression levels relative to negative control (black bar, set to 100%) and SD from three experiments performed were determined.  $P$  values were calculated using  $t$  test. \*\* $P < .01$ . \*\*\* $P < .001$ . n.s. indicates not significant. (B) Model of the positive feedback loop regulating p130<sup>Cas</sup> expression in response to environmental stimuli in tamoxifen-sensitive MCF-7 and tamoxifen-resistant TAM-R breast cancer cells. Solid arrows depict direct interactions or up-regulation/activation; dashed arrows indicate additional undefined steps in between leading to an up-regulation of expression. Right panel: Red color indicates enhanced activity or expression (as shown by larger size symbols) of the depicted molecule or process in TAM-R versus MCF-7 cells.

higher p130<sup>Cas</sup> expression in TAM-R cells correlates with increased NAB2 levels.

In summary, these findings suggest that in MCF-7 cells p130<sup>Cas</sup> signaling induces the expression of EGR1 and NAB2, thereby establishing a positive feedback loop to activate its own expression. Importantly, this mechanism seems to be partially altered in the tamoxifen-resistant derivative TAM-R (as compared in Figure 8B).

#### Discussion

Here, we studied the regulation of p130<sup>Cas</sup>/BCAR1 expression in mammary carcinomas. We show for the first time that p130<sup>Cas</sup> mRNA and protein are upregulated in response to PMA treatment, mimicking growth factor signaling. Our results indicate that, in breast cancer cells,

p130<sup>Cas</sup> mediates the induction of the TF EGR1 and its coregulator NAB2. Moreover, EGR1 and NAB2 are, in turn, involved in the up-regulation of p130<sup>Cas</sup>/BCAR1, pointing to the existence of a positive feedback loop in mammary cancers. Importantly, we discovered significant differences in the p130<sup>Cas</sup>/EGR1/NAB2 network among tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. We demonstrated that NAB2 expression is elevated in tamoxifen-resistant breast cancer cells and in advanced and more aggressive human breast cancers, suggesting that NAB2 contributes to the enhanced levels of p130<sup>Cas</sup> found in these cancers.

Previously, we showed that p130<sup>Cas</sup> protein levels are elevated *in vitro* in tamoxifen-resistant breast cancer cells (TAM-R) [19]. Our experiments now revealed increased BCAR1 mRNA expression in the same tamoxifen-resistant TAM-R model system compared with the parental tamoxifen-sensitive MCF-7 cells [19,20]. These data suggest that increased expression of BCAR1 may contribute to the previously observed elevated p130<sup>Cas</sup> protein levels in tamoxifen-resistant cells and possibly in breast cancer. Corresponding to the alternative first exon variants Cas<sub>a</sub> and Cas<sub>b</sub> of murine p130<sup>Cas</sup> that did not show altered activity in binding to focal adhesion kinase [41], we identified the human mRNA orthologs, referred to as BCAR1-1 and BCAR1-1', respectively. The large genomic distance between the first exon of each variant supports the potential usage of alternative promoters.

The *in silico* analysis of the 5' regions of both BCAR1 variants revealed several prominent clusters of overlapping EGR/SP1 sites. Similar *cis* regulatory elements present in the promoters of, for example, the *vascular endothelial growth factor* (VEGF) [28], *PDGF-A* [29], and NAB2 [32], have been shown to be essential for driving EGR1-induced gene expression. With knockdown approaches and overexpression studies, we showed that EGR1 and its coregulator NAB2 control BCAR1/p130<sup>Cas</sup> expression in breast cancer cells. The EGR1 target NAB2 can act as both a corepressor [31,32] and a coactivator [30] of EGR1-mediated transcription. Our studies suggest that NAB2 coactivates the EGR1-mediated increase in p130<sup>Cas</sup> levels and that complex formation of EGR1 and NAB2 may be required to positively regulate p130<sup>Cas</sup> expression. More potent NAB2 regulatory effects on BCAR1/p130<sup>Cas</sup> and EGR1 levels in TAM-R *versus* MCF-7 cells were revealed by NAB2 knockdown experiments. In MCF-7 cells, NAB2 depletion primarily affected BCAR1-1' levels, whereas in TAM-R cells, BCAR1-1, BCAR1-1' mRNAs, and EGR1 mRNA and protein were significantly reduced. This suggests that NAB2 is important for BCAR1-1' expression in both cell lines and, in addition, in TAM-R cells for maintaining EGR1 and BCAR1-1 levels. Importantly, higher basal NAB2 expression was found in TAM-R *versus* MCF-7 cells, and consistently, in ChIP assays performed with these cell lines, significantly increased binding of EGR1 to the BCAR1 5' regions in TAM-R cells was observed. Therefore, our studies suggest that high constitutive NAB2 levels may result in sustained EGR1/NAB2 complex formation important for the recruitment of EGR1 to the BCAR1 regulatory 5' regions and subsequently enhanced BCAR1/p130<sup>Cas</sup> levels in tamoxifen-resistant TAM-R cells. Correspondingly, elevated total BCAR1 mRNA levels and a similar increase in the expression of both exon 1 variants were found in TAM-R cells in comparison to MCF-7 cells (Figure 1, C and D). This suggests that, in this tamoxifen-resistant model, both BCAR1 variants are regulated by likewise mechanisms. However, the different expression patterns of the two mRNA variants in additional breast tissues and a breast cancer cell line (Figure 1C) suggest that additional mechanisms may distinctly regulate the expression of the two p130<sup>Cas</sup> isoforms by, for example, tissue-specific TFs or in response

to different signaling pathways altered during tumor development/progression. Such mechanisms have been observed for the aromatase (*Cyp19*) [47] and ER $\alpha$  genes (*ESR1*) [48]. Because increased NAB2 expression was also found in breast cancer specimens derived from higher-grade tumors and metastases [43–45], this mechanism may contribute not only to the elevated BCAR1/p130<sup>Cas</sup> levels observed in tamoxifen-resistant but also in aggressive/advanced mammary carcinomas [14,15,40].

Binding sites for members of additional TF families, such as NF- $\kappa$ B, p53, and HIF, that are inducible or activated by multiple signaling pathways, were predicted in the 5' regions of the alternative first BCAR1 exons by *in silico* analysis. At this point, their importance in BCAR1 regulation cannot be ruled out, and these sites further point to the inducibility of p130<sup>Cas</sup>/BCAR1 expression. However, p130<sup>Cas</sup>/BCAR1 levels have not yet been linked to cell cycle progression or to be upregulated by extracellular stimuli such as growth factors as it is known for the family member HEF1 [7]. Previous studies primarily focused on the early posttranslational modifications of the p130<sup>Cas</sup> protein in response to different stimuli. Our comprehensive investigation of the expression kinetics in response to phorbol esters, mimicking growth factor signaling, indicated for the first time that p130<sup>Cas</sup> protein and BCAR1 mRNA are inducible following the kinetics of a late response gene in MCF-7 cells. However, BCAR1/p130<sup>Cas</sup> levels were not majorly influenced in TAM-R cells, which may in part be explained by the high basal BCAR1/p130<sup>Cas</sup> expression in these cells. In addition, this implies that the elevated BCAR1/p130<sup>Cas</sup> levels in TAM-R cells might be maintained by intrinsic mechanisms. These altered activities in MCF-7 and TAM-R suggest changes in the regulation of BCAR1 expression during acquired tamoxifen resistance.

In summary, based on the results presented here, we propose the following models for the regulation of BCAR1 expression in tamoxifen-sensitive and -resistant cells (Figure 8B). In the tamoxifen-sensitive MCF-7 cells, upon extracellular stimuli, p130<sup>Cas</sup> is activated/phosphorylated, leading to the activation of downstream signaling pathways [7] that induce the expression of the immediate early gene EGR1 [46]. Subsequently, EGR1 activates the transcription of the delayed early response gene NAB2 [33]. In turn, the EGR1/NAB2 complex mediates the late induction of BCAR1, thereby establishing a positive feedback loop. In contrast, in the tamoxifen-resistant TAM-R cells, p130<sup>Cas</sup> levels are not majorly influenced by phorbol esters, mimicking growth factor signaling. Therefore, we suggest that the constitutively high expression and phosphorylation levels of p130<sup>Cas</sup> [19] lead to enhanced downstream signaling and possibly to a constant induction of NAB2. Because p130<sup>Cas</sup> signaling does not influence EGR1 levels in TAM-R cells, other TFs of, for example, the EGR family [32] may induce NAB2 expression. High NAB2 expression is important for maintaining basal EGR1 levels in these cells and, subsequently, results in enhanced EGR1/NAB2 complex formation and thus the activation of BCAR1 expression. This reciprocal circuit may in part explain why p130<sup>Cas</sup> and NAB2 expression is constitutively upregulated in TAM-R cells.

The results of our studies in identifying EGR1 and NAB2 as regulators of p130<sup>Cas</sup>/BCAR1 may have an impact not only on breast cancer but also on other malignancies. Recently, p130<sup>Cas</sup>/BCAR1 was identified as a key player in prostate carcinomas, and increased levels were associated with aggressiveness and progression of this disease [49,50]. Because EGR1 overexpression has been described in multiple studies to be crucial for driving prostate cancer progression [51–53], it may also contribute to the up-regulation of p130<sup>Cas</sup> in this type of cancer. It would be interesting to see whether the p130<sup>Cas</sup>/EGR1/NAB2 circuit

or alterations of this network may play a role in promoting these carcinomas as well.

Further deciphering of the precise mechanism of the p130<sup>Cas</sup>/EGR1/NAB2 feedback loop may help to understand the molecular biology not only of breast cancers but also of other malignancies and to identify new targets for the development of novel treatment strategies.

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