

Disruption of the Y-Box Binding Protein-1 Results in Suppression of the Epidermal Growth Factor Receptor and HER-2

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Abstract

The overexpression of the epidermal growth factor receptor (EGFR) and HER-2 underpin the growth of aggressive breast cancer; still, it is unclear what governs the regulation of these receptors. Our laboratories recently determined that the Y-box binding protein-1 (YB-1), an oncogenic transcription/translation factor, induced breast tumor cell growth in monolayer and in soft agar. Importantly, mutating YB-1 at Ser¹⁰², which resides in the DNA-binding domain, prevented growth induction. We reasoned that the underlying cause for growth attenuation by YB-1(Ser¹⁰²) is through the regulation of EGFR and/or HER-2. The initial link between YB-1 and these receptors was sought by screening primary tumor tissue microarrays. We determined that YB-1 ($n = 389$ cases) was positively associated with EGFR ($P < 0.001$, $r = 0.213$), HER-2 ($P = 0.008$, $r = 0.157$), and Ki67 ($P < 0.0002$, $r = 0.219$). It was inversely linked to the estrogen receptor ($P < 0.001$, $r = -0.291$). Overexpression of YB-1 in a breast cancer cell line increased HER-2 and EGFR. Alternatively, mutation of YB-1 at Ser¹⁰² > Ala¹⁰² prevented the induction of these receptors and rendered the cells less responsive to EGF. The mutant YB-1 protein was also unable to optimally bind to the EGFR and HER-2 promoters based on chromatin immunoprecipitation. Furthermore, knocking down YB-1 with small interfering RNA suppressed the expression of EGFR and HER-2. This was coupled with a decrease in tumor cell growth. In conclusion, YB-1(Ser¹⁰²) is a point of molecular vulnerability for maintaining the expression of EGFR and HER-2. Targeting YB-1 or more specifically YB-1(Ser¹⁰²) are novel approaches to inhibiting the expression of these receptors to ultimately suppress tumor cell growth. (Cancer Res 2006; 66(9): 4872-9)

Introduction

The Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor that was isolated by screening an expression library for DNA-binding proteins that interact with the epidermal growth factor receptor (EGFR) enhancer and the HER-2 promoter

(1). Curiously, this was reported nearly two decades ago, but follow-up studies confirming the interaction *in vivo* or correlating expression in patient samples have not been published. Since this time, however, YB-1 has become widely appreciated for its role in cancer. For example, the YB-1 protein is highly expressed in cancers of the breast (2), prostate (3), colon (4), ovary (5), lung (6), and bone (7). YB-1 functions as a newly described breast cancer oncogene (8). Interestingly, the expression of YB-1 in the mammary gland results in the development of hyperplastic nodules and the eventual development of tumors with 100% genetic penetrance (8). To understand how YB-1 may alter the cancer phenotype, Royer et al. expressed it in preneoplastic breast epithelial cells, which caused them to be resistant to doxorubicin (2). The presence of YB-1 correlated with an increase in the mRNA expression of multidrug resistant gene-1 (*MDR-1*) and its protein product, P-glycoprotein. Taking this one step further, they showed that the expression of YB-1 correlated with *MDR-1*/P-glycoprotein expression in primary breast cancers. They also concluded that YB-1 was not detected in normal breast tissue but was highly expressed in tumors (2), a finding that was subsequently confirmed (9, 10). Therefore, YB-1 is preferentially expressed in breast tumors relative to normal tissue and may play a role in drug resistance. More recently, YB-1 was reported to stimulate the proliferation of preneoplastic breast cancer cells by inducing cyclin A and B mRNA (11). Direct evidence for the role of YB-1 in cell proliferation came from gene knockdown experiments where the loss of one allele in chicken lymphocytic cells inhibited growth by >70% (12). More recently, silencing YB-1 with antisense or small interfering RNA (siRNA) inhibited the growth of rat mesangial cells by >50%, at least in part through the suppression of DNA polymerase α (13). It, therefore, seems that YB-1 is centrally important for positively regulating cell growth and the development of mammary tumors. However, the underlying mechanisms are still not understood.

Our laboratory recently reported that the serine/threonine kinase Akt binds to YB-1 and phosphorylates it on Ser¹⁰² (14). The functional importance of this observation was revealed in studies showing that the expression of YB-1 in MCF-7 breast cancer cells enhanced tumor cell growth in monolayer as well as in soft agar (14). However, expression of YB-1 mutated to Ala¹⁰² failed to enhance growth under the aforementioned conditions. These data indicated that the Ser¹⁰² residue of YB-1 was essential for providing a growth advantage to breast cancer cells. The Ser¹⁰² site is located in the cold shock domain, which is involved in nuclear transport (11) and DNA binding (15). We further determined that the loss of this phosphorylation site on YB-1 attenuated nuclear transport (14). Because the Ser¹⁰² site resides in the DNA-binding motif of YB-1, we have not ruled out the possibility that this site could also

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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be important for regulating protein/DNA interactions. To understand how YB-1 promotes breast cancer cell growth, we revisited the possibility that YB-1 may act as a transcription factor that regulates genes involved in cell proliferation. Since YB-1 has been shown to bind to the regulatory regions of EGFR and HER-2 (1), which are established proteins involved in breast cancer, we suspect that the growth advantage afforded by YB-1 is via the induction of these receptor tyrosine kinases.

The human EGFR family (HER-1 or EGFR, HER-2, HER-3, and HER-4) is important for sustaining the growth of breast cancer cells (16). EGFR and HER-2 are overexpressed in ~15% (17) and ~20% of breast cancers (18), respectively. In the case of EGFR, overexpression is generally due to transcriptional activation (19). HER-2 on the other hand is more commonly overexpressed as a result of amplification, but transcriptional up-regulation also occurs (20). The underlying reasons for transcriptional activation of EGFR and/or HER-2 have not been fully elucidated. The transcription factors AP-2 (21) and PEA3 (22) seem to contribute to the expression of HER-2 in breast cancer. In other types of cancer, however, HER-2 overexpression does not correlate with the presence of AP-2 or gene amplification, suggesting that additional factors are involved (23). Despite the clinical importance of EGFR, the molecular mechanisms responsible for its expression are still being discovered, although a number of cis- and trans-activating elements, including the EGF-responsive DNA-binding protein-1 (24), c-jun (25), and SP-1 (26). Recently, YB-1 has also been shown to regulate EGFR in human mammary epithelial cells (27). Berquin et al. found that elevated expression of YB-1 consequent to induced EGFR and provided an obvious growth advantage to cells representing an early stage of breast cancer. This discovery led us to address whether YB-1 is correlated with EGFR or HER-2 in primary breast tumors, and if YB-1(Ser¹⁰²) was specifically needed for the regulation of these receptors.

Materials and Methods

Tumor tissue microarray. The patient characteristics and their tumors were previously reported by us (14). Tissue microarrays were constructed from 438 consecutive cases of breast carcinoma from the archives at Vancouver General Hospital, as described previously (28). Duplicate 0.6-mm cores of formalin-fixed, paraffin-embedded cores were used for array construction. Tissue microarray slides were immunostained for EGFR (29) and HER-2 as previously described (30). To detect YB-1, the tissues underwent antigen retrieval by heating to 100°C in a citrate buffer for 30 minutes, and the slides were incubated with anti-YB-1 (1:2,000 dilution, COOH-terminal epitope, from Dr. Colleen Nelson, British Columbia Cancer Agency Vancouver, Canada) overnight. YB-1 was then detected using the LSAB+ system (DAKO, Carpinteria, CA) according to our previously published method (14). For interpretation of immunostaining, only cores containing invasive carcinoma were considered interpretable. Results from duplicate cores were combined to give a single result per case. Where there were discordant results for the duplicate cores, the higher result was accepted for that case as described previously (28). For YB-1, tumors showing no staining or weak focal staining were considered to be YB-1 negative, whereas moderate or intense staining was considered positive. YB-1 immunostaining was predominantly found in the cytoplasm. The statistical analyses were based on the amount of YB-1 expressed in the cytoplasm. Although nuclear YB-1 was only obvious in 7 of 389 cases by immunostaining, it was clearly detectable when cytoplasmic and nuclear fractions were purified from breast cancer cell lines. Given these data, we expect that the epitope is masked during formalin fixation; therefore, the antibody is not able to detect YB-1 in the nucleus.

Tissue microarray slides were digitized using Bliss slide scanner based on Olympus BX61 microscope (Bacus Laboratories, Inc., Lombard, IL). Nine

pictures were taken from each core, and the pixel dimensions were tiled to create a composite image. The images were viewed using the WebSlide Browser program (Bacus Laboratories), and all immunostained slides are available on our web site.⁶ The Kaplan-Meier curve was generated using a nonparametric method for survival. Survival estimates are based on the weighed differences between actual and expected numbers of deaths at the observed time points. The log-rank test factors in all deaths equally, whereas Breslow tests give more weight to early deaths. All statistical analyses were done using the SPSS 11.0 statistical package. Cox regression model is used for multivariate survival analysis. Tumor collection and analyses were done in accordance with the guidelines established by the Vancouver General Hospital Research Ethics Board.

Cell lines and reagents. The MCF-7, MDA-MB-231, and MDA-MB-453 breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). The COOH-terminal polyclonal antibody to YB-1 was provided by Dr. Colleen Nelson. The NH₂-terminal chicken anti-human YB-1 polyclonal antibody was custom raised to the NH₂ terminus MSSEAEYQPPAA (27). Flag:YB-1 transgenes were detected using the M2 mouse monoclonal antibody to Flag (Sigma, St. Louis, MO). Flag:YB-1 (Ser¹⁰²) was mutated to Flag:YB-1(Ala¹⁰²) by site-directed mutagenesis as previously reported by us (14).

Western blotting. Proteins were isolated from MCF-7 cells expressing either the empty vector, Flag:YB-1, or Flag:YB-1(Ala102). Cells were 80% confluent when harvested by scraping. Cells were lysed in ELB buffer [5 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8), 1% Triton X-100, 1% deoxycholate, and 0.1% SDS] with protease inhibitors and sheared through a 21-gauge needle. Proteins (100 µg/lane) were run on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane at 40 V overnight at 4°C. The membrane was then probed with anti-EGFR (1:275; StressGen, San Diego, CA) and anti-HER-2 (1:500; Abcam, Cambridge, MA) antibodies. Vinculin (1:2,000; Sigma clone Vin 11-5, V4505 antibody) was used as a loading control. MCF-7 cells expressing the various constructs were also serum starved overnight then stimulated with EGF (40 ng/mL) for 30 minutes. The extracts were then evaluated for activation of signaling through the mitogen-activated protein kinase (MAPK) pathway using phospho-extracellular signal-regulated kinase 1/2 (p-Erk 1/2) antibody (Thr²⁰²/Tyr²⁰⁴, 1:1,000; Cell Signaling, Beverly, MA). Activation of EGFR was reversed using Iressa (1.0 or 10 µmol/L). In this case, MCF-7(Flag:YB-1) cells were pretreated with either Iressa or DMSO for 30 minutes and then stimulated with EGF for 30 minutes. Signal transduction through the MAPK pathway was evaluated using the p-Erk antibody as described above.

Real-time quantitative reverse transcription-PCR. RNA was isolated from MCF-7 cells [empty vector, Flag:YB-1, or Flag:YB(Ala102)] grown in log phase. The RNA was reverse transcribed and amplified using HER-2- or EGFR-specific primers and probes (Applied Biosystems, Foster City, CA). TATA box binding protein mRNA was measured as a housekeeping gene (Applied Biosystems) as previously described (31). Each sample was analyzed in replicates of four on two separate occasions.

Chromatin immunoprecipitation. Chromatin immunoprecipitation assays were done using the ChIP-IT kit (Active Motif 101198). The protocol can be downloaded from the Active Motif web site.⁷ Buffers and reagents were provided by the ChIP-IT kits unless otherwise indicated. In brief, MCF-7(Flag:YB-1) and MCF-7[Flag:YB-1(Ala102)] cells were grown on 15-cm plates in phenol red-free RPMI with 5% fetal bovine serum and 400 µg/mL G418. At 80% confluency (8 × 10⁶ cells), cells were fixed for 10 minutes with 1% formaldehyde. After quenching the reaction with glycine for 5 minutes, cells were treated with lysis buffer and extracted using a dounce homogenizer. The nuclei were isolated and chromatin was sonicated in shearing buffer using a Cole Parmer Ultrasonic Processor at 25% power with 10 pulses of 20 seconds each and a 30-second rest on ice between each pulse to give chromatin fragments between 200 bp and 1 kb. The input DNA was evaluated in several ways before chromatin immunoprecipitation. First, a sample of the sheared DNA was evaluated from each sample. The degree of

⁶ <http://www.gpec.ubc.ca>.

⁷ <http://www.activemotif.com>.

shearing and the amount of input DNA seemed equal. Genomic DNA was amplified with each of the primer sets to produce a single gene product of the expected size. To ensure that the input DNA was equal before performing chromatin immunoprecipitation, aliquots of the input DNA from each sample was titrated (1, 3, 5 μ L). The input DNA was amplified with primers to EGFR2a, and the amount of PCR products from MCF-7/Flag:YB-1 and MCF-7/Flag:YB-1(Ala102) seemed the same. Given these data, we were confident that our chromatin immunoprecipitation starting material was unbiased.

Chromatin immunoprecipitation was then done using an antibody to Flag. Chromatin was then precleared with salmon sperm DNA/protein G agarose and incubated with 3 μ g anti-FLAG M2 monoclonal antibody (Sigma F3165) overnight at 4°C. Chromatin incubated with 3 μ g mouse IgG overnight at 4°C was used as a negative control. Salmon sperm DNA/protein G agarose was subsequently incubated with the chromatin and antibody for 1.5 hours at 4°C. The immunoprecipitated material was then washed and eluted from the beads. To reduce nonspecific binding, the washing steps were modified so that the immunoprecipitated material was washed twice with chromatin immunoprecipitation buffer, five times with wash buffer 1, twice with wash buffer 2, and thrice with wash buffer 3. The immunoprecipitated material was incubated in 200 mmol/L NaCl and 10 μ g RNase A overnight at 65°C, to reverse cross-link and to remove RNA. The proteins were then removed by proteinase K treatment, and DNA was purified using the DNA purification minicolumns provided with the kit. Eluted DNA (5 μ L) was amplified by PCR with the following primers: EGFR1b, 5'-TCGCCCAACGCCACAAC-3' (forward) and 5'-ACACGCCCTTACCTTTCTTTCTCCAG-3' (reverse); EGFR2a, 5'-CCGCGAGTTTCCCTCGCATTTCT-3' (forward) and 5'-CCTTCCCCTTCCCTTCTTTGTTTAC-3' (reverse); EGFR2b, 5'-TCCCATTTGCC-TTCTCTAGTTTGTTC-3' (forward) and 5'-GTCCACCCATCCCC-ACTGTTCTTCTC-3' (reverse); EGFR3, 5'-TTCAGAAACCATTTCTTCT-3' (forward) and 5'-GCTTCTGCACACCTGGGCTGAG-3' (reverse). The PCR program was set with an initial melting step at 94°C for 3 minutes, then 35 cycles of (94°C for 20 seconds, 59°C for 30 seconds, and 72°C for 30 seconds). The PCR products were then analyzed on agarose gel by electrophoresis. PCR products were taken out after 32, 36, and 40 cycles to make sure that the reaction was in the linear range. We determined that 35 cycles was sufficient to produce amplicons in the linear range; therefore, we used this to establish optimal PCR conditions for the EGFR primers.

Endogenous YB-1 was subjected to chromatin immunoprecipitation using a chicken anti-human polyclonal antibody (27). The MDA-MB-231 cells were selected for part of this study because they express high levels of EGFR (32), and the MDA-MB-453 cells were used because they express high levels of HER-2 in the absence of gene amplification (33). Our protocol was adapted from the *Upstate Cell Signaling Solutions Chromatin Immunoprecipitation Assay Kit Manual*. In brief, 7.5×10^6 cells per plate were treated with 1% formaldehyde in growth media then harvested and washed with PBS. Pellets were resuspended in lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris (pH 8.1)] and sheared on ice by sonication (25% power, 10 cycles of 20 seconds on, 30 seconds off). Chromatin solution was diluted 5-fold in immunoprecipitation buffer [0.01% SDS, 1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris (pH 8.1), 500 mmol/L NaCl] and precleared with PrecipHen beads (Aves Labs, Tigard, OR) in lysis buffer (1:3). The solution was then incubated with anti-YB-1, chicken IgY, or preimmune chicken IgY overnight. Complexes were incubated with 100 μ L of PrecipHen beads in lysis buffer (1:3) for 1.5 hours. Immunoprecipitates were washed once with low salt buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris (pH 8), 150 mmol/L NaCl], once with high salt buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris (pH 8), 500 mmol/L NaCl], once with LiCl buffer [0.25 mol/L LiCl, 1% IGEPAL, 1% deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris (pH 8)], and twice with Tris-EDTA buffer [10 mmol/L Tris (pH 8), 1 mmol/L EDTA]. Protein/DNA complexes were eluted twice with elution buffer (1% SDS, 0.1 mol/L NaHCO₃) and brief vortex. Eluates and input controls were treated with 5 mol/L NaCl at 65°C overnight followed by proteinase K at

45°C for 1 hour. DNA was purified by phenol/chloroform extraction and alcohol precipitation. PCR was done with primers to EGFR as described above or to HER-2: HER-2 primers, 5'-AGGGGCTCCAAATAGAATGT (forward), 5'-AATTTGGGAGGAGACAGTCA (reverse). The HER-2 primers produced a 464-bp product that spanned -978 to -514. The DNA was amplified using the following PCR conditions: 95°C for 2 minutes, 40 cycles of 95°C for 20 seconds, 57°C for 30 seconds, and 72°C for 30 seconds.

siRNA. siRNA targeting YB-1 was expressed in pSuperDuper as previously described (13). This targeting vector was used to silence YB-1 in MCF-7(Flag:YB-1) cells. The MCF-7(Flag:YB-1) cells were plated in a six-well dish and transfected with 0.8 μ g of plasmid DNA. The plasmid DNA was added to the transfection reagent (LipofectAMINE 2000, Invitrogen, Carlsbad, CA) at a ratio of 1:12.5 (siYB-1 1) or 1:25 (siYB-12); 96 hours later, the cells were lysed in ELB buffer (described above) and evaluated for changes in YB-1, EGFR, and HER-2 by immunoblotting. YB-1 was also silenced using the pSuper vector (generous gift from Dr. Thomas Iftner, Tuebingen, Germany; ref. 34) in MDA-MB-453 cells. To extend the effect of siRNA targeting YB-1 even further, we also used oligonucleotides to inhibit its expression. The oligonucleotides (AGAAGGUCAUCGCAACGAA; Dharmacon, Lafayette, CO) were transfected into MDA-MB-231 cells at a concentration of 20 nmol/L into the cells using Oligofectamine (Invitrogen), and proteins were isolated 72 hours later. The negative control oligonucleotide (Dharmacon) was also transfected at a concentration of 20 nmol/L. The effect of tumor cell growth was assessed after 1 week using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Each of the conditions was evaluated in replicates of 12.

Results

YB-1 correlates with EGFR and HER-2 in primary breast cancers. In our tissue microarrays of 389 invasive breast cancer cases, YB-1 protein was coordinately expressed with HER-2, EGFR, and the proliferation marker Ki67 (Table 1). YB-1 expression was also inversely correlated with the estrogen receptor (ER). However, nodal status and tumor size did not relate to high levels of YB-1. YB-1 was moderately to highly expressed in 43% (167 of 389) of cases, which showed decreased patient survival based on Breslow ($P = 0.0065$) and log-rank ($P = 0.01$) analyses, respectively (Supplementary Fig. S1). It is noteworthy that the Breslow test is predictive of early deaths. Thus, our data indicate that YB-1

Table 1. Expression of YB-1 protein as it relates to clinicopathologic markers of breast cancer

Marker	Fishers exact	Patient no.
EGFR	0.000*	337
HER-2	0.008*	319
ER	0.000*	304
Ki67	0.002*	304
Nodal status	0.909	350
Size (>2 cm)	0.57	321

NOTE: The expression of YB-1 was inversely correlated with the ER and positively associated with Ki67, HER-2, and EGFR. There was no relationship between YB-1 and the tumor size at the time of diagnosis using ± 2 cm as a cutoff. YB-1 also did not correlate with the presence of breast cancer in the lymph nodes. Statistical significance was determined by the Fisher's exact test using SPSS software.

*Correlations were considered significant if $P < 0.05$ (Fisher's exact test).

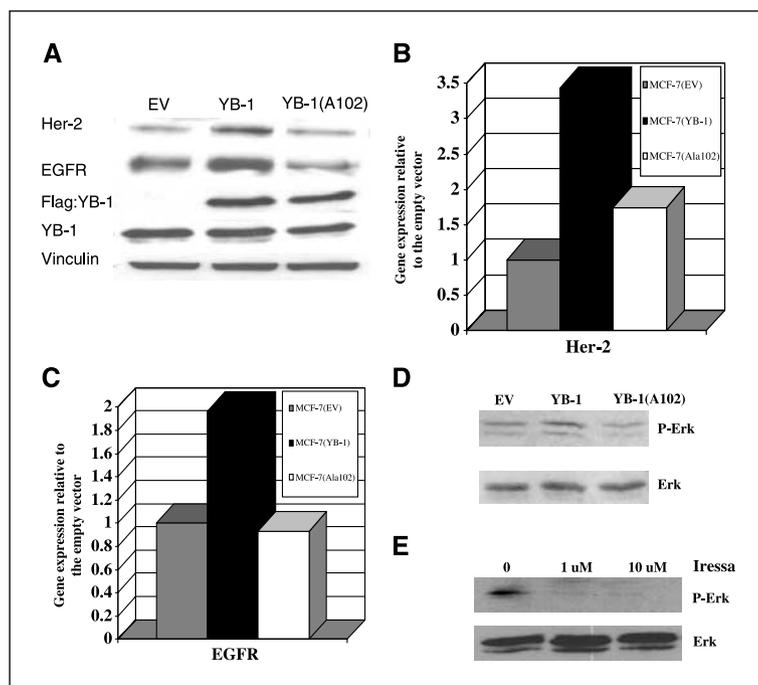
expression is associated with early death due to breast cancer. We then sought to determine whether YB-1 is an independent prognostic factor for breast cancer. In a multivariate analysis, YB-1 had independent prognostic significance ($P = 0.019$; Supplementary Table S2); the Cox regression model included HER-2 ($P = 0.018$), lymph node status ($P < 0.001$), and tumor size ($P = 0.022$), which are also known to be independently associated with poor survival. YB-1 had a comparable effect on the relative risk of dying from breast cancer to expression of either HER-2 or tumor size. The relative risk ratios for YB-1, HER-2, lymph node status, and tumor size were 1.792, 1.994, 2.436, and 1.788, respectively. With this information, we began to explore the possibility that YB-1 may have a direct effect on the regulation of EGFR and/or HER-2.

EGFR and HER-2 are regulated by YB-1. To understand whether there was a mechanistic link between YB-1 and the expression of EGFR and HER-2, we stably transfected Flag:YB-1 into MCF-7 cells. Comparisons were made to cells expressing mutant YB-1(Ala¹⁰²) where the Ser¹⁰² site was mutated by site-directed mutagenesis. Interestingly, HER-2 and EGFR proteins were induced as a result of YB-1 overexpression (Fig. 1A). However, mutating YB-1 on its DNA-binding domain from Ser¹⁰² to Ala¹⁰² prevented the induction of those proteins (Fig. 1A). Furthermore, the expression of YB-1 caused an increase in EGFR and HER-2 mRNA, whereas the mutant did not (Fig. 1B and C). Cells expressing Flag:YB-1 were also more responsive to EGF because there was more signaling through the MAPK pathway (Fig. 1D, lane 2) compared with the cells expressing either the YB-1(Ala¹⁰²) (Fig. 1D, lane 3) or empty vector (Fig. 1D, lane 1). Conversely, when the MCF-7(Flag:YB-1) cells were pretreated with the EGFR inhibitor Iressa (using either 1 or 10 $\mu\text{mol/L}$) and then treated with EGF, signaling through the MAPK pathway was inhibited (Fig. 1E).

YB-1 binds directly to the EGFR and HER-2 promoters. Given that YB-1 is a transcription factor, we addressed the question of whether it binds directly to the EGFR promoter. The first 2 kb of the EGFR promoter was inspected, and nine putative YB-1-responsive elements (YRE) were identified (Fig. 2A). DNA/protein

complexes were isolated from MCF-7/Flag:YB-1 and MCF-7/Flag:YB-1(Ala¹⁰²) expressing cells. The anti-Flag:YB-1 antibody was able to precipitate DNA that was amplified with each of the EGFR primer sets, suggesting that YB-1 bound directly to the EGFR promoter region (Fig. 2B, lanes 1-4). However, Flag:YB-1(A102) did not bind to the EGFR promoter amplified by the 1b and 2a primers (Fig. 2B, lanes 5-6). There also seemed to be somewhat less binding to the regions amplified by the 2b and 3 primers (Fig. 2B, lanes 7-8). We confirmed that Flag:YB-1 specifically bound to DNA because there was virtually undetectable amplification of the IgG controls (Fig. 2B, lanes 9-12). Furthermore, we established that the input DNA for each sample was equal by amplifying the input DNA. MCF-7(YB-1) and MCF-7(A102) input DNA was titrated (1, 3, and 5 μL) and amplified with the EGFR2a primers, yielding the same amount of PCR products (Fig. 2B, lanes 13-18). These data show that the input was the same and notably that the EGFR2a primers amplified the DNA from the MCF-7(A102) sample, which rules out the possibility that the lack of amplification in the YB-1 chromatin immunoprecipitation procedure was because the primers were unable to amplify this sample. We therefore concluded that the loss of YB-1(Ser¹⁰²) disrupted binding to both the EGFR 1b and EGFR 2a sites located in the proximal promoter. These data indicated that YB-1(Ala¹⁰²) does not optimally bind to the first 1 kb of the EGFR promoter, which could explain why loss of Ser¹⁰² prevents the induction of EGFR. Following this series of experiments, chromatin immunoprecipitation was done on the MDA-MB-231 cells to confirm that endogenous YB-1 bound to the EGFR promoter. The primers EGFR1b, EGFR2a, and EGFR2b amplified products of the expected size; however, EGFR3 did not (Fig. 2C, lanes 1-3). Amplification was not due to nonspecific binding to the IgY, given that the control reactions produced little or no product (Fig. 2C, lanes 5-8). The sheared input DNA (Fig. 2D, lanes 1-4) and the no-template DNA reactions (Fig. 2D, lanes 5-8) served as positive and negative controls for chromatin immunoprecipitation, respectively. The primers were also used to amplify genomic DNA (Fig. 2D lanes 9-12). We concluded that Flag:YB-1 and endogenous

Figure 1. Overexpression of YB-1 but not YB-1(Ala¹⁰²) results in induction of HER-2 and EGFR. **A**, the stable expression of Flag:YB-1 increased levels of HER-2 and EGFR protein, whereas the mutant Flag:YB-1(Ala¹⁰²) did not. Proteins were isolated from cells stably expressing either the empty vector (EV), Flag:YB-1, or Flag:YB-1(Ala¹⁰²). The relative levels of the transgenes were assessed using an antibody to Flag. Vinculin was examined to ensure that the samples were equally loaded. Expression of Flag:YB-1 increases **(B)** EGFR mRNA and **(C)** HER-2 mRNA. The mRNA was harvested from cells growing in log phase, reverse transcribed, and then amplified for HER-2 or EGFR expression by quantitative reverse transcription-PCR. Each cell line was evaluated in quadruplicates, and the data were normalized to TATA box binding protein. The relative level of induction was compared with the MCF-7(EV) cell line. **D**, cells that overexpress YB-1 are more responsive to EGF stimulation. MCF-7 cells expressing the empty vector, Flag:YB-1, or Flag:YB-1(Ala¹⁰²) were serum starved for 24 hours then stimulated with EGF (40 ng/mL) for 30 minutes. The cell lines were compared for relative amounts of p-Erk1/2. Total Erk was included as a control for sample loading. **E**, the signaling through Erk was EGFR dependent because it was reversible with Iressa. MCF-7(Flag:YB-1) cells were serum starved overnight, then stimulated with EGF (40 ng/mL) for 30 minutes or pretreated with Iressa (1 or 10 $\mu\text{mol/L}$), and then stimulated with EGF at the same concentration. Proteins were evaluated for p-Erk.



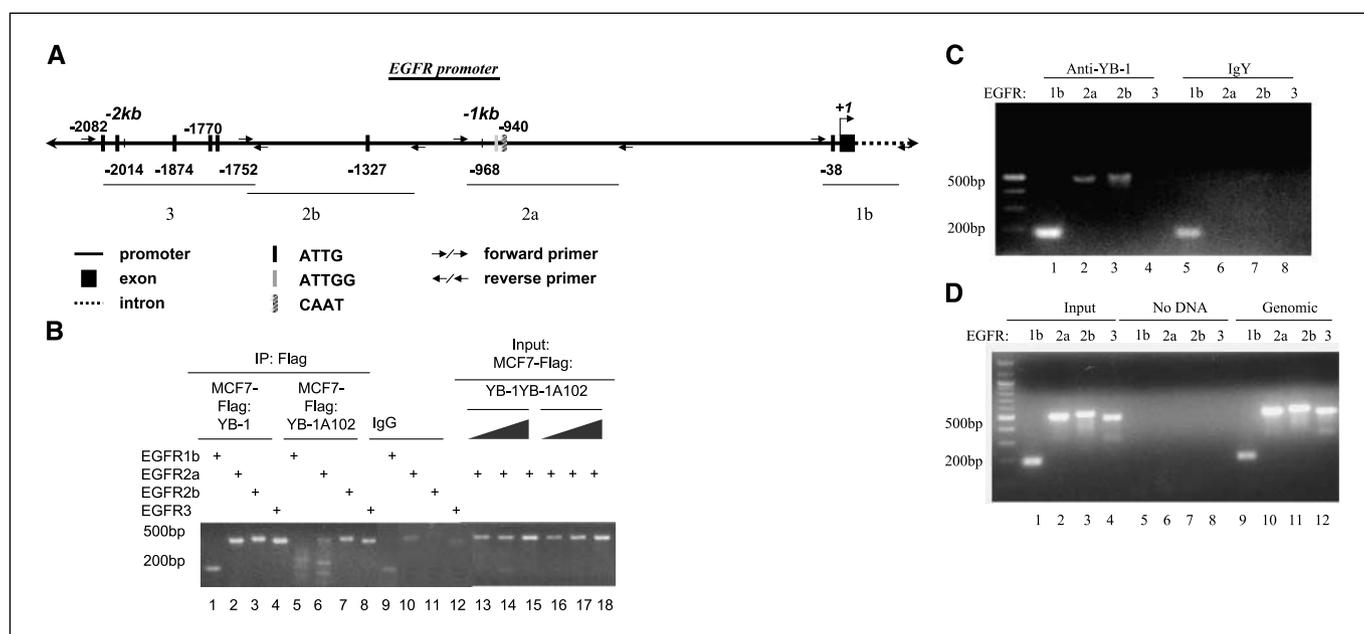


Figure 2. YB-1 binds to the EGFR promoter. *A*, schematic representation of potential YB-1 binding sites and the location of the primers used for chromatin immunoprecipitation. *B*, DNA/protein complexes were cross-linked from MCF-7/Flag:YB-1 and MCF-7/Flag:YB-1(A102) cells. Flag:YB-1 bound to the EGFR promoter at all loci (*lanes 1-4*), respectively, whereas Flag:YB-1(A102) did not bind to the 1b or 2a sites (*lanes 5-6*). Flag:YB-1(A102) also bound to the 2b and 3 sites but with less affinity than Flag:YB-1 (*lanes 7-8*). Nonspecific binding to the IgG was negligible (*lanes 9-12*). The input DNA for each sample was also titrated (1, 3, and 5 μ L) and then amplified using EGFR2a primers to show that the sample input was equivalent (*lanes 13-18*). *C*, endogenous YB-1 was immunoprecipitated from MDA-MB-231 cells, and chromatin immunoprecipitation was done using primers to EGFR1b, EGFR2a, EGFR2b, EGFR3 (*lanes 1-4*). The chicken IgY chromatin immunoprecipitation was also done as a negative control (*lanes 5-8*). *D*, input DNA (*lanes 1-4*), and no DNA control reactions (*lanes 5-8*) were amplified for EGFR. The EGFR primers were also used to amplify genomic DNA from the MDA-MB-231 cells (*lanes 9-12*).

YB-1 bound to the EGFR promoter, and that loss of the Ser¹⁰² site on YB-1 destabilized the interaction. To extend this observation further, we questioned whether there would be differences in EGFR reporter activity by comparing the levels in MCF-7(YB-1) cells to MCF-7(A102) cells. An EGFR reporter construct spanning -1109 to -16 was used because this was the region that encompassed the most relevant YREs based on chromatin immunoprecipitation. We found that disruption of the YB-1 at S102 also showed a trend toward less EGFR reporter activity (data not shown). These data indicated that the loss of the S102 site on YB-1 correlated with an inhibition in EGFR reporter activity. We concluded that *EGFR* is an YB-1-responsive gene that depends on the S102 site for optimal DNA binding.

Similarly, we investigated whether disrupting YB-1 at Ser¹⁰² has an effect on the binding of this transcription factor to HER-2. There are four potential YREs on the HER-2 promoter (Fig. 3A). Primers were designed to capture three of four binding sites, excluding the one that was juxtaposed to the AP-2 and Ets sites. Flag:YB-1 bound to HER-2 (Fig. 3B, lane 1), whereas the Flag:YB-1(Ala¹⁰²) mutant did not (Fig. 3B, lane 2). We further questioned whether endogenous YB-1 also bound to HER-2. To interrogate this possibility, we used the MDA-MB-453 cells because they express very high levels of HER-2 in the absence of gene amplification. Endogenous YB-1/DNA complexes were pulled down, and the precipitated DNA was amplified for HER-2. In this breast cancer cell line, YB-1 specifically bound the HER-2 promoter (Fig. 3C, lane 1), further supported by the absence of binding to the IgG (Fig. 3C, lane 2). Input sheared DNA served as a positive control (Fig. 3C, lane 3).

Knocking down YB-1 expression decreases HER-2 and EGFR. We determined whether the expression of HER-2 or EGFR was dependent on YB-1. The expression of YB-1 was silenced using

siRNAs. MCF-7(Flag:YB-1) cells were transfected with pSuperDuper vector expressing siRNA targeting YB-1, and proteins were harvested 96 hours later. The targeting vector suppressed the expression of Flag:YB-1 and endogenous YB-1 by ~50% to 70% (Fig. 4A, lanes 2-3) compared with the empty vector (Fig. 4A, lane 1). Loss of YB-1 expression correlated with a decrease in EGFR protein expression (Fig. 4A, middle). Two different concentrations of the targeting vector were used (siYB-1 #1 and siYB-1 #2), and both resulted in suppression of EGFR (Fig. 4A) and HER-2 (Fig. 4B). Likewise, knocking down YB-1 with oligonucleotides attenuated the expression of EGFR in MDA-MB-231 cells (Fig. 4C). Furthermore, silencing its expression with a pSuper vector suppressed the expression of HER-2 in MDA-MB-453 cells (Fig. 4D). These data indicated that three different targeting sequences against YB-1 inhibited the expression of HER-2 and EGFR. Finally, knocking down YB-1 ultimately resulted in a remarkable 46% suppression in tumor cell growth (Fig. 4E). This was done to specifically show that EGFR overexpressing cells are vulnerable to growth inhibition by disrupting YB-1.

Discussion

In this study, we show for the first time that YB-1 is coordinately expressed with EGFR and HER-2 in primary human breast cancer specimens. Moreover, the expression of these receptors is dependent on functional YB-1. Using siRNA to knock down the expression of YB-1, we were able to silence the expression of the receptors and ultimately inhibit tumor cell growth. We also determine that YB-1 is an important independent prognostic factor for breast cancer, further supporting it as a molecular target for cancer therapy. The latter was previously proposed by Janz et al.

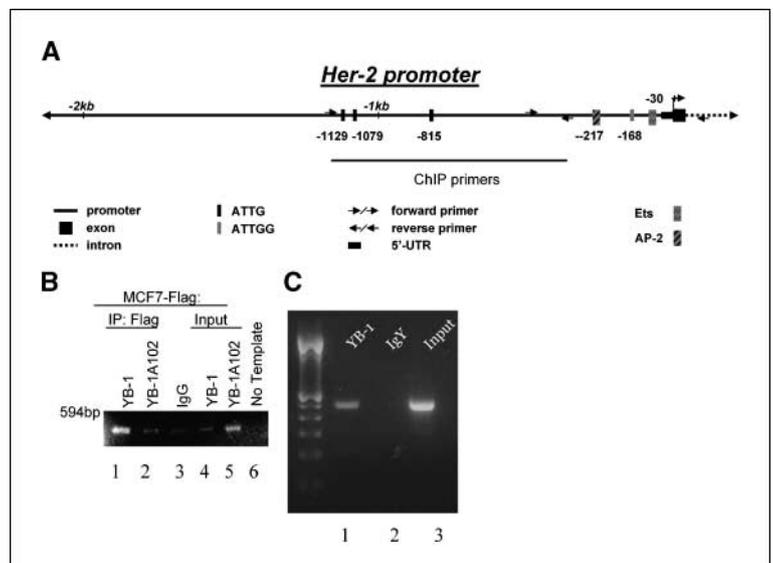
in a pilot study ($n = 42$ cases) reporting on a correlation of YB-1 expression and poor survival for patients with low-risk breast cancer (35); however, their median follow-up time was only >5 years. With a much larger cohort and longer follow-up (20 years), we are able to validate this trend. Although our studies are similar in this respect, we observe contrary to their findings (a) a strong inverse correlation between YB-1 and ER, and (b) a positive correlation of YB-1 with the expression of HER-2. These inconsistencies could be due to differences in the way the proteins were detected, as the antibodies used to detect YB-1 target different domains of the protein (35) and antigen retrieval may affect the results. Alternatively, the differences may relate to the patient populations examined. Nevertheless, both data sets indicate that YB-1 is a powerful risk group discriminator. Janz et al. were able to show that YB-1 is particularly valuable in predicting which low-risk patients would subsequently relapse and die from breast cancer. Our data broaden the prognostic value of YB-1 in patients at high risk (i.e., those treated with chemotherapy and radiation), by showing that YB-1 expression is a predictive marker using univariate and multivariate analyses.

Consistent with our data, the gene expression of YB-1 (also referred to as nucleosome sensitive element protein-1) clusters in cDNA microarrays with highly aggressive types of breast cancer that are almost exclusively ER negative (10, 36). To understand the role of YB-1 in breast cancer further, one may consider whether this protein falls into defined subtypes of disease. It is now well recognized that there exist subtypes of breast cancer beyond the simple classification based on the ER status. A study by Sorlie et al. used gene expression profiles to classify breast cancer into luminal A, luminal B, normal breast-like, ErbB2 (HER-2), and basal-like subtypes (10). In a study conducted by van't Veer using microarrays from primary breast tumors, YB-1 was found in a cluster of genes that were basal-like; this was observed in a cohort of women who had tumors that also possessed BRCA-1 mutations (36). In a second study, YB-1 did not fall into any of those categories; rather, it was placed in a novel unknown cluster (10). Upon closer inspection, it is evident that YB-1 is most intensely expressed in cluster groups representing basal-like tumors, which are known to express high levels of

EGFR (17). One possible explanation for this apparent contradiction and failure of clear-cut classification into the basal-like group in the study conducted by Sorlie relates to the observation that unlike basal markers, YB-1 is not expressed in normal breast tissue (10). Validated basal-like markers, such as cytokeratin 5 (17), are highly expressed in both normal and basal-like breast cancer tissues, whereas YB-1 is only found in tumors (10). This feature supports the notion that YB-1 is an attractive molecular target for cancer treatment, especially for basal-like breast cancers with inherent poor prognosis for relapse-free survival (10, 37). Data collected from breast cancer cell line profiling also point toward a connection between the basal-like phenotype and YB-1. By this approach, Bertucci et al. determined that SUM149 cells display the gene expression signature of basal-like breast cancer (38). Those cells also express high levels of EGFR (39) and YB-1 (27), which may be no coincidence. Thus, it is inviting to consider that perhaps YB-1 would also be a viable target for basal-like cancer as it regulates EGFR.

The regulation of EGFR and HER-2 is unquestionably complex. For example, HER-2 is commonly overexpressed due to amplification (18), and to complicate matters further, it is thought that there are resident transcription factors expressed in the breast cancer cells that regulate the additional copies of the gene (40, 21). The SkBr-3 and BT474 breast cancer cell lines have eight copies of HER-2 but overexpress the mRNA 80 times higher than normal breast (41). Thus, a simple increase in copy number cannot account for the fold increase in HER-2 mRNA. In this regard, the resident transcription factors AP-2 (21) and Ets, also referred to as PEA3 (22), induce the expression of HER-2 in breast cancer cell lines. In other types of cancer, however, HER-2 overexpression does not correlate with the presence of AP-2, suggesting that additional factors are involved (23). The role of the Ets in regulating HER-2 is equally controversial. In one case, the expression of a dominant-negative competitive inhibitor to PEA3 blocked the development of MMTV-neu mammary tumors (42). This is in contrast to a report indicating that the expression of PEA3 in fact inhibits HER-2 expression *in vitro*, and that this transcription factor suppressed the development of tumors derived from two different ovarian cancer cell lines (22). PEA3 also repressed HER-2 expression in pancreatic cancer (43). To account for these differences, it is

Figure 3. YB-1 binds to the HER-2 promoter. **A**, schematic of potential YB-1 binding sites on the HER-2 promoter. Primers were designed to amplify the -815 , -1079 , and -1129 sites. **B**, MCF7/Flag:YB-1 and MCF7/Flag:YB-1(A102) cells were subjected to chromatin immunoprecipitation, and the DNA was amplified for HER-2. Flag:YB-1 bound to the HER-2 promoter (lane 1), whereas Flag:YB-1(A102) did not (lane 2). There was no nonspecific binding to the IgY-negative control (lane 3). The sheared input DNA from the Flag:YB-1 (lane 4) and Flag:YB-1(A102) (lane 5) served as a positive controls. A no-template control was also included to ensure that the PCR reaction was not contaminated with template DNA (lane 6). **C**, YB-1:DNA complexes were isolated from MDA-MB-453 cells and amplified for HER-2 to confirm endogenous YB-1 binding. YB-1 bound to the HER-2 promoter (lane 1), whereas there was no nonspecific binding to the IgG (lane 2). The input DNA was also amplified with the primers as a positive control (lane 3).



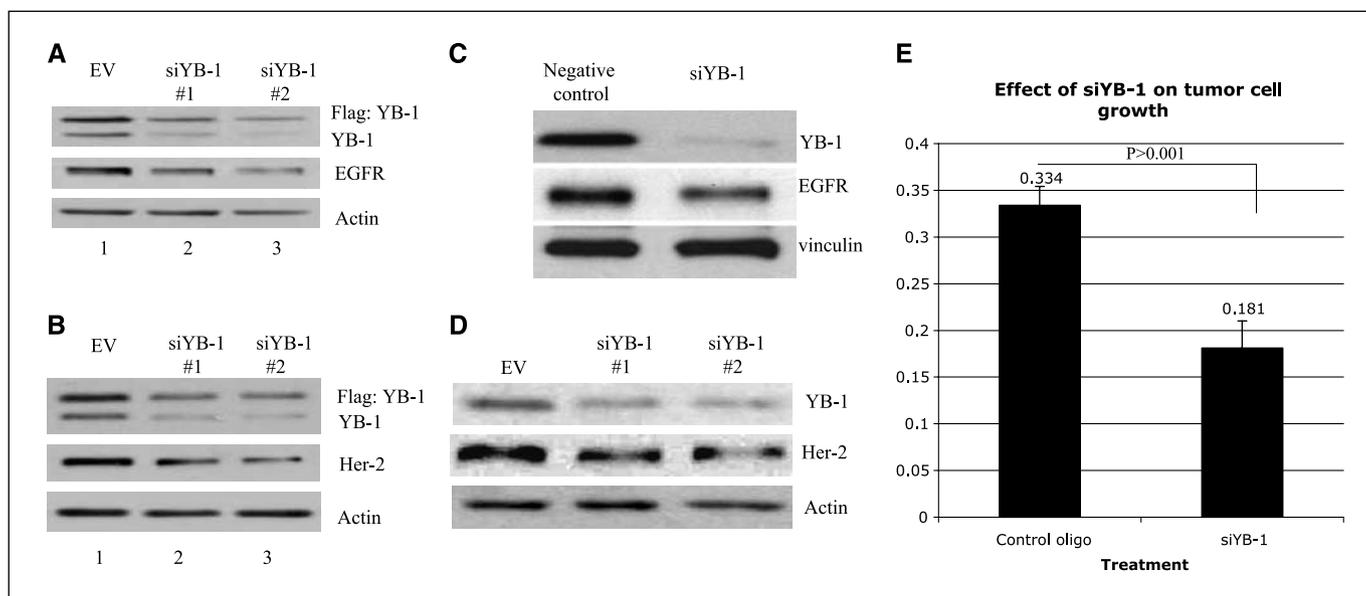


Figure 4. siRNA targeting YB-1 results in loss of EGFR and HER-2. Knock down was achieved using three different targeting strategies each designed to silence YB-1. **A**, MCF-7(Flag:YB-1) cells were transfected with siRNA targeting YB-1 for 96 hours, where the targeting sequence was expressed in a pSuperDuper vector. Compared with the empty vector (*lane 1*), the targeting vector silenced YB-1 (*lane 2*, siYB-11, ~50%; *lane 3*, siYB-12, ~70%). Knocking down YB-1 inhibited the expression of EGFR (*middle*) but had no effect on the housekeeping protein actin (*bottom*). **B**, inhibition of YB-1 using siRNA pSuperDuper system (*top*) also correlated with a loss of HER-2 protein expression (*middle*) but did not suppress actin (*bottom*). **C**, oligonucleotide siRNAs directed at inhibiting YB-1 silenced its expression (*top*) and similarly attenuated the expression of EGFR (*middle*) in MDA-MB-231 cells. MDA-MB-231 cells were treated with either the negative control (*lane 1*) or siYB-1 oligonucleotides (20 μ mol/L, *lane 2*) for 72 hours. **D**, knocking down YB-1 with a pSuper expression vector silenced its expression (*top*) and inhibited HER-2 expression (*middle*) in MDA-MB-453 cells. The cells were transiently transfected with the pSuper vector or the siYB-1 vector and proteins for harvest 48 hours later. **E**, knocking down YB-1 expression resulted in a 46% reduction in tumor cell growth after 7 days in culture. MDA-MB-231 cells were transfected with siYB-1 oligonucleotides, and cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The experiment was done in replicates of 12 per treatment group.

conceivable that PEA3 plays a specific role in regulating the expression of HER-2 in breast cancer but not in other types of malignancy. Our data indicate that YB-1 regulates the expression of HER-2; this is consistent with an earlier study defining this relationship *in vitro* (1). By searching the HER-2 promoter, we noted four putative YREs located within 2 kb from the start site, suggesting that YB-1 could directly regulate gene expression by binding to these sites. It is also possible that YB-1 could bind to another transcription factor, such as AP-2, to regulate HER-2. For example, it was previously reported that YB-1 binds to AP-2 (44). However, the region of the HER-2 promoter that we focused on was outside of the AP-2 and Ets sites. We attempted to amplify this region using sequence specific primers. Unfortunately, we were not able to study that region because we could not detect a PCR product probably due to the high GC content of the area. It is unlikely that this region is part of the amplicons produced because the sheared DNA was <400 bp (data not shown). Our results, therefore, indicate that YB-1 regulates HER-2 directly rather than by coupling to AP-2 or Ets, although this remains to be definitively proven. Regardless of where YB-1 binds, we find that HER-2 expression can be down-regulated by either silencing YB-1 or by specifically mutating YB-1(Ser¹⁰²). Thus, YB-1 is among the resident transcription factors that is highly expressed in breast tumors and has the propensity to regulate HER-2.

The possibility that YB-1 regulates EGFR was first proposed in 1988 (1) through studies showing that it is a DNA-binding protein that binds to the EGFR enhancer. By examining the EGFR promoter, we identify several YB-1 binding sites (YRE) within the EGFR promoter. Using chromatin immunoprecipitation, we are

able to show that Flag:YB-1 binds to EGFR at several loci and that loss of the Ser¹⁰² site disrupts binding within the proximal 1 kb of the regulatory sequence. Similarly, our data confirm that endogenous YB-1 binds to HER-2 and EGFR cis-elements. Future studies will be required to fine-map the relevant sites for transcriptional regulation, as our data indicate that YB-1 binding activities are not per se disrupted by mutagenesis of Ser¹⁰², and rather the DNA sequence context is of relevance. In line with our observation, a recent study (27) shows that YB-1 induces EGFR in mammary epithelial cells representing an early stage of breast cancer progression. Our approach of RNA interference to reduce endogenous YB-1 levels resulted in the down-regulation of EGFR, which indicates that its expression is to a large extent dependent upon functional YB-1.

In closing, our data indicate for the first time that YB-1 is associated with the expression of EGFR and HER-2 in primary breast cancer. We also show that YB-1 binds directly to the EGFR and HER-2 promoters, and that the Ser¹⁰² site is necessary for optimal promoter occupancy. These studies thereby suggest that inhibiting YB-1 and specifically YB-1(Ser¹⁰²) may be novel approaches to decrease the expression of EGFR or HER-2.

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Disruption of the Y-Box Binding Protein-1 Results in Suppression of the Epidermal Growth Factor Receptor and HER-2

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