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Advances in Brief

Recurrent Rearrangement of the Ewing's Sarcoma Gene, *EWSR1*, or Its Homologue, *TAF15*, with the Transcription Factor *CIZ/NMP4* in Acute Leukemia[1]

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Abstract

Fusions of the TET-proteins (TLS/FUS, EWSR1, and TAF15/RBP56) to different transcription factors are involved in various malignancies including Ewing's sarcoma, primitive neuroectodermal tumors, and acute myeloid leukemia. These are thought to arise through transcriptional deregulation, with the transcription factor defining the tumor phenotype. We show that, as result of a t(12;17)(p13;q11) or its variant t(12;22)(p13;q12), the transcription factor gene *CIZ/NMP4* is recurrently involved in acute leukemia through fusion with either *EWSR1* or *TAF15*. The fusions possess transforming properties in NIH3T3 cells but do not affect the expression of *CIZ* target genes, suggesting a contribution to oncogenesis that is independent of the transactivating properties of the fusion protein. These results also extend the involvement of TET-protein fusions to acute lymphoblastic leukemia and suggest a role for *CIZ/NMP4* in lymphoid and myeloid development.

Introduction

Oncogenic fusions involving the RNA-binding proteins of the TET[3] family have been identified in both solid tumors and acute myeloid leukemia and are characterized by a common structure in which the COOH-terminal RNA-binding domain of the TET proteins is replaced by a transcription factor or its DNA-binding domain. The systematic and interchangeable presence of the NH_2-terminal transactivation domain of the different TET proteins suggests a common oncogenic mechanism. The tumor phenotype, however, is defined by the transcription factor. Fusions to Ets factors are found in Ewing's sarcoma. ERG fusions are found in both Ewing's sarcoma and acute myeloid leukemia. The C/EBP factor CHOP fusions are responsible for myxoid liposarcoma, and fusions with WT1 are found in desmoplastic round cell tumors, ATF1 in clear cell sarcomas, and TEC/NOR1 in extraskeletal myxoid chondrosarcoma (1). The molecular mechanisms of transformation by TET fusions are not yet resolved. The TET fusions were reported to bind to the same DNA sequences as the wild-type transcription factors and to act as transcriptional activators of reporter constructs carrying those sequences, a property that is dependent on the presence of the TET-transactivating domain (2, 3). EWSR1-FLI1 induces expression of *manic fringe* (4) and represses expression of *TGF-β type II receptor* (5), and both events are linked to transformation. Other target genes of EWSR1-FLI1 include *MMP3*, *EAT2*, and *CK15* (6). However, evidence was presented that also DNA binding domain-independent pathways are involved in *EWSR1-FLI1*-mediated oncogenesis (7, 8). More recently, it was reported that TET proteins are involved in pre-mRNA splicing, a process that is affected by oncogenic TET fusions (9–11).

We have collected seven acute leukemia cases with a novel translocation t(12;17)(p13;q11) or its variant t(12;22)(p13;q12) and show that the rearrangements affect the *CIZ/NMP4* transcription factor gene on chromosome 12 and the *EWSR1* or *TAF15* genes on chromosome 17 and 22, respectively. The recurrent *TAF15-CIZ* and *EWSR1-CIZ* fusions described here expand the oncogenic properties of TET fusions toward lymphoid malignancies, a phenotype that must be defined by *CIZ/NMP4*, a transcription factor not yet implicated in hematopoiesis.

Materials and Methods

Patient Material. Seven patients presented with a diagnosis of acute leukemia: case 1, acute undifferentiated leukemia; case 2, acute myeloid leukemia, FAB-M1; cases 3–6, acute lymphoblastic leukemia with pro-B phenotype (CD19+, CD10−); and case 7, a common B-ALL. Their bone marrow karyotype showed either a t(12;17)(p13;q11) in cases 2–6 or a t(12;22)(p13;q12) in cases 1 and 7 in the majority of the metaphases. The patients were diagnosed in Bordeaux, France (case 1); Nantes, France (cases 2); Italy (cases 3–6); and Leuven, Belgium (case 7).

5′ RACE and RT-PCR. 5′-RACE and RT-PCR experiments were performed according to established protocols. mRNA was reverse transcribed with the CIZ-Ex4R1 (5′-GGTCAGCTGGTCTGACTTGGA) primer. The specific primers used for the 5′-RACE were CIZ-Ex4R2 (5′-TCTGGCAACAGCTGATCCTTC) and CIZ-Ex3R (5′-GAAGGCCAGAAGTACGGGTTAGA). The *TAF15-CIZ* fusion was confirmed by heminested RT-PCR on patient cDNA using primers CIZ-Ex4R1 and CIZ-Ex4R2, in combination with primer TAF2N-Ex3F (5′-GGCTATGGACAAGCATCACA). The *EWSR1-CIZ* fusion transcript was detected with CIZ-Ex4R1 and CIZ-Ex4R2 in combination with EWSR1bf (5′-CCCAAACTGGATCCTACAGC).

Plasmid Constructs. *CIZ* was amplified from human bone marrow cDNA with the primers CIZflF1 (5′-GAGATCTAATGCCGGCAGAATGGAAGAATCTCACTTC) and CIZfl-R1 (5′-AGGGCCCAGAGCTGGCCAGGTGCTCCAC); the NH_2-terminal region of *TAF15* was amplified with TAF2N-F1 (5′-GAGATCTGCCACCATGTCGGATTCTGGAAGT) and TAF2N-R1 (5′-AGCCGGCATCTGTTCTGGGTCCATAATC); and the NH_2-terminal region of *EWSR1* was amplified with primers EWS-F1 (5′-GAGATCTGAGAAAATGGCGTCCACGGATTAC) and EWS-R1b (5′-TGCCGGCCTGCCCGTAGCTGCTGCTCTGT). All PCR products were cloned in pGEM-Teasy, and

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[3] The abbreviations used are: TET, TLS/FUS, EWSR1, and TAF15/RBP56; RACE, rapid amplification of cDNA ends; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription; NMP4, nuclear matrix protein 4; ALL, acute lymphoblastic leukemia; MMP, matrix metalloproteinase.

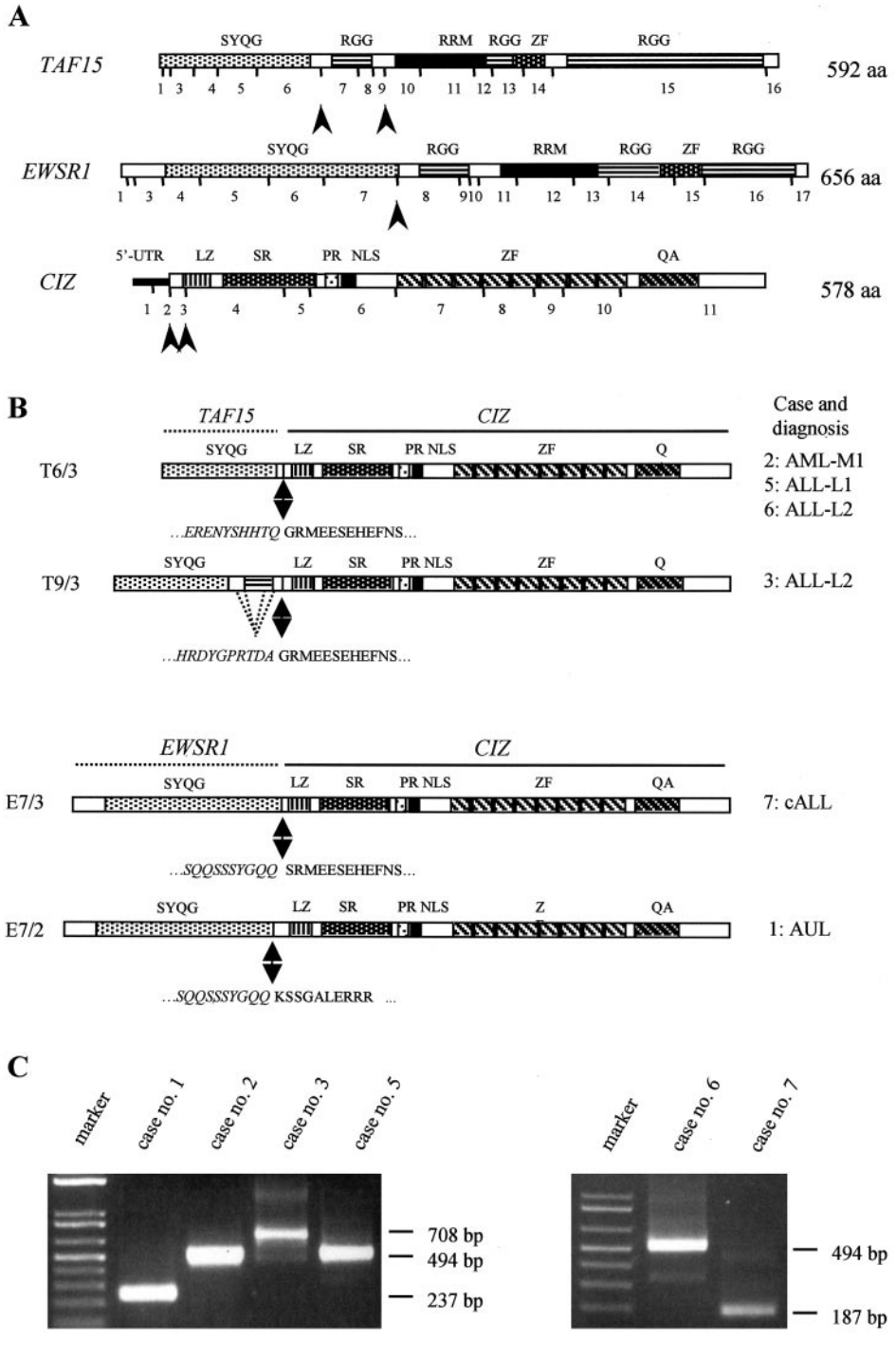

Fig. 1. Structure of the TET-CIZ fusions. *A*, structure of the CIZ, TAF15, and EWSR1 proteins. Both TET-proteins have a Ser-Tyr-Gln-Gly-rich (SYQG) transactivating domain at their NH_2 terminus, an RNA-binding domain composed of three Arg-Gly-Gly-rich (RGG) regions and an RNA recognition motif (*RRM*) at the COOH terminus. A zinc finger (*ZF*) is present, flanked by two of the RGG boxes. CIZ contains leucine-rich (*LZ*), serine rich-(*SR*), and proline-rich (*PR*) domains at the NH_2 terminus, followed by a nuclear localization signal (*NLS*), eight Krüppel-type C2H2 zinc finger domains, and a Gln-Ala repeat (*QA*) at the COOH terminus. Exon numbers and boundaries are marked *below* the protein structures. *Arrowheads*, breakpoints observed in the t(12;17) and t(12;22) leukemia cases. *B*, structure of the fusion proteins. The fusions are named after the TET-protein present (*T* or *E*), followed by the last exon number of the *TET* gene and the first exon of *CIZ* present in the fusion. The *double arrows* show the point of fusion; part of the amino acid sequence of the fusion proteins is shown underneath (TET-protein sequences are in *italic*, and CIZ sequences are roman). The leukemia cases in which a particular fusion was found are indicated on the *right*. The *dashed lines* for the T9/3 fusion show alternative splice forms detected for this fusion in the leukemic cells. *C*, RT-PCR detection of *TAF15-CIZ* and *EWSR1-CIZ* fusion cDNAs. Two rounds of RT-PCR were performed with primers located in the relevant exons of *TAF15*, *EWSR1*, and *CIZ* genes. In case 3, several bands are amplified because of the alternative splicing of *TAF15*. Product lengths are indicated to the *right*.

both strands were sequenced. Expression constructs for CIZ, EWSR1-CIZ, and TAF15-CIZ were constructed in pcDNA3.1–6HysMyc, introducing a COOH-terminal 6His-Myc-tag. The inserts were then moved to pMSCV-puro (a gift from G. Gilliland, Harvard University, Boston, MA).

EMSAs. HEK293T cells were transiently transfected with the pMSCV plasmids using the Fugene 6 Transfection Reagent (Roche) and lysed after 24 h as described (12). DNA probes were prepared by annealing the oligonucleotides 5′-CGCGTCAACCTTTTTCAAAAAGACCAG and 5′-CTAGCTG-GTCTTTTTGAAAAAGGTTGA with the −320 to −305 region of the human *MMP1* promoter and filling in the 5′-overhangs with Klenow polymerase and [α-^{32}P]dCTP. Mutant probe was prepared by annealing the oligonucleotides 5′-CCTGTGTCAGAGAGA and 5′-TCTCTCTGACACAGG.

Promoter Reporter Assays. The −296HMAT-Luc reporter construct with the human *MMP7* promoter in pGL2Basic was a gift from L. Matrisian (Vanderbilt University, Nashville, TN; Ref. 13). HEK293T cells at 35% confluence were cotransfected with the reporter construct (150 ng), together with each of the expression vectors (or empty pMSCV-puro) and 15 ng of a β-galactosidase expression plasmid, using FuGene-6 Reagent (Roche). After 36 h, cells were lysed in passive lysis buffer (Promega) and assayed for luciferase activity using a commercial assay (Promega). All values were normalized for β-galactosidase activity. Experiments were performed three times, each point in triplicate.

Transformation Assays. Viral supernatants were produced in HEK293T cells cotransfected with the pMSCV-puro constructs and the pIK6.1MCV packaging vector (a gift from G. Gilliland, Harvard University). NIH3T3 cells were seeded at 2×10^5 cells/well of a 6-well plate 24 h before adding the viral supernatants containing 8 μg/ml Polybrene for 4 h. Twenty-four h later, selection with puromycin (2 μg/ml) was started. For the focus formation assay,

the transduced NIH3T3 cells were seeded at a density of 10^6 cells per 9-cm culture dish in DMEM/10% FCS and cultured for 3 weeks. Medium was replaced every 3.5 days. Soft agar assays were performed as described (7).

RT-PCR Analysis of Gene Expression. RNA was isolated form NIH3T3 or Ba/F3 cells expressing the different fusion genes and treated with DNaseI (Invitrogen). RT-PCR was performed with oligonucleotides 5′-GAGGAGCTAGCAGGTTATCCTAA and 5′-AGCTACACAGTGCTTCTGAACATC for *Mmp3*, 5′-AAGGAGAGATCATGGAGACAGCTT and 5′-GATGTCTCGCAACTTCATGTTACC for *Mmp7*, 5′-CCCTCAGCTACGGTGTCT and 5′-TCGTAGCCTTTCCTGTCA for *manic fringe*, and 5′-CTGACCAAGCGAGAGTGTGA and 5′-TCCCTCTTTGGCATAGGTTG for *Eat2*. Thirty-five cycles of denaturation for 30 s at 95°C, annealing for 30 s at 56°C, and extension for 40 s at 72°C were performed. The EWSR1/FLI1 expression plasmid was obtained from C. Denny (UCLA, Los Angeles, CA).

Results and Discussion

Cloning of t(12;17) and t(12;22). Metaphases of an acute myeloid leukemia with t(12;17)(p13;q11) and an acute undifferentiated leukemia with t(12;22)(p13;q12) as the sole anomaly were analyzed by fluorescence *in situ* hybridization using bacterial artificial chromosome clones mapped to chromosome region 12p13. Clone 433J6 (accession number AC079387) showed split signals in both cases. The 12p breakpoint was narrowed by Southern blot experiments, which, combined with the sequence from 433J6, indicated that a gene highly similar to rat *Ciz* (p130CAS interacting zinc finger, *rCIZ*; accession number AB019281) was disrupted by both translocations. rCIZ was identified as a p130CAS interacting protein (14) and is identical to the NMP4 (15). Next, we obtained two cDNAs of 1.55 and 1.74 kb, respectively, for human *CIZ* by RT-PCR on bone marrow cDNA. The longer cDNA has an open reading frame coding for 578 amino acids showing 92% identity to *rCIZ* and is identical to the human *CIZ* cDNA sequence reported recently (accession number XM_033227; Fig. 1*A*). The shorter transcript is a splice variant coding for a 517 amino acid protein, similar to splice variants described for *rCIZ/NMP4* (14, 15). The gene has 16 exons spanning ~60 kb of genomic DNA.

To identify the fusion partner of *CIZ*, 5′-RACE experiments were performed on cDNA from the t(12;17) case. A fusion fragment linking exon 6 of the *TAF15* gene (also known as *RBP56* or *TAF2N*) to exon 3 of *CIZ* was detected (Fig. 1, *B* and *C*). This *TAF15-CIZ* rearrangement was confirmed by RT-PCR and results in an in-frame fusion of the 5′ part of *TAF15*, encoding its transactivating domain, to the entire *CIZ* sequence. TAF15 is a member of the TET family of proteins, which also includes EWSR1 and TLS/FUS. We thus investigated whether the t(12;22) involved EWSR1. Using RT-PCR with *EWSR1* and *CIZ* primers, an in-frame fusion linking exon 7 of *EWSR1* to exon 2 of *CIZ* was detected. As the start codon of *CIZ* is located in exon 3, this fusion protein contains some novel sequences derived from the 5′ UTR of *CIZ*, as well as the complete CIZ amino acid sequence (Fig. 1, *B* and *C*). Attempts to amplify possible reciprocal transcripts were negative.

Four additional leukemias with a t(12;17) were collected; interestingly, these were all ALLs. A *TAF15-CIZ* fusion cDNA was detected by RT-PCR cDNA in the 3 cases for which cDNA was available. The structure of the fusion was identical to the one described above for two cases, whereas in the third case, a fusion of exon 9 of *TAF15* to exon 3 of *CIZ* was found (Fig. 1, *B* and *C*). Fluorescence *in situ* hybridization analysis detected a break within the *CIZ* gene in the case for which no RNA was available, strongly suggesting that this also harbors a *TAF15-CIZ* rearrangement. One additional case, also an ALL, with a t(12;22) was found. RT-PCR detected a variant *EWSR1-CIZ* fusion (*EWSR1* exon 7-*CIZ* exon 3; Fig. 1, *B* and *C*).

These results show that the *TET-CIZ* fusions are a recurrent rearrangement, mainly in ALL, but also occurring in acute myeloid leukemia. TET fusions were not described previously in acute lymphoblastic malignancies, and this novel phenotype must be defined by the COOH-terminal partner, *CIZ*. Bone metabolism is the only physiological process in which *CIZ* was implicated to date, despite its rather ubiquitous expression (14, 15). Our data indicate that *CIZ* is also implicated in normal and aberrant lymphoid and myeloid development.

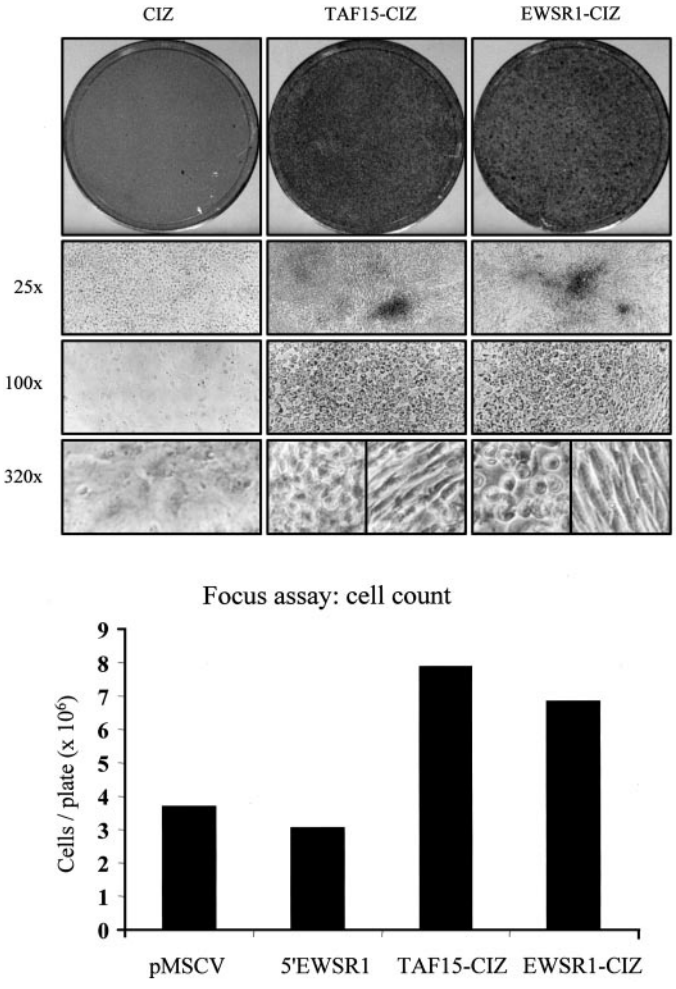

Fig. 2. Transforming properties of TET-CIZ fusions. *A*, focus formation assay. Microscope pictures (*three bottom rows*) were taken at three different magnifications, indicated at the *left*). For *TAF15-CIZ* and *EWSR1-CIZ*, two different areas of the same dish are shown at highest magnification to illustrate both the small round cell (*left*) and the spindle shape (*right*) morphology of the cells. *B*, cultures of cells transduced with the empty pMSCV-puro vector and with pMSCV-puro carrying either the 5′-end of *EWSR1*, the *EWSR1-CIZ*, or the *TAF15-CIZ* fusion in 100-mm dishes were trypsinized at confluency after 27 days and counted using a Coulter Multisizer.

Transforming Properties of the TET-CIZ Fusions. The oncogenic properties of the TET-CIZ fusions were analyzed by overexpression in NIH3T3 and Ba/F3 cell lines. NIH3T3 cells stably expressing TAF15-CIZ or EWSR1-CIZ displayed a clearly transformed phenotype, compared with cells transduced with empty pMSCV-puro vector or cells overexpressing CIZ (Fig. 2). The cells are smaller, grow to a higher density, and form multiple foci. Two distinct populations were observed: cells with a small round cell morphology and spindle-like cells with a "herring-bone" appearance, reminiscent of fibrosarcoma. Interestingly, a similar round cell phenotype was described for EWSR1-FLI1 and EWSR1-ETV1 (16). Controls with constructs expressing exclusively the EWSR1 or TAF15 part of the fusion, or wild-type CIZ, resulted in a normal phenotype (not shown), demonstrating that the transformation properties of the fusions are

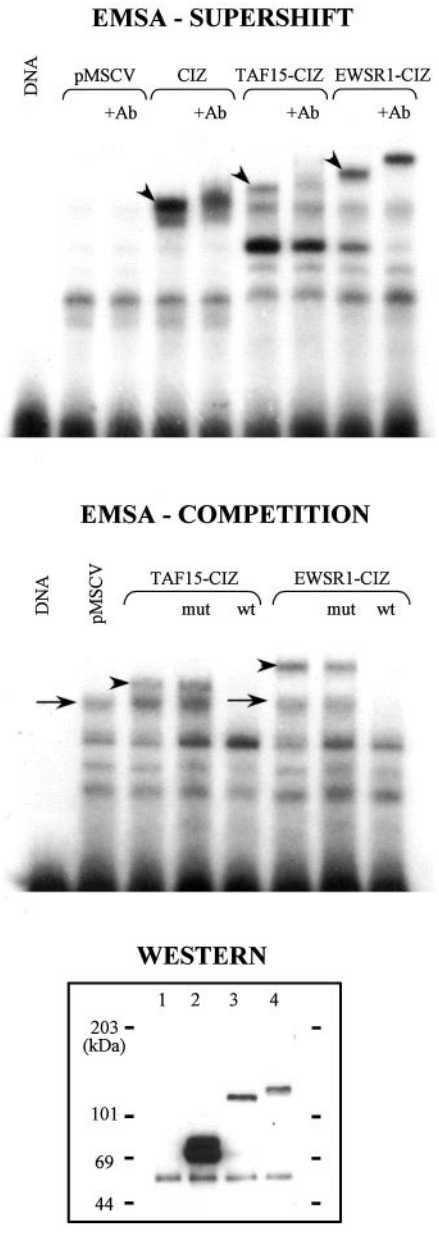

Fig. 3. DNA binding of the TET-CIZ fusion proteins. Total lysate of HEK293T cells transiently transfected with *CIZ*, *TAF15-CIZ*, or *EWSR1-CIZ* expression constructs in pMSCV-puro was capable of forming a gel shift complex with a ^{32}P-labeled oligonucleotide with the CIZ DNA binding consensus sequence. This complex was supershifted with a monoclonal anti-myc antibody (*Ab*) and disappeared in the presence of a 1000-fold excess of cold wild-type probe (*wt*), whereas the same amount of cold mutant probe (*mut*) did not compete for the binding. *Arrowheads*, shifted complexes. *Arrows* in the *competition panel* indicate signals resulting from endogenous CIZ expression. Western blot analysis of the cell lysates is shown at the *bottom* of the figure. *Lane 1*, pMSCV−; *Lane 2*, CIZ; *Lane 3*, TAF15-CIZ; *Lane 4*, EWSR1-CIZ. Proteins were detected using the anti-myc monoclonal.

dependent on the presence of both the NH_2-terminal domain of TAF15 or EWSR1 and CIZ, respectively. Expression of TAF15-CIZ or EWSR1-CIZ, however, could not induce substrate-independent growth of NIH3T3 cells in soft agar (whereas a control with BCR-ABL expressing RAT-1 cells was positive; result not shown). It was shown previously that EWSR1-FLI1 supports anchorage-independent growth of NIH3T3 cells, but that EWSR1-ETV1 does not, showing that soft agar growth is a transformed phenotype not shared by all TET fusions (16). This shows clearly that the transcription factor determines the strength of transformation and suggests that CIZ defines the weak transforming potential of the TET-CIZ fusions.

Expression of the fusion constructs in the interleukin 3-dependent murine pre-B lymphoid cell line Ba/F3 failed to induce growth factor independence or increased resistance to apoptosis (not shown).

Transactivating Properties of the TET-CIZ Fusions. CIZ has the properties of a transcription factor: it localizes in focal adhesions and in the nucleus of adherent cell lines, it binds specifically to the (G/C)AAAAA(A) sequence, and overexpression of CIZ was shown to activate *MMP1*, *MMP3*, and *MMP7* promoters that contain the CIZ consensus sequence (14). To investigate the transactivating properties of TET-CIZ fusions, EMSAs were performed with extracts of HEK293T cells transiently expressing myc-tagged TAF15-CIZ or EWSR1-CIZ. These showed specific binding of the chimeric proteins to DNA with the CIZ consensus binding sequence; a retarded labeled band is observed that can be supershifted with antibodies against the myc-tag and disappears upon addition of unlabeled probe (Fig. 3). Next, reporter assays were performed. Overexpressed CIZ in HEK293T cells had a weak but reproducible transactivating activity

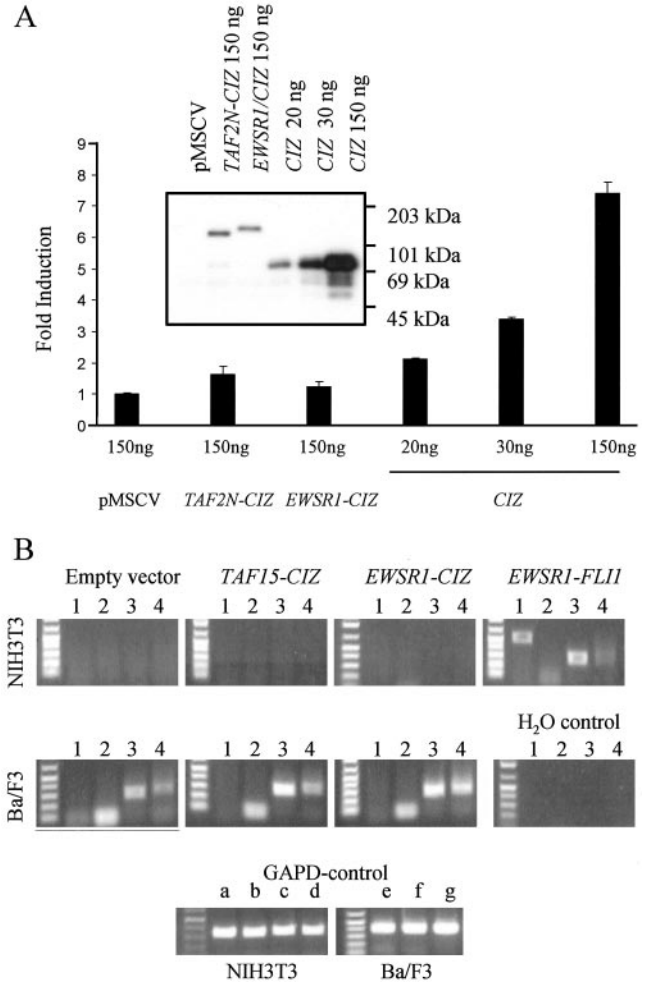

Fig. 4. Analysis of transactivation by TET-CIZ fusion proteins. *A*, activation of luciferase reporter constructs carrying the *MMP7*-promoter. HEK293T cells were cotransfected with a human *MMP7* promoter −296HMAT-Luc reporter construct together with each of the expression vectors (amount of DNA indicated) and a β-galactosidase expression plasmid. After 36 h, luciferase and β-galactosidase activities were measured. Luciferase values, normalized for β-galactosidase, are the means of representative experiments in triplicate; *bars*, SD. To evaluate protein expression levels, a Western blot was performed (*inset*). Equal volumes of total cell lysate (~30 μg of protein) were separated by 7% SDS-PAGE and visualized with mouse monoclonal anti-myc antibodies. *B*, RT-PCR analysis of *mmp3* (*Lane 1*), *mmp7* (*Lane 2*), *eat2* (*Lane 3*), and *manic fringe* (*Lane 4*) transcripts. NIH3T3 or Ba/F3 cells were transduced with different pMSCV-puro constructs. The negative control contained H_2O instead of cDNA. A positive control amplifying *GAPD* is also shown, *a* and *e*, pMSCV-puro; *b*, *EWSR1-FLI1*; *c* and *f*, *TAF15-CIZ*; *d* and *g*, *EWSR1-CIZ*.

on a luciferase reporter construct carrying the −301 to +35 region of the human *MMP7* promoter as described previously (14). Surprisingly, TAF15-CIZ and EWSR1-CIZ and CIZ did not show enhanced (or decreased) transactivation of this promoter at similar expression levels (Fig. 4). We were not able to increase the expression levels of the EWSR1 and TAF15 fusions, possibly because these interfere with proliferation of the cells, as described for EWSR1-FLI1 (17).

It is puzzling that the TET-CIZ fusions do not transactivate reporter constructs carrying CIZ binding sites, although they do bind to the CIZ DNA recognition sequence in EMSA experiments. It is possible that HEK293T cells lack some CIZ- or EWSR1/TAF15-interacting factors, preventing the full transactivating activity of the TET-CIZ proteins. However, it should be noted that HEK293T cells do express endogenous CIZ, and that increasing the concentration of CIZ is able to enhance the activation of the *MMP7* promoter.

The transactivation properties of the TET-CIZ fusions were thus further explored, and the transcriptions of the CIZ target *mmp7* and of the EWSR1-FLI1 targets *mmp3, eat2* (6), and *manic fringe* (4) were analyzed in NIH3T3 and BA/F3 cells by RT-PCR (Fig. 4). No transcription of any target was seen in NIH3T3 cells expressing either TET-CIZ fusion, whereas a strong induction of *mmp3, eat2,* and *manic fringe* transcription was observed in the EWSR1-FLI1-expressing controls. Similarly, no induction of *mmp3* or *mmp7* was visible in the Ba/F3 cells expressing the TET-CIZ fusions. Interestingly, *eat2* and *manic fringe* were found to be constitutively expressed in this cell line. The semiquantitative character of the RT-PCR did not allow us to determine whether the transcription of these genes was increased.

Taken together, these results suggest that the CIZ fusions do not act as transcriptional activators (or repressors) for the *MMP3* or *MMP7* promoters, although they do bind the CIZ consensus sequence, or that MMPs are not the true targets of CIZ. On the other hand, it has been shown that some of the transforming activities of *EWSR1-FLI1* fusions are not dependent on DNA binding and transactivation (7, 8). In this regard, the observation that TET proteins do affect pre-mRNA splicing (11, 18), a process that is affected by EWSR1-FLI1 (11, 19), TLS-ERG (9), and EWSR1-NOR1 (10), is of particular interest. Alternative splicing of particular genes can lead to modified functional properties of the gene products, *e.g.*, dominant-negative isoforms of *ikaros* and a decrease of *ikaros* activity is associated with blast crisis in chronic myeloid leukemia (20). If transcriptional activation is not the major mechanism of action of TET-CIZ fusions, these could provide an interesting model to investigate alternative oncogenic pathways for TET fusions, such as the effect on pre-mRNA splicing.

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