Transcriptional repression plays a critical role in development and homeostasis. The ETO family represents a group of highly conserved and ubiquitously expressed transcriptional regulatory proteins that are components of a diverse range of multiprotein repressor complexes. ETO proteins function as transcriptional repressors by interacting with a number of transcription factors that bind to their cognate consensus DNA binding sequences within the promoters of target genes. We previously reported that the classical C2H2 zinc finger DNA-binding sequences within the promoters of target genes. We transcription factors that bind to their cognate consensus DNA regulatory proteins that are components of a diverse range of highly conserved and ubiquitously expressed transcriptional factors. The ETO family represents a group of sequence. Our results show that the E-box gene expression of breast oncogenesis. Here we report the identification with the ETO protein CBFA2T3 and has a role in the suppression of breast oncogenesis. Here we report the identification and validation of the ZNF652 consensus DNA binding sequence. Our results show that the E-box gene of HEB is a direct target of CBFA2T3-ZNF652-mediated transcriptional repression. The CBFA2T3-ZNF652 complex regulates HEB expression by binding to a single ZNF652 response element located within the promoter sequence of HEB. This study also shows that the NHR3 and NHR4 domains of CBFA2T3 interact with a conserved proline-rich region located within the C terminus of ZNF652. Our results, together with previous reports, indicate that HEB has a complex relationship with CBFA2T3; CBFA2T3 interacts with ZNF652 to repress HEB expression, and in addition CBFA2T3 interacts with the HEB protein to inhibit its activator function. These findings suggest that CBFA2T3-ZNF652-mediated HEB regulation may play an important role in hematopoiesis and myogenesis.

Gene regulation occurs through the balanced activity of transcriptional activators and repressors and is critical for the ordered development and maintenance of homeostasis. Inherited or acquired defects in transcription factor structure and function can result in irreversible alterations in this balance. The ensuing aberrant expression of target genes can lead to developmental abnormalities or to the initiation and promotion of cancer (1). Spatial and temporal gene regulation by transcriptional activation has been intensely investigated, whereas the critical role of transcriptional repression in development and disease has only been recently recognized (2).

CBFA2T1 (RUNX1T1, MTG8) together with CBFA2T2 (MTG11) and CBFA2T3 (MTG16) form a group of ubiquitously expressed transcriptional regulatory proteins called the “ETO” family. The ETO proteins have modular structures and are characterized by the presence of four regions, NHR1 to NHR4, so named because of their homology to the Drosophila nervous protein (3). ETO proteins are primarily nuclear-localized and are capable of oligomerization and interaction with other proteins through their NHR domains (4–6). The NHR1 (also called eTAFH) domain of CBFA2T1 has been shown to bind the repression domain I of nuclear receptor co-repressor N-CoR in vitro and repress transcription in a reporter assay (7). NHR1 mediates ETO interactions with a conserved activation domain of the E-box proteins HEB and E2A and inhibits their transactivational functions (8). Therefore, the NHR1 motif can interchange negative and positive coregulatory proteins to control transcription (9). The second domain, NHR2, is described as a hydrophobic heptad repeat domain and is required for homo- and heterotetramerization of the ETO proteins (6). The tetramerization of the ETO proteins is not essential for interaction with the corepressors N-CoR, SMRT, mSin3A, and HDAC1–3. However, NHR2-mediated tetramerization of the AML1-CBFA2T1 fusion protein contributes to the development of leukemia in t(8;21) translocation carriers (6). The third domain in CBFA2T1, NHR3, has been shown to interact with the regulatory subunit of type II cyclic AMP-dependent protein kinase (PKA RIIα) in lymphocytes (10). The last of these domains, NHR4, also called the MYND (Myeloid-Nervy-DEAF-1) domain, contains two nonclassical zinc fingers and, in conjunction with NHR1 and NHR3, interacts with the core...

2 The abbreviations used are: NHR, nervous homology region; AML, acute myeloid leukemia; BSA, bovine serum albumin; ChIP, chromatin immunoprecipitation; CHO, Chinese hamster ovary; dsDNA, double-stranded DNA; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; Gfi-1, growth factor independence 1; HA, hemagglutinin; HDAC, histone deacetylase; HEB, HelA E-box-binding protein; HEK, human embryonic kidney; HPRT, hypoxanthine-guanine phosphoribosyltransferase; HuT78, human T-lymphoblastoid; Luc, luciferase; MBP, maltose-binding protein; MT, mutant; MYND, Myeloid-Nervy-DEAF-1; N-CoR, nuclear receptor co-repressor; RE, response element; SCL, stem cell leukemia; siRNA, small interfering RNA; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; TK, thymidine kinase; WT, wild type; ZNF652, zinc finger protein 652.
CBFA2T3-ZNF652 Mediates Transcriptional Repression of HEB

CBFA2T3 is located in the 16q24.3 loss-of-heterozygosity region in breast cancer, and our functional studies are consistent with a role of CBFA2T3 as a breast cancer tumor suppressor (22, 23). We have previously shown that, or reduced levels, of CBFA2T3 expression is associated with breast oncogenesis (22). CBFA2T3 has also been shown to interact with the soluble intracellular domain, termed s80, of ERBB4 and to have a role in ERBB4-dependent differentiation (24). Reporter assays have shown ERBB4 also regulates CBFA2T3-mediated transcriptional repression. ERBB4 and CBFA2T3 are both expressed in normal breast tissue, and changes in their levels are associated with breast cancer. Interaction between CBFA2T3 and ERBB4 may therefore have an important role in the normal development of the mammary gland, and their deregulation may lead to breast cancer (24). On the whole, although the original description of the ETO family was based on their involvement in leukemia, these proteins have now been shown to have a range of other functions (14).

The ETO proteins act as transcriptional repressors by forming multiprotein complexes with the corepressor proteins N-CoR, SMRT, mSin3A, and ATN1, which act as scaffolds for recruiting various combinations of HDACs (14). The recruited HDACs are critical for the potent repressor function of the ETO complexes (14). The three ETO proteins exhibit variations in their direct interactions with HDACs, with CBFA2T1 interacting directly with HDAC1, -2, and -3 (25), CBFA2T2 only with HDAC3 (17), and CBFA2T3 with HDAC1, -2, -3, -6, and -8 (25).

ETO proteins do not bind DNA. Gene-specific repression by ETO-containing multiprotein complexes is mediated through their interaction with transcription factors that can bind directly to the promoters of target genes. These DNA-binding transcription factors are predominantly classical C2H2 zinc finger proteins. It is remarkable that all of the identified ETO-interacting DNA-binding zinc finger proteins are involved in cancer. For example, BCL6 (B-cell lymphoma 6 protein) is frequently translocated and mutated in diffuse large cell lymphoma (26), and PLZF (promyelocytic leukemia zinc finger) is disrupted by a t(11;17) rearrangement in acute promyelocytic leukemia (27). The zinc finger protein Gfi-1 is required for the development of neuroendocrine cells, sensory neurons, and both T- and B-lymphocytes (28). Germ-line mutations in the Gfi-1 coding sequence are associated with severe congenital neutropenia or non-immune chronic idiopathic neutropenia in adults (29). Recent findings also suggest that Gfi-1 can act as either an oncogene or tumor suppressor gene (28, 30). Therefore, the ETO family of proteins generates a diverse constellation of transcriptional repressor complexes through interaction with various transcription factors and corepressors.

We have recently shown that the classical C2H2 zinc finger DNA-binding protein ZNF652 specifically and functionally interacts with the ETO protein CBFA2T3 to repress transcription (31). ZNF652 has seven classical C2H2 zinc finger motifs conforming to the consensus CXXC12H3H0 sequence, with three of these joined by part or all of a consensus TGEKP linker sequence, suggesting a role as a DNA-binding protein (32, 33). The CBFA2T3-ZNF652 complex was proposed to repress transcription of genes that have roles in the oncogenesis of breast (31). As an approach to discovering ZNF652 target genes, we have identified the ZNF652 consensus DNA binding sequence and subsequently have determined that the promoter of the HEB gene contains a functional ZNF652 DNA binding motif. We present data that HEB expression is regulated by the CBFA2T3-ZNF652 repressor complex. In addition, we also have determined the minimal interaction domains for ZNF652 and CBFA2T3.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—HEK293T, CHO, HuT78, Jurkat (human T-cell leukemia), MCF7 and ZR75-1 (breast cancer), and HeLa (cervical carcinoma) cells were purchased from the American Type Culture Collection and grown in the recommended media at 37 °C in 5% CO2. Antibodies used were: rabbit affinity-purified anti-ZNF652 (31); rat anti-HA (12CA5, Roche...
Diagonistics); rabbit anti-HEB (sc-357, Santa Cruz Biotechnology); mouse anti-Myc (9E10; sc-40, Santa Cruz Biotechnology); and rabbit anti-rat-IgG-HRP (DakoCytomation), sheep antimouse-IgG-HRP, and donkey anti-rabbit-IgG-HRP (Amer sham Biosciences). A rabbit polyclonal anti-CBFA2T3 antobody was generated using regions of the protein that diverged among the ETO family (15). CBFA2T3 DNA fragments expressing amino acids 354–392 and 505–567 were combined by an overlap-PCR method and cloned in-frame into the bacteri al expression vector pET14b (Novagen). The His6-bya overlap-PCR method and cloned in-frame into the bac sham Biosciences). A rabbit polyclonal anti-CBFA2T3 anti- mouse-IgG-HRP, and donkey anti-rabbit-IgG-HRP (Amer- sham Biosciences). A rabbit polyclonal anti-CBFA2T3 antibo dy was generated using regions of the protein that diverged among the ETO family (15). CBFA2T3 DNA fragments expressing amino acids 354–392 and 505–567 were combined by an overlap-PCR method and cloned in-frame into the bacte rial expression vector pET14b (Novagen). The His6- by an overlap-PCR method and cloned in-frame into the bac sham Biosciences). A rabbit polyclonal anti-CBFA2T3 antibo dy was generated using regions of the protein that diverged among the ETO family (15). CBFA2T3 DNA fragments expressing amino acids 354–392 and 505–567 were combined by an overlap-PCR method and cloned in-frame into the bacterial expression vector pET14b (Novagen). The His6- by an overlap-PCR method and cloned in-frame into the bac sham Biosciences). A rabbit polyclonal anti-CBFA2T3 antibo dy was generated using regions of the protein that diverged among the ETO family (15). CBFA2T3 DNA fragments expressing amino acids 354–392 and 505–567 were combined by an overlap-PCR method and cloned in-frame into the bacterial expression vector pET14b (Novagen). The His6- by an overlap-PCR method and cloned in-frame into the bacteria l expression vector pET14b (Novagen). The His6- by an overlap-PCR method and cloned in-frame into the bacterial expression vector pET14b (Novagen). The His6-bya overlap-PCR method and cloned in-frame into the bacterial expression vector pET14b (Novagen). The His6-bya overlap-PCR method and cloned in-frame into the bacterial expression vector pET14b (Novagen). The His6-
ZNF652 (31) were isolated according to the published method (36). Nuclei were resuspended in cold buffer containing 20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 25% glycerol (37) and sonicated on a Vibra-Cell VCX 130 (Sonics) with three 10-s pulses at 30% amplitude. Nuclear lysates were then centrifuged, and the supernatants were assayed for protein content using a BCA protein assay kit (Pierce). Aliquots were stored at −80 °C until required. Nuclear extracts were also prepared from Jurkat and HEK293T cells transiently expressing HA-ZNF652.

Electrophoretic Mobility Shift Assay (EMSA)—All EMSA gels were 4% polyacrylamide made in TGE buffer (12.5 mM Tris-HCl, pH 8.5, 85 mM glycerine, 0.5 mM EDTA). DNA fragments from 3–5 rounds of CASTing were end-labeled with [γ-32P]ATP in the presence of T4 DNA polynucleotide kinase (New England Biolabs), and purified on QIAquick nucleotide removal columns (Qiagen). Equal amounts of DNA from the three rounds were incubated with either MBP or MBP-ZNF652 in 1× DNA-binding buffer (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 10 μM ZnSO₄, 0.5 mM DTT, 0.2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 250 μg/ml BSA, 5 μg/ml sheared salmon sperm DNA) in a final volume of 20 μl and loaded onto gel. For remaining EMSAs, annealed oligonucleotides with wild type (5’-GATCCGTGTCGAGCAG-3’ and 5’-GATCCGTGTCGCGAATTC-3’) and mutated sequences were incubated with different amounts of proteins in 20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 85 mM NaCl, 10 μM ZnSO₄, 10% glycerol, 100 μg/ml BSA, and 50 μg/ml sheared salmon sperm DNA in a final volume of 10 μl. For super-shift EMSA, appropriate antibodies were added to binding reactions 5 min before loading onto gels.

Co-immunoprecipitations—Approximately 2 × 10⁶ HEK293T cells were transiently transfected with 4 μg of each relevant plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h, cells were harvested and lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Triton X-100 supplemented with complete protease inhibitor mixture (Roche Applied Science). Lysates were sonicated and cell debris removed by centrifugation at 16,000 × g. Supernatants were incubated with 500 ng of mouse monoclonal 9E10 anti-Myc antibody (Roche Applied Science) overnight at 4 °C followed by the addition of 15 μl of sheep anti-mouse IgG Dynabeads (Invitrogen). Beads were washed three times with lysis buffer, twice with wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), and then twice with low salt buffer (20 mM Tris-HCl, pH 7.5). Proteins were eluted with 50 μl of SDS protein-loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) (38) for 5 min at 95 °C. Inputs and co-immunoprecipitates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C-Extra, Amersham Biosciences).

Western Blotting—Western blots carrying input and immunoprecipitated samples were first incubated with primary antibody (rat anti-HA or mouse anti-Myc) followed by the appropriate HRP-conjugated antibodies. Proteins were visualized using the enhanced chemiluminescence kit (ECL, Amersham Biosciences).

Dual-Luciferase Reporter Assays—Approximately 1.75 × 10⁵ CHO cells were transiently transfected using Lipofectamine 2000 with 200 ng of pGL2-4×ZNF652-TK-Luc, constructs expressing varying amounts of ZNF652 and/or Myc-CBFA2T3, and 25 ng of pRL-TK plasmid (Promega) as an internal transfection control. The cells were harvested after 16 h, lysed, and assayed using the Dual-Luciferase reporter assay system (Promega). Luciferase values were normalized to Renilla luciferase activity and expressed as relative light units. For reporter assays on the HEB promoter, HeLa cells (1.5 × 10⁵) were transfected with 200 ng of luciferase reporter together with varying amounts of constructs expressing Myc-ZNF652 and/or Myc-CBFA2T3 and 50 ng of pRL-TK plasmid (Promega). Cells were harvested and analyzed as described above. All luciferase reporter assays were performed in triplicate and repeated at least three times with the data presented as mean ± S.E. ZNF652 and CBFA2T3 expression was confirmed by Western blot analysis using the appropriate primary and secondary antibodies.

Reverse Transcription Real-time PCR—Total RNA was extracted using an RNeasy mini kit (Qiagen) along with Oligo(dT)₂₄ and Moloney murine leukemia virus reverse transcriptase (H⁻) (Promega). Real-time PCR was performed on these cDNA samples to measure the levels of HEB transcript using forward (5’-GTCACCAGTACACAGACACTG-3’) and reverse (5’-CCTCTAGCCCAGGACAGACACAG-3’) primers and of the housekeeping gene cyclophilin A using forward (5’-GCCAATGATGGACCAACAGACAGACAC-3’) and reverse (5’-CTTACAGATTCCAGTACAGGTGCCTGAA-3’) primers. Real-time PCRs were carried out using the SYBR Green Supermix (Bio-Rad) on an iQ real-time PCR detection system under the following conditions: 95 °C for 3 min; 40 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. HEB expression was normalized against the levels of cyclophilin A.

Promoter Precipitation—Wild type and mutant HEB promoter fragments were PCR-amplified from the pGL3-HEB-E-Luc and pGL3-HEB-mut-E-Luc constructs, respectively, using HEB-F (5’-TGCTTCTCAGGTGTTTCCAGCAGACACTG-3’) and the biotinylated GLprimer2-R (5’-CTTTACAGATTCCATGTCGCGAATTC-3’) primers and of the housekeeping gene cyclophilin A using forward (5’-GCCAAATGATGGACCAACAGACAGACAC-3’) and reverse (5’-CTTACAGATTCCAGTACAGGTGCCTGAA-3’) primers. Real-time PCRs were carried out using the SYBR Green Supermix (Bio-Rad) on an iQ real-time PCR detection system under the following conditions: 95 °C for 3 min; 40 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. HEB expression was normalized against the levels of cyclophilin A.
sequence were incubated with the appropriate cell extracts in binding buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol, 6 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 0.01% Nonidet P-40) (20) for 2 h at room temperature and then washed three times with the same buffer. The proteins bound to the immobilized DNA were recovered by incubating the beads with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM NaCl buffer, resolved on SDS-PAGE, and analyzed by Western blotting using the appropriate primary and secondary antibodies.

Chromatin Immunoprecipitation (ChIP) Assay—A modified ChIP assay protocol (Upstate Biotechnology) was used. Approximately 2 × 10⁷ HEK293T cells were cross-linked for 9 min at room temperature using 1% formaldehyde in suspension on a rotor, terminated with cold 0.125 M glycine, and washed twice with cold phosphate-buffered saline. Cells were lysed on ice with lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) for 10 min and then sonicated on a Vibra-Cell VCX 130 (Sonics) using a 3-mm tip with seven 15-s pulses at 30% amplitude. Cells were centrifuged and the supernatant diluted with 1% formaldehyde in suspension buffer, incubated for 30 min with 500 μg/ml sonicated salmon sperm DNA and 1.25 mg/ml BSA in ChIP wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.7 mM EDTA, 1% Triton-X-100, 0.05% SDS), were used to collect the protein-DNA complexes. Beads were washed three times with the ChIP wash buffer and twice with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Protein-DNA cross-links were reversed by treating them with 200 mM NaCl at 50°C for 30 min, and DNA was isolated according to a published method (39). PCRs were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 94°C for 12 min; 56 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s for the HEB promoter (forward, 5’–GGACAGGCAAGGCTTATCC–3’ and reverse, 5’–GAGCAGGCAAGGCTTATCC–3’) or MCF7 (lanes 1–12), or MBP (lanes 13–15) or with increasing amounts of MBP-ZNF652 fusion protein (HEB GenBank™ accession number NC_000015)) and 31 cycles for the negative control β-globin sequence (forward, 5’–GAAAGACCAAGGACGGGTAT–3’ (nucleotides 671–690), and reverse, 5’–GAAAGACCAAGGACGGGTAT–3’) or scrambled control (Qiagen) siRNA at 100 nM using lipotransfection reagent according to the published method (40). Cells were collected 48 h after transfection and analyzed for levels of ZNF652 and HEB protein using Western blotting. Protein loading was confirmed by probing with anti-β-actin antibody.

RESULTS

Identification and Validation of ZNF652 Consensus DNA Binding Sequence—The binding sequence for ZNF652 was identified using a CASTing protocol (34). Bacterially expressed MBP-ZNF652 fusion protein was used in five sequential rounds of binding and elution cycles to enrich for ZNF652 binding sequences from a random pool of dsDNA. This random pool was generated by annealing two complementary oligonucleotides with 18 degenerate bases (see “Experimental Procedures”). The enrichment of the ZNF652 DNA binding sequence was monitored by performing EMSA on equal amounts of the pools of DNA amplified from rounds 3 to 5 of selection (Fig. 1A). Binding of MBP-ZNF652 protein to the enriched DNA

| Enrichment cycles | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|-------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MBP (ng)          | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| MBP-ZNF652 (ng)   | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

MBP-ZNF652-DNA Complex

Free Probe

FIGURE 1. ZNF652 recognizes a specific DNA response element. A, sequential enrichment of ZNF652 DNA binding sequences was performed using the CASTing protocol, where a pool of dsDNA containing 18 bp of randomized sequence was incubated with purified recombinant MBP-ZNF652 fusion protein. DNA bound to the MBP-ZNF652 fusion protein was PCR-amplified and used in subsequent cycles of selection and amplification. An equivalent amount of PCR-amplified DNA derived from the third, fourth, and fifth cycles of selection was 32P-labeled, incubated alone (lanes 1–3) or with increasing amounts of MBP-ZNF652 fusion protein (lanes 4–12) or MCF7 (lanes 13–15), and subjected to EMSA. Alignment of ZNF652 DNA binding sequences. A schematic diagram showing relative frequency of nucleotides at each position within this consensus sequence are presented.
To confirm that MBP-ZNF652 specifically binds to this consensus sequence, complementary oligonucleotides with wild type or mutant ZNF652 DNA binding sequences were designed. These oligonucleotides were annealed to generate short DNA fragments and used in EMSA. Whereas MBP-ZNF652 bound to the short dsDNA carrying the wild type sequence, no such binding was detected with the mutated sequence (Fig. 2A). In addition, binding to the wild type probe decreased in correspondence to the presence of increasing amounts of unlabeled wild type probe DNA (Fig. 2A, lanes 4–6). However, no change in the binding to the wild type probe was observed in the presence of increasing amounts of unlabeled mutant probe DNA (Fig. 2A, lanes 9–11). MBP protein alone did not bind to the wild type probe (Fig. 2A, lane 2). In a separate EMSA, both the recombinant MBP-ZNF652 and endogenous ZNF652 proteins (from ZR75-1 breast cancer cells) were observed to bind to the wild type probe. The specificity of this binding was evident as both the MBP-ZNF652-DNA and endogenous ZNF652-DNA complexes supershifted in the presence of an anti-ZNF652 antibody (Fig. 2B). However, such a supershift was not observed in the presence of a non-specific antibody. These results show that the ZNF652 consensus DNA binding sequence identified from the CASTing protocol binds the ZNF652 protein.

To further confirm that endogenous mammalian nuclear ZNF652 binds to the identified consensus sequence, competitive EMSA experiments were performed using ZR75-1 nuclear extracts (Fig. 2C). These results were similar to those obtained using the bacterially expressed MBP-ZNF652 fusion protein (Fig. 2A). Finally, the specificity of nuclear ZNF652 protein binding to the consensus ZNF652 DNA sequence was confirmed by supershift EMSA using nuclear extracts of ZR75-1 and HEK293T cells transiently expressing HA-ZNF652 (Fig. 2D). ZNF652-DNA complexes underwent a supershift in the presence of an anti-ZNF652 antibody but not in the presence of a non-specific antibody (Fig. 2D, compare lane 3 with 5 and 9

pools sequentially increased with the three successive cycles of selection (Fig. 1A, compare lanes 6, 9, and 12). No such binding was detected with the MBP protein alone (Fig. 1A, lanes 13–15). PCR products from the fifth round of selection were cloned and sequenced. Sequence alignment of 27 individual clones revealed the consensus binding sequence CGAAAGGGT-TAAT (Fig. 1B).
CBFA2T3-ZNF652 Mediates Transcriptional Repression of HEB

The CBFA2T3-ZNF652 Complex Mediates Transcriptional Repression through a ZNF652 RE—Dual-Luciferase assays were used to determine whether the CBFA2T3-ZNF652 interaction complex was able to mediate transcriptional repression through the identified ZNF652 RE. For these assays, the pGL2-4×ZNF652-TK-Luc construct carrying four tandem copies of the ZNF652 RE was used. Luciferase activity of the pGL2-4×ZNF652-TK-Luc reporter was considered as the basal transcriptional activity. Reporter assays showed a dose-dependent decrease in transcription of the luciferase gene in response to increasing levels of ZNF652; this repression was enhanced in the presence of increasing levels of CBFA2T3 (Fig. 3). However, expression of CBFA2T3 alone had no significant effect on luciferase activity from the pGL2-4×ZNF652-TK-Luc reporter. These results show that ZNF652 and CBFA2T3 act as functional corepressors of transcription.

HEB (TCF12) Is a Target of CBFA2T3-ZNF652-mediated Transcriptional Regulation—The Eukaryotic Promoter Database (EPD release 84) was used to search for gene promoters that contain the consensus ZNF652 RE and thus are potentially regulated by ZNF652. The human HEB promoter contains a putative ZNF652 RE (AAGGGTTAA) located at position −306 upstream of the translational start site that is conserved in the mouse HEB promoter (located at position −332).

Reportor assays using a construct in which this HEB promoter sequence drives the luciferase gene (pGL3-HEB-E-Luc) showed a dose-dependent transcriptional repression in the presence of ectopically expressed ZNF652. Ectopic expression of CBFA2T3 further increased this repression (Fig. 4). ZNF652-mediated repression was abrogated in the construct pGL3-HEB-mut-E-Luc carrying the mutated ZNF652 RE (Fig. 4). These results suggest that ZNF652 protein binds the ZNF652 RE within the HEB promoter sequence to induce transcriptional repression, which is enhanced in the presence of the CBFA2T3 corepressor.

The CBFA2T3-ZNF652 Complex Specifically Binds to the ZNF652 RE within the HEB Promoter Region—A polyclonal rabbit anti-CBFA2T3 antibody was raised and affinity-purified. This antibody was seen to specifically detect the CBFA2T3 protein from the HEK293T cells ectopically expressing Myc-CBFA2T1, Myc-CBFA2T2, or Myc-CBFA2T3 protein (Fig.

FIGURE 3. ZNF652 transcriptional repression is enhanced by CBFA2T3. Increasing levels of ZNF652 result in a dose-dependent decrease in reporter gene expression; this is further reduced in the presence of CBFA2T3. Dual-Luciferase reporter assays were performed to assess the effect of ZNF652 on transcriptional activity of the pGL2-4×ZNF652-TK-Luc construct (schematic shown below the graph). CHO cells were transfected with pGL2-4×ZNF652-TK-Luc together with other expression constructs as indicated below each column. The total DNA in each treatment was equalized by supplementing it with varying amounts of empty vector. pRL-TK-Renilla luciferase vector was used as a transfection control. The -fold repression of transcription was calculated from the ratio of firefly to Renilla luciferase activities. Data shown are representative of three independent experiments and are presented as mean ± S.E. (n = 3).

FIGURE 4. ZNF652-mediated transcriptional repression of HEB is enhanced in the presence of CBFA2T3. Increasing levels of ZNF652 are inversely related to the luciferase reporter gene activity driven by the HEB promoter; this activity is further reduced in the presence of CBFA2T3. Dual-Luciferase reporter assays were performed to assess the effect of ZNF652 on the transcription of the luciferase gene driven by the 1043-bp HEB promoter containing either a single WT (pGL3-HEB-E-Luc) or MT ZNF652 DNA-binding site (pGL3-HEB-mut-E-Luc). Reporter constructs are diagrammed below the graph. HeLa cells were transfected with pGL3-HEB-E-Luc (bars 1 and 2), pGL3-HEB-mut-E-Luc (bars 3–7), or pGL3-HEB-mut-E-Luc (bars 8–10) reporters with varying amounts of ZNF652 and CBFA2T3 expression constructs as indicated. The total DNA in each treatment was equalized by supplementing with varying amounts of empty vector. The pRL-TK-Renilla luciferase vector was used as a transfection control. The -fold repression of transcription was calculated in relative light units (RLU) from the ratio of firefly to Renilla luciferase activities. The transcriptional activity of each reporter plasmid was used as base line. The activity of the pGL3-HEB-E-Luc was significantly repressed in the presence of ZNF652 (lanes 2–4) or in combination with CBFA2T3 (lanes 6 and 7) but was not repressed in the presence of CBFA2T3 alone (bar 8). The levels of ZNF652 and CBFA2T3 expression were verified by Western blotting with antibodies against the appropriate tags (inset). Data shown are representative of three independent experiments and are presented as mean ± S.E. (n = 3).
CBFA2T3-ZNF652 Mediates Transcriptional Repression of HEB

FIGURE 5. Affinity-purified rabbit anti-CBFA2T3 antibody specifically detects the CBFA2T3 protein, and ZNF652 and CBFA2T3 bind to the single ZNF652 RE within the HEB promoter. A, affinity-purified rabbit anti-CBFA2T3 polyclonal antibody specifically detects the CBFA2T3 protein. Lysates from HEK293T cells ectopically expressing Myc-CBFA2T1, Myc-CBFA2T2, or Myc-CBFA2T3 were Western blotted with either anti-CBFA2T3 (upper panel) or anti-Myc (lower panel) antibody. Anti-CBFA2T3 antibody detected only the Myc-CBFA2T3 protein (upper panel). B, affinity-purified rabbit anti-CBFA2T3 polyclonal antibody specifically detects the endogenous CBFA2T3 protein. Nuclear extracts of MCF7 (9 µg) and Jurkat (6 µg) cells were Western blotted with anti-CBFA2T3 antibody. Lysate from HEK293T cells ectopically expressing Myc-CBFA2T3 was used as a positive control. C, exogenously expressed ZNF652 and CBFA2T3 proteins bind to the ZNF652 RE located within the HEB promoter. WT and MT HEB promoter sequences were PCR-amplified from the appropriate reporter constructs using a pair of primers (one of which was biotinylated at the 5' end) and immobilized to streptavidin-coated magnetic beads. Magnetic beads charged with promoter sequences (lanes 2 and 3) or uncharged beads (lane 4) were incubated with lysates from HEK293T cells ectopically expressing FLAG-ZNF652 and Myc-CBFA2T3 and washed, and bound proteins were eluted with 1 M NaCl. Input (lane 1) and eluted (lanes 2–4) proteins were analyzed by Western blotting with anti-ZNF652 (upper panel) and anti-Myc (lower panel) antibodies. D, endogenous ZNF652 and CBFA2T3 bind to the ZNF652 RE located within the HEB promoter. Promoter precipitation assay was performed as described in C except that total protein lysates from HuT78 cells were used. Input (lane 1) and protein eluted from the beads charged with WT (lane 2) or MT (lane 3) promoter sequence or uncharged beads (lane 4) were analyzed by Western blotting with anti-ZNF652 (upper panel) and anti-CBFA2T3 (lower panel) antibodies. Minor background bands (lanes 3–4) were due to a low level of nonspecific binding of the ZNF652 and CBFA2T3 to the magnetic beads. E, ZNF652 binds in vivo to the ZNF652 RE located within the HEB promoter. A ChIP assay was performed on HEK293T cells using either rabbit IgG control (lane 1) or an anti-ZNF652 (lane 2) antibody. DNA isolated from the chromatin immunoprecipitates (IP; lanes 1 and 2) or input (lane 3; positive control) were PCR-amplified using primers flanking the ZNF652 RE within the HEB promoter (upper panel). Amplification of a β-globin sequence was used as a negative control (lower panel). Non-template negative controls are also shown (lane 4).

5A). Anti-CBFA2T3 antibody could also specifically detect the endogenous CBFA2T3 protein from MCF7 and Jurkat nuclear extracts (Fig. 5B).

In vitro promoter binding assays were used to show the binding of CBFA2T3-ZNF652 to the single ZNF652 RE within the HEB promoter. Firstly, promoter binding assays were performed using protein lysates from HEK293T cells exogenously expressing FLAG-ZNF652 and Myc-CBFA2T3. Both ZNF652 and CBFA2T3 bound to the HEB promoter carrying the wild type ZNF652 RE but did not bind to the HEB promoter when.
this RE was mutated (Fig. 5C). Secondly, promoter binding assays were performed using protein lysates from the human T-cell line that expresses both endogenous ZNF652 and CBFA2T3. Endogenous CBFA2T3 and ZNF652 also specifically bound to the HEB promoter carrying the wild type ZNF652 RE. However, no such binding was observed with the HEB promoter containing a mutated ZNF652 RE (Fig. 5D). Furthermore, the stoichiometric level of ZNF652 binding was higher than that of CBFA2T3. Finally, ChIP assay was used to confirm in vivo binding of ZNF652 to its cognate RE located within the HEB promoter region (Fig. 5E). PCR data showed that the HEB promoter sequence was enriched with an anti-ZNF652 antibody but not with rabbit IgG. Negative control β-globin sequences were also not enriched by either anti-ZNF652 antibody or control IgG (Fig. 5E). These results suggested that ZNF652 binds to the HEB promoter in vivo. As CBFA2T3 does not directly bind DNA, these assays confirmed that ZNF652 binds to the single wild type ZNF652 RE within the HEB promoter region and is associated with CBFA2T3 (31).

**HEB Is a Direct Transcriptional Target of ZNF652**—To further confirm that ZNF652 mediates transcriptional repression of HEB in vivo, HEK293T cells ectopically expressing FLAG-ZNF652 alone or with Myc-CBFA2T3 were used. The relative levels of HEB transcription were assayed using real-time reverse transcription-PCR. The HEK293T cells expressing exogenous ZNF652 had a reduced level of HEB expression compared with the empty vector-transfected HEK293T cells (Fig. 6A). ZNF652-mediated repression of HEB was enhanced in the presence of CBFA2T3. Exogenous expression of CBFA2T3 alone in the HEK293T cells also resulted in a minimal repression of HEB, an observation that can be attributed to the presence of endogenous ZNF652 (Fig. 6A, lane 4). Similar to the transcript levels, HEB protein levels were also repressed in HEK293T cells ectopically expressing either ZNF652 or CBFA2T3 or both (Fig. 6A). These results show that ZNF652 mediates transcriptional repression of HEB in vivo and that this repression is enhanced in the presence of CBFA2T3.

To further demonstrate that ZNF652 represses HEB transcription, the effect of knockdown of ZNF652 expression on levels of HEB protein was investigated. MCF7 cells were transfected with either scrambled or two different ZNF652-specific siRNAs, and HEB and ZNF652 protein levels were determined subsequently by Western blot analysis. As predicted, knockdown of ZNF652 expression on levels of HEB protein was detected in cells with ablated ZNF652 expression, but no such increase was detected in the presence of scrambled siRNA (Fig. 6B).

**NHR3 and NHR4 Motifs of CBFA2T3 Are Both Required for Interaction of CBFA2T3 with ZNF652**—We have shown previously that CBFA2T3 interacts with ZNF652 to repress transcription (31). To more precisely define the regions of CBFA2T3 that interacted with ZNF652, various fragments of CBFA2T3 were used in co-immunoprecipitation assays (Fig. 7A). Full-length CBFA2T3 and the fragment CBFA2T3-3, containing both the NHR3 and NHR4 domains, interacted with ZNF652 (Fig. 7B, lanes 9 and 11), whereas interaction was not observed with the fragments CBFA2T3-1 (containing NHR2 alone), CBFA2T3-2 (containing NHR3 alone) and CBFA2T3-4 (containing NHR4 alone) (Fig. 7B, lanes 7, 8, and 10). These results suggest that both the NHR3 and NHR4 domains of CBFA2T3 are required for interaction with ZNF652.

**ZNF652 Interacts with the CBFA2T3 via Its C-terminal Region**—Immunoprecipitation experiments were undertaken to define the region of ZNF652 that interacts with the CBFA2T3-3. Immunoprecipitations on HEK293T cells expressing CBFA2T3-3 and either full-length or two different C-terminal fragments of ZNF652 were performed (Fig. 8A). ZNF652-2 weakly interacted with CBFA2T3-3, but no such interaction was detected with ZNF652-4 (Fig. 8B), suggesting...
that CBFA2T3-ZNF652 interaction is stabilized in the presence of the zinc finger region (31). These results further confirm that ZNF652 interacts with CBFA2T3 via the NHR3 and NHR4 domains.

**ZNF652 Interacts with CBFA2T3 via a Proline-rich Stretch within its C-terminal Region**—Recently it was demonstrated that a proline-rich motif of N-CoR/SMRT has high affinity for the MYND domain located within the NHR4 motif of CBFA2T1. This finding was consistent with the data on the solution structure of the NHR4-SMRT/N-CoR peptide complex (12) and an earlier report showing that NHR3-4 domains of CBFA2T1 interact with N-CoR repression domain III, a region of N-CoR that contains a conserved PPLXP binding motif for corepressor proteins (7). We aligned the amino acid sequences of ZNF652, N-CoR, and SMRT (Fig. 9A). We reasoned that CBFA2T3 interacts with ZNF652 through a similar proline-rich region (amino acids 565–574) within the C terminus of ZNF652. To test this possibility, we determined the interaction of ZNF652 with either PPVP mutated to AAAA (amino acids 565–568; HA-ZNF652-mut1) or PPPP mutated to AAAA (amino acids 571–574; HA-ZNF652-mut2) and CBFA2T3 using co-immunoprecipitation assays. Consistent with this premise, neither of the ZNF652 mutants interacted with the CBFA2T3 protein, suggesting that a proline-rich region located within the C terminus of ZNF652 (amino acids 565–574) is the minimal region of interaction with CBFA2T3 (Fig. 9B). To confirm that the two ZNF652 mutant proteins retained their three-dimensional structure, we determined their ability to bind the ZNF652 RE within the HEB promoter using promoter precipitation assays. Both the wild type and mutant ZNF652 proteins were able to bind the ZNF652 RE, indicating that alanine substitutions do not impair the structure or DNA binding ability of the ZNF652 mutants (Fig. 9C). Taken together, these results suggest that a C-terminally located proline-rich region of ZNF652 interacts with the NHR3-NHR4 domain of CBFA2T3.

**DISCUSSION**

DNA-binding activator or repressor proteins play a critical role in regulation of gene expression. Dysregulation of their functional activity can lead to a number of malignancies, including breast cancer (1). The identification of transcription factor binding sequences is necessary to identify target genes and determine their precise biological function.

ETO proteins do not directly bind DNA but perform their repressive role through interaction with transcription factors that are capable of binding their cognate response elements located within promoter sequences of the target genes. The ETO family of proteins has been shown to interact with the zinc finger proteins Gfi-1, BCL6, PLZF, and GATA-1, as well as with transcription factors such as HEB and SCL/TAL-1 (stem cell leukemia/T-cell acute lymphocytic leukemia-1), to repress gene expression. In addition, we have shown that ZNF652 is a novel ETO-interacting protein that functions as a DNA-binding transcription factor and provides further complexity to the ETO family of repressor complexes (31). The major established function of these ETO-based complexes is in hematopoiesis, with dysregulation leading to leukemia (41). However, the expression of the ETO proteins and their interacting DNA-binding transcription factors are not limited to the hematopoietic lineages, as more diverse roles are currently emerging. For example, the CBFA2T3-ZNF652 complex has a role in suppressing breast oncogenesis (31), and the BCL6 oncoprotein is implicated in the pathogenesis of B-cell lymphomas (42). In addition, BCL6 is expressed in a higher proportion of clinically aggressive breast tumors and has been shown to prevent mammary epithelial differentiation (43).

The novel classical zinc finger protein ZNF652 specifically interacts with the breast tumor suppressor CBFA2T3 (31). This is a transcriptional repressor, because a GAL4-ZNF652 fusion protein, when tethered to DNA via a GAL4 DNA binding sequence, represses transcription in reporter assays, and this repression is enhanced in the presence of the CBFA2T3 protein
CBFA2T3-ZNF652 Mediates Transcriptional Repression of HEB

A. N-terminal Zinc Fingers of ZNF652

| N-CoR  | 606-615 | EPPPELPPP   |
| N-CoR  | 1031-1040 | REPPELIESS  |
| SMRT   | 1103-1112 | SNNPPLISSA |
| SMRT   | 1664-1673 | LLPYELINGY |

B. Input and Promoter Precipitates

| Input | IP: anti-myc |
|-------|-------------|
| myc-CBFA2T3 | + + + |
| HA-ZNF652 | - - - |
| HA-ZNF652-mut1 | + - - |
| HA-ZNF652-mut2 | - + + |

C. Beads with HEB promoter

| Beads with HEB promoter | + + + |
| Uncharged beads | - - - |
| HA-ZNF652 | + - - |
| HA-ZNF652-mut1 | + - - |
| HA-ZNF652-mut2 | - + + |

FIGURE 9. ZNF652 interacts with the CBFA2T3 through a C-terminal proline-rich region. A, diagram showing structure of the ZNF652 protein and alignment of the proline-rich regions of ZNF652, N-CoR, and SMRT proteins. Amino acids (aa) identical to those of ZNF652 are shaded. B, HEK293T cells were transfected with constructs expressing HA-tagged wild type ZNF652 (lanes 1, 4, 7, and 10), ZNF652-mut1 (lanes 2, 5, 8, and 11; PPPV converted to AAAA, amino acids 571–574), or PPPV converted to AAAA, amino acids 571–574) or with Myc-CBFA2T3 (lanes 4–6 and 10–12). The authenticity of the identified ZNF652 DNA binding sequence was confirmed by competitive and supershift EMSAs using purified recombinant MBP-ZNF652 and endogenous and ectopically expressed mammalian ZNF652 proteins (Fig. 2). Furthermore, CBFA2T3 and ZNF652 form a transcriptional repressor complex on this ZNF652 RE (Fig. 3).

Our in vivo assays show that ZNF652 represses transcription of HEB by binding to a single ZNF652 RE within the HEB promoter sequence, and this repression is enhanced in the presence of CBFA2T3 (Fig. 4). This result was further supported by our in vivo and in vitro promoter binding assays showing that the CBFA2T3-ZNF652 complex is recruited to the HEB promoter (Fig. 5). Furthermore, ZNF652 ablation resulted in an increased level of HEB expression (Fig. 6). Based on these findings, it is proposed that CBFA2T3 and ZNF652 form a complex on the HEB promoter, subsequently recruiting corepressors and HDACs and resulting in repression of transcription.

Our results show that HEB is a direct target of CBFA2T3-ZNF652-mediated transcriptional repression. It is of interest that all of the ETO family proteins can interact directly with HEB (along with E2A) plays an essential role by positively regulating a number of target genes critical in B- and T-cell lineage differentiation and development (48). Systematic gene replacement experiments show that signals leading to transcriptional activation of HEB versus E2A are crucial for B- and T-cell lineage commitment and differentiation (49). Our results show that CBFA2T3-ZNF652 complex represses HEB expression. Therefore, we predict that CBFA2T3-ZNF652-mediated HEB repression may determine the relative levels of HEB and thus the ratio of E2A-E2A homodimers to E2A-HEB heterodimers, critical in B- and T-cell development.

CBFA2T3 performs its transcriptional repressor function in erythropoiesis as a component of a multiprotein complex. Within this complex, CBFA2T3 associates with the hematopoietic-specific E-box SCL protein and a number of other transcription factors (for example, HEB, GATA-1, E2A, E12, E47). The stoichiometry of CBFA2T3 relative to the level of SCL, E2A, or HEB activators within this multiprotein complex varies during erythroid differentiation, and levels of CBFA2T3 determine both the ability to activate or repress transcription of essential SCL target genes and the timing of their expression (20). Therefore, it is evident that HEB has a complex relationship with CBFA2T3, as the CBFA2T3-ZNF652 transcriptional complex represses HEB.
expression, and the CBFA2T3 protein directly interacts with the HEB protein to inhibit its activator function.

Although CBFA2T3 does not interact with the ZNF652 zinc finger region (amino acids 243–491), this region is required in order to stabilize the interaction of the C-terminal region of ZNF652 (amino acids 498–606) with CBFA2T3 (31). Findings presented in this study define the NHR3 and NHR4 domains of CBFA2T3 as the regions that interact with ZNF652 (Fig. 7). It has been shown for CBFA2T1 that both the NHR3 and NHR4 domains were also required for its interaction with the corepressor N-CoR (7, 50). Additional studies have shown that CBFA2T1 interacts through a conserved proline-rich PPLXP motif within N-CoR, and reporter assays confirmed that this interaction is functionally important (7). A number of proteins have been reported to interact with MYND domains through their PPLXP peptide motif. MYND domains are defined by a C\(_4\)-C\(_4\)HC consensus and are frequently implicated in transcriptional repression (12, 51). Therefore, we predict that the MYND domain of CBFA2T3 interacts with ZNF652 through a proline-rich region of CBFA2T1 and CBFA2T3 with these proteins are depicted by the amino acid (aa) numbers above or below the horizontal lines, respectively.

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We are currently working toward genome-wide identification of ZNF652 gene targets, particularly ZNF652-responsive genes directly associated with breast oncogenesis, using a ChIP-ChIP approach. Such investigations are critical in determining how ZNF652 regulates normal gene expression in cell fate, development, and differentiation and how ZNF652-mediated transcriptional alterations of target genes can lead to cell proliferation and/or apoptosis.

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