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14. ABSTRACT

Efforts to identify genes that contribute to breast cancer lead to the discovery of the human TREX complex, a group of proteins that work together to accurately process and transport messenger RNAs from the nucleus to the cytoplasm in a cell. A member of this complex, referred to as p84N5 (more recently named hTREX84) was found to be a culprit of aggressive human breast cancers. hTREX84 is expressed at very low levels in normal breast epithelial cells, but is highly expressed in breast tumors. hTREX84 expression correlates with tumor size and the metastatic state of the tumor progression. Inhibition of hTREX84 levels via RNAi approaches blocks breast tumor cell growth and causes the cells to die. Thus, hTREX84 appears to be a prognostic marker for determining the aggressiveness of breast cancer and may also be an ideal target for therapeutic drugs against breast cancer.

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Transcriptional Elongation, mRNA Export, Breast Cancer

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INTRODUCTION:

A major challenge to breast cancer researchers has been and continues to be the ability to distinguish genetic alterations that are critical to tumor initiation from those that are epiphenomena of genetic instability. A small percentage of total breast cancer cases (~10%) are attributed to inherited mutations in highly penetrant breast cancer susceptibility genes, such as *BRCA1* and *BRCA2* [reviewed in (1)]. However, the majority of the tumors occur in women with little or no family history and the molecular basis of these sporadic breast cancers is still poorly defined. Amplification or over-expression of oncogenes (for example c-*MYC*, *ERBB2*, cyclin D1, *EGFR*, γ-synuclein) and loss of *TP53*, *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), *PTCH* (patch), *MKK4* (MAP kinase kinase 4), *BRCA1*, *BRCA2*, and *HIN*-1 (high in normal 1) have been shown to be present in sporadic disease (1-14). Epigenetic changes, such as inactivation of *BRCA1* due to promoter hypermethylation, have also been described in a portion of breast carcinomas (15-17). During the course of this study, we discovered that the protein, originally referred to as p84N5 (or hTREX84) is abnormally expressed in the vast majority of breast cancers (Guo et al., Cancer Research, 2005).

1.c *Nuclear Matrix Protein, hTREX84*

The *hTREX84* gene, located on chromosome 18p11.32, was originally isolated on the basis of its ability to encode a protein that specifically associates with the N-terminal half of $p110^{RB}$ (18). This study demonstrated that hTREX84 is a nuclear matrix protein that localizes to subnuclear regions associated with RNA processing and binds preferentially to the functionally active, hypophosphorylated form of $p110^{RB}$. The relevance of this interaction for RB function is not completely understood. The hTREX84 protein has a region of structural similarity to the death domains of several well-characterized proteins involved in apoptosis, including tumor necrosis factor receptor 1 (TNFR-1) (19). It is thought that hTREX84 functions in an apoptotic-signaling pathway initiated from within the nucleus in response to DNA damage (19-21). In addition, the hTREX84 protein in cell lines has a specific subcellular nuclear localization that gives a characteristic punctate staining pattern in cells (18). Furthermore, we have found that hTREX84 does not appear to be expressed in normal breast ductal epithelial cells, but is expressed in the majority of breast tumors and tumor cell lines. In a survey of the various hereditary cancer syndromes, at least four proto-oncogenes are found to be activated (i.e., *RET*, *MET*, c-*KIT*, *CDK4*) (22, 23). In general, such activation of oncogenes in the germline is embryonic lethal; however, this is not the case with these proteins. Instead, a second hit is observed in the cancer, which leads to two mutant copies (loss of heterozygosity) and/or trisomy (two mutant and one wild-type allele). We hypothesize that hTREX84 may be a proto-oncogene, and when over-expressed or mutated contributes to the development of both sporadic and familial forms of breast cancer.

BODY

Final Report

Task 1. To evaluate the expression of p84N5 (hTREX84) in clinical breast tumor samples and correlate with predictive factors and clinical outcomes.

We have exceeded the initial expectations of this first task. Our findings were published in a manuscript in April 2005 (Guo, et al. Cancer Res. 2005, 65:3011-3016) and presented at the 96th annual AACR meeting in Anahiem, CA. We were also selected to present our studies as an oral report at the ERA of HOPE meeting in June 2005. Furthermore, we have written a manuscript entitled "Translational Regulation of hTREX84: A Link Between Transcriptional Elongation, mRNA Export and Cancer," which explores the mechanism of aberrant hTREX84 expression in cancer. Although not accepted in its current form, we have recently modified and have resubmitted the manuscript. Overall, our results indicated that hTREX84 is not only a marker of breast cancer progression, but may also contribute to other forms of

cancer, including ovarian. Since, over-expression of and not mutations in hTREX84 were observed in breast tumors (Task 2), we investigated the mechanisms regulating gene expression. We found that aberrant methylation was not involved in regulating *hTREX84* expression; however, we demonstrated that RelA/p65 might play a pivotal role. In summary, our results suggest that hTREX84 could be a predictive marker of tumor progression and a potential therapeutic target for treatment of breast cancer. We have included some of the results from our published and unpublished studies.

Task 2. To evaluate *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *p84N5* (*hTREX84*) mutations.

We have completed the proposed goals outlined in this objective. Unfortunately, no germline or somatic mutations were found in *hTREX84* leading us to explore other mechanisms regulating its expression in breast tumors (as outlined above, and shown below). Specifically, we first screened DNA isolated from blood of affected probands in *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *hTREX84* mutations by direct DNA sequencing. In total, we screened 70 women affected with breast cancer who reported at least one first degree relative with breast or ovarian cancer, and observed no deleterious mutations. Additionaly, 72 cases of sporadic breast cancer and 30 cases of ovarian cancer were screened, as well as 35 cases of EBV-transformed lymphocytes, which were generated from *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds by Western blot analysis. We used this approach as a means to identify tumor samples potentially carrying a truncated form of the protein. However, all of the samples tested only expressed wild-type hTREX84 protein. Examples of these studies are included below.

Project Results

Task 1-"Evaluate the expression of p84N5 (hTREX84) in clinical breast tumor samples and correlate with predictive factors and clinical outcomes."

A. Expression of hTREX84 is also associated with human ovarian cell proliferation

As indicated above, once Task 1 was completed we decided to expand our studies. We observed that the expression of hTREX84 in breast tumors was inversely related to hormone receptor status (Guo et al, 2005). Therefore, we decided to compare *hTREX84* mRNA expression in 6 reduction mammoplasty specimens, including 3 nulliparous premenopausal and 3 parous premenopausal women. We observed that *hTREX84* mRNA levels were substantially higher in the nulliparous specimens (data not shown). These results indicated that *hTREX84* is not only deregulated in breast tumors, but also regulated during normal human breast lobular differentiation and might be modified by some hormones, such as human chorionic gonadotropin (hCG). We next asked whether *hTREX84* might also be aberrantly expressed in other hormone-dependent tumors, such as ovarian tumors. As expected, hTREX84 was highly expressed in all 30 cases of ovarian epithelial tumors examined (data not included). Further, we determined hTREX84 protein expression (hTREX84/beta-actin ratio) in primary human ovarian surface epithelial (HOSE) cell cultures (n=10), SV40 Tag immortal HOSE cell lines (n=10) and ovarian tumor cell lines (n=11) by Western blotting analysis. We found that hTREX84 expression is significantly elevated in immortal cell lines (average value, 0.51) as compared to primary epithelial cells (average value, 0.125; p=0.00024) and reaches its highest level in cancer cell lines (average value, 2.10; p=0.0022) (**Figure 1a, b**).

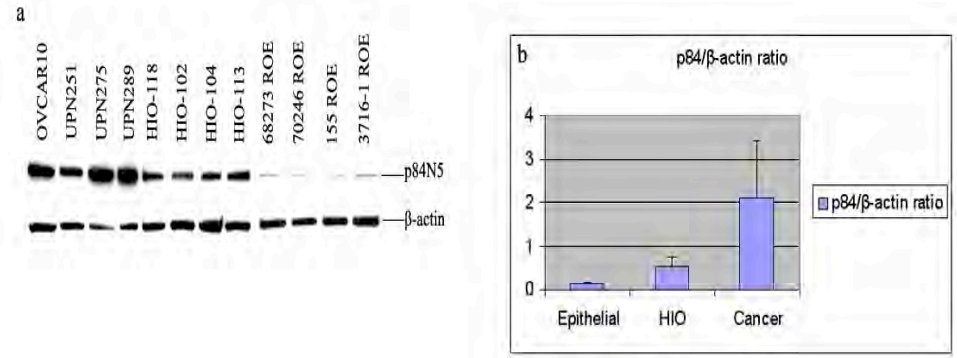


Figure 1. hTREX84 is aberrantly expressed in ovarian cancer cells. *(a),* hTREX84 protein expression in representative ovarian cancer cell lines (OVCAR10, UPN251, UPN275, UPN289), immortal epithelial cell lines (HIO-118, HIO-102, HIO-104, HIO-113), primary human surface epithelial cells (ROE). Protein samples were separated on a SDS-polyacrylamide gel and transferred proteins immunoblotted using anti-hTREX84 or ß-actin monoclonal antibodies. (*b),* hTREX84/ß-actin ratio in primary ovarian epithelial cell cultures (epithelial), immortal epithelial cell lines (HIO) and cancer cell lines (cancer).

To further elucidate the biological significance of *hTREX84* in cancer, siRNA against *hTREX84* was transfected into OVCAR10 cells. RT-PCR analysis using oligonucleotide primers specific to the *hTREX84* gene showed that the expression level of the *hTREX84* transcript decreases 70~80% by following transfection of hTREX84 siRNA as compared to cell transfected with control siRNA (**Figure 2a**). *hTREX84*-targeted siRNAs effectively reduced the protein levels of hTREX84 without affecting levels of non-targeted transcripts, such as β-actin (**Figure 2b**). Immunostaining confirmed that hTREX84 protein was drastically decreased in the majority of the treated cells (**Figure 2c**). Visually, the total number of cells decreased significantly following treatment with *hTREX84*-siRNAs as compared to cells treated with transfection reagent or control-siRNA (**Figure 2d**). We observed that cell growth was reduced in cells treated with *hTREX84*-siRNA as compared to control (**Figure 2e**). The GuavaNexin assay showed that there was also a reduction of Annexin V-PE- and 7-AAD-positive cells upon treatment with *hTREX84*-siRNAs compared to control siRNA, although the differences were not significant ($p>0.05$) (data not shown). In order to evaluate the mechanism of *hTREX84* siRNA action, we further determined the cell cycle distribution by flow cytometry and found that the number of cells in G2-M phase was decreased while the number of cells in G1 phase was increased in OVCAR10s treated with *hTREX84* siRNA versus control siRNA, indicating that hTREX84 may be necessary for entry into the G2-M phase (**Figure 2f**). These results indicated that aberrant expression of *hTREX84* may contribute to ovarian cancer, as well as breast cancer, by promoting cell proliferation.

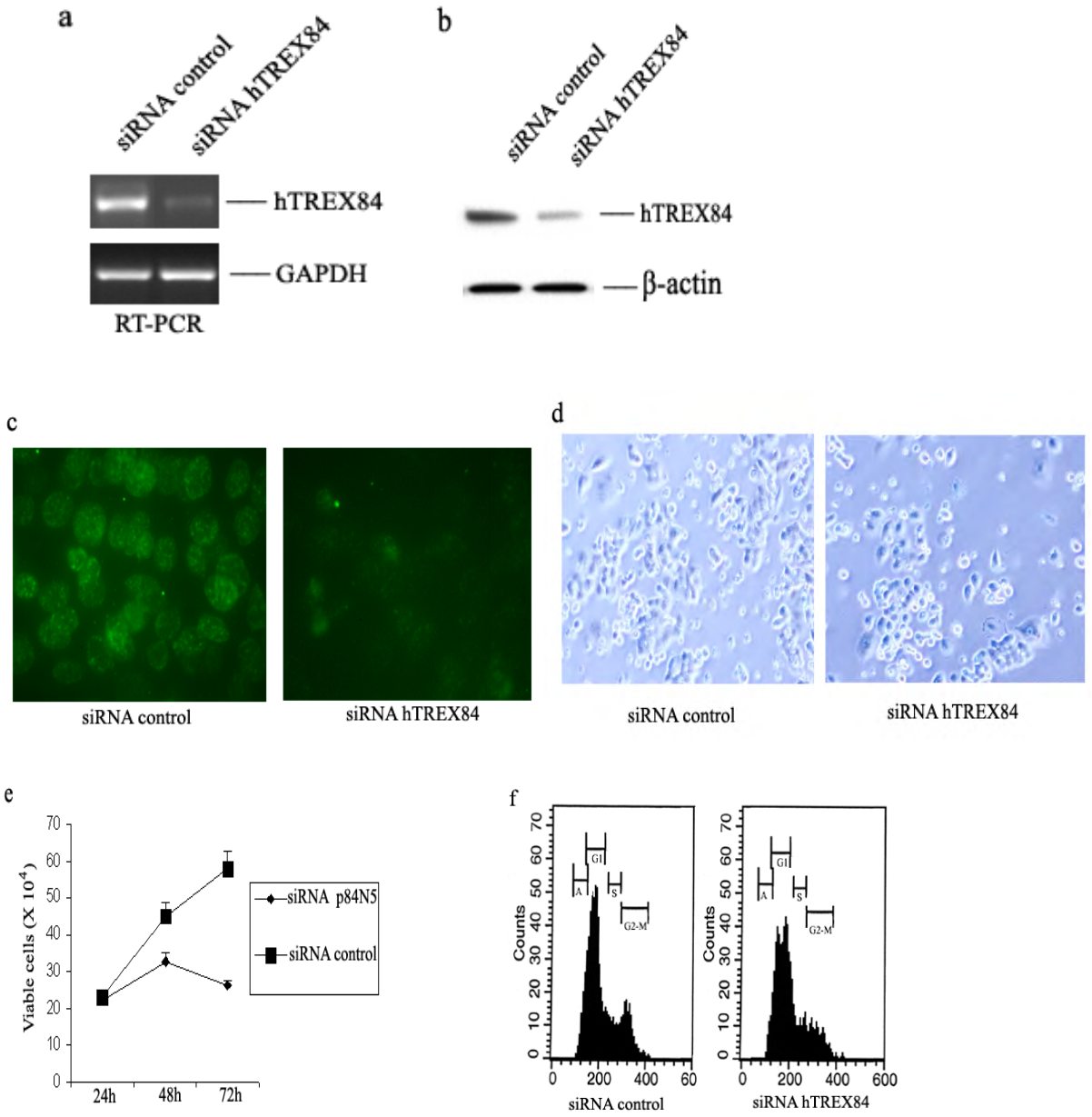


Figure 2. Knock-down of *hTREX84* leads to defects in cellular proliferation of OVCAR10. *(a),* Analysis of *hTREX84* and *GAPDH* mRNA levels following treatment of cells with siRNA against *hTREX84* or control siRNA. *(b),* Analysis of hTREX84 and β-actin protein levels after treatment of cells with siRNA against *hTREX84* or control siRNA. *(c),* Analysis of hTREX84 expression following siRNA treatment for 72 h by immunofluorescent staining of cells (*left,* cells transfected with control siRNA; *right,* cells treated with *hTREX84*-siRNA). *(d),* Photomicrographs show the morphology of the cells following abrogation of hTREX84 expression (*left,* tumor cells transfected with control siRNA; *right,* cells treated with *hTREX84*-siRNA). *(e),* Tumor cell proliferation following abrogation of hTREX84. Cell proliferation and apoptosis (data not shown) were examined using Guava ViaCount and Nexin assays, respectively. The number of viable cells ($x10^4$) at 24, 48, and 72 hrs after treatment with control or *hTREX84*-siRNA is shown; data are from three independent experiments. *(f)*, FACS analysis of the cells following down-regulation of hTREX84 levels. The comparison of cell cycle distribution after 72 h of treatment with either control siRNA (*left*) or *hTREX84*-siRNA (*right*).

B. hTREX84 is a subunit of the TREX complex

To gain insight into the biological role of hTREX84, we isolated a hTREX84-containing multiprotein complex from mammalian cells. This was accomplished by developing a 293-derived stable cell line expressing Flag-tagged hTREX84. **Figure 3A** depicts the purification of Flag-hTREX84 using anti-Flag antibodies followed by the analysis of the Flag-hTREX84 eluate using gel filtration chromatography. This analysis revealed the specific association of hTREX84 with polypeptides of 125, 120, 90, 45, 40, and 30K molecular mass (**Figure 3B and C**). Interestingly, mass spectrometric sequencing of hTREX84-associated polypeptides revealed the identity of hTREX84 associated proteins as the human counter parts of the yeast TREX complex reported to couple transcriptional elongation and mRNA export (**Figure 3D**). Therefore, we have termed this complex human TREX and hTREX84 as hTREX84. Importantly, in contrast to the yeast TREX complex, the human complex was devoid of the RNA export and splicing factors ALY and UAP56. We therefore asked whether endogenous ALY and hTREX84 form a stable complex, which would be reflected by coelution of the two proteins via gel filtration. Analysis of HeLa nuclear extract by Superose 6 sizing fractionation showed distinct chromatographic elution profiles for hTREX84 and ALY proteins indicating that the two proteins are not stably associated (**Figure 3E**). However, consistent with a previous report (24), we observed the association of hTREX and ALY through the UAP56 protein (**Figure 3F**), and the colocalization of hTREX and ALY in breast tumor cells as determined by immunofluorescence assays (data not shown). These results indicated that whereas hTREX and ALY may not be stably associated, their interaction is promoted by the UAP56 protein.

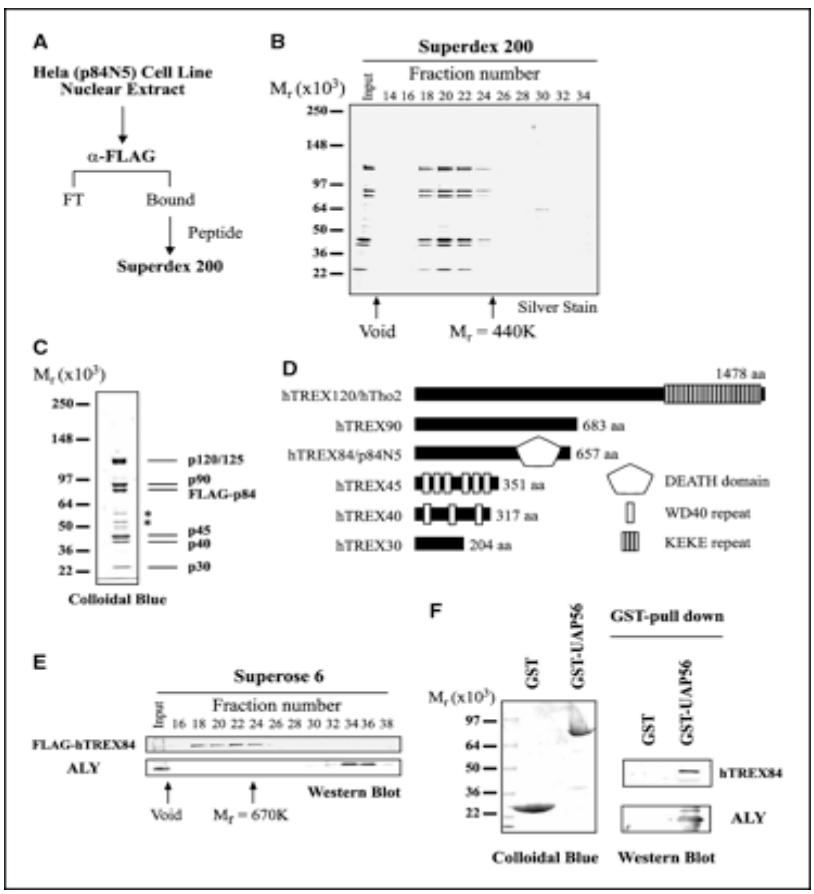


Figure 3. hTREX84 is a component of the human TREX complex. *A,* Schematic of hTREX84 isolation using a 293-derived Flag-tagged cell line. *B,* The human TREX complex, isolated using the protocol shown in (*A*), was analyzed by silver staining following fractionation on the Superset 200. *C,* Colloidal blue analysis of Flag-affinity eluate shown in (*A*). Individual bands

were excised and subjected to mass spectrometric sequence analysis. *D,* Diagrammatic representation of human TREX subunits. hTREX120, hTREX90, hTREX45, hTREX40, and hTREX30 correspond to Genbank accession numbers AL030996, XM_037945, NM_032361, NM_024339, and BC020599, respectively. *E,* Analysis of nuclear extract using Superose 6 gel filtration. Column fractions were analyzed by Western blotting using antibodies (*right? left*). *D,* GST or GST-UAP56 were used for affinity-purification of human TREX84 and ALY proteins.

C. Methylation status of hTREX84 promoter and exon1 in cancer cells

In our previous report, we found that *hTREX84* mRNA was more highly expressed in breast tumors of grade III than those of grade II by quantitative real-time PCR (qPCR). Moreover, *hTREX84* mRNA was also higher in malignant epithelial cells than in normal mammary ductal epithelial cells as shown by qPCR analysis on samples obtained by laser capture microdissection (LCM). Therefore, we speculated that deregulated transcription of *hTREX84* mRNA may be one of the mechanisms of hTREX84 protein over-expression in cancer cells. It is well known that methylation of DNA at CpG dinucleotides has been recognized as an important mechanism for regulation of gene expression in mammalian cells (25, 26). Methylation of cytosines in the CpG sequence, located in either the promoter region or exon 1, is thought to ensure the silencing of certain tissue-specific genes in non-expressing cells. Aberrant methylation is now considered an important epigenetic alteration occurring in human cancer. Hypermethylation of normally unmethylated tumor suppressor genes correlates with a loss of expression in cancer cell lines and primary tumors. On the other hand, failure to repress genes appropriately, through abnormal demethylation of tissue-restricted genes or by hypomethylation of proto-oncogenes, could result in the loss of tissue specificity and could promote cancer formation. To elucidate the molecular mechanisms underlying the abnormal transcription of *hTREX84* in carcinogenesis, a cell line expressing low levels of hTREX84 were treated with a demethylating agent, 5-aza-2'-deoxycytidine (5-aza-C), at concentrations of 1, 5, 10, 50 μM for 5 days. Total RNAs were isolated, and RT-PCR with specific primers to *hTREX84* or *β-actin* cDNA was conducted. The results showed that the intensities of RT-PCR product of *hTREX84* were increased by the 5-aza-C treatment in a dose-dependent manner. By contrast, the products of *β-actin* were evenly amplified from all the samples, illustrating that the expression of *β-actin* was not altered by the 5-aza-C treatment (**Figure 4a**). hTREX84 protein was also increased in the same manner as assayed by Western blotting (**Figure 4b**). Similar results were obtained when using breast tumor cell lines, in which endogenous hTREX84 was expressed at low basal levels (data not shown). Genomic DNA was subsequently isolated from these 5-aza-C treated cells and sodium bisulfite DNA sequencing was performed. Surprisingly, the results demonstrated that all the CpG dinucleotides located within *hTREX84* promoter and exon1 regions from treated and untreated cells were demethylated, indicating hypomethylation was likely not the cause for increasing expression of *hTREX84* mRNA and protein in these cells after 5-aza-C treatment (data not shown).

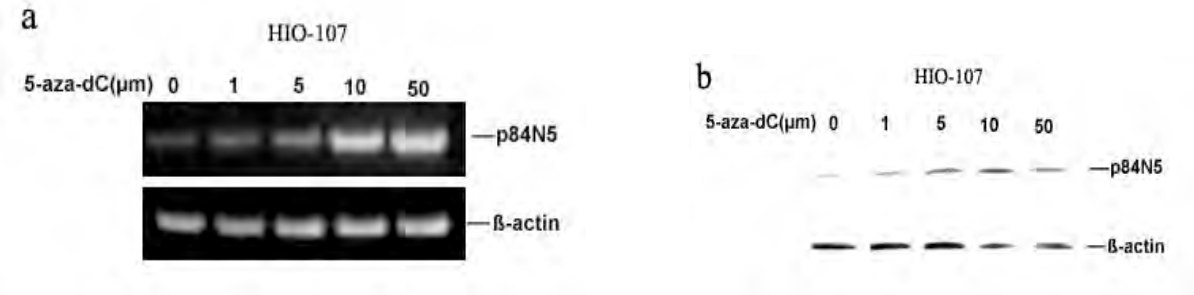
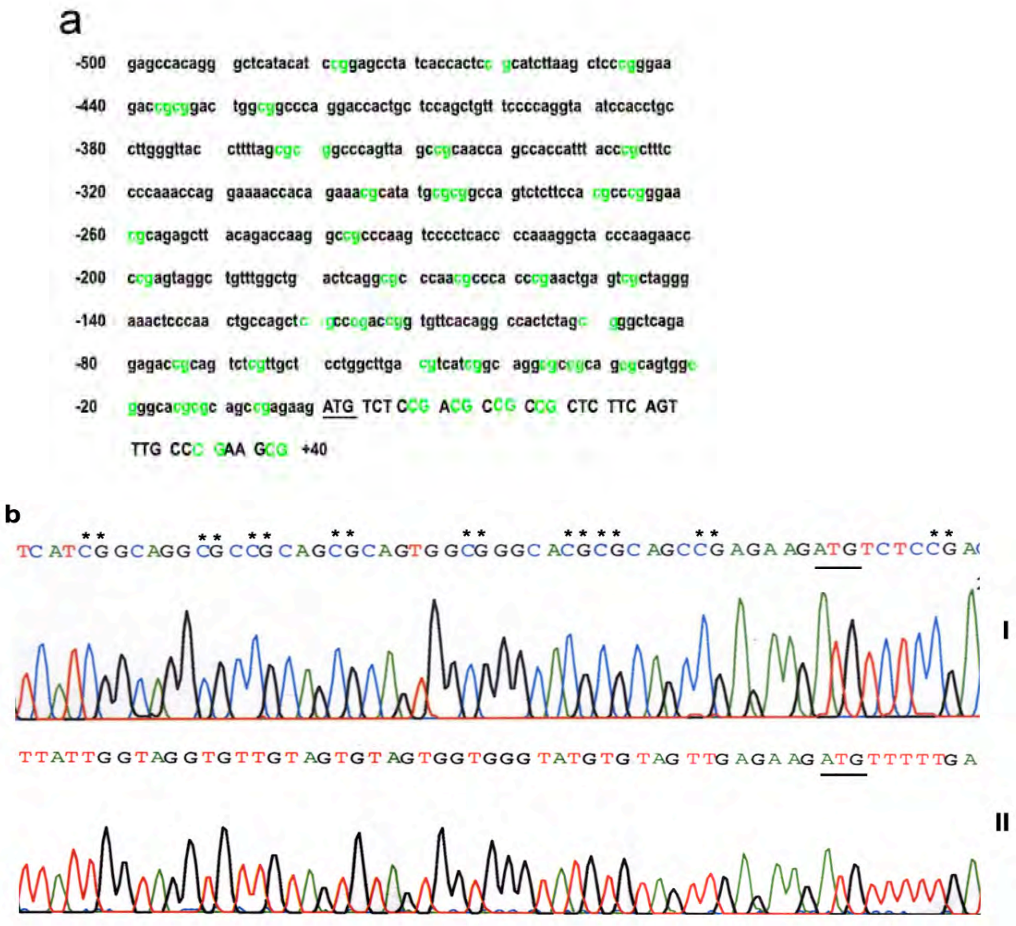


Figure 4. Methylation as a potential regulatory mechanism for *hTREX84* gene expression. Cells were treated with a demethylating agent, 5-aza-C, at concentrations of 1, 5, 10, and 50 μM for 5 days. RT-PCR *(a),* and Western blot analysis *(b),* showed *hTREX84* mRNA and protein expression, respectively.

To further rule out a role for methylation in regulating hTREX84 expression, we analyzed the promoter and exon 1 regions of *hTREX84* in 8 breast and 7 ovarian cancer cell lines, 10 primary mammary epithelial cell cultures, 20 cases of invasive breast ductal carcinoma, and 10 cases of ovarian tumors, as well as their paired normal tissues, by sodium bisulfite DNA sequencing. The results showed that the *hTREX84* promoter and exon 1 regions in almost all cell lines were unmethylated (**Figure 5a and b**) and that the methylation status did not correlate with *hTREX84* expression. The *hTREX84* promoter and exon 1 regions in most normal tissues were also unmethylated, although we observed occasional methylation of a few CpG dinucleotides (**Figure 5c**). Overall, our results suggested that aberrant methylation of *hTREX84* is not likely to contribute to its abnormal expression in the majority of breast tumors. There are several possibilities, which could explain why 5-aza-C can induce *hTREX84* expression independently of promoter methylation. For example, 5-aza-C may have dramatic effects on chromosomes, leading to decondensation of chromatin structure, thus enhancing specific gene expression (27). Another possibility is that 5-aza-C might lead to activation of transcription factors or repression of inhibitors that regulate *hTREX84* expression. Although not yet resolved, we have observed that NF-κB is upregulated in many breast tumors and that *hTREX84* contains consensus NF-κB binding motifs in its promoter (see below).



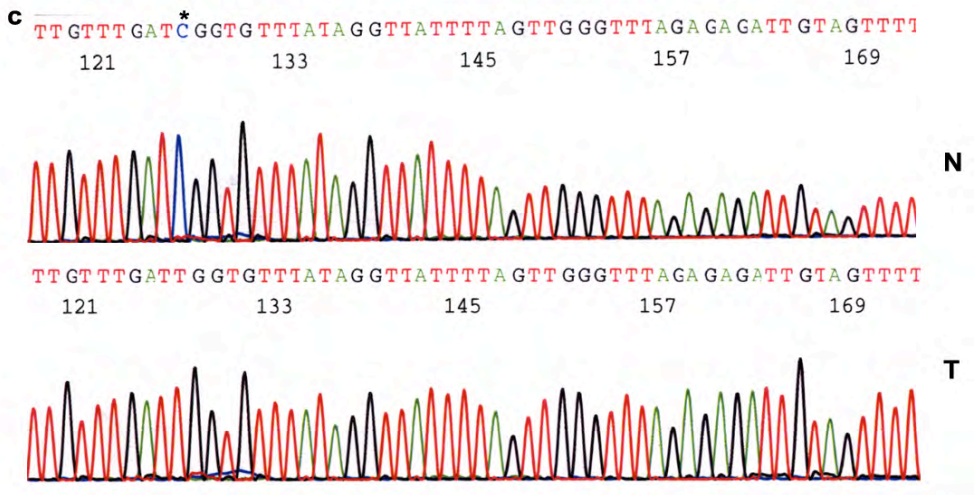


Figure 5. (a). hTREX84 promoter and exon 1 region, the nucleotides in green color are CpG sites. Nucleotides are numbered on the right from the ATG translation start codon which is underlined. (b). sodium bisulfite DNA sequencing, from untreated (I) and treated DNA samples (II). Star marker indicates CpG sites. (c). Sodium bisulfite DNA sequencing, from a normal breast tissue (N) and a carcinoma tumor (T). Star maker indicates CpG sites.

D. NF-κB activation enhances hTREX84 expression in immortal and/or cancer cells

To provide a better understanding of the molecular basis of hTREX84 over-expression in cell immortalization and carcinogenesis, we took the approach to identify the transcription factor binding sites in the *hTREX84* promoter (32) using the AliBaba2 tool, available at http://wwwiti.cs.uni-magdeburg.de/grabe/alibaba2. 9-SP1, 7-NF1, 4-AP1, 2-NF-κB, together with other transcriptional factor consensus binding sequences, were found in the *hTREX84* promoter by this program. We focused on and validated nuclear factor of κB NFκB for several reasons. NFκB is not a single protein, but a small menagerie of closely related protein dimers that bind a common sequence motif known as the κB site (33). According to Hanahan and Weinberg, tumorigenesis requires six essential alterations to normal cell physiology: self-sufficiency in growth signals; insensitivity to growth inhibition; evasion of apoptosis; immortalization; sustained angiogenesis; and tissue invasion and metastasis (34). NF-κB is able to induce several of these cellular alterations (35), and has been shown to be constitutively activated in some types of cancer cells, including breast cancer. Previous studies have documented elevated or constitutive NF-κB DNA-binding activity both in mammary carcinoma cell lines and primary breast cancer cells of human and rodent origin (36-38). This could be correlated with the increased level of epithelial growth factor family receptors (EGFR) (39). The chromatin immunoprecipitation (ChIP) assay is a powerful technique to determine true *in vivo* binding of transcription factors and other nucleosomal proteins to chromatin (40, 41). We used this assay to determine status of RelA/p65, one subunit of NF-κB, at the promoter of *hTREX84*. After the ChIP protocol, hTREX84 gene promoter regions were amplified and analyzed by semiquantitative PCR using specific primer pairs around NF-κB binding regions on the promoter of *hTREX84* (**Figure 6a**). MDA-MB-231, OVCAR10, OVCAR5 cells cultured for 3 days were subjected to ChIP with and without antibody to p65. Enrichment of specific DNA sequences in the chromatin immunoprecipitates, indicating association of p65 to DNA strands within intact chromatin, were visualized by PCR amplification. No binding was seen for immunoprecipitated samples without p65 antibody (**Figure 6b**). These results were further confirmed when we transiently transfected p65 expression plasmid into MCF-10F cells and hTREX84 protein was also increased (**Figure 6c**). Moreover, when we knocked down p65 expression by siRNA targeted *p65*, hTREX84 protein also decreased, as

predicted (**Figure 6d**). In summary, these results show for the first time that RelA/p65 plays a pivotal role in regulating the hTREX84 expression.

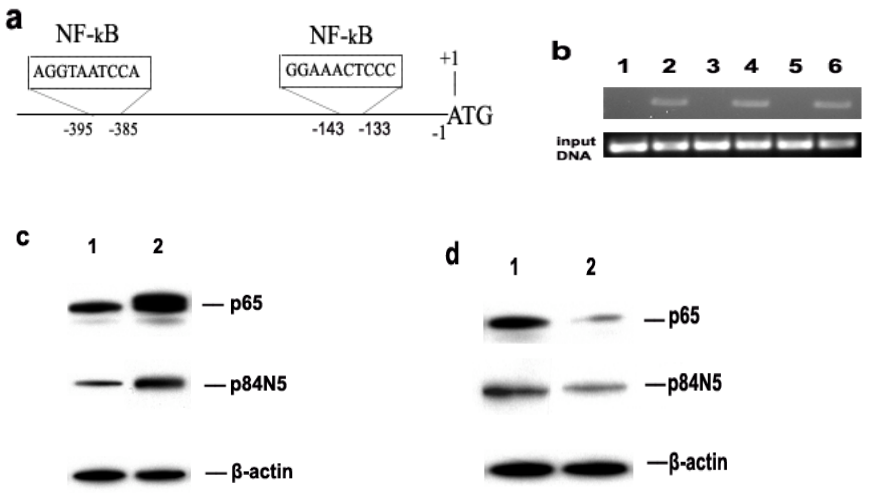


Figure 6. The role of NF-κB in regulation hTREX84 expression. *(a),* Schematic diagram of the *hTREX84* promoter, indicating the NF-kB DNA binding motif. *(b)*, ChIP assays of p65 binding to hTREX84 gene promoter in MDA-MB-231 (lanes 1, 2); OVCAR5 (lanes 3, 4); OVCAR 10 (lanes 5, 6). Cells were cultured for 72 h. ChIP assays were then performed with anti-p65 antibody. PCR analysis was performed on immunoprecipitation samples without antibody (lane 1, 3,5), with p65 antibody (lane 2, 4, 6). *(c)*, Western blot analysis of p65, hTREX84 and β-actin after MCF-10F cells were transiently transfected with control vector (lane 1), or with p65 plasmid (lane 2), for 48 h. *(d)*, Western blot analysis of p65, hTREX84 and β-actin protein levels after treatment of MDA-MB-231 cells with control siRNA (lane 1) or siRNA against p65 (lane 2) for 72 h.

Table 1. RelA/p65 expression in human normal breast tissue and tumors

	N	0/+	++	+++
Normal breast tissue	5	4		1
Tumor histologic grade				
1 (well differentiated)	22	11	7	4
2 (moderately differentiated)	33	7	16	10
3 (poorly differentiated)	34	3	10	21

Since our previous studies demonstrated that hTREX84 was highly expressed in the cell nucleus, especially in poorly differentiated and more aggressive human breast cancers, we asked whether RelA/p65 might also be expressed in a similar manner. We examined the protein expression of RelA/p65 by immunohistochemical analysis in 89 cases of human breast cancer, as well as 5 normal breast tissues (**Table 1**). This tumor panel includes 22, 33 and 34 cases of well, moderately and poorly differentiated tumors, respectively. RelA/p65 was weakly (0/+1) detected in normal breast epithelial cells (4 of 5) and protein staining indicated cytoplasmic localization (**Figure 7a**). Staining for RelA/p65 was also observed mainly in the cytoplasm in well-differentiated tumors (**Figure 7b**). Distinctly granular staining with an increased number of positively stained nuclei was observed in the poorly differentiated tumor specimens (+2/+3, 31 of 34) (**Figure 7c**). Similar expression patterns of RelA/p65 and hTREX84 in normal and malignant cells suggests a correlation between expression and tumor progression and metastasis.

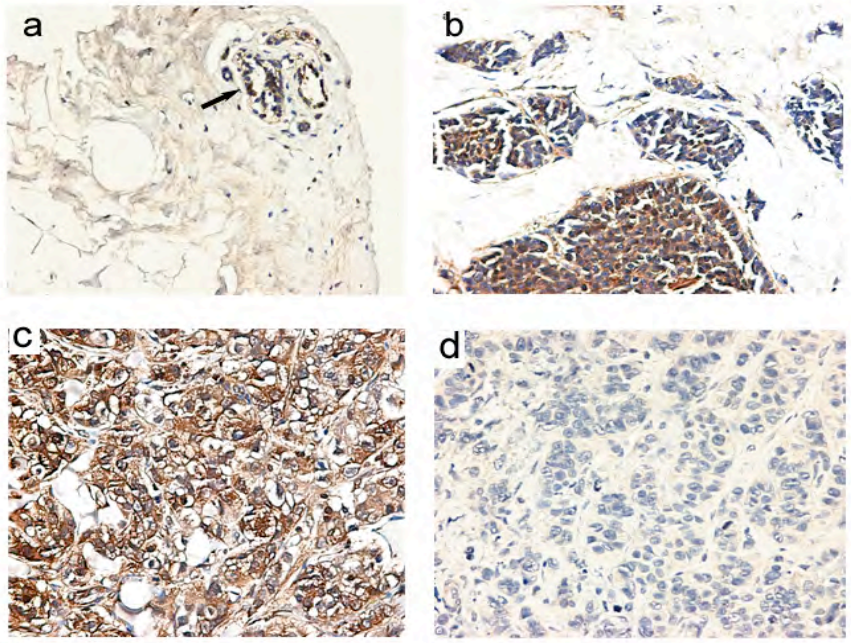


Figure 7. Immunohistochemical staining of RelA/p65 proteins in representative breast cancer tissue specimens. *A*, RelA/p65 was weakly detected in normal breast epithelial cells and positive products were located in the cell cytoplasm. *B*, Staining for RelA/p65 was detected mainly in the cytoplasm in high differentiated tumors. *C*, Intense and distinctly granular staining for RelA/p65 was detected in stained nuclei of low differentiated tumor specimens. *D*, Tumor section evaluated without the primary antibody to serve as a negative control. Magnification 200x.

Additional findings relevant to hTREX84's role in the pathogenesis of breast cancer.

In our previous report, we observed that hTREX84 protein is undetectable in most normal breast tissues by Western blot analysis. However, *hTREX84* mRNA is detected by RT-PCR analysis. We speculated that a mechanism of hTREX84 protein level regulation, which is independent of RNA level regulation, might exist in the normal cell. Since we lacked appropriate cultured normal breast epithelial cells for this study, we selected a model of 32D cells, which are well-characterized diploid murine hemopoietic cells. 32D cells have an absolute requirement for interleukin-3 (IL-3), and undergo apoptosis when IL-3 is withdrawn. When we induced the cell to differentiate by Granulocytic-Colony Simulating Factor (G-CSF), hTREX84 protein rapidly disappeared on the second day; however mRNA levels decreased but were still present even after 5 days. When we treated cells with Lactacystin, a proteasome inhibitor, hTREX84 degradation was significantly delayed. Further, hTREX84 protein was not able to bind ubiqutin, indicating hTREX84 protein degradation during cell differentiation might occur through a ubiqutin-independent pathway.

Task 2 "To evaluate *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *hTREX84* mutations."

We designed four sets of primers specific for *hTREX84* cDNA and performed DNA sequence analysis (**Table 1**). The results showed that all cancer cell lines have several nuclear base changes that are different from the original cloned hTREX84 cDNA sequence (GenBank NM_005131), but identical to the later predicted hTREX84 cDNA sequence (GenBank XM_008756), by automated computational analysis using a gene prediction method, BLAST, supported by mRNA and EST evidence as well as our cDNA sequence (**Table 2**). To further confirm this, we sequenced cDNA from 7 SV40 Tag immortal HOSE cell lines (HIO-102, HIO-113, HIO-114, HIO-117, HIO-121, HIO-135, HIO-166), which were derived and maintained in our laboratory, as well as cDNA from 6 ovarian cancer cell lines (OVCAR2, OVCAR3, OVCAR4, UPN251, UPN275 and A2780). All the ovarian cell lines showed cDNA sequence results identical to those of breast cancer cell lines. Based on these data, we concluded that there are no somatic mutations in hTREX84 in these cell lines.

We also tested 70 cases of *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *hTREX84* mutations by EMD and direct DNA sequencing. Again, no germline mutations were detected. Interestingly, we failed to observe polymorphisms in *hTREX84* sequence. Additionally, 72 cases of sporadic breast cancer and 30 cases of ovarian cancer, as well as 35 cases of EBV-transformed lymphocytes, which were generated from *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds, were screened by Western blot analysis. No aberrant protein bands were observed (data not shown). Therefore, activation of hTREX84 is independent of mutations and is associated with overexpression.

Table 1. Oligonucleotide primer pairs for PCR amplification of the entire cDNA sequence of hTREX84

Primer pairs [1]	Oligonucleotide sequences (5'-3')
47U/515L	5'-CTCTTCAGTTTGCCCGAAGC/AAAAGAGCTGAATCCGTCCA-3'
Exons 1-7, fragment length 469 bp	
506U/1033L	5'-CAGTCTTCTGTGGACGGATTC/ATGCCCCTTGAGATATTGGA-3'
Exons 7-12, fragment length 548 bp	
1026U/1576L	5'-CAAGTGAAAAGCTGATGGATTT/TTTAAACTGCTGGTTGGTTGG-3'
Exons 11-19, fragment length 641 bp	
1442U/2083L	5'-GAACAGGCAGACCCTGAAAA/CCAAAACCAGTGGACCTCTT-3'
Exons 18-21, fragment length 642 bp	

[1] Primers were designated by nucleotide position to hTREX84 (GenBank XM_00876) corresponding to the 5' position, followed by the letter U for upper (*i.e.,* sense strand) or L for lower (*i.e* ,anti-sense strand).

Table 2. The nuclear base and corresponding amino acid differences of cDNA in the cell lines from the original hTREX84 cDNA sequence

Exon	Nucleotide position-database (encoded amide acid)	Nucleotide position-cell lines (encoded amino acid)
6	388G (A)	388T (S)
9	657T	657C
16	1298C (T)	1298T (M)
18	1438C (A)	1438T (V)
19	1458A (M)	1458G (V)
19	1493A (K)	1493G (R)
19	1556A (Q)	1556C (P)

C. KEY RESEARCH ACCOMPLISHMENTS:

C.1. "The nuclear death domain protein hTREX84; a candidate breast cancer susceptibility gene"

1.a. Demonstrated that p84N5 (hTREX84) is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.

1.b. Report that TREX84 expression correlates with tumor size and the metastatic state of the tumor progression, i.e., identify TREX84 as a prognostic marker for aggressive forms of human breast cancer.

1.c. Identified that TREX84 is the human counter part of the yeast TREX complex reported to couple transcriptional elongation and mRNA export.

1.d. Demonstrated that TREX84 is over-expressed in both breast and ovarian tumors.

1.e. Abrogation of *TREX84* expression leads to growth arrest in both breast and ovarian tumor cell lines.

1.f. Found that 5-aza-C can induce TREX84 expression that is not dependent on hTREX84 promoter methylation.

1.g. Identified several transcriptional factors, including NF-κB binding sites in the *hTREX84* gene promoter and demonstrated by chromatin immunoprecipitation (ChIP) and site directed mutagenesis that RelA/p65 binds the NF-kB binding sites and stimulates *hTREX84* expression.

1.h. Demonstrated by immunohistochemistry (IHC) that RelA/p65 is abundantly expressed in malignant cells that aberrantly express hTREX84 indicating that RelA/p65 might play a pivotal role in regulating hTREX84 expression in breast cancer.

D. REPORTABLE OUTCOMES (5/2004 to present):

D.1. "The nuclear death domain protein hTREX84; a candidate breast cancer susceptibility gene"

1.a. Abstracts

Shan-Chun Guo and A.K. Godwin. Accumulation of hTREX84 domain protein is associated with an aggressive phenotype of human breast tumors. Proceedings of American Association of Cancer Research, 44:2421, 2003.

Guo, S., Farber, M.J., Shiekhattar, R. and Godwin, A.K. Over-expression of death domain containing protein-hTREX84 in human ovarian cancer cell lines is associated with cell proliferation. Proceedings of American Association of Cancer Research, 44:1805, 2004.

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Coupling transcriptional elongation and mRNA export to metastatic breast cancers. Proceedings of American Association of Cancer Research, 46:5588, 2005.

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Linking transcriptional elongation and mRNA export to metastatic breast cancers. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, June 8-11, 2005, P40-6 (selected for oral presentation)

1.b. Publications

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Linking Transcriptional Elongation and mRNA Export to Metastatic Breast Cancers. Cancer Res. 65(8):3011-3016, 2005.

Guo, S., Vanderveer, L., Farber, M.J. Godwin, A.K. Translational Regulation of hTREX84: A Link Between Transcriptional Elongation, mRNA Export and Cancer. The manuscript has been modified and resubmitted, 2007.

Book chapters and review articles:

Pan, Z-Z., Godwin, A.K. Oncogenes. Encyclopedia of Molecular Cell Biology and Molecular Medicine. Edited by R.A. Meyers, Second Edition, Volume 9. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp., 435-495, 2005.

Bui, C.T., Nicolas, E., Sallmann, G., Chiotis, M., Lambrinakos, A., Rees, K., Trounce, I., Cotton, R.G.H., Hancock, L., Godwin, A.K., Yeung, A.T. Enzymatic and Chemical Cleavage Methods to Identify Genetic Variation. In Molecular Diagnostics (Ed. G. Patrinos and W Ansorge) in press 2006.

Chen, X., Arciero, C., Godwin, A.K. BRCA1-associated complexes: New targets to overcome breast cancer radiation resistance. Expert Review of Anticancer Therapy, 6:187-196, 2006.

E. CONCLUSIONS:

E.1. "The nuclear death domain protein hTREX84; a candidate breast cancer susceptibility gene"

THO/TREX is a conserved eukaryotic complex containing Tho2, HPR1, MFT1 and Thp2, as well as proteins involved in mRNA metabolism and export such as Aly and UAP56. In the present work, we identified human hTREX84 (referred to as hTREX84) as a conserved counterpart of yeast protein HPR1 (Tho1) and for the first time, demonstrated that aberrant hTREX84 expression is associated with not only human breast cancer, but also ovarian cancers. We report that the demethylation agent, 5-aza-2-deoxycytidine, significantly induced hTREX84 mRNA and protein expression, suggesting that abnormal expression of hTREX84 might be mediated by epigenetic mechanisms. We also have found that RelA/p65 plays a pivotal role in regulating the hTREX84 expression. Other mechanisms such as protein abnormal degradation might also contribute hTREX84 over-expression in cancer cells. Thus hTREX84 might be served as good tumor proliferation maker as well as an ideal target for therapeutic drugs against cancer.

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APPENDICES

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Linking Transcriptional Elongation and mRNA Export to Metastatic Breast Cancers. Cancer Res. 65(8):3011-3016, 2005.

Guo, S., Vanderveer, L., Farber, M.J. Godwin, A.K. Translational Regulation of hTREX84: A Link Between Transcriptional Elongation, mRNA Export and Cancer. The manuscript has been modified and resubmitted, 2007.

Linking Transcriptional Elongation and Messenger RNA Export to Metastatic Breast Cancers

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Abstract

The biochemical pathways that are disrupted in the genesis of sporadic breast cancers remain unclear. Moreover, the present prognosticating markers used to determine the prognosis of node-negative patient leads to probabilistic results, and the eventual clinical course is far from certain. Here we identified the human TREX complex, a multiprotein complex that links transcription elongation to mRNA transport, as culprit of aggressive human breast cancers. We show that whereas p84N5 (called hTREX84) is expressed at very low levels in normal breast epithelial cells, it is highly expressed in breast tumors. Importantly, hTREX84 expression correlates with tumor size and the metastatic state of the tumor progression. Reduction of hTREX84 levels in breast cancer cell lines by small interfering RNA result in inhibition of cellular proliferation and abrogation of mRNA export. These results not only identify hTREX84 as a prognosticator of breast cancer but also delineate human TREX complex as a target for therapeutic drugs against breast cancer. (Cancer Res 2005; 65(8): 3011-6)

Introduction

Metastatic tumors are the most prevalent cause of death in cancer patients. A major aim in studying metastasis is to understand the mechanism by which cancer cells acquire distinct genetic and epigenetic changes that result in their progression through metastatic states. Recent experiments using microarray studies have expanded our understanding of metastasis in various human tumor samples (1, 2). Although such studies have been powerful for producing gene expression fingerprints of metastatic tumor cells, it has been difficult to assess the contribution of individual genes to the metastasis progression. Breast cancer is the most common malignancy in women and it could be effectively cured if diagnosed at an early stage. The most commonly used predictive molecular markers for breast cancer include Ki-67, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (3). We searched for new prognostic markers that not only could be predictive of the more aggressive forms of breast cancers but also could further provide mechanistic insight into the molecular mechanism underlying metastasis. In this study, we describe the increased expression of TREX84, a subunit of a multiprotein complex involved in transcriptional elongation and mRNA export, in human breast cancer and its intimate association with breast cancer progression and metastasis.

Note: S. Guo and M-A. Hakimi contributed equally to this work.
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Materials and Methods

Primary breast cancer specimens. Human breast tissue specimens used in this study were collected following NIH guidelines and using protocols approved by the Institutional Review Board at Fox Chase Cancer Center. These specimens were surgically obtained from breast cancer patients at Fox Chase from 1991 to 2002. A total 72 primary breast cancer were examined which included 69 invasive ductal carcinomas and 3 invasive lobular carcinomas. Seventy females and two males were included in the study. Ninety percent (65 of 72) of the patients were Caucasian (i.e., white non-Hispanic), 8% (6 of 72) were African American, and 1% (1 of 72) were Asian. The age range was 31 to 97 years with a median age of 56 years. Grading of histologic malignancy of each specimen was assessed according to the system as reported previously (4, 5). Lymphonodal metastatic status was determined by histopathologic examination in each case according to the pTNM classification as proposed by the American Joint Committee on Cancer. Thirty-seven paired normal breast tissues were also obtained from the above patients. All of the samples were snap frozen in liquid nitrogen and kept at −80°C until used. Tissue extracts were prepared as previously described (6).

Affinity purification of Flag-p84. Flag-p84 and a selectable marker for puromycin resistance were cotransfected into HeLa cells. Transfected cells were grown in the presence of 5 mg/mL puromycin, and individual colonies were isolated and analyzed for Flag-p84 expression. To purify the p84 complex, nuclear extract from the Flag-p84 cell line was incubated with anti-Flag M2 affinity gel (Sigma, St. Louis, MO), and after extensive washing with buffer A [20 mmol/L Tris-HCl (pH 7.9), 0.5 mol/L KCl, 10% glycerol, 1 mmol/L EDTA, 2 mmol/L $MgCl_2$, 5 mmol/L DTT, and 0.5% NP40], the affinity column was eluted with buffer A containing Flag peptide (500 mg/mL) according to manufacturer's instructions (Sigma). p84-containing eluate were fractionated on a Superdex 200 (Pharmacia, Peapack, NJ) equilibrated in 0.5 mol/L KCl in buffer A containing 0.1% NP40 and 1 μg/mL aprotinin, leupeptin, and pepstatin. Analysis of nuclear extract on Superose 6 was as described previously (7).

Glutathione *S*-transferase pulldown with UAP56. Control glutathione *S*-transferase (GST, lanes 1) or GST-UAP56 (lane 2) was incubated with HeLa nuclear extract. After washing with BC500 buffer [20 mmol/L Tris-HCl (pH 8), 500 mmol/L KCl, 10% glycerol, 0.2 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride] proteins bound to GST-UAP56 or GST were analyzed by Western blot with p84 antibodies.

Organoid isolation, cell lines, and cell culture. Media and cell culture reagents were prepared by the Cell Culture Facility at Fox Chase Cancer Center. Eighteen cases of organoids were separated and prepared by using collagenase digestion as described previously (8, 9). Six primary cultures of human breast epithelial cells were established and cultured in 199 Medium with 15% fetal bovine serum and insulin (290 units per 500 mL). Six primary cultures of human breast fibroblast cells were cultured in DMEM supplemented with 20% FBS and 1× antibiotic-antimycotic solution. Human breast cancer cell lines MDA-MB-231, MDA-MB-435, MDA-MB-468, MCF-7, BT-20, and ZR-75-1 were cultured in DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic solution. T47D cells were maintained in RPMI supplemented with 10% FBS and 0.2 unit/mL of pork insulin. SKBP-3 cells were maintained in McCoy's 5a medium supplemented with 15% FBS.

Immunofluorescence. Cells grown in monolayer cultures were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100,

and blocked with 10% FCS before antibody staining. Staining by anti-p84 antibodies was visualized with corresponding fluorescein-labeled secondary antibody. All images were acquired with a bio-Rad MRC1000 confocal microscope.

Western blotting assay. After cell lysates were obtained from cell lines or tissues, 30 μg of total protein from each sample were analyzed by Western blotting. Protein extracts were electrophoresed on a 4% to 20% Tris-glycine gel, and the separated proteins were electrophoretically transferred to nitrocellulose for immunodetection. The membrane was blocked in 5% nonfat dry milk in TBST for 1 hour at room temperature and incubated with monoclonal antibody to human p84N5 at a dilution of 1:2000 in TBST + 2.5% nonfat dry milk, followed by horseradish peroxidase-conjugated antimouse secondary antibody (Amersham, Piscataway, NJ) at a dilution of 1:10,000. Immunoblots were reprobed with β-actin monoclonal antibody to confirm equal loading. MDA-MB-435 cell extracts were used as a control sample in each of the experiments. The expression levels of p84 and β-actin detected by immunoblotting were quantitated using the program IMAGE (NIH) for the integrated density of each band. Western blot assays were conducted in duplicate for each sample and the mean value was used for the calculation of protein expression levels.

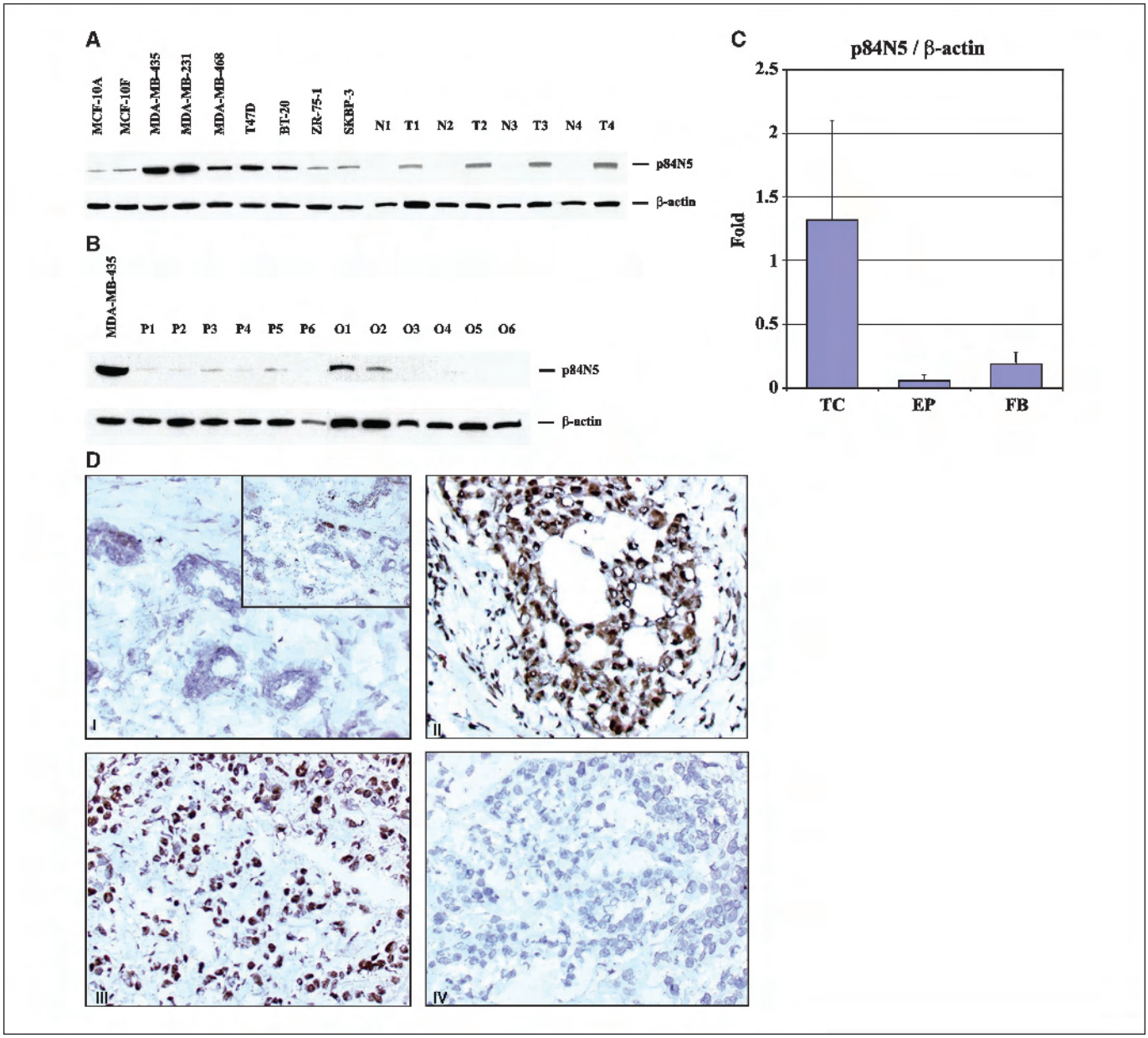

Figure 1. p84N5 is aberrantly expressed in breast cancer. *A,* p84N5 protein expression in immortal breast epithelial cell lines (MCF-10A and MCF-10F), breast tumor cell lines, paired normal (N1-4), and breast cancer (T1-4) tissues. Protein samples were separated on a SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 or β-actin monoclonal antibodies. *B,* p84N5 protein expression in primary breast epithelial cell cultures (P1-P6) and purified organoids (O1-O6) by Western blotting. *C,* p84N5/β-actin ratio in breast cancer cell lines (*TC*), primary breast epithelial cell cultures (*EP*), fibroblast cell cultures (*FB*). *D,* immunohistochemical analysis of frozen sections of normal breast tissue and breast tumor specimens for the p84N5 protein. *I,* p84N5 is weakly expressed in the cytoplasm and nuclei of normal ductal epithelia and lobular epithelia. A few epithelial structures showed moderate immunostain. *Inset,* same region at lower magnification to show overall staining pattern with only few moderately stained ductal structures. *II,* p84N5 is intensively expressed in the cytoplasm and nuclei of a grade 1 invasive ductal carcinoma. *III,* p84N5 is expressed at high levels exclusively in the nuclei of a grade 3 invasive ductal carcinoma. *IV,* previous tumor section evaluated without the primary antibody to serve as a negative control. Magnification 200×.

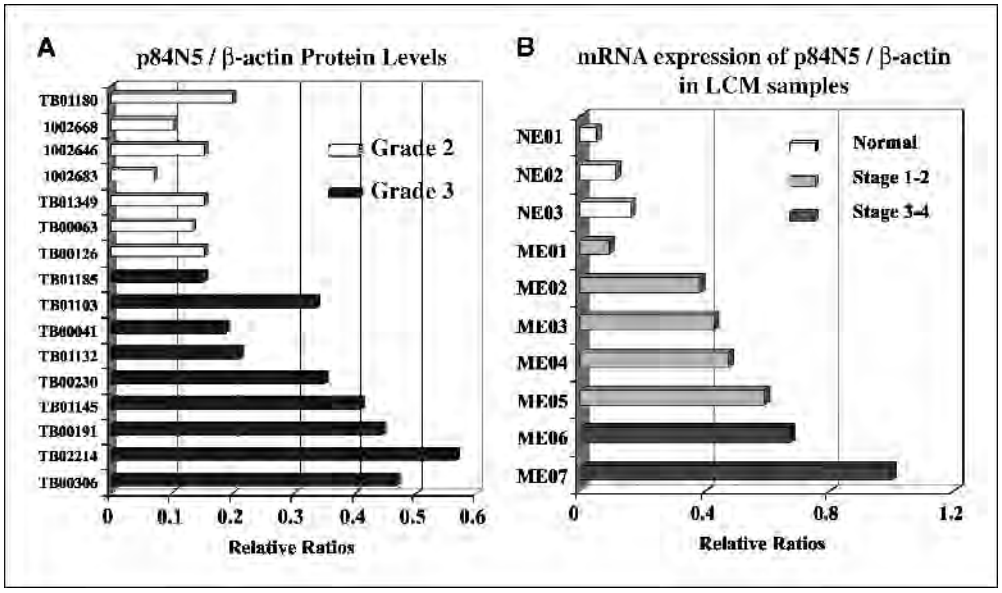

Figure 2. p84N5 displays increased expression in late-stage tumors. *A,* expression of p84N5 by Western blot analysis in the same grade 2 and 3 breast tumors as evaluated. *B,* quantitative real-time PCR analysis of normal mammary lobular epithelial cells (*NE*) and malignant epithelial (*ME*) cells captured by laser capture microdissection. All tumors were grade 3 and were separated based on clinical staging [i.e., combined primary tumor staging (*Tis*), nodal staging (*N0*), and metastatic staging (*M0*)]. ME01-05 were determined to be stage I and II breast tumors, whereas ME06-07 were stage III and IV tumors according to the *AJCC Staging Manual*.

Immunohistochemistry. p84N5 protein immunostaining was carried out with mouse monoclonal p84N5 antibody (Novus Biologicals, Littleton, CO), at a dilution of 1:100. Because the antibody available does not recognize p84N5 in formalin-fixed, frozen sections were used. For frozen section immunohistochemistry, the sections were fixed in cold acetone for 10 minutes and rinsed in cold PBS for 5 minutes. The sections were then incubated in methanol/0.3% hydrogen peroxide for 10 minutes, washed with PBS, and treated with 0.1% Triton X-100 in PBS for 5 minutes and washed with PBS again. The sections were then incubated at 4°C overnight with p84N5 antibody. Reaction products were visualized by immersing the glass slides in 3,3-diaminobenzidine tablet sets (Sigma Fast, Sigma) and counterstained with hematoxylin. A positive control was included in each experiment. As negative controls, either the p84N5 antibody was omitted or sections were washed in 1× PBS.

Laser capture microdissection. Laser capture microdissection (LCM) was done as previously described with minor modification (7). In brief, frozen normal and tumor breast tissue samples were embedded in ornithine carbamyl transferase medium, sectioned in a cryostat at 8-μm thickness, and mounted on nonadhesive glass slides. Fixation was done in 70% ethanol for 60 seconds. Breast epithelial cells were visualized by H&E staining. H&E-stained frozen sections were dehydrated for 30 seconds in 70%, 95%, and 100% ethanol with a final 2-minute dehydration step in xylene. Air-dried sections were then laser captured and microdissected by a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). The normal or malignant mammary epithelial cells to be selectively microdissected away from stroma were identified and targeted through a microscope, and a 15-μm laser beam pulse activated the film on a CapSure LCM Cap (Arcturus Engineering). Approximately 5×10^3 cells were captured for each specimen. Based on careful review of the histologic sections, each microdissection is estimated to contain ~90% of the desired cells. After microdissection, 100 μL of guanidinium isothiocyanate-containing lysis buffer with 0.7 μL mercaptoethanol were applied directly to the microdissected cells adhered on the CapSure LCM cap, samples were placed into a 0.5-mL microfuge tube, and vortexed vigorously. Total RNAs were extracted using the Strata Prep Total RNA Microprep Kit (Stratagene, La Jolla, CA). A DNase treatment was done according to the manufacturer's recommendations. The RNA was resuspended in 20 μL of RNA elution buffer. After being reconcentrated by vacuum without heat, total RNA from each LCM sample was reverse transcribed in a 20-μL reaction as described above.

Quantitative real-time PCR analysis. cDNA mixture (0.63 μL) above was used in a real-time PCR reaction (25 μL total volume) done with Smart Cycle TD (Cepheid, Sunnyvale, CA) following methods recommended by the manufacturer. Optimal conditions were defined as step 1, 95°C for 10 minutes; step 2, 95°C for 15 seconds and 60°C for 60 seconds with Optics, repeated for 50 cycles. The relative mRNA expressions of *p84N5* were adjusted with *ACTB*. The primer and probe sets used for real-time PCR were as follows: *p84N5*, forward primer 5′-GGAACCCTGTGCAATGCTATG-3′ and reverse primer 5′-ACATGTTCTCCTCCTGTTTTCAATT-3′; Taqman probe, (FAM) 5′-ATAAATTAGATGATACTCAGGCCTCAAGAAAAAAGATGGA-3′ (BHQ1). *ACTB*: forward primer 5′-GCCAGGTCATCACCATTGG-3′ and reverse primer 5′-GCGTACAGGTCTTTGCGGAT-3′; Taqman probe, (Cal red) 5′-CGGTTCCGCTGC CCTGAGGC-3′ (BHQ2).

Small interfering RNA transfection and cell proliferation. The small interfering RNA (siRNA) sequences targeting *p84N5* corresponded to the coding region 1652 to 1672 (5′-AATGATGCTCTACTGAAGGAA-3′) relative to

Table 1. Relationship between p84 protein expression and clinicopathologic variables

	n	Mean	Lower bound	Upper bound	*P*
Menopausal status					
Premenopausal	27	0.280	0.165	0.396	0.375
Postmenopausal	45	0.180	0.139	0.260	
Tumor size (cm)					
≤2	21	0.134	0.078	0.191	0.015
>2	50	0.285	0.234	0.363	
Lymph node metastasis					
Negative	31	0.131	0.077	0.185	0.002
Positive	36	0.329	0.232	0.425	
Histologic grade					
2	18	0.143	0.053	0.233	0.033
3	51	0.283	0.213	0.354	
Estrogen receptor					
Negative	23	0.32	0.193	0.448	0.063
Positive	36	0.177	0.122	0.247	
Progesterone receptor					
Negative	28	0.331	0.219	0.442	0.011
Positive	31	0.147	0.092	0.219	

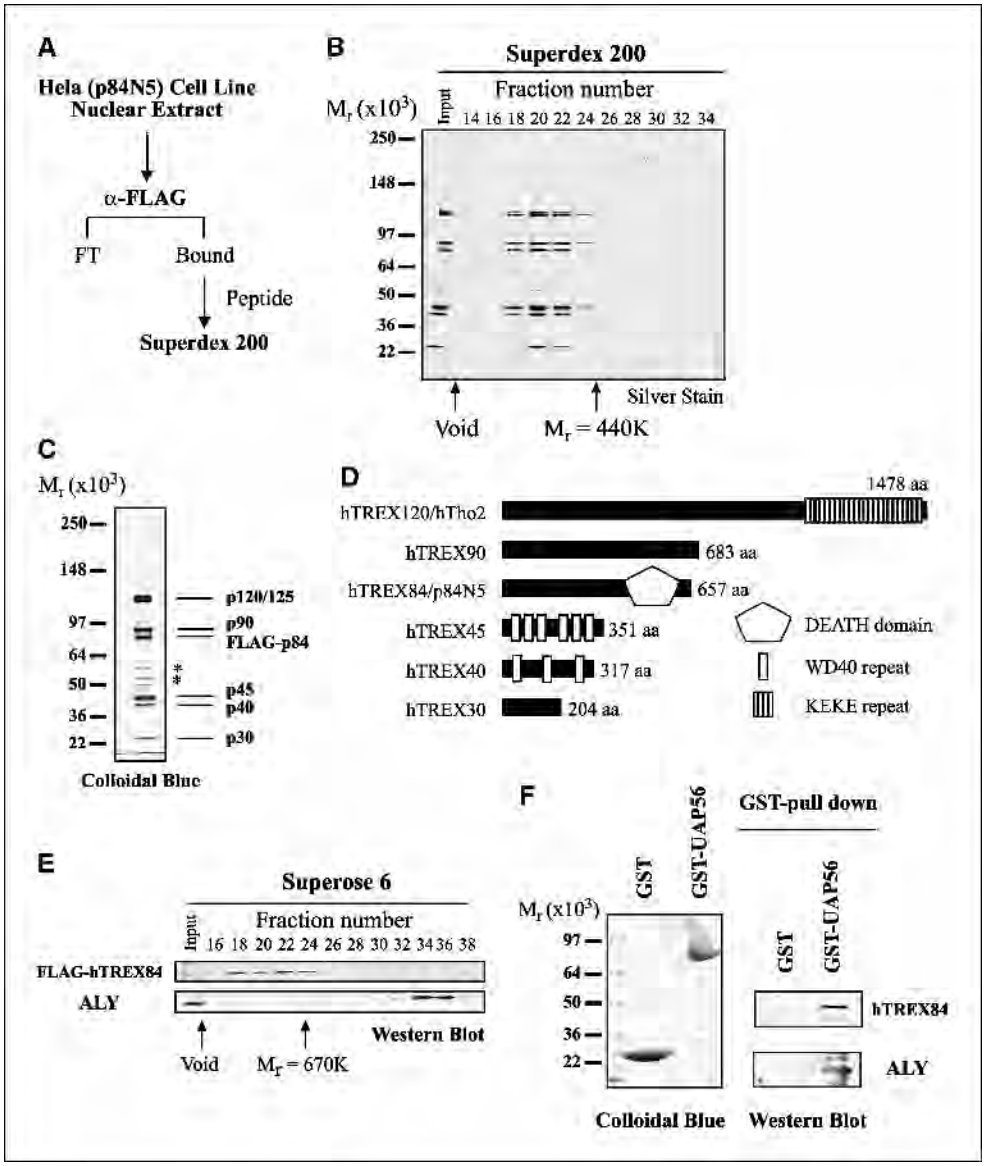

Figure 3. p84N5 is a component of the human TREX complex. *A,* schematic of p84N5 isolation using a 293-derived Flag-tagged cell line. *B,* human TREX complex isolated using the protocol shown in (*A*) was analyzed by silver staining following fractionation on the Superset 200. *C,* colloidal blue analysis of Flag-affinity eluate shown in (*A*). Individual bands were excised and subjected to mass spectrometric sequence analysis. *D,* diagrammatic representation of human TREX subunits. hTREX120, hTREX90, hTREX45, hTREX40, and hTREX30 correspond to Genbank accession nos. AL030996, XM_037945, NM_032361, NM_024339, and BC020599, respectively. *E,* analysis of nuclear extract using Superose 6 gel filtration. Column fractions were analyzed by Western blotting using antibodies (*right*). *D,* GST or GST-UAP56 were used for affinity-purification of human REX and ALY proteins.

the start codon. The corresponding siRNA duplexes with the following sense and antisense sequences were used: 5′-UGAUGCUCUACUGAAGGAAdTdT (sense) and dTdTACUACGAGAUGACUUCCUU-5′ (antisense). A nonspecific control XI siRNA duplex had the following sequences: 5′-AUAGAUAAG-CAAGCCUUACUU (sense) and UUUAUCUAUUCGUUCGGAAUGP-5′ (antisense). All of the siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry.

Cells in the exponential phase of growth were plated at 30% confluence in 6-cm plates, grown for 24 hours, and transfected with siRNA (p84N5 siRNA: 200 nmol/L) using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose-response studies. Silencing was examined 24, 48, and 72 hours after transfection. Control cells were treated with oligofectamine (mock) or transfected using a control siRNA. Cell proliferation and apoptosis was examined using Guava ViaCount and Nexin assays, respectively as previously described (10). All studies were done in triplicates.

Statistical methods. Statistical analyses, including χ^2 and t test, were done using Microsoft Excel software. All statistical tests were two sided, and Ps < 0.05 were considered to be statistically significant. Error bars represent 95% confidence intervals.

Results and Discussion

To identify novel genes whose aberrant regulation may result in sporadic breast cancer, we analyzed the expression profiles of genes in breast tumors using public databases. We focused on p84N5, a nuclear protein containing a DEATH-domain previously reported to associate with *Rb* (11, 12), as one of the genes that displayed increased expression in breast cancers. To directly analyze the expression of p84N5 in breast cancers, we compared the p84N5 protein levels in the breast cancer tissues and the surrounding normal tissues using Western blot analysis. As Fig. 1*A* indicates, whereas cancerous tissues displayed high levels of p84N5 expression, the levels of p84N5 in normal tissues were nearly undetectable (compare N1 through N4 and T1 through T4). Similar increased expression of p84N5 is evident comparing breast cancer cell lines and normal primary epithelial cells or breast organoids (Fig. 1*A* and *B*). We substantiated these results by examining the expression of p84N5 using real-time PCR and immunohistochemistry. Using frozen sections, we detected by immunohistochemistry that normal breast tissue displayed a heterogeneous expression

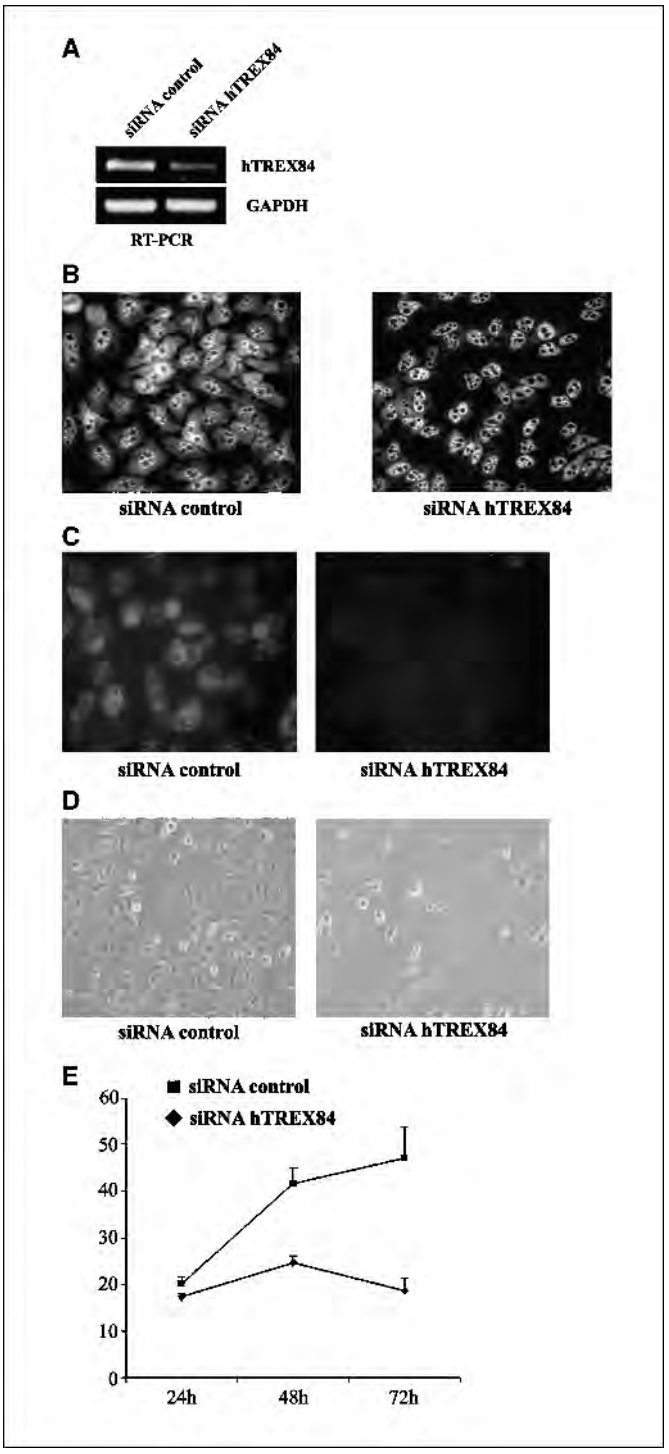

Figure 4. Knock down of TREX84 leads to defects in mRNA export and cellular proliferation. *A,* analysis of TREX84 and GAPDH mRNA levels following treatment of HeLa cells with siRNA against TREX84 or control siRNA. *B,* treatment of HeLa cells with siRNA against TREX84 or control siRNA; treatment of cells with siRNA against TREX84 results in accumulation of mRNA in the nucleus. *C,* analysis of TREX84 expression following siRNA treatment for 72 hours by immunofluoresence staining in MDA-MB-231 tumor cells (*left,* cells transfected with control siRNA; *right,* cells treated with TREX84-siRNA). *D,* photomicrographs show the morphology of the MDA-MB-231 cells following abrogation of TREX84 expression (*left,* tumor cells transfected with control siRNA; *right,* cells treated with TREX84-siRNA). *E,* cell proliferation of breast tumor cells following abrogation of TREX84. Cell proliferation and apoptosis (data not shown) was examined using Guava ViaCount and Nexin assays, respectively. Plotted is the number of viable cells ($\times 10^4$) at 24, 48, and 72 hrs after treatment with control siRNA or with TREX84-siRNA. Three independent experiments.

pattern with a few ductal and lobular epithelial structures exhibiting moderate expression of p84N5, whereas most of the normal breast showed mild or negative expression of the protein (Fig. 1*D*, *I*). Conversely, ductal carcinomas showed an intense and homogeneous expression of p84N5, which is consistent with the Western blot analysis (Fig. 1*C* and *D*, *II-IV*).

We next asked whether p84N5 expression levels were indicative of the aggressive nature of the breast cancers. Comparison of early-stage tumors (grade 2) and those of later stages (grade 3) revealed a marked elevation of p84N5 RNA and protein levels in late-stage tumors (Fig. 2*A* and data not shown). Importantly, analysis of p84N5 levels in a large number of tumors revealed a strong relationship between p84N5 expression levels and lymph node metastasis ($P = 0.002$) and tumor size ($P = 0.015$; Table 1). Other prognostic indicators, including ER positivity ($P = 0.063$) and histologic grade ($P = 0.033$) were also found to be associated with increased p84N5 protein levels. To further confirm these results, lobular epithelial cells from normal breast tissues and malignant epithelial cells from grade 3 tumors were captured by laser capture microdissection and p84N5 levels were analyzed by quantitative real-time PCR (Fig. 2*B*). As Fig. 2*B* attests, the expression levels of p84N5 transcripts are elevated in all but one of the tumors as compared with histologically normal epithelial cells. When these tumors were subdivided based on clinical staging [combined T (tumor size), N (nodal involvement), M (metastatic) classification], p84N5 levels correlated with more aggressive tumors (stage I-II versus III-IV). Taken together, these data indicate that p84N5 is highly expressed in breast cancers and its expression is strongly associated with an aggressive phenotype of human breast tumors.

To gain insight into the biological role of p84N5, we isolated a p84N5-containing multiprotein complex from mammalian cells. This was accomplished by developing a 293-derived stable cell line expressing Flag-tagged p84N5. Figure 3*A* depicts the purification of Flag-p84N5 using anti-Flag antibodies followed by the analysis of the Flag-p84N5 eluate using gel filtration chromatography. This analysis revealed the specific association of p84N5 with polypeptides of 125, 120, 90, 45, 40, and 30K (Fig. 3*B* and *C*). Interestingly, mass spectrometric sequencing of p84N5-associated polypeptides revealed the identity of p84N5 associated proteins as the human counter parts of the yeast TREX complex reported to couple transcriptional elongation and mRNA export (Fig. 3*D*; refs. 13, 14). Therefore, we have termed this complex human TREX and p84N5 as hTREX84. Importantly, in contrast to the yeast TREX complex, the human complex was devoid of the RNA export and splicing factors ALY and UAP56 (13). We therefore asked whether endogenous ALY and hTREX84 form a stable complex which is reflected by coelution of the two proteins on gel filtration. Analysis of HeLa nuclear extract by Superose 6 sizing fractionation showed distinct chromatographic elution profiles for hTREX84 and ALY proteins indicating that the two proteins are not stably associated (Fig. 3*E*). However, consistent with a previous report (13), we observed the association of hTREX and ALY through the UAP56 protein (Fig. 3*F*), and that hTREX and ALY colocalize in breast tumor cells as determined by immunofluorescence assays (data not shown). These results indicate that whereas hTREX and ALY may not be stably associated, their interaction is promoted by the UAP56 protein.

The yeast TREX complex was shown to be intimately involved in the export of mRNA to the cytoplasm (13, 14). We therefore, asked whether human TREX also plays a role in mRNA export.

mRNA was visualized using immunofluorescent analysis using oligo-dt as probes. To address the role of human TREX in mRNA export, hTREX84 protein was depleted using siRNA against hTREX84 following which mRNA levels were analyzed (Fig. 4*A*). Whereas the mRNA in cells treated with control siRNA could be visualized in both the cytoplasmic and the nuclear domains, treatment of cells with siRNA against hTREX84 resulted in the accumulation of mRNA in the nucleus and the loss of cytoplasmic mRNA (Fig. 4*B*). These results indicate that similar to the role for yeast TREX complex, hTREX plays a pivotal function in mRNA export.

Because hTREX84 is highly expressed in aggressive forms of breast cancer, we asked whether reduction of hTREX84 concentrations may slow the proliferative capacity of breast cancer cells. Human breast cancer cell lines express high levels of hTREX84 compared with that of primary breast epithelial cells and organoids (Fig. 1*A* and *B*). To address the proliferative potential of hTREX84, we treated MDA-MB-231 breast cancer cell line with siRNA against hTREX84 (Fig. 4*C*). Treatment of breast cancer cells with siRNA against hTREX84 potently and specifically reduced the proliferative potential of these cells (Fig. 4*D* and *E*). Analyses of these cells using a GuavaNexin assay found no statistically difference for Annexin V-PE and 7-AAD positive cells in siRNA treated cells, indicating the absence of induction of apoptosis (data not shown). Taken together, our finding suggest a role for the hTREX complex in cellular proliferation and following confirmation by other studies conducted among different populations in the future, hTREX84 may serve as a prognostic marker for aggressive forms of human breast cancer. Furthermore, therapeutic interventions that target human TREX should be of tremendous value in the fight against breast cancer.

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TRANSCRIPTIONAL REGULATION OF hTREX84: A LINK BETWEEN TRANSCRIPTIONAL ELONGATION, mRNA EXPORT AND CANCER

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Running title: Transcriptional regulation of *hTREX84*

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TREX (transcription/export) is a multiprotein complex that plays a key role in the transcriptional elongation and transport of mRNA from the nucleus to the cytoplasm. We recently reported the purification of the human TREX and found that expression of a member of this complex, p84N5 (referred to as hTREX84 or hHPR1), a retinoblastoma (RB) binding protein, correlated with breast tumor size and metastasis. Here we examine the mechanisms of aberrant expression of hTREX84 in breast and ovarian cancer cells and evaluate its role in tumorigenesis. We show that ovarian tumor cells over-express hTREX84 4-fold and 10-fold relative to immortal, non-tumorigenic and primary ovarian surface epithelial cells, respectively and that reduction of hTREX84 levels by small interfering RNA results in inhibition of cellular proliferation and $G_{0/1}$ arrest. Even though we observed that hTREX84 expression was induced following treatment with a demethylation agent, 5-aza-2'-deoxycytidine (5-aza-dC), sodium bisulfite DNA sequencing and methylation specific PCR found no evidence of changes in DNA methylation in the CpG islands in the regulator region of *hTREX84*. We did identify several transcriptional factors, including NF-κB binding sites in the *hTREX84* gene promoter and demonstrate by chromatin immunoprecipitation (ChIP) and site directed mutagenesis that RelA/p65 binds the NF-kB binding sites and stimulates *hTREX84* expression. Finally, we show by immunohistochemistry (IHC) that RelA/p65 is abundantly expressed in malignant cells that aberrantly express hTREX84 indicating that RelA/p65 might play a pivotal role in regulating hTREX84 expression in cancer.

INTRODUCTION

The TREX (transcription/export) complex plays a key role in the transcriptional elongation and transport of mRNA from the nucleus to the cytoplasm (1). This complex is conserved from yeast to human. In yeast, TREX complex is composed of the THO complex and the mRNA export factors Sub2 and Yra1 (2-5). THO complex contains heterotetrameric subunits, Tho2, Hpr1, Mft1 and Thp2 (6,7). Additionally, Tex1, a protein of unknown function, was found to co-purify with the THO complex, albeit in substoichiometric amounts (3,4). TREX complex components are also associated with Gbp2 and Hrb1 (4,8). These two proteins are hallmarks of the serine-arginine-rich family of splicing factors and are recruited to nascent mRNAs through a physical interaction with TREX complex during transcription elongation (9). Functionally, the THO complex plays a role in transcription-dependent recombination

and transcription (7). It has been shown that the THO complex is recruited to actively transcribed genes (4) and is required for efficient transcription elongation (5). Null mutations in each of the genes encoding the subunits of the THO complex lead to an mRNA export defect (4,10).

Recently, the *Drosophila* and human TREX complexes were characterized by several groups including ours (4,11-14). Human TREX complex includes THO2 (yeast component Tho2), TREX84/HPR1 (yeast Hpr1), UAP56 (yeast Sub2) and ALY (yeast Yra1). Additionally, the human TREX complex contains other components, TREX90 (fSAP79), TREX40 (fSAP35) and TREX30 (fSAP24), which have counterparts in *Drosophila* (THOC5, 6 and 7, respectively), but these are not present in yeast (1,12,14). Both the *Drosophila* and the human TREX complex lack homologs of Mft1 or Thp2. Nevertheless, *Drosophila* and human RNA interference studies of THO2 and/or HPR1 indicate that the metazoan and human THO complex, like its yeast counterpart, functions in mRNA export (11,12). TREX84/HPR1 associates with elongating RNA polymerase II, indicating human THO complex also functions in transcriptional elongation (13).

Our interests in human HPR1 resulted from our observation that p84N5 was aberrantly expressed in human breast cancer (15). In fact, p84N5 is not new and was discovered as a binding protein that associates with the RB tumor suppressor protein (16). For a long time, it served as a nuclear protein marker (17,18). Surprisingly, we recognized that p84N5 is human HPR1, a yeast counterpart in the TREX complex (12). This finding was further confirmed by other groups independently (13,14) and the protein is referred to as hTREX84/HPR1.

The mechanism of regulation of the human TREX complex, including hTREX84 in normal and transformed cells is not well studied. We report that hTREX84 is aberrantly expressed in both breast and ovarian cancer and its expression is regulated, in part, by RelA/p65.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture.

Media and cell culture reagents were prepared by the Cell Culture Facility at Fox Chase Cancer Center. 10 cases of human ovarian surface epithelial (HOSE) cell cultures and 10 cases of SV40 Tag immortal HOSE cell lines were established and cultured in 199 Medium with 15% FBS and Insulin (290 units/per 500 ml) in our laboratory as previously described (19). The immortalized human breast epithelial cells MCF-10F (HMECs), which were grown in DMEM/F12 medium supplemented with 5% horse serum, insulin, hydrocortisone, epidermal growth factor, cholera toxin, and antibiotics, were established from a patient with fibrocystic disease and do not display characteristics of a malignant phenotype (20,21). Human ovarian cancer cell lines OVCAR2, OVCAR3, OVCAR4, OVCAR5, OVCAR8, OVCAR10, UPN251, UPN275, UPN289, UPN300, A2780, and breast cancer cell lines, MDA-MB-231, MDA-MB-435 were cultured in DMEM supplemented with 10% FBS and 1x antibiotic-antimycotic solution.

Immunoflorescence

Cells grown in monolayer cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.2% Triton X-100, and blocked with 10% fetal calf serum prior to antibody staining. Staining by anti-hTREX84 antibody (Novus Biologicals, Littleton, CO) was visualized with corresponding fluorescein-labeled secondary antibody. All images were acquired with a Bio-Rad MRC1000 confocal microscope.

Western Blotting Assay

After cell lysates were obtained from cell lines, 30 μg of total protein from each sample was analyzed by Western blotting. Protein extracts were electrophoresed on a 4–20% Tris-glycine gel, and the separated proteins were electrophoretically transferred to nitrocellulose for immunodetection. The membrane was blocked in 5% nonfat dry milk in TBST for 1 h at room temperature and incubated with mAB to human hTREX84 at a dilution of 1:2000 in TBST + 2.5% nonfat dry milk, followed by horseradish peroxidase-conjugated antimouse secondary antibody (Amersham) at a dilution of 1:10,000. Immunoblots were reprobed with β-actin monoclonal antibody to confirm equal loading. The expression levels of hTREX84 and β-actin detected by immunoblotting were quantitated using the program IMAGE (National Institutes of Health) for the integrated density of each band. Western blot assays were conducted in duplicate for each sample and the mean value was used for the calculation of protein expression levels.

siRNA Transfection and Cell Proliferation

The small interfering RNA (siRNA) sequences targeting *hTREX84* corresponded to the coding region 1652–1672 (5'-AATGATGCTCTACTGAAGGAA-3') relative to the start codon. The corresponding siRNA duplexes with the following sense and antisense sequences were used: 5'-UGAUGCUCUACUGAAGGAAdTdT (sense) and dTdTACUACGAGAUGACUUCCUU-5' (antisense). A non-specific control XI siRNA duplex had the following sequences: 5'-AUAGAUAAGCAAGCCUUACUU (sense) and UUUAUCUAUUCGUUCGGAAUGP -5' (antisense). All of the siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2'-ACE protection chemistry. Cells in the exponential phase of growth were plated at 30% confluence in 6-cm plates, grown for 24 h and then transfected with siRNA (hTREX84 siRNA: 200 nM) using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose-response studies. Silencing was examined 24, 48, and 72h after transfection. Control cells were treated with oligofectamine (mock) or transfected using a control siRNA. Cell proliferation and apoptosis were examined using Guava ViaCount and Nexin assays, respectively, as previously described (22). All studies were done in triplicate.

Cell Cycle Analysis

Cells were trypsinized, centrifuged, and fixed in 70% ethanol at 4°C. Cell pellets were resuspended in 50μg/ml propidium iodide in PBS for 30 min at 4°C. The stained cells were analyzed by flow cytometry performed on a FACScan, and the data were analyzed with Cell Quest software (Becton Dickinson).

Isolation of Genomic DNA from Cell Lines and Tissues

The genomic DNA was isolated from various cell lines using Promega's wizard DNA isolation kit according to the manufacturer's instructions. Primary breast tumor tissues and normal breast tissues were obtained after surgical resection and stored frozen at -80°C. The tissues were incubated at 55°C in homogenization buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 0.5% Tween 20, and 5mg/ml proteinase K for 3 h, and then genomic DNA was isolated using Promega's DNA isolation kit. Donors of tissue specimens agreed to allow their specimens to be used for research purposes.

Genomic Bisulfite DNA Sequencing

Two μg of genomic DNA from each sample was modified by sodium bisulfite as described previously (23). The modified

DNA was amplified with primer hTREX84-G2F and hTREX84-G2R covering the region -275 to +140. PCR reactions were performed in a volume of 50 μl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pM of each primer, and 2.5 units of platinum Taq polymerase (Life Technology Inc.). PCR reaction was carried out at 94°C for 1 min, and 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and finally 72˚C for 5 min. The 415-bp PCR product was gel purified and ligated into PCR2.1 Topo cloning vector (Invitrogen, Carlsbad, CA). After transformation, individual colonies were picked, and the insert was PCR amplified as described above and sequenced using hTREX84-G2F as the primer.

Chromatin Immunoprecipitation

The ChIP protocol used in this study was adapted from Weinmann *et al*., (24) and from the protocol recommended by Upstate Biotechnologies. The cells were grown on three 10-cm plates to 85% confluence. Formaldehyde was added to a final concentration of 1%, and the plates were incubated 10 min at 37˚C. The cross-linking reaction was stopped by the addition of 100 mM glycine containing protease inhibitors (Complete; Roche Applied Science). Cells were washed in dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 150 mM NaCl, pH 8.0 plus protease inhibitors), resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0 plus protease inhibitors) and sonicated to shear the DNA into 0.3–3-kb fragments. Insoluble material was removed by centrifugation, and the extract was precleared by incubation with blocked protein A-Sepharose to reduce nonspecific interactions. The precleared chromatin was split into two samples, one in which 3 μg of anti-RelA/p65 antiserum (Santa Cruz Biotechnology) was added, and one in which no antibody was added (negative control). Both samples were treated identically in every other respect. Samples were incubated overnight at 4°C and blocked protein A-Sepharose was then added. The immunoprecipitated complexes were washed twice in dilution buffer, once in high salt dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 500 mM NaCl, pH 8.0), once in LiCl buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 250 mM LiCl, pH 8.0) and once in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Following treatment of the samples with RNase A (Roche Applied Science) and proteinase K (Roche Applied Science), cross-links were reversed by incubation at 65˚C overnight. The DNA was purified using the Qiagen MinElute kit. ChIP and input DNA were analyzed by PCR using hTREX84 promoter primers (For-, 5'-ACC ACT GCT CCA GCT GTT TC-3'; Rev-, 5'-AGA CTG CGG TCT CTC TGA GC-3') to amplify a 351-bp fragment. The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and quantified using the program IMAGE (NIH). The linear range of PCR product amplification was determined, and the amount of ChIP-DNA template was optimized.

Reporter Plasmid Construction

To assay the promoter activity, the 5'-flanking region of the *hTREX84* gene was inserted into the firefly luciferase reporter vector, pGL3-Basic (Promega), which contained no eukaryotic promoter or enhancer element. The strategy for cloning of the fragment of the *hTREX84* gene promoter into pGL3-Basic vector was as follows: PCR was performed using the PCR2.1 Topo cloning plasmid which contains the hTREX84 gene promoter fragment as a template and 5'- and 3'-primer pairs (the newly synthesized XhoI and HIDIII sites in the primers are underlined),

5'-ATC GCT CGA GCG GGA TGA CCG CGG ACT G-3', 5'-ATG CA AGC TTC TTC TCG GCT GCG CGT G-3'. The PCR product was then cloned into pGL3-Basic vector. The correct orientation and sequences of plasmid construct were verified by sequence analysis. The unaltered plasmid, pGL3-Basic, was used as a promoterless control, and the plasmid, pGL3-SV40 (Promega) contained the firefly luciferase gene driven by the SV40 promoter as a positive control.

Site-directed Mutagenesis

PCR-based site-directed mutagenesis (the QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA) technique was used for the generation of reporter gene constructs with NF-kB binding sites' mutations following the manufacturer's instructions. The two NF-kB binding sites were mutated from 5'-GGAAACTCCC-3' to 5'-CCAAACTCCC-3'and from 5'-AGGTAATCCA-3' to 5'-ACCTAATCCA-3', respectively. The constructs were verified by DNA sequencing.

Transfection and Luciferase Assay

One day prior to transfection, the cell lines (1 x 10^5 cells each) were seeded in 35-mm tissue culture dishes. Cells were transfected and/or co-transfected with 1 μg of the reporter plasmid and CMV-RelA/p65 and the FuGENE6 transfection reagent (Roche Applied Science). Cells were harvested 48 h after transfection, lysed in 200 μl of lysis buffer, and subjected to freeze-thaw lysis. *Renilla* luciferase activity in 10 μl of cell lysate was determined with a Dual-Luciferase Reporter Assay System (Promega) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The results show the mean values of three experiments with standard errors.

Immunohistochemistry

Breast tumor tissue microarrays (TMA) were provided by the Tissue Bank Core Facility at Fox Chase Cancer Center. Grading of histologic malignancy of each specimen was assessed according to the system as reported previously (25,26). Slides containing formalin-fixed, paraffin-embedded samples were deparaffinized, hydrated in water, and subjected to antigen retrieval in 10 mM citrate buffer, pH 6.0. Immunostaining was performed as described previously but with a slight modification (27). Briefly, slides were probed with RelA/p65 antibody (sc-109; Santa Cruz Biotechnology, Santa Cruz, CA), at a dilution of 1:150. Then, the slides were incubated with secondary antibody. Finally, reaction products were visualized by immersing slides in 3, 3-diaminobenzidine tablet sets (Sigma Fast, Sigma) and counterstained with hematoxylin. A positive control was included in each experiment. As negative controls, either the RelA/p65 antibody was omitted or sections were washed in 1x PBS.

Statistical Methods

Statistical analyses, including chi-square and t-test, were performed using Microsoft Excel software. All statistical tests were two sided, and *P* values less than 0.05 were considered to be statistically significant. Error bars represent 95% confidence intervals.

RESULTS

Over-Expression of hTREX84 in Human Ovarian Cancer Cells.

Previously, we reported that the expression of hTREX84 in breast tumors is inversely related to hormone receptor status (12). Moreover, when we compared hTREX84 mRNA expression in 6 representative reduction mammoplasty specimens, including 3 nulliparous premenopausal and 3 parous premenopausal women, *hTREX84* mRNA expression was significantly elevated in the nulliparous specimens [(12) and data not shown]. These

results indicate that hTREX84 is not only deregulated in breast tumors, but also highly regulated during normal human breast lobular differentiation and might be modified by certain hormones, such as human chorionic gonadotropin (hCG). Therefore, we asked whether hTREX84 is also aberrantly expressed in other hormone-dependent tumors, such as ovarian tumors. As expected, hTREX84 was highly expressed in all 30 cases of ovarian epithelial tumors. We also determined hTREX84 expression (hTREX84/beta-actin ratio) in primary human ovarian surface epithelial (HOSE) cell cultures (n=10), SV40 Tag immortal HOSE cell lines (n=10), and ovarian tumor cell lines (n=11) by Western blotting analysis. We found that hTREX84 expression is significantly elevated in immortal cell lines (average value, 0.51) as compared to primary epithelial cells (average value, 0.125; p=0.00024) and reaches its highest level in cancer cell lines (average value, 2.10; p=0.0022) (**Figure 1a, b**).

To further elucidate the biological significance of hTREX84 in ovarian cancer cells, the siRNA against *hTREX84* was transfected into an OVCAR10 cells. RT-PCR analysis using oligonucleotide primers specific to the *hTREX84* gene showed that the expression level of the hTREX84 transcript decreases ~70 to 80% from the transfection of the siRNA into OVCAR10 cells when compared with that of the control cells. Under these conditions, the constant expression levels of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene were obtained in both cells (**Figure 2a**). The hTREX84-targeted siRNAs effectively reduced the levels of hTREX84, but did not affect the levels of non-targeted transcripts such as β-actin (**Figure 2b**). Immunostaining confirmed that the hTREX84 protein was drastically decreased in a majority of the treated cells (**Figure 2c**). The total numbers of cells decreased significantly following treatment with hTREX84-siRNAs as compared to cells treated with transfection reagent or control-siRNA (**Figure 2d**). We observed that cell growth was reduced in cultures treated with hTREX84-siRNA as compared to control (**Figure 2e**). Guava Nexin assays showed that there was also a reduction of Annexin V-PE and 7-AAD positive cells with hTREX84-siRNA treatment as compared to controls, and but the differences were not significant ($p>0.05$) (data not shown). In order to look at the mechanism of hTREX84 siRNA action, we further determined the cell cycle distribution by flow cytometry and found that the cell numbers in G2-M phase were decreased and cell numbers in G1 phase were increased in OVCAR10 treated with hTREX84 siRNA as compared to the cells treated with control siRNA, indicating that hTREX84 may be necessary for entry into the G2-M phase (**Figure 2f**). These results indicate that aberrant expression of hTREX84 may contribute to ovarian cancer by promoting cell proliferation. Similar results were obtained using additional tumor cell lines (data not shown).

Increased hTREX84 Expression by 5-aza-dC *in Ovarian Immortal Cells*

Previously, we reported that *hTREX84* mRNA was aberrantly expressed in the vast majority of high grade and invasive ductal carcinomas of the breast (12). Moreover, *hTREX84* mRNA levels were elevated in malignant epithelial cells as compared to normal mammary ductal epithelial cells, as demonstrated by laser captured micro-dissection and qPCR analysis. Therefore, we speculated that deregulation of transcription of *hTREX84* mRNA may be one of the mechanisms of hTREX84 protein over-expression in cancer cells. To help elucidate the molecular mechanisms underlying the abnormal transcription of *hTREX84* in tumorigenesis, an immortal,

non-malignant ovarian surface epithelial cell line, HIO-107, was treated with a demethylating agent, 5-aza-dC at concentrations of 1, 5, 10 or 50 μM for 5 days. Total RNAs were isolated and RT-PCR with specific primers to hTREX84 cDNA or β-actin cDNA was conducted. The results showed that the intensities of RT-PCR product of *hTREX84* were increased by the 5-aza-dC treatment in a dose-dependent manner. By contrast, the products of β-actin were evenly amplified from all the samples, illustrating that the expression of β-actin was not altered by the 5-aza-dC treatment (**Figure 3a**). hTREX84 protein was also found to be increased in the same manner using Western blotting analysis (**Figure 3b**). Similar results were obtained when we used an ovarian cancer cell line, OVCAR2, which exhibited low expression of endogenous hTREX84 (data not shown).

Sodium Bisulfite DNA Sequencing of promoter and exon1 of hTREX84 gene

We identified in the GenBank™ a human genomic clone (RP11-70501) derived from chromosome 18p containing the human TREX84 cDNA sequence reported initially by Durfee, et al (16) (DDBJ/GenBank™ /EMBL Data Bank, accession number AAA53571), as well as other groups (4,28,29) (Accession number NM_005131). The alignment of the human *TREX84* cDNA and the genomic clone in the GenBank™ allowed us to determine the exon-intron organization of the gene. Genomic DNA was subsequently isolated from these 5-aza-dC-treated cells and sodium bisulfite DNA sequencing was performed. Surprisingly, the results demonstrated that all CpG dinucleotides located in the hTREX84 promoter and exon 1 regions from treated and untreated HIO107 and OVCAR2 cells were demethylated, indicating that changes in promoter methylation are not associated with increasing expression of *hTREX84* mRNA and protein after 5-aza-dC treatment.

To further correlate the methylation status of the *hTREX84* promoter and exon 1 region with hTREX84 expression, we analyzed 15 cases of breast and ovarian cancer cell lines, 10 cases of breast and ovarian immortal cell lines, 6 cases of invasive breast ductal carcinoma and 13 cases of ovarian tumors, as well as their paired normal tissues, by sodium bisulfite DNA sequencing. The results showed that *hTREX84* promoter and exon 1 regions in almost all cell lines were demethylated (**Figure 4a, b**). *hTREX84* promoter and exon 1 regions in most normal tissues were also demethylated. There were sporadic methylated CpG sites in normal tissues (**Figure 4c**); however, aberrant promoter methylation of *hTREX84* did not appear to be the major epigenetic mechanism associated with abnormal expression of hTREX84 in breast and ovarian tumors and tumor cell lines.

Identification of Transcription Factors Bound to the hTREX84 Gene Promoter

To provide a better understanding of the molecular basis of hTREX84 over-expression in breast and ovarian cancer, we evaluated transcription factor binding sites in *hTREX84* regulator regions (30) using AliBaba2 (http://www.iti.cs.uni-magdeburg.de/grabe/alibaba2). Nine SP1, 7 NF1, 4 AP1, 2 NF-κB, together with other transcriptional factors binding sites, were predicted by this program. We initially focused on two NF-κB binding sites in the transcriptional regulation of *hTREX84*. First, we utilized the chromatin immunoprecipitation (ChIP) assay to determine the status of RelA/p65, one of the subunits of NF-κB, at the promoter of *hTREX84*. Following the ChIP protocol, *hTREX84* gene promoter regions were amplified and analyzed by semiquantitative PCR using specific primer pairs around NF-

κB binding regions on the promoter of *hTREX84* (**Figure 5a**). One breast (MDA-MB-231) and two ovarian (OVCAR10, OVCAR5) tumor cell lines were cultured and subjected to ChIP with an antibody (Ab) to RelA/p65. Enrichment of specific DNA sequences in the chromatin immunoprecipitates, which indicates an association of RelA/p65 with DNA strands within intact chromatin, were visualized by PCR amplification. No binding was seen on immunoprecipitation samples without RelA/p65 antibody (**Figure 5b**). These results were further confirmed when we transiently transfected RelA/p65 expression plasmids into a non-tumorigenic breast epithelial cell line, MCF-10F, and stimulated hTREX84 protein expression (**Figure 5c**). Moreover, when we knocked down RelA/p65 expression using siRNA targeted against RelA/p65, the hTREX84 protein levels were, in turn, decreased (**Figure 5d**).

To further determine whether NF-κB directly regulates *hTREX84* promoter activity through these two NF-kB binding sites, we performed a transient transfection assay in MCF-10F cells with hTREX84/pGL3 reporters. Three reporters contain mutated NF-kB binding sites each and both, respectively (**Figure 6a, b**), which were generated by PCR-directed mutagenesis, were compared to wild type in the presence of RelA/p65. The results showed that all the reporters containing NF-kB mutated binding site(s) had reduced promoter activity (**Figure 6c**).

Since our previous studies found that hTREX84 was highly expressed in the cell nucleus, especially in poorly differentiated and more aggressive human breast cancers (12), we asked whether RelA/p65 may also be expressed in a similar manner. We examined the protein expression of RelA/p65 by immunohistochemical analysis in 89 cases of human breast cancer, as well as 5 normal breast tissues (**Table 1**). This tumor panel includes 22, 33 and 34 cases of well, moderately and poorly differentiated tumors, respectively. RelA/p65 was weakly (0/+1) detected in normal breast epithelial cells (4 of 5) and protein staining indicated cytoplasmic localization (**Figure 7a**). Staining for RelA/p65 was also observed mainly in the cytoplasm in well-differentiated tumors (**Figure 7b**). Distinctly granular staining with an increased number of positively stained nuclei was observed in the poorly differentiated tumor specimens (+2/+3, 31 of 34) (**Figure 7c**). Similar expression patterns of RelA/p65 and hTREX84 in normal and malignant cells suggests a correlation between expression and tumor progression and metastasis.

DISCUSSION

In this report, we extended our previous observation that over-expression of hTREX84 is not only associated with aggressive breast cancer, but is also associated with aberrant cell proliferation in ovarian cancer. We explored the molecular mechanisms governing over-expression of hTREX84 in cancer cells. Since *hTREX84* mRNA levels are significantly elevated in breast tumors and tumor cell lines, we speculated that epigenetic mechanisms may contribute to this phenotype. It is well known that methylation of DNA at CpG dinucleotides is an important mechanism for regulation of gene expression in mammalian cells (30,31). Methylation of cytosines in the CpG sequence located in regulator regions of some genes is thought to ensure the silencing of certain tissue-specific genes in nonexpressing cells. Aberrant methylation is now considered an important epigenetic alteration occurring in human cancer (32,33). Hypermethylation of normally unmethylated tumor suppressor genes correlates with a loss of expression in

cancer cell lines and primary tumors (34-36). On the other hand, failure to repress genes appropriately by abnormal demethylation of tissue-restricted genes or by hypomethylation of proto-oncogenes could result in the loss of tissue specificity and could promote cancer formation (37-39). In previous studies, we have shown that the γ-synuclein promoter, which has a similar pattern of CpG sites as *hTREX84*, is hypomethylated in many human solid tumors that aberrantly express this protein (40,41). We hypothesized that *hTREX84* might be regulated by a similar mechanism. In fact, 5-aza-dC induced hTREX84 in all cells treated, but indirectly as evidenced by a lack of methylation changes at the CpG sites, indicating that hypomethylation is not directly associated with increased expression of *hTREX84* mRNA and protein. These results were further confirmed when we analyzed a series of breast and ovarian tumors and tumor cell lines, and normal tissues for evidence of aberrant methylation by sodium bisulfite DNA sequencing. The CpG sites in the *hTREX84* promoter and exon 1 regions were universally demethylated regardless of the level of *hTREX84* expression. The results suggest that abnormal *hTREX84* methylation is not associated with elevated hTREX84 expression in breast and ovarian tumors and may be regulated by other epigenetic mechanisms.

There are several possibilities which could explain why 5-aza-dC does not induce hTREX84 expression directly through *hTREX84* gene methylation status. For example, 5-aza-dC may have dramatic effects on chromosomes, leading to decondensation of chromatin structure, thus enhancing specific gene expression (42,43). Another possibility is that 5-aza-dC might affect some transcription factors that subsequently influence hTREX84 expression.

To provide a better understanding of the molecular basis of hTREX84 over-expression in cell immortalization and tumorigenesis, we identified transcription factor binding sites in the *hTREX84* promoter. We focused on nuclear factor of κB (NF-κB) and validate it for several reasons. NF-κB is not a single protein, but a small group of closely related protein dimers that bind to a common sequence motif known as the κB site (44). According to Hanahan and Weinberg, tumorigenesis requires six essential alterations to normal cell physiology: self-sufficiency in growth signals; insensitivity to growth inhibition; evasion of apoptosis; immortalization; sustained angiogenesis; and tissue invasion and metastasis (45). NF-κB is able to induce several of these cellular alterations (46), and it has been shown to be constitutively activated in some types of cancer cells, including breast cancer. Previous studies have documented elevated or constitutive NF-κB DNA-binding activity both in mammary carcinoma cell lines and in primary breast cancer cells of human and rodent origin (47-49). This could be correlated with the increased level of epithelial growth factor family receptors (EGFR) (50). Using ChIP (51,52) and functional assays we clearly demonstrated that RelA/p65, one of the subunits of NF-κB, binds to the promoter of *hTREX84* and influences *hTREX84* mRNA expression. Moreover, when specifically depleted by siRNA approaches, loss of RelA/p65 blocked hTREX84 expression. To further determine whether NF-κB directly regulates *hTREX84* promoter activity via the NF-κB binding sites, we performed a luciferase promoter assay in which the NF-κB binding sites were mutated individually or in combination. The results showed that the NF-κB binding sites were essential for maximum promoter activity. We further examined the protein expression of

RelA/p65 by IHC in human breast cancer specimens and showed a consistent pattern of over-expression in more aggressive, poorly differentiated tumors. Therefore, RelA/p65 is expressed in a manner similar to hTREX84 (12), indicating that these two proteins may cooperatively contribute to tumor progression and metastasis.

NF-κB, and its RelA/p65 subunit in particular, can promote tumorigenesis through its ability to induce the expression of anti-apoptotic genes such as *Bcl*-xL, *XIAP* and *IEX*-1L (53,54). NF-κB can also stimulate tumor proliferation through induction of certain oncogenes, such as *cyclin D1* (55) and *c-MYC* (56). Additional NF-κB target genes that contribute to tumor cell migration and/or metastasis include cellular adhesion molecules, such as ICAM-1 and VCAM-1 (57), matrix metalloproteinases, such as MMP-9, chemokine receptors, such as CXCR4, and vascular endothelial growth factor (VEGF) (58,59). In a recent report, NF-κB was shown to regulate a large network of genes; many more than originally estimated (60). The regulation of *hTREX84* by NF-κB is significant since hTREX84 is essential for transcriptional elongation and mRNA export (12), and it is interesting to speculate that NF-κB might directly mediate mRNA metabolism through regulation of *hTREX84* in cancer cells. NF-κB is also known to induce the expression of some cytokines and chemokines and, in turn, is induced by them (56,61). This positive feedback mechanism is usually kept in check to inhibit aberrant activation of NF-κB and prevent a chronic or excessive reaction associated with certain diseases (61). The hTREX84 protein and RelA/p65 subunit of NF-κB could also be mutually activated in aggressive breast cancer. In previous studies, hTREX84 stimulated transcription of RelA/p65 (62); however, the role of hTREX84 as a transcriptional factor has not been well-established. We also observed that hTREX84 expression is enhanced in estrogen receptor (ER) negative breast cancer (12), while constitutive activation of NF-κB has been shown to be associated with more aggressive breast cancers (49,50,63). Breast cancers often progress from a hormone-dependent, nonmetastatic, antiestrogen-sensitive phenotype to a hormone-independent, antiestrogen- and chemotherapy-resistant phenotype with highly invasive and metastatic growth properties (49). As such, the lack of molecular targets in ER-negative breast cancer remains a major therapeutic hurdle. Like NF-κB, hTREX84 could be considered an ideal therapeutic target for ER-negative breast cancers.

Other transcriptional factors are likely to contribute to the regulation of *hTREX84*. Four AP-1 binding sites were identified in the *hTREX84* promoter region. The AP-1 transcription factor is a dimeric complex that contains members of the JUN, FOS, ATF and MAF protein families (64). Elevation of AP-1 activity was also found in human breast tumors and drug resistant breast tumor cell lines (65-67). NF-κB can indirectly increase the expression of AP-1-regulated genes by physically associating with AP-1 (68). The potential role of AP-1 and other transcription factors in regulating *hTREX84* expression remains to be determined; however, our recent studies have shown that RelA/p65 plays a pivotal role in regulating *hTREX84* expression in breast and ovarian cancer.

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FOOTNOTES

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FIGURE LEDENDS

Figure 1. hTREX84 is aberrantly expressed in ovarian cancer cells. *A,* hTREX84 protein expression in representative ovarian cancer cell lines (OVCAR10, UPN251, UPN275, UPN289), immortal epithelial cell lines (HIO-118, HIO-102, HIO-104, HIO-113), primary epithelial cells (ROE). Protein samples were separated on a SDS-polyacrylamide gel and proteins were immunoblotted using anti-hTREX84 or ß-actin monoclonal antibodies. *B,* hTREX84/ß-actin ratio in primary ovarian epithelial cell cultures (epithelial), immortal epithelial cell lines (HIO) and cancer cell lines (cancer).

Figure 2. Depletion of hTREX84 leads to defects in cellular proliferation of OVCAR10**.** *A*, analysis of hTREX84 and GAPDH mRNA levels following treatment of cells with siRNA against hTREX84 or control siRNA. *B,* Analysis of hTREX84 and β-actin protein levels after treatment of cells with siRNA against hTREX84 or control siRNA. *C,* Analysis of hTREX84 expression following siRNA treatment for 72 hours by immunofluoresence staining in the cells (*left,* cells transfected with control siRNA; *right,* cells treated with hTREX84-siRNA). *D,* Photomicrographs showing the morphology following depletion of hTREX84 (*left,* tumor cells transfected with control siRNA; *right,* cells treated with hTREX84-siRNA). *E,* Cell proliferation of tumor cells following depletion of *hTREX84*. Cell proliferation and apoptosis (data not shown) was examined using Guava ViaCount and Nexin assays, respectively. Plotted are the number of viable cells ($x10^4$) at 24, 48, and 72 hrs after treatment with control siRNA or with hTREX84-siRNA. Shown are the results of three independent experiments. *F*. FACS analysis of the cells following down-regulation of hTREX84 levels. The comparison of cell cycle distribution after 72 hour of treatment with either siRNA (left panel) or hTREX84-siRNA (right panel).

Figure 3. HIO-107 cells were treated with 5-aza-dC to example changes in DNA methylation in CpG sequences in *hTREX84*. *A,* HIO-107 were treated with 5-aza-dC at concentrations of 1, 5, 10, 50 μM, respectively, for 5 days. RT-PCR show *hTREX84* mRNA expression and *B*, western blot analysis show hTREX84 protein levels.

Figure 4. Sodium bisulfite DNA sequencing of CpG sites in the *hTREX84* promoter and exon 1 regions. *A*, DNA sequence of *hTREX84* regulator regions. CpG sites are shown in green color. Nucleotides are numbered on the right from the AUG translation start code which is underlined. *B*, Sodium bisulfite sequencing of DNA isolated from untreated (I) and treated (II) cells. The stars indicate CpG sites. *C*. Sodium bisulfite sequencing of DNA from a normal breast tissue (N) and an invasive ductal carcinoma (T). The stars indicate CpG sites.

Figure 5. NF-κB activation enhances hTREX84 expression in immortal and/or cancer cells. *A*, Schematic diagram of the hTREX84 promoter indicating the conserved NF-κB DNA binding motif. *B*, ChIP assay of RelA/p65 binding to *hTREX84* gene promoter in MDA-MB-231 (lane 1, 2); OVCAR5 (lane 3, 4); OVCAR 10 (lane 5, 6). Cells were cultured for 48 h. ChIP assays were then performed with anti-RelA/p65 antibody. PCR analysis was performed on immunoprecipitation samples without antibody (lane 1, 3, 5), with RelA/p65 antibody (lane 2, 4, 6). *C*, MCF-10F cells were transiently transfected with a control vector (lane 1) or a RelA/p65 cDNA expression construct for 48 hours. Western blot analysis for RelA/p65, hTREX84 and β-actin. *D*, Sestern blot analysis of RelA/p65, hTREX84 and β-actin protein levels after treatment of MDA-MB-231 cells with control siRNA (lane 1) and siRNA against RelA/p65 (lane 2) for 72 hours.

Figure 6. Determination of *hTREX84* promoter activities in MCF-10F cells with hTREX84/pGL3 reporters. *A,* DNA sequence of NF-κB1M demonstrating that one of the two NF-kB binding site is mutated from 5'-GGAAACTCCC-3' to 5'-CCAAACTCCC-3'. *B,* DNA sequence of NF-κB2M, demonstrating that the second NF-kB binding site is mutated and from 5'-AGGTAATCCA-3' to 5'-ACCTAATCCA-3'. *C*, Promoter activities among the three

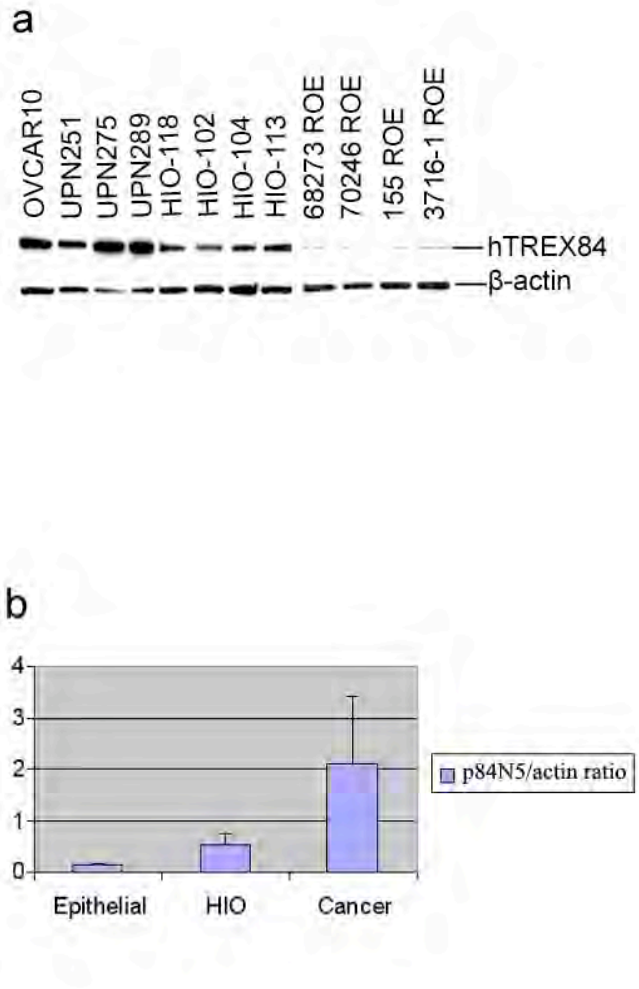
reporters constructs containing either a single mutated NF-κB binding sites or both (NF-κB1/2M) as determined by a luciferase assay. 1, Wild type NF-κB binding sites; 2, N5-κB1M; 3, N5-κB2M; 4, N5-κB1/2M.

Figure 7. Immunohistochemical staining of RelA/p65 proteins in representative breast cancer tissue specimens. *A*, RelA/p65 was weakly detected in normal breast epithelial cells and positive products were located in the cell cytoplasm. *B*, Staining for RelA/p65 was detected mainly in the cytoplasm in high differentiated tumors. *C*, Intense and distinctly granular staining for RelA/p65 was detected in stained nuclei of low differentiated tumor specimens. *D*, Tumor section evaluated without the primary antibody to serve as a negative control. Magnification 200x.

Table 1. RelA/p65 expression in human normal breast tissue and tumors

	N	0/+	++	+++
Normal breast tissue	5	4		1
Tumor histologic grade				
1 (well differentiated)	22	11	7	4
2 (moderately differentiated)	33	7	16	10
3 (poorly differentiated)	34	3	10	21

Figure 1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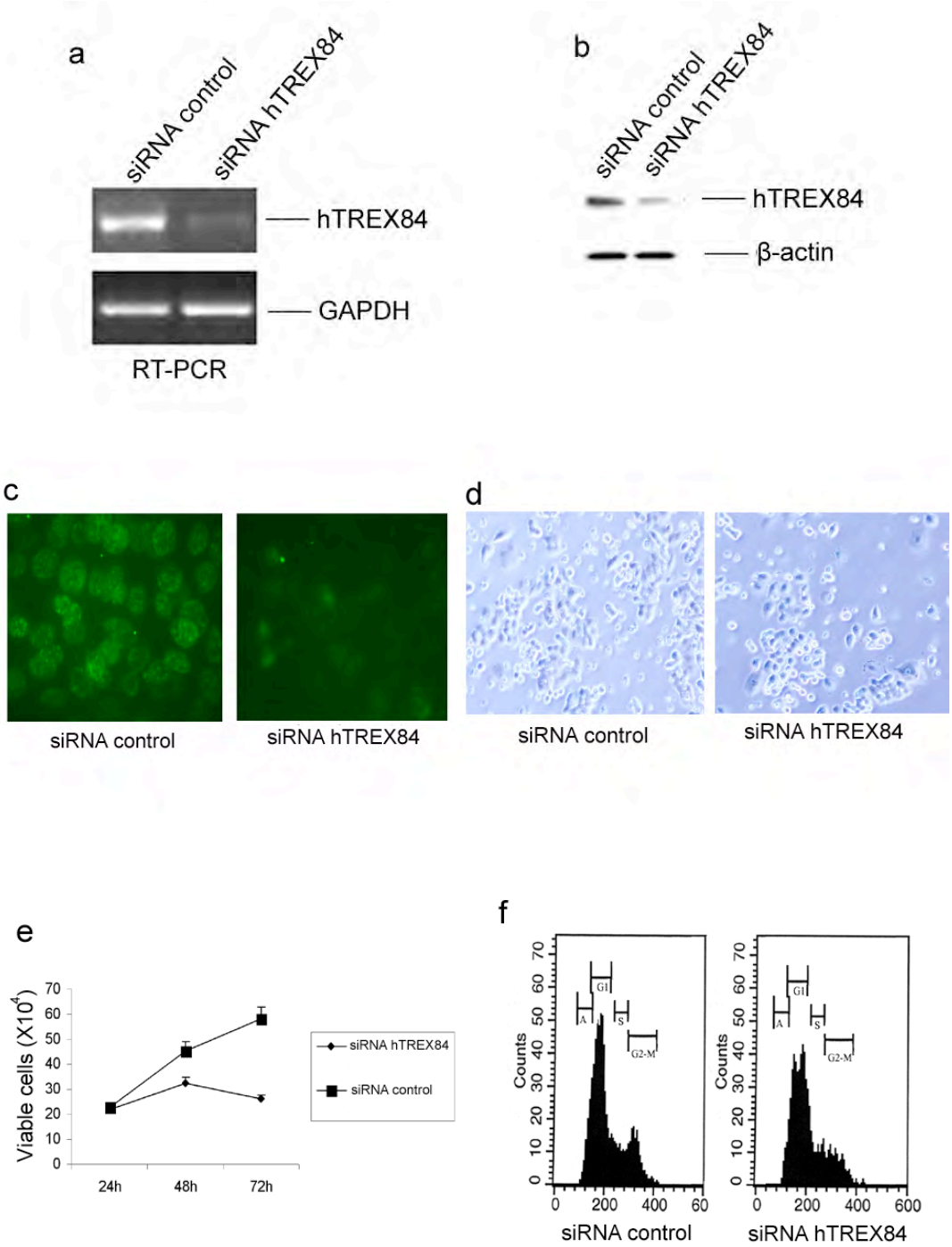
UPN275
UPN289
HIO-118
HIO-102
HIO-104
HIO-113
68273 ROE
70246 ROE
155 ROE
3716-1 ROE
hTREX84
β-actin
b
4
3
2
1
0
p84N5/actin ratio
Epithelial
HIO
Cancer

Figure 2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siRNA control
siRNA hTREX84
hTREX84
GAPDH
RT-PCR
b
siRNA control
siRNA hTREX84
hTREX84
β-actin
c
siRNA control
siRNA hTREX84
d
siRNA control
siRNA hTREX84
e
Viable cells (X10^4)
24h
48h
72h
siRNA hTREX84
siRNA control
f
Counts
G1
S
G2-M
siRNA control
siRNA hTREX84

Figure 3

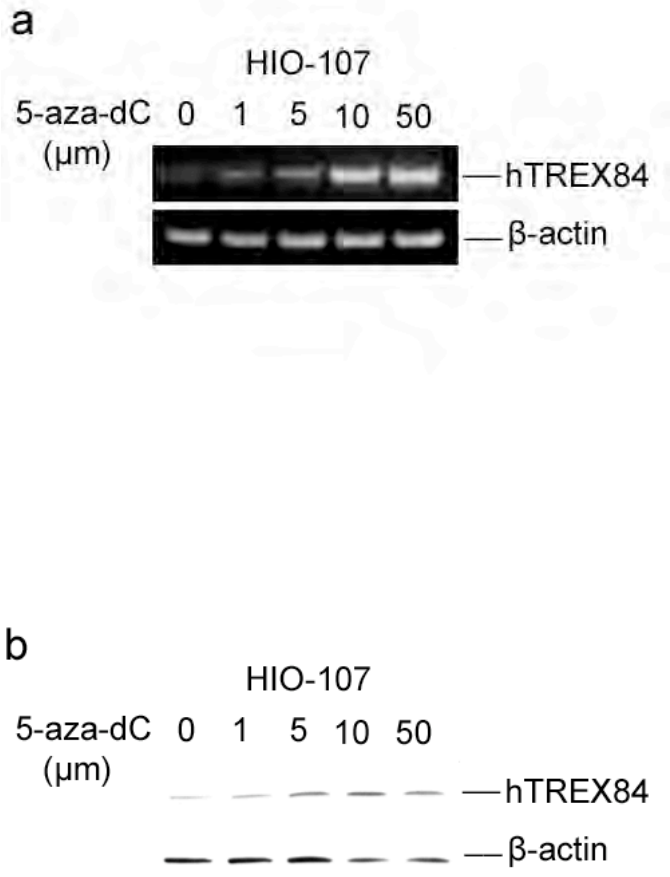
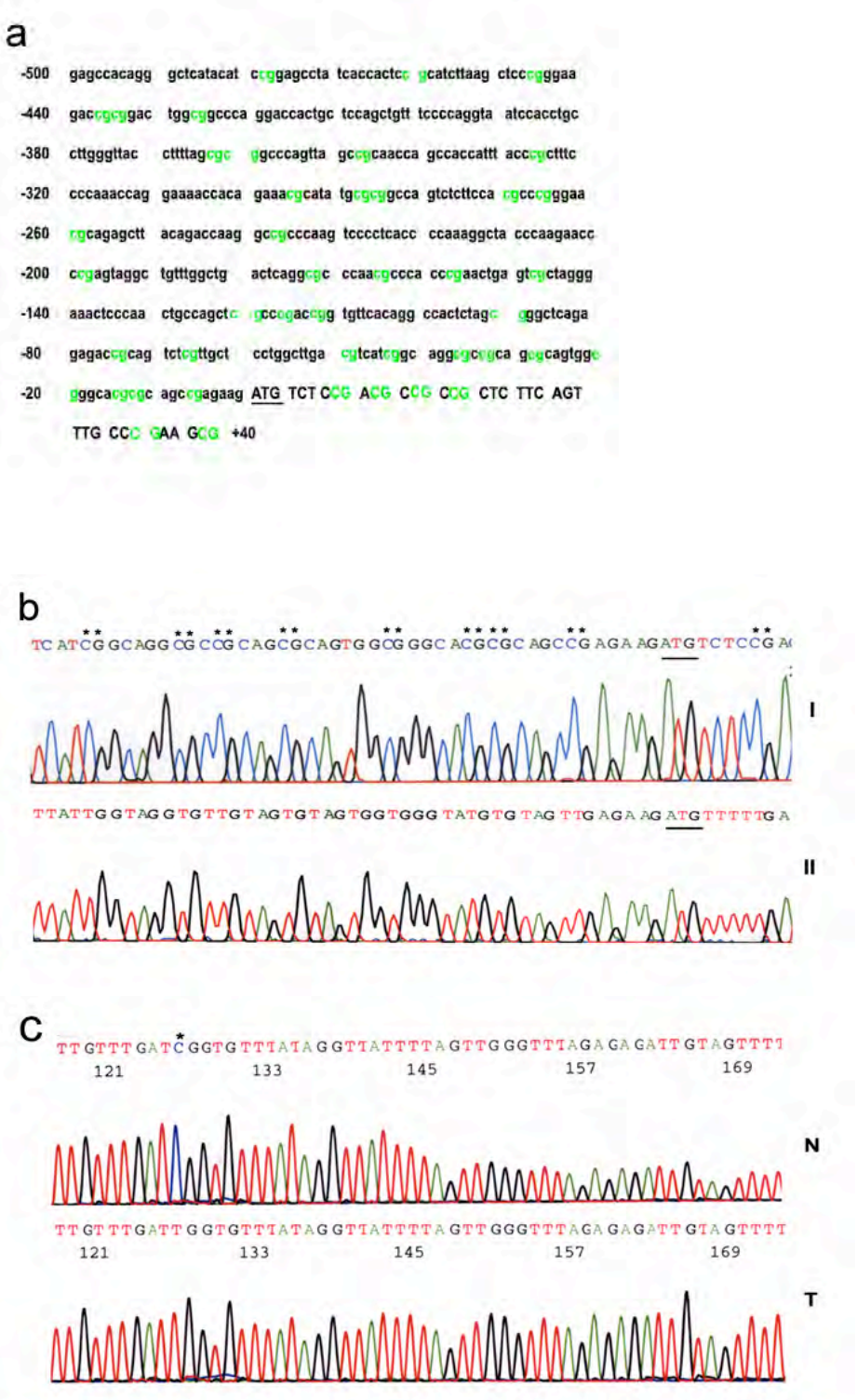

a
HIO-107
5-aza-dC (μm)
0 1 5 10 50
hTREX84
β-actin
b
HIO-107
5-aza-dC (μm)
0 1 5 10 50
hTREX84
β-actin

Figure 4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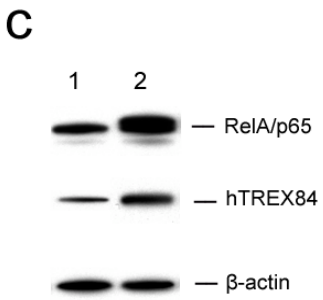
TTG CCC GAA GCG +40
b
TC ATCG GCAGG CG CCG CAGCGCAGTG GCG GGC ACGCGC AGCCG AGA AGATGTCTCCG A
I
TTATTGGTAGGTGTTGTAGTGTAGTGGTGGG TATGTG TAG TTG AGA AG ATGTTTTTG A
II
C
TTGTTTGATC GGTGTTTATAGGTTATTTTAGTTGGGTTTAGAGAGATTGTAGTTTT
121 133 145 157 169
N
TTGTTTGATTGGTGTTTATAGGTTATTTTAGTTGGGTTTAGAGAGATTGTAGTTTT
121 133 145 157 169
T

Figure 5


a
NF-kB
AGGTAATCCA
NF-kB
GGAAACTCCC
+1
ATG
-395
-385
-143
-133
-1


b
1 2 3 4 5 6
input DNA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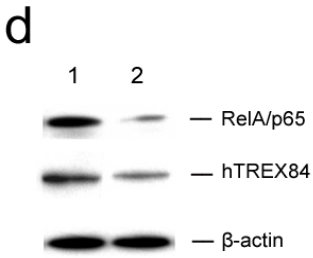
RelA/p65
hTREX84
β-actin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1 2
RelA/p65
hTREX84
β-actin

Figure 6

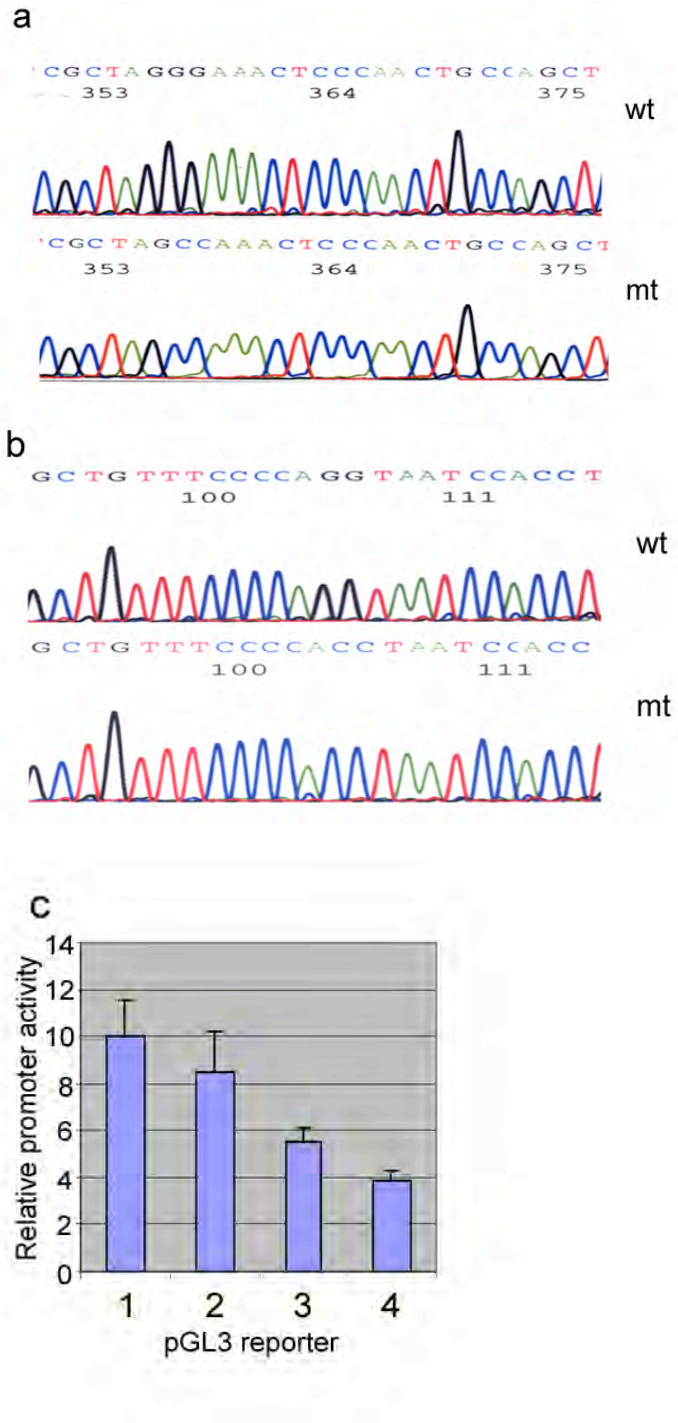
a
353
364
375
wt
mt
b
100
111
wt
mt
c
Relative promoter activity
0
2
4
6
8
10
12
14
1
2
3
4
pGL3 reporter

Figure 7