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The hTAF$_{II}$68-TEC fusion protein functions as a strong transcriptional activator

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Human extraskeletal myxoid chondrosarcoma (EMC) is caused by a chromosomal translocation that involves *TEC* (translocated in extraskeletal myxoid chondrosarcoma), and either *EWS* (Ewing's sarcoma) or *hTAF$_{II}$68* (human TATA-binding protein-associated factor II 68), which generates EWS-TEC or hTAF$_{II}$68-TEC fusion proteins, respectively. Although there has been a great deal of progress in characterizing EWS-TEC, there is relatively little known about the biological function of hTAF$_{II}$68-TEC. We have examined the functional consequences of the fusion of the amino terminal domain (NTD) of hTAF$_{II}$68 to TEC in EMC. The chimeric gene encodes a nuclear protein that binds DNA with the same sequence specificity as parental TEC. Nuclear localization of hTAF$_{II}$68-TEC was dependent on the DNA binding domain, and we identified a cluster of basic amino acids in the DNA binding domain, KRRR, that specifically mediate the nuclear localization of hTAF$_{II}$68-TEC. The transactivation activity of hTAF$_{II}$68-TEC was higher than TEC towards a known target promoter that contained several TEC binding sites. Finally, deletion analysis of hTAF$_{II}$68-TEC indicated that the hTAF$_{II}$68 NTD, and the AF1 and AF2 domains of hTAF$_{II}$68-TEC are necessary for full transactivation potential. These results suggest that the oncogenic effect of the t(9;17) translocation may be due to the hTAF$_{II}$68-TEC chimeric protein and that fusion of the hTAF$_{II}$68 NTD to the TEC protein produces a gain of function chimeric product.

Key words: hTAF$_{II}$68-TEC; chromosomal translocation; fusion protein; transactivation; nuclear localization signal; human extraskeletal chondrosarcoma

The loss of cellular growth regulation that gives rise to tumorigenesis originates with genetic changes in the cell. The t(9;17)(q22;q11.2) chromosomal translocation is associated with human extraskeletal myxoid chondrosarcoma (EMC), and results in the generation of a chimeric molecule consisting of the NTD of hTAF$_{II}$68 (also named TAF2N and RBP56) fused to full-length translocated in extraskeletal myxoid chondrosarcoma (TEC).[1,2] Human EMC is a soft tissue tumor of uncertain histogenetic origin that arises primarily in the musculature, most commonly in the thigh and knee.[3,4]

hTAF$_{II}$68 encodes a putative RNA/single stranded DNA-binding protein that associates with TFIID and RNA polymerase II.[5] The gene for hTAF$_{II}$68 was originally identified because of its homology to the proto-oncogenes *EWS* and *TLS* (Translocated in lymphosarcoma), a member of the *EWS* gene family.[5,6] *EWS* and *TLS* are involved in several tumor-related chromosomal translocations that result in gene fusions with a number of putative transcription factors, such as *Fli-1, ERG, ETV1, E1A-F, FEV, WT1, ATF1, CHOP* and *TEC*.[7,8] In each case, the translocation produces a chimeric molecule containing the NTD of EWS or TLS fused to the DNA binding domain of its partner. Recently, a new translocation involving the *EWS* gene was identified in some bone and soft-tissue tumors.[9] In these tumors, a t(6;22)(p21;q12) chromosomal translocation gave rise to a fusion protein in which EWS was fused to the DNA binding domain of the embryonic POU transcription factor Oct-4. It has also been reported that EWS-Oct-4 functions as a gain-of-function oncogene, and activates Oct-4-target genes and tumor formation in nude mice.[10]

TEC (also known as *CHN* and *MINOR*) is the human homologue of rat *NOR-1*.[11] It encodes a novel orphan nuclear receptor that belongs to the steroid/thyroid receptor gene superfamily.[3,4] The structural features of TEC include an N-terminal AF1 domain, a centrally located DNA binding domain (DBD), a putative ligand binding domain and a C-terminal AF2 domain. TEC is involved in a chromosome translocation with *hTAF$_{II}$68* on chromosome 17,[1,2,12] as well as *EWS* on chromosome 22,[3,4] *TCF12* on chromosome 15[13] and TFG on chromosome 3.[14]

In comparison with EWS-TEC, the biological function of hTAF$_{II}$68-TEC is not well characterized. To begin to understand the role of hTAF$_{II}$68-TEC in human EMC, we analyzed the biochemical and transcriptional activity of the protein. We showed that hTAF$_{II}$68-TEC is a nuclear protein that binds DNA with the same sequence specificity as TEC. We showed that the transcriptional activity of hTAF$_{II}$68-TEC is much greater than TEC, and that full transactivation potential requires the hTAF$_{II}$68 (NTD), and the AF1 and AF2 domains of hTAF$_{II}$68-TEC. These results indicate that hTAF$_{II}$68-TEC plays a critical role in human EMC *via* transcriptional regulation of its target genes.

Material and methods

Materials and general methods

Restriction endonucleases, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs. PfuTurbo polymerase was purchased from Stratagene and [γ-^{32}P] ATP (3,000 Ci/mmol) was obtained from PerkinElmer. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations and SDS-polyacrylamide gel electrophoresis of proteins were carried out by standard methods.[15] Subclones generated from PCR products were sequenced by the chain termination method using double-stranded DNA templates to ensure the absence of mutations.

Constructs

Details on the construction of pCMV-Tag2A/hTAF$_{II}$68-TEC and pCMV-Tag2A/TEC have been previously reported.[16] The hTAF$_{II}$68-TEC reporter plasmid p(B1a)^8-Luc has been previously described.[17]

The GST-EGFP-hTAF$_{II}$68-TEC deletion mutants were generated as follows: (*i*) pGST-EGFP: to generate pGST-EGFP, the gene encoding glutathione *S*-transferase (GST) was amplified from pGEX (4T-1) (GE Healthcare) by PCR using the primers 5′-GST(+Met) (5′-GATCGAATTCATGTCCCCTATACTAGGT-3′; underline indicates the *Eco*RI site) and 3′-GST (BamHI) (5′-GATCGGATCCAGATCCGATTTTGGAGGA-3′; underline indicates the *Bam*HI site), and subjected to restriction enzyme digestion with *Eco*RI and *Bam*HI. The resultant fragment was cloned into the corresponding sites of pEGFP-N1 vector (Clontech). (*ii*) pGST-EGFP-hTAF$_{II}$68 (NTD): to generate pGST-EGFP-hTAF$_{II}$68 (NTD), pCMV-Tag2A/hTAF$_{II}$68 (NTD) was subjected to

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digestion with *Hin*dIII, the restriction ends were blunted with Klenow, and the construct was subjected to digestion using *Nhe*I. The resultant fragment was cloned into the *Nhe*I/*Hin*dIII (blunted) sites of pEGFP-N1 to generate pEGFP-hTAF$_{II}$68 (NTD). GST was amplified from pGEX (4T-1) by PCR using the primers 5′-GST(-Met) (5′-GATCGGATCCATCCCCTATACTAGGTTA-3′; underline indicates the *Bam*HI site) and 3′-GST(BamHI), subjected to digestion with *Bam*HI, and cloned into the corresponding site of pEGFP-hTAF$_{II}$68 (NTD). (*iii*) pGST-EGFP-AF1: to generate pGST-EGFP-AF1, an AF1 fragment was amplified from pCMV-Tag2A/hTAF$_{II}$68-TEC by PCR using the primers 5′-AF1 (5′-GATCGAATTCATGCCCTGCGTCCAAGCC-3′; underline indicates the *Eco*RI site) and 3′-AF1 (5′-GATCGGATCCGCGC CAGACGACGAGCTC-3′; underline indicates the *Bam*HI), digested with *Eco*RI and *Bam*HI, and cloned into the corresponding sites of pEGFP-N1 to generate pEGFP-AF1. GST was amplified from pGEX (4T-1) by PCR using the primers 5′-GST(-Met) and 3′-GST(BamHI), digested with *Bam*HI, and cloned into the corresponding site of pEGFP-AF1. (*iv*) pGST-EGFP-DBD: to construct pGST-EGFP-DBD, a DBD fragment was amplified from pCMV-Tag2A/hTAF$_{II}$68-TEC by PCR using the primers 5′-DBD (5′-GATCGAATTCATGGAGGGCACGTGTGCC-3′; underline indicates the *Eco*RI site) and 3′-DBD (5′-GATCGGATCCGCT GGGCTCTTTGGTTTG-3′; underline indicates the *Bam*HI site), digested with *Eco*RI and *Bam*HI, and cloned into the corresponding sites of pEGFP-N1 vector to generate pEGFP-DBD. GST was amplified from pGEX (4T-1) by PCR using the primers 5′-GST (-Met) and 3′-GST(BamHI), digested with *Bam*HI, and cloned into the corresponding site of pEGFP-DBD. (*v*) pGST-EGFP-DBD (AAAA): to generate pGST-EGFP-DBD (AAAA), pEGFP-DBD was digested with *Eco*RI and *Bam*HI and cloned into the corresponding sites of pBluscript II KS+ (Stratagene) to generate pKSII/EGFP-DBD. To generate pKSII/DBD (AAAA), in which the amino acids KRRR were substituted with AAAA, we used the QuikChange^{TM} site-directed mutagenesis kit (Stratagene) and the mutagenic primer set 5′-mNLS (5′-CTGCCCAGTAGACGCGG CAGCTGCAAACCGATGTCAG-3′) and 3′-mNLS (5′-CTGAC ATCGGTTTGCAGCTGCGGCGTCTACTGGGCAG-3′). pKSII/DBD (AAAA) was digested with *Eco*RI and *Bam*HI to isolate the DBD (AAAA) fragment, which was then cloned into the corresponding sites of pEGFP-N1 to generate pEGFP-DBD (AAAA). GST was amplified from pGEX (4T-1) by PCR using the primers 5′-GST(-Met) and 3′-GST(BamHI), digested with *Bam*HI, and cloned into the corresponding site of pEGFP-DBD (AAAA). (*vi*) pGST-EGFP-LBD-AF2: to generate pGST-EGFP-LBD-AF2, an LBD-AF2 fragment was amplified from pCMV-Tag2A/hTAF$_{II}$68-TEC by PCR using the primers 5′-LBD (5′-GATCGAATTC ATGTTACAACAGGAACCT-3′; underline indicates the *Eco*RI site) and 3′-AF2 (5′-GATCGGATCCGCGAAAGGTAGGGTGT CC-3′; underline indicates the *Bam*HI site) and cloned into the corresponding sites of pEGFP-N1 to generate pEGFP-LBD-AF2. GST was amplified from pGEX (4T-1) by PCR using the primers 5′-GST(-Met) and 3′-GST(BamHI), digested with *Bam*HI, and cloned into the corresponding site of pEGFP-LBD-AF2.

To generated pCMV-Tag2A/hTAF$_{II}$68-TEC (ΔAF1), pCMV-Tag2A/TEC was digested with *Bal*I, repaired using Klenow, and redigested with *Xho*I. The excised fragment was ligated directly into the blunt-ended *Eco*RI and *Xho*I sites of pCMV-Tag2A/hTAF$_{II}$68 (NTD). To generate pCMV-Tag2A/hTAF$_{II}$68-TEC (ΔAF2), pCMV-Tag2A/hTAF$_{II}$68-TEC was digested with *Bgl*II and *Apa*I, repaired using Klenow, and self-ligated using T4 DNA ligase.

In vitro *transcription and translation*

In vitro transcription and translation of hTAF$_{II}$68-TEC and TEC was carried out using the TNT kit (Promega) and pCMV-Tag2A/hTAF$_{II}$68-TEC and pCMV-Tag2A/TEC, respectively, as described by the manufacturer (Promega). *In vitro* translation products were subjected to electrophoresis by 8% SDS-PAGE and analyzed by Western blot using an anti-Flag antibody (Sigma). Quantitation of *in vitro* translated proteins was performed using the ChemiDoc^{TM} XRS System (Bio-Rad).

Electrophoretic mobility shift assay (EMSA)

The sequences of the synthetic oligonucleotide probes used in the electrophoretic mobility shift assay (EMSAs) were described previously.[17] Probes (0.5 ng each) were prepared by end-labeling of annealed complementary oligonucleotides with [γ-^{32}P] ATP using T4 polynucleotide kinase. DNA binding reactions were performed with *in vitro* translated hTAF$_{II}$68-TEC and TEC for 30 min (min) at 4°C in binding buffer containing 10 mM Tris · HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1 mM DTT, 0.05% NP-40, and 10 ng/μl of poly (2′-deoxyinosinic-2′-deoxycytidylic acid). Following the binding reaction, the mixtures were separated on 4% polyacrylamide gels (acrylamide/bisacrylamide ratio. 37:1) in 0.5 × TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA) buffer at 150 V for 2 to 3 hr at 4°C. The gels were dried and exposed to Kodak X-Omat film at −70°C with an intensifying screen.

Subcellular localization

Immunohistochemistry was performed as previously described.[18] Briefly, 293T cells were grown on glass coverslips and transfected with pCMV-Tag2A/hTAF$_{II}$68-TEC and pCMV-Tag2A/TEC using VivaMagic Reagent (Vivagen). After 24 hr, the cells were washed in phosphate-buffered saline (PBS) and fixed for 10 min at −20°C in a mixture of acetone and methanol (1:1, v/v). To detect Flag fusion proteins of hTAF$_{II}$68-TEC or TEC, we used an anti-Flag antibody (M2, Sigma), followed by a TRITC-conjugated secondary antibody (Sigma). Fluorescence was detected using a fluorescence microscope (Olympus, IX71) equipped with DP71 digital camera (Olympus).

To examine the localization of GST-EGFP fusion proteins of the hTAF$_{II}$68-TEC truncation mutants, cells were transfected with pGST-EGFP, pGST-EGFP-hTAF$_{II}$68 (NTD), pGST-EGFP-AF1, pGST-EGFP-DBD, pGST-EGFP-DBD(AAAA) or pGST-EGFP-LBD-AF2, washed in PBS and then fixed for 10 min at −20°C in a mixture of acetone and methanol (1:1, v/v). Coverslips were mounted with 50% glycerol/PBS, and the green fluorescence of EGFP was detected with a fluorescence microscope (Olympus, IX71) equipped with DP71 digital camera (Olympus).

Transfection and reporter gene assays

Cells were transiently transfected with the indicated plasmids by electroporation using a Gene Pulser II RF module system (Bio-Rad). Luciferase assays were performed using the Dual-luciferase Assay System (Promega). Renilla luciferase activity was used to normalize for transfection efficiency.

Results

hTAF$_{II}$68-TEC binds to a consensus TEC binding sequence

One of the chromosomal translocation events in EMC results in the in-frame fusion of *hTAF$_{II}$68* to *TEC*.[1] The breakpoint in *hTAF$_{II}$68* is in intron 6, and the breakpoint in *TEC* is 2 nucleotides upstream of the ATG initiation codon. The predicted chimeric protein consists of the amino-terminal transactivation domain of hTAF$_{II}$68 fused to full-length TEC (Fig. 1).

The DBD of TEC is a conserved DNA-binding domain that binds to the NGFI-B Response Element (NBRE) sequence motif (5′-AAAGGTCA-3′).[17] Although there is considerable structural variation between hTAF$_{II}$68-TEC and TEC, the DBD of TEC is intact in both proteins. To determine whether hTAF$_{II}$68-TEC binds the physiological targets of TEC, we performed an EMSA using the NBRE sequence motif as a target in the binding reaction. Synthetic RNAs produced by *in vitro* transcription of full-length *hTAF$_{II}$68-TEC* and *TEC* were used to program cell-free rabbit reticulocyte lysates, and the resultant *in vitro* translated hTAF$_{II}$68-TEC and TEC proteins were quantified by SDS-PAGE and Western blot using an anti-Flag antibody (M2, Sigma-Aldrich)

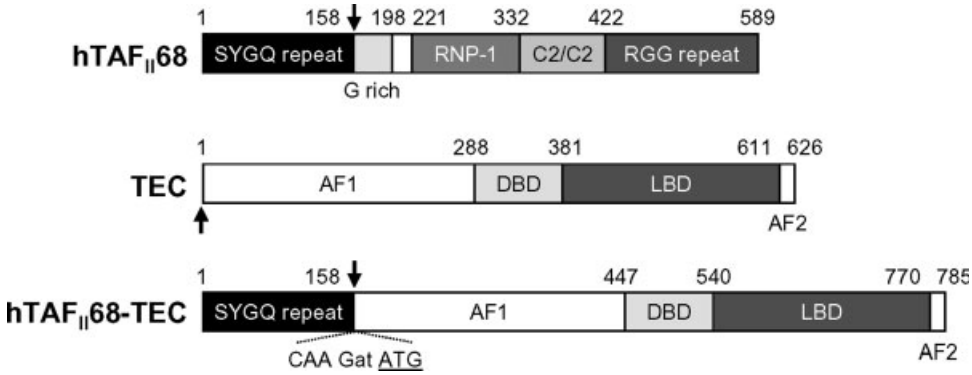

FIGURE 1 – Schematic illustration of the functional domains of the products of *hTAF$_{II}$68*, *TEC* and *hTAF$_{II}$68-TEC*. Amino acid position is indicated above the schematic of each protein. The first 158 amino acids (aa) (residues 1–158) of hTAF$_{II}$68 are fused to residues 1–626 of TEC *via* one additional aa encoded by the 5′ UTR of *TEC* in *hTAF$_{II}$68-TEC*. Functional domains of hTAF$_{II}$68, TEC and hTAF$_{II}$68-TEC are indicated: SYGQ repeat, Ser, Tyr, Gly, Gln repeat domain; G rich, Gly-rich domain; RNP-1, a putative RNA binding RNP-1 motif; C2/C2, a putative DNA-binding zinc finger motif; RGG repeat, Arg, Gly, Gly-repeat sequence. The break points of the translocation are indicated by vertical arrows. The fusion region of exon 6 of *hTAF$_{II}$68* is indicated by capital letters; the 2 nucleotides upstream of the ATG initiation codon of *TEC* are represented by small letters; and the ATG initiation codon of *TEC* is indicated by underlined capital letters in hTAF$_{II}$68-TEC.

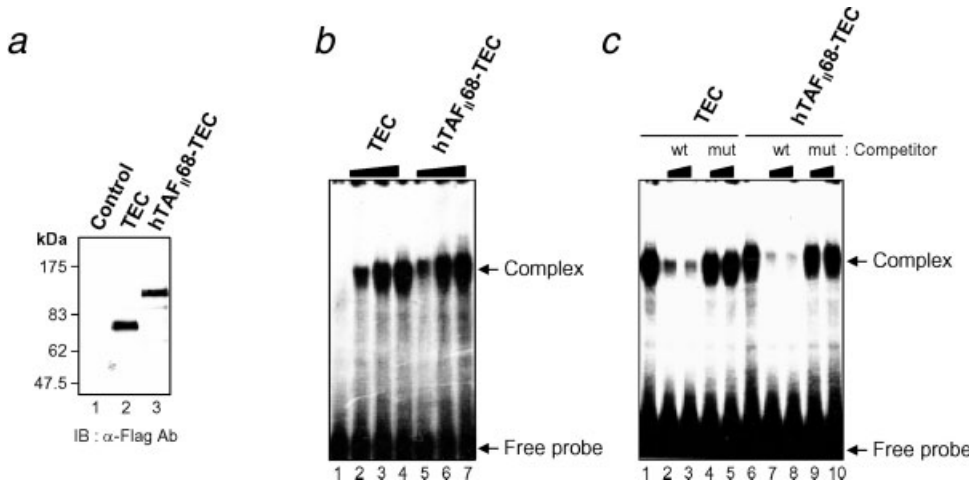

FIGURE 2 – Specific DNA binding of hTAF$_{II}$68-TEC. (*a*) Western blot analysis of *in vitro* translated hTAF$_{II}$68-TEC and TEC. Rabbit reticulocyte lysates were programmed with no RNA (lane 1), *TEC* mRNA (lane 2) or *hTAF$_{II}$68-TEC* mRNA (lane 3). Three microliters of the translation reaction product were resolved by 8% SDS-PAGE and analyzed by Western blot using an anti-Flag antibody (M2). Migration of the prestained molecular weight markers (New England Biolabs) is indicated to the left. (*b*) EMSAs of the DNA binding properties of hTAF$_{II}$68-TEC and TEC. Equal moles of *in vitro* translated hTAF$_{II}$68-TEC and TEC were present in each assay. EMSAs were performed with either unprogrammed reticulocyte lysate (lane 1), reticulocyte lysate programmed with *TEC* mRNA (lane 2, 1 μl; lane 3, 3 μl; lane 4, 5 μl) or reticulocyte lysate programmed with *hTAF$_{II}$68-TEC* mRNA (lane 5, 1 μl; lane 6, 3 μl; lane 7; 5 μl) and radiolabeled probe (0.5 ng), as described in Material and methods. For all reactions, the total amount of lysate was adjusted to 5 μl with unprimed control lysate. The proteins used in each assay are indicated above the panel. Protein–DNA complexes were resolved on a nondenaturing 4% polyacrylamide gel. The positions of free probe and protein-DNA complexes are indicated. (*c*) Sequence-specific DNA binding by hTAF$_{II}$68-TEC. Competition experiments were performed with 3 μl of normalized *in vitro* translation product and either 5-fold (lanes 2 and 7) or 10-fold (lanes 3 and 8) excess of wild-type TEC oligonucleotide or 5-fold (lanes 4 and 9) or 10-fold (lanes 5 and 10) excess of a mutated TEC oligonucleotide. The positions of free probe and protein–DNA complexes are indicated by arrows.

(Fig. 2*a*). Equal molar amounts of *in vitro* translated hTAF$_{II}$68-TEC and TEC were added to each assay. Quantitation of *in vitro* translated proteins was performed using the ChemiDoc^{TM} XRS System (Bio-Rad). EMSAs were performed using a single concentration of probe, and increasing amounts of *in vitro* translated protein. Protein–DNA complexes were formed in the presence of both hTAF$_{II}$68-TEC (Fig. 2*b*, lanes 5–7) and TEC (Fig. 2*b*, lanes 2–4), whereas unprogrammed reticulocyte lysate exhibited a very low level of binding (Fig. 2*b*, lane 1). The interaction was specific, as the complexes were disrupted by a 5- and 10-fold excess of unlabeled oligonucleotide containing the NBRE sequence motif, but not by an oligonucleotide containing a mutated NBRE sequence motif that is not recognized by the TEC DBD (Fig. 2*c*). These results indicated that the DNA binding specificity of hTAF$_{II}$68-TEC is similar to that of TEC.

The DBD targets hTAF$_{II}$68-TEC to the nucleus

hTAF$_{II}$68-TEC contains the NTD of hTAF$_{II}$68 fused to full-length TEC. To determine the subcellular localization of hTAF$_{II}$68-TEC, we carried out indirect immunofluorescence analysis using several different cell lines (293T, COS-7 and C28/I2 human chondrocyte cells), because human EMCs are tumors of uncertain histogenetic origin.[3,4] In addition, hTAF$_{II}$68-TEC-positive human EMC cell lines or their derivative are not currently available. 293T cells were transfected with an empty expression vector

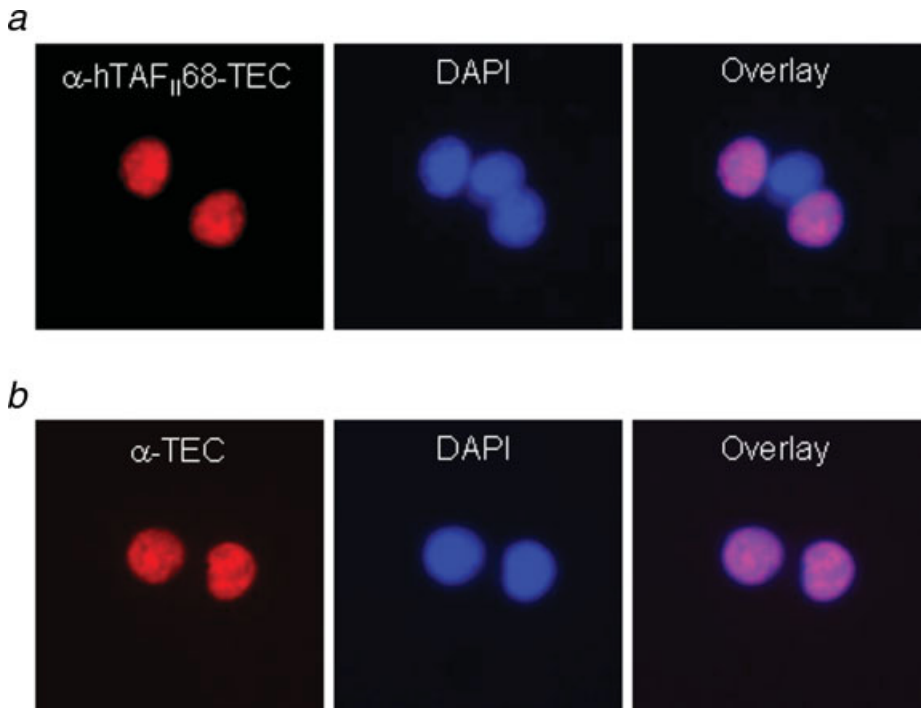

FIGURE 3 – Subcellular localization of hTAF$_{II}$68-TEC. 293T cells grown on coverslips were transfected with mammalian expression vectors encoding (*a*) Flag-tagged hTAF$_{II}$68-TEC or (*b*) Flag-tagged TEC. Cells expressing either Flag-hTAF$_{II}$68-TEC or Flag-TEC were subjected to fluorescence immunohistochemistry with an anti-Flag antibody (M2). DNA was visualized using 4′,6-diamidino-2-phenylindol (DAPI).

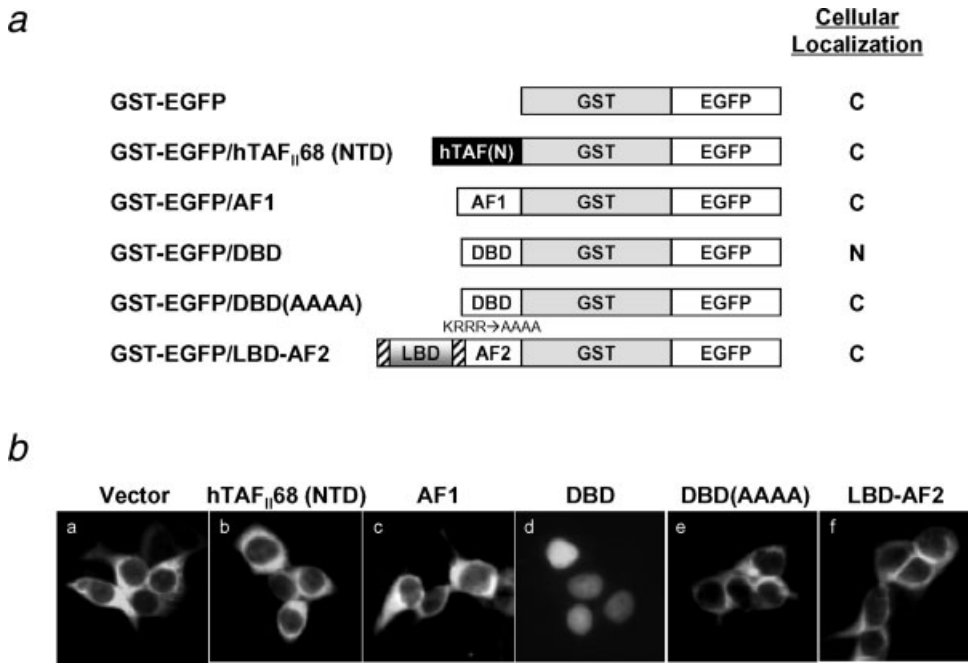

FIGURE 4 – Mapping of the nuclear localization signal of hTAF$_{II}$68-TEC to the DNA-binding domain. (*a*) Schematic diagram of GST-EGFP-fusion proteins of hTAF$_{II}$68-TEC truncation mutants. Subcellular localization of the indicated truncation mutants was determined by monitoring the location of green fluorescence, and is indicated as N (nuclear localization) or C (cytoplasmic localization). (*b*) Subcellular distribution of hTAF$_{II}$68-TEC deletion mutants. 293T cells were grown on coverslips under low density conditions and transfected with expression plasmids for the indicated GST-EGFP-hTAF$_{II}$68-TEC deletion mutants. The cells were fixed with an acetone/methanol mixture and EGFP was analyzed by fluorescence microscopy.

(pCMV-Tag2A, data not shown), pCMV-Tag2A/hTAF$_{II}$68-TEC (Fig. 3*a*) or pCMV-Tag2A/TEC (Fig. 3*b*) and analyzed by fluorescence immunohistochemistry. TEC localized to the nucleus (Fig. 3*b*) in transiently-transfected 293T cells. Chimeric hTAF$_{II}$68-TEC also localized to the nucleus (Fig. 3*a*). These data indicated that both hTAF$_{II}$68-TEC and TEC are nuclear proteins.

To map the region of hTAF$_{II}$68-TEC responsible for its nuclear localization, we generated a set of hTAF$_{II}$68-TEC deletion mutants (Fig. 4*a*). Because polypeptides with molecular masses of less than 40–50 kDa can passively diffuse into the nucleus,[19] we fused the isolated functional domains of hTAF$_{II}$68-TEC to GST and EGFP. 293T cells were transfected with expression vectors

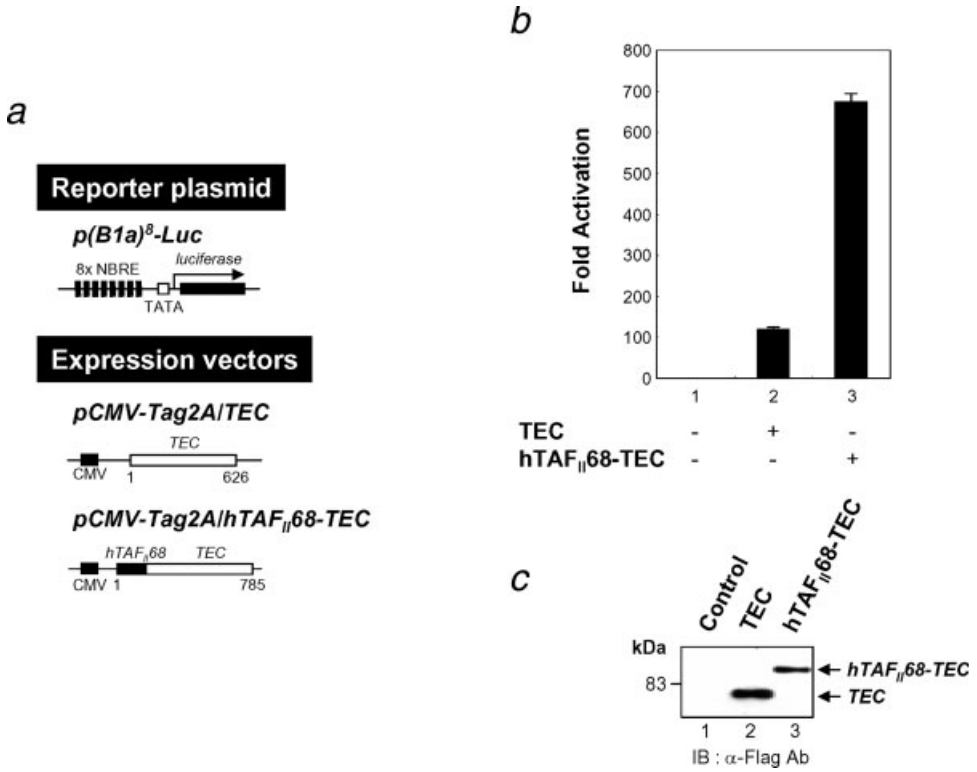

FIGURE 5 – Transactivation potential of hTAF$_{II}$68-TEC. (*a*) Schematic representation of the reporter and expression plasmids. The p(B1a)^{8}-Luc reporter plasmid contained eight copies of the NBRE (solid bars) upstream of a basal promoter-luciferase construct. The TATA box is represented by an open box, and the luciferase gene by a solid bar. The expression vectors driving the production of TEC or hTAF$_{II}$68-TEC are also shown. The positions of the first and last amino acids are indicated below each construct. (*b*) Transcriptional activity of hTAF$_{II}$68-TEC and TEC. 293T cells were co-transfected with expression vectors encoding hTAF$_{II}$68-TEC or TEC, the p(B1a)^{8}-luc reporter plasmid, and the Renilla luciferase expression vector. Reporter activity was normalized with to Renilla luciferase activity to correct for different transfection efficiencies. Fold-induction is expressed relative to the empty expression vector. Data represents the means and standard errors (±S.E., vertical bars) of at least 3 independent transfection experiments. (*c*) Immunoblot analysis of hTAF$_{II}$68-TEC and TEC expression in transiently transfected cells. Total cell lysates were fractionated by 8% SDS-PAGE and analyzed by Western blot using an anti-Flag (M2) antibody.

for GST-EGFP fusion proteins of the hTAF$_{II}$68-TEC truncation mutants, and the localization of EGFP was analyzed by fluorescence microscopy. GST-EGFP-hTAF$_{II}$68 (NTD) (Fig. 4*b*; refer to *b*′), AF1 (Fig. 4*b*; refer to *c*′) and LBD-AF2 (Fig. 4*b*; refer to *f*′) localized to the cytoplasm, whereas GST-EGFP-DBD (Fig. 4*b*; refer to *d*′) clearly localized to the nucleus. GST-EGFP alone also localized to the cytoplasm of 293T cells (Fig. 4*b*; refer to *a*′). We obtained similar results using COS-7 and C28/I2 human chondrocyte cells (data not shown).

To further define the nuclear localization signal in the DBD of hTAF$_{II}$68-TEC, we generated a DBD mutant in which several highly conserved basic amino acids, ^{338}KRRR^{341}, were replaced with alanine residues using site-directed mutagenesis. This region of basic amino acids was chosen as a the putative nuclear localization signal, based on prior evidence that positively-charged sequences are good candidate nuclear targeting signals.[20] Substitution of ^{338}KRRR^{341} with alanine residues resulted in the cytoplasmic accumulation of GST-EGFP-DBD (Fig. 4*b*; refer to *e*′). This result suggested that this cluster of basic amino acids in the DBD functions as a nuclear localization signal (NLS) of hTAF$_{II}$68-TEC.

hTAF$_{II}$68-TEC is a more potent transcriptional activator than TEC

To assess the transcriptional effects of the hTAF$_{II}$68 NTD of hTAF$_{II}$68-TEC, we carried out a reporter gene assay of hTAF$_{II}$68-TEC and TEC using a reporter plasmid that contained 8 copies of the NBRE sequence motif and a TATA box upstream of the luciferase gene.[17] The control plasmid in these experiments consisting of a cytomegalovirus-driven *Renilla luciferase* gene (Fig. 5*a*). As shown in Figure 5*b*, transfection of 293T cells with an expression vector for hTAF$_{II}$68-TEC resulted in a 690-fold increase in *luciferase* gene expression (Fig. 5*b*, bar 3). Transfection with an expression vector for TEC resulted in an approximately 110-fold increase in reporter gene activity (Fig. 5*b*, bar 2). Thus, in this system, hTAF$_{II}$68-TEC was a much more potent transcriptional activator than TEC. Western blot analysis confirmed that the difference in transactivation potential between hTAF$_{II}$68-TEC and TEC was not due to differences in the amounts of these proteins in cells (Fig. 5*c*). Similar results were obtained using COS-7 cells (data not shown).

Three functional domains are important for transactivation by hTAF$_{II}$68-TEC

To define the regions of hTAF$_{II}$68-TEC that are critical for transactivation, we performed a series of reporter gene assays using hTAF$_{II}$68-TEC deletion mutants fused to the Flag epitope tag. The structures of the deletion mutants are shown schematically in Figure 6*a*. Deletion of the hTAF$_{II}$68 NTD [right panel, hTAF$_{II}$68-TEC (ΔNTD)] or the AF1 domain [hTAF$_{II}$68-TEC (ΔAF1)] drastically reduced the transactivation activity of hTAF$_{II}$68-TEC, while deletion of the AF2 domain of [hTAF$_{II}$68-TEC (ΔAF2)] resulted in a partial reduction of transactivation activity. As shown in Figure 6*b*, Flag-hTAF$_{II}$68-TEC (ΔNTD), -hTAF$_{II}$68-TEC (ΔAF1) and -hTAF$_{II}$68-TEC (ΔAF2) localized to the nucleus. In addition, Western blot analysis showed that equivalent

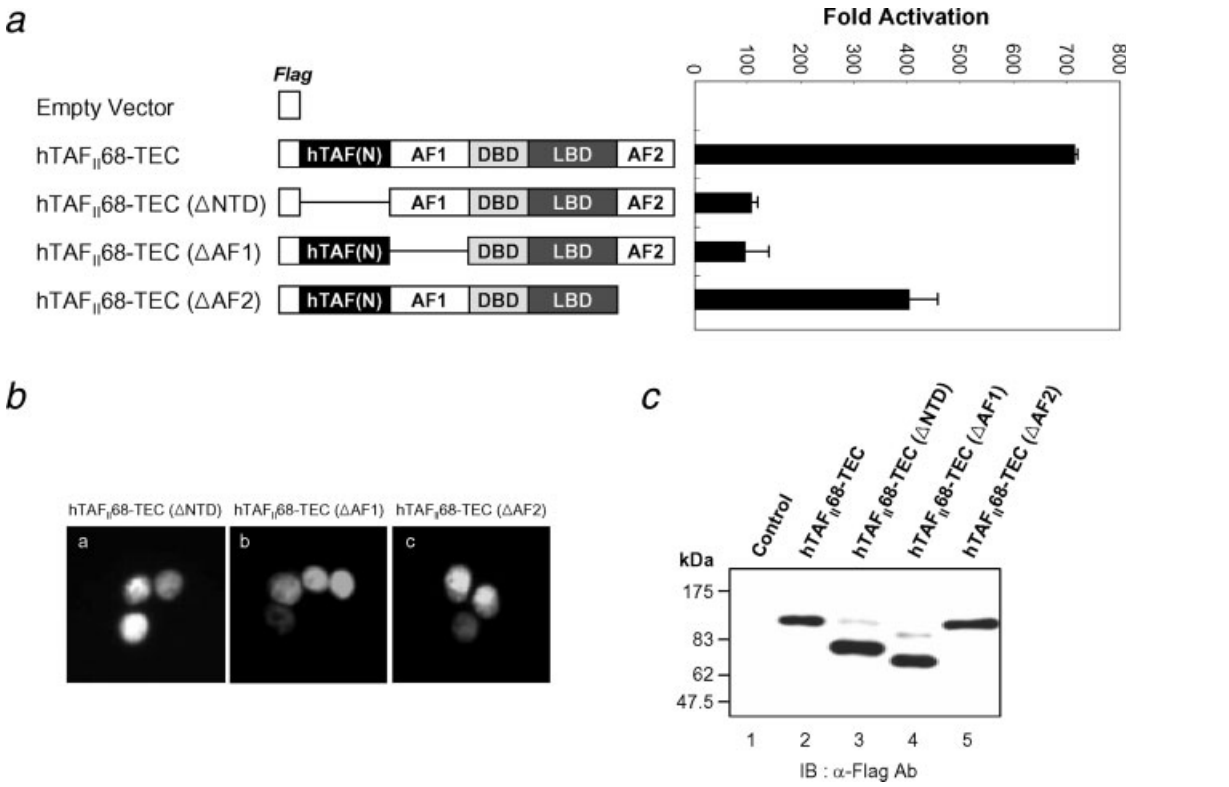

FIGURE 6 – Functional regions of hTAF$_{II}$68-TEC. (*a*) Schematic representation of the deletion constructs of hTAF$_{II}$68-TEC. 293T cells were transfected with p(B1a)[8]-luc, and expression vectors for the indicated Flag-tagged hTAF$_{II}$68-TEC deletion mutants. Fold-induction is expressed relative to the empty expression vector. Data represents the means ± S.E. (vertical bars) of at least 3 independent transfection experiments performed in duplicate. (*b*) Subcellular localization of hTAF$_{II}$68-TEC (ΔNTD), hTAF$_{II}$68-TEC (ΔAF1) and hTAF$_{II}$68-TEC (ΔAF2). 293T cells were cultured on coverslips under low density conditions and transfected with expression vectors for Flag-hTAF$_{II}$68-TEC (ΔNTD) (panel a), Flag-hTAF$_{II}$68-TEC (ΔAF1) (panel b) and Flag-hTAF$_{II}$68-TEC (ΔAF2) (panel c). The cells were fixed with an acetone/methanol mixture and stained with anti-Flag antibody (M2). (*c*) Immunoblot analysis of hTAF$_{II}$68-TEC deletion mutants expression in transiently transfected cells. Total cell lysates were fractionated by 8% SDS-PAGE and analyzed by Western blot using an anti-Flag (M2) antibody.

levels of each of the deletion mutants were expressed in cells (Fig. 6*c*). Thus, the differences in transactivation potential of the various hTAF$_{II}$68-TEC deletion mutants were not due to differences in subcellular localization or protein expression levels. These results indicated that the hTAF$_{II}$68 NTD and the AF1 and AF2 domains of hTAF$_{II}$68-TEC are important for full transactivation potential. Similar results were obtained in COS-7 cells (data not shown).

Discussion

In this study, we have characterized hTAF$_{II}$68-TEC, a fusion protein generated by a chromosome translocation in human EMC. In EMC, there is a characteristic t(9;17)(q22;q11.2) translocation that results in the fusion of the NTD of hTAF$_{II}$68 and full-length TEC.[1,2] We showed that hTAF$_{II}$68-TEC localizes to the nucleus, and binds DNA with a similar sequence specificity as TEC. We also showed that the EMC fusion gene encodes a more potent transcriptional activator than *TEC*. These results indicate that hTAF$_{II}$68-TEC may be oncogenic in humans.

hTAF$_{II}$68 encodes a putative RNA binding protein with similarity to *EWS* and *TLS*.[6] The structural characteristics of TAF$_{II}$68 includes a serine-, tyrosine-, glycine- and glutamine-rich (SYGQ repeat) region in its N-terminal domain; an RNA binding domain (RNP-1) and a C_2C_2 finger motif (C2/C2) in its central region; and degenerate repeats of DR(S)GG(G)YGG sequences (RGG repeats) in its C-terminal region (Fig. 1). Fusion products of *EWS* and *TLS* most likely function as abnormal transcription factors.[21] The NTDs of EWS and TLS can function as potent transactivators when fused to the 3′ DBD of a heterologous transcriptional regulator.[10,22–27] We demonstrated that the NTD of hTAF$_{II}$68-TEC also appears to contribute to the transcriptional activation function of hTAF$_{II}$68-TEC by providing a novel activation domain (Figs. 5 and 6). However, although the overall structure of hTAF$_{II}$68 is similar to EWS and TLS, the amino acid sequence of the N-terminal region of hTAF$_{II}$68 does not show extensive similarity to the corresponding regions of EWS and TLS,[6] which indicates that these regions may not be functionally identical. Consistent with this, we have recently shown that the coactivator GAPDH specifically interacts with the hTAF$_{II}$68 NTD, but not with the NTD of EWS or TLS.[18] In addition, hTAF$_{II}$68, EWS, and TLS associate with the TFIID complex, but they are each present in a distinct population of TFIID.[5,28] This raises the question of whether hTAF$_{II}$68-TEC and EWS-TEC function similarly in human EMC. The NTDs of hTAF$_{II}$68-TEC and EWS-TEC may also play a role in the cross-talk between the transcript and transcriptional apparatus. Additional experiments are needed to explore this possibility.

TEC is the human homologue of the rat *NOR-1*,[11] and represents a new member of the nuclear receptor gene superfamily.[3,4] Like *EWS-TEC*, *hTAF$_{II}$68-TEC* consists of the entire coding sequence of the *TEC* gene.[1,2] Along with the PML/RARα fusion[29,30] and the EWS-TEC fusion,[3,4] this is the third example to date of the oncogenic conversion of a nuclear receptor in human cancer. The DNA binding domain of TEC shares 98% and 91% amino acid identity with the orphan nuclear receptors NURR1 and

NGFI-B (also known as Nur77 or TR3), respectively.[4] Interestingly, it has been shown that the Akt kinase [also known as protein kinase B (PKB)] phosphorylates NGFI-B directly *in vitro* and *in vivo*, and that phosphorylation reduces its DNA binding activity, and stimulates its association with 14-3-3 in a phosphorylation site-dependent manner.[31,32] Similarly, nerve growth factor (NGF) induces the phosphorylation of Ser316 of NGFI-B, which is located within the DBD, and phosphorylation results in the transcriptional deactivation of NGFI-B.[33,34] Because the DNA binding specificity of $hTAF_{II}68$-TEC resembles that of TEC (Fig. 2), and the DNA binding domain of $hTAF_{II}68$-TEC contains a conserved putative phosphorylation site, it would be interesting to examine whether $hTAF_{II}68$-TEC activity is modulated by NGF.

It is well-known that NLS sequences contain regions rich in basic amino acids (lysines or arginines), and generally conform to 1 of 3 motifs.[35] The first type of NLS consists of a continuous stretch of 4 basic amino acids or 3 basic amino acids together with a histidine or proline. The second type of NLS starts with a proline and is followed within 3 residues by an amino acid sequence containing 3 of 4 basic residues. The third type of NLS, known as a bipartite motif, consists of 2 basic amino acids, a 10 amino acid spacer and a 5 amino acid sequence containing at least 3 basic residues. In this study, we showed that $hTAF_{II}68$-TEC localizes to the nucleus (Fig. 3), and contains a conserved nuclear localization signal, KRRR, in its DBD (Fig. 4). Substitution of this motif with alanine residues resulted in cytoplasmic accumulation of $hTAF_{II}68$-TEC. Interestingly, it has been demonstrated that NGF promotes the translocation of NGFI-B out of the nucleus, and this is regulated by phosphorylation through the TrkA/Ras/MAP kinase pathway.[33] We are currently investigating whether $hTAF_{II}68$-TEC is also a target for NGF-induced nuclear export, and whether its activity is regulated by NGF.

In conclusion, we have provided evidence that $hTAF_{II}68$-TEC is a more potent transcriptional activator than TEC, and may be necessary for tumorigenesis in human EMC. Because the *$hTAF_{II}68$-TEC* gene encodes a strong transcriptional activator, it likely contributes to tumorigenesis *via* the deregulation of its target genes. Thus, additional genes may cooperate with *$hTAF_{II}68$-TEC* and/or be required for tumor progression. The identification of these downstream target genes will help us to understand the role of the chromosomal translocation fusion products in human EMC.

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