Elongation Factor ELL (Eleven-Nineteen Lysine-rich Leukemia) Acts as a Transcription Factor for Direct Thrombospondin-1 Regulation

Jiangang Zhou†, Xi Feng§, Bin Ban†, Jingxia Liu†, Zhou Wang‡, and Wuhan Xiao§

From the †Key Laboratory of Biodiversity and Conservation of Aquatic Organisms, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China and the §Department of Urology, University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15232

The eleven-nineve lysine-rich leukemia (ELL) gene undergoes translocation and fuses in-frame to the multiple lineage leukemia gene in a substantial proportion of patients suffering from acute forms of leukemia. Studies show that ELL indirectly modulates transcription by serving as a regulator for transcriptional elongation as well as for p53, U19/Eaf2, and steroid receptor activities. Our in vitro and in vivo data demonstrate that ELL could also serve as a transcriptional factor to directly induce transcription of the thrombospodin-1 (TSP-1) gene. Experiments using ELL deletion mutants established that full-length ELL is required for the TSP-1 up-regulation and that the transcriptional activation domain likely resides in the carboxyl terminus. Moreover, the DNA binding domain may localize to the first 45 amino acids of ELL. Not surprisingly, multiple lineage leukemia-ELL, which lacks these amino acids, did not induce expression from the TSP-1 promoter. In addition, the ELL core-response element appears to localize in the −1426 to −1418 region of the TSP-1 promoter. Finally, studies using zebrafish confirmed that ELL regulates TSP-1 mRNA expression in vivo, and ELL could inhibit zebrafish vasculogenesis, at least in part, through up-regulating TSP-1. Given the importance of TSP-1 as an anti-angiogenic protein, our findings may have important ramifications for better understanding cancer.

ELL4 was first identified in acute myeloid leukemia as a translocation partner of MLL (1). Wild-type MLL positively regulates expression of Hox genes, important in embryonic development and hematopoiesis (2–4), but the formation of MLL chimeras dysregulates this expression, contributing to leukemogenesis (4–14). MLL partners also likely contribute to leukemogenesis. One such partner is ELL, which fuses with MLL as a result of the frequent translocation event t(11;19) (q23; p13.1) that occurs in acute myeloid leukemia (1). In vitro studies showed that wild-type ELL can increase the rate of polymerase II transcriptional elongation by suppressing transient pausing (15, 16), an activity shared by the two paralogs of ELL found in mammalian cells, ELL2 and ELL3 (17, 18). The targeted knock-out of ELL in mice caused embryonic lethality, suggesting an essential role for this protein in early embryonic development (19). Further functional analyses revealed that ELL regulates cell growth and survival (20) and serves as a selective co-regulator for steroid receptor functions (21). In all, these studies point to the importance of ELL in normally functioning cells.

Like other MLL translocation products, the chimera MLL-ELL appears to play an important role in leukemogenesis (22, 23). The MLL-ELL fusion product contains the amino-terminal region of MLL fused to amino acids 46–621 of ELL that include the elongation domain and a lysine-rich region (1). Transplantation of blood progenitor cells transduced with MLL-ELL into sublethally irradiated normal mice resulted in morphological and clinical disease manifestations closely resembling those observed in patients suffering from leukemia (24). Although the precise mechanism of how MLL-ELL participates in leukemogenesis remains unknown, evidence suggests that MLL-ELL contributes to the transformation of blood progenitor cells and the development of leukemia, at least in part, through the repression of p53 activity (25–27). Both MLL-ELL and wild-type ELL, but not wild-type MLL, inhibited p53 transcriptional activity, whereas MLL-ELL inhibits p53 more efficiently than ELL (25–28). Furthermore, the ELL extreme carboxyl terminus (amino acids 508–621), which is responsible for p53 repression, is both necessary and sufficient for the malignant transformation of bone marrow cells and the onset of leukemia in animals (24).

To gain a more complete picture of how ELL functions in the cell, two-hybrid screens and biochemical purification have been performed to identify ELL-binding partners. These studies led to the discovery of Eaf1 and Eaf2 (29, 30). Both Eaf1 and Eaf2 co-localize with ELL in the nucleus in a stippled pattern, supporting the physiological relevance of their association with ELL (29, 30). Interestingly, the carboxyl-terminal domains of Eaf1 and Eaf2 share similarity with AF4 and ENL, other MLL translocation partners, suggesting that interactions between ELL and Eaf1 may contribute to the leukemic phenotype of cells expressing the MLL-ELL chimera (29). Indeed, expression of
ELL Acts as Transcription Factor for Direct TSP-1 Regulation

exogenous MLL-Eaf1 chimeras can immortalize hematopoietic progenitor cells and induce the development of acute myeloid leukemia in mice (23). Concomitantly, we identified Eaf2, which we named U19, as a novel testosterone-regulated protein capable of inducing apoptosis in the prostate (31). Our studies indicated that the association between ELL and U19/Eaf2 regulated U19/Eaf2 intracellular localization, stability, and transcription activity, and the binding domain was required for U19/Eaf2 apoptotic activity (32–34). Moreover, our studies provided evidence that ELL alone has transactivation capability (32). In vitro and in vivo studies revealed U19/Eaf2 as a potential tumor suppressor (31, 35), further supporting a role for ELL in tumor progression.

Whereas studies point to an important role for ELL in normal cells as well as cancer, the exact mechanism of ELL action remains unclear, due in part to the embryonic lethality of ELL knock-out mice (19). Studies suggest that the protein acts as a regulator of p53, U19/Eaf2, steroid receptors, and transcriptional elongation (21, 25–27, 32); however, our observation that knock-out mice (19). Studies suggest that the protein acts as a tumor suppressor (31, 35), further supporting a role for ELL in tumor progression.

ELL provides evidence that ELL alone has transactivation capability (32). Moreover, our studies provided evidence that ELL alone has transactivation capability (32). In vitro and in vivo studies revealed U19/Eaf2 as a potential tumor suppressor (31, 35), further supporting a role for ELL in tumor progression.

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmid Construction—HEK 293 cells were obtained from ATCC. HA1E, a derivate of human embryonic kidney cells, was provided by Randolph S. Watnick. Cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin (HyClone) at 37 °C in a humidified atmosphere containing 5% CO₂.

The cDNA of human ELL, provided by Ali Shilatifard, was cloned into the CMV-Myc and PM vectors (Clontech) by PCR. Deletion mutants of ELL were generated by PCR and cloned into CMV-Myc, PM (Clontech), and pVP16 vectors (Clontech). The promoter region (−2033 to +750) of TSP-1, provided by Olga Volpert, was cloned into the pGL3-Basic vector (Promega) by PCR. Deletion mutants of the TSP-1 promoter were generated by restriction endonuclease digestion or PCR and cloned into the pGL3-Basic vector. The region of −1658 to −1270 of the TSP-1 promoter was cloned into pGL2 promoter vector (Promega) by PCR. Myc-Uaf1, Myc-Uaf2, and Myc-p53 were described previously (32). pcDNA-MLL-ELL was generated by restriction digestion or PCR and cloned into the pGL2 promoter, pGL3-Basic vector, respectively, at KpnI and BglII sites.

Deletion mutants of ELL were generated by PCR and cloned into the pGL3-Basic vector (Promega) by PCR. Deletion mutants of the TSP-1 promoter were generated by restriction endonuclease digestion or PCR and cloned into the pGL3-Basic vector. The region of −1658 to −1270 of the TSP-1 promoter was cloned into pGL2 promoter vector (Promega) by PCR. Myc-Eaf1, Myc-Uaf2, and Myc-p53 were described previously (32). pcDNA-MLL-ELL was generated by restriction digestion or PCR and cloned into the pGL2 promoter, pGL3-Basic vector, respectively, at KpnI and BglII sites.

In vitro

To functionally map the DNA-binding sites of ELL in the TSP-1 promoter, seven deletion mutants (−1604 to 1270, −1554 to 1270, −1504 to 1270, −1454 to 1270, −1404 to 1270, −1354 to 1270, and −1248 to 1270) of the TSP-1 promoter were generated by PCR and cloned into pGL2 promoter vector at KpnI and BglII sites. The primers were as follows:

- TSP-1(−1270), 5′-tatcagacttcatcaataaaagatctccagc-3′
- TSP-1(−1427), 5′-tatcagacttttctatctccagc-3′
- TSP-1(−1454), 5′-tatcagactttcattcagc-3′
- TSP-1(−1504), 5′-tatcagactttcattcagc-3′
- TSP-1(−1604), 5′-tatcagacttttctatctccagc-3′
- TSP-1(−1658), 5′-tatcagacttttctatctccagc-3′
- TSP-1(−1805), 5′-tatcagacttttctatctccagc-3′
- TSP-1(−2033), 5′-tatcagacttttctatctccagc-3′

Western blot—Anti-α-tubulin monoclonal antibody was purchased from Upstate. Anti-β-actin and anti-Myc antibodies were purchased from Santa Cruz Biotechnology. Western blots were performed as described previously (32). FujiFilm LAS4000 mini luminescent image analyzer was used to photograph blots. GraphPad Prism 5.0 was used for graph preparation and statistical analysis (t test).

Semi-quantitative RT-PCR—Total RNA was isolated with RNasy mini kit (Qiagen) from 293 cells transfected with 4 μg of CMV-Myc or 4 μg of Micy-ELL, and then 2 μg of RNA reverse-transcribed using RevertAid™ first strand cDNA synthesis kit (Fermentas, Burlington, Ontario, Canada) and random primers. An amount of cDNA first strands corresponding to 50 ng of total RNA was used as the template for PCR amplification on a Chromo4™ detector for PTC DNA Engine™ system (Bio-Rad) in the presence of SYBR green. All PCRs were
ELL Acts as Transcription Factor for Direct TSP-1 Regulation

run in triplicate and repeated at least three times. Differences were calculated according to the ΔΔCt relative quantitation method using 18 S RNA gene as calibrators. The primers for human TSP-1 were 5′-AACAAGTGTGGATGCTTGTTCC-3′ and 5′-GCCAATGTAGTATGCGGAT-3′ as designed by Primer Express Program (Applied Biosystems, Foster City, CA). The primers for human 18 S RNA gene were 5′-TCAAC-TGCGATGTTAGCGCCGT-3′ and 5′-TCCTTGGATGTG-TGACGCTTCT-3′.

Northern Blot—293 cells were transfected with the pCMV-Myc empty vector (Clontech) or vectors expressing Myc-ELL. Total RNA was isolated with TRIzol® reagent (Invitrogen). Electrophoresis, transfer, and hybridization were performed as described previously (31). The membrane was probed using synthesized oligonucleotides corresponding to human TSP-1 (5′-aacaagcaccacatttccagctgccat-3′) and human β-actin (5′-atgtcaataaagctctggccaca-3′) labeled with biotin at the 3′ end. The signal was detected using the North2South nucleus labeling and detection kit (Pierce). Photography and data analysis were done as described for the Western blot analysis.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation assays were performed according to the protocol modified from the user manual of an acetyl-histone H3 immunoprecipitation kit (Upstate). 293 cells were cross-linked with 1% formaldehyde in phosphate-buffered saline for 2 min at room temperature and scraped off the plate following three washes with cold phosphate-buffered saline. Subsequently, cells were lysed with lysis buffer containing proteinase inhibitors (Sigma) and 1 mM dithiothreitol. Cell lysates were sonicated to break down chromatin into fragments 200–500 bp in length. Chromatin was diluted 1:10 and pre-cleared with protein A-agarose beads (Upstate) for 1 h at 4 °C. Approximately 3% of the chromatin from each sample was saved as input. The rest of the chromatin was used for immunoprecipitation with either polyclonal rabbit anti-ELL or preimmune serum as a control at 4 °C overnight. Immunoprecipitated antibody-protein-DNA complexes were collected by protein A-agarose beads at 4 °C for 1 h and extensively washed. The complex was eluted and reverse cross-linked at 65 °C for 6 h, and then proteinase K was added for further incubation at 45 °C for 1 h. DNA samples were extracted using phenol/chloroform. The primers specific for TSP-1 promoter region were 5′-CTGCTTCCCA-ACATCTGAAATCCA-3′ and 5′-GACTGTCTGCAAAACGCC-CAAGTTGT-3′. PCR was performed for 35 cycles with the following cycling conditions: 94 °C, 30 s; 53 °C, 30 s; 72 °C, 1 min. The promoter region of β-actin was used as internal control. The primers used for amplifying promoter region of β-actin were 5′-CAGGGCGCTGATGGTGCGCA-3′ and 5′-CATA-CATGATCTGGTACTTCTC-3′.

Whole-mount in Situ Hybridization—In situ hybridization was performed on whole-mount zebrafish embryos as described (36). For the TSP-1 probe, the fragment of zebrafish TSP-1 cDNA was amplified from total RNA isolated from zebrafish embryos at 24 h post-fertilization (hpf) by RT-PCR using the following primers: zTSP-1-F, 5′-GTGCGAATACTAGCCCGCGCTCAAG, and zTSP-1-R, 5′-CTCGAAGCTAGACGCCCCACACCGG. Subsequently, the fragment was subcloned into vector pTA2 (Toyoba), and riboprobes were synthesized using the digoxigenin RNA labeling mix (Roche Applied Science).

ELL Antisense MO and Its Validation—Zebrafish ELL antisense morpholino (MO) was designed and synthesized in Gene Tools, LLC, based on the sequence of the full-length zebrafish ELL gene. The sequence of 25-mer ELL-MO was ACTGCTCT-TCCTTTCAACGGCCCAT and covered open reading frame bases +1 to +25. Oligonucleotides were resuspended in sterile water and injected into the one-cell stage of fertilized zebrafish eggs. Each egg was injected with about 6 ng of MO. Whole-mount in situ hybridization for TSP-1 was performed at 12 and 24 hpf.

The wild-type zebrafish ell cDNA and mismatched zebrafish ell cDNAs were cloned into pEGFP-N1 (Clontech) to generate wild-type ELL and mismatched ELL tagged with GFP at the carboxyl terminus to validate the specificity and efficiency of ELL-MO. The GFP-tagged mismatched zebrafish ELL was generated by PCR using a forward primer with five mismatched nucleotides, 5′-TGTCGACATGGGCGGCTTA-AAGAAAGAGCAGTGTACG-3′ (mismatched nucleotides are underlined).

Zebrafish ell mRNA and Ectopic Expression—The open reading frame sequences of zebrafish ell and GFP cDNA were subcloned into the Pspe64 poly(A) vector (Promega). Capped mRNAs were synthesized using the Ambicat SP6 High Yield message maker kit (Epicenter). Capped mRNA (200–500 pg) was injected into one-cell stage embryos. Flk-GFP transgenic zebrafish was provided by Jianfang Gui, originally obtained from Suo Lin (37).

RESULTS

Carboxyl Terminus of ELL Contains a Transactivation Domain—Previous work from us and others has demonstrated the ability of ELL to both indirectly modulate transcription by binding to p53 and U19/Eaf2 (26, 32). At the same time, we have also shown that ELL alone has transactivation activity (32). These findings prompted us to further investigate the role of ELL in transcription. We cloned full-length ELL and various ELL deletion mutants into the PM vector to evaluate transactivation activity as well as to map the potential transactivation domain of ELL. The vectors expressed the GAL4 DNA binding domain fused with full-length ELL or ELL deletion mutants. Each of these constructs was co-transfected with the luciferase reporter plasmid pER-luc, which contains the Photinus pyralis luciferase gene under the control of five tandem repeats of the yeast GAL4-binding site into HA1E cells, a derivative of human embryonic kidney cells. Cells were also transfected with the Renilla luciferase expression vector as an internal control. A Western blot using anti-ELL polyclonal antibody demonstrated that the HA1E cells expressed full length and each mutant efficiently (Fig. 1A, panel b). Fig. 1A, panel a, shows that co-transfection of the reporter construct with full-length ELL resulted in 7-fold greater luciferase activity than co-transfection with the GAL4-DNA binding domain alone (PM vector). The deletion mutant containing amino acids 447–621 produced the highest level of luciferase activity, indicating that the main transactivation domain resides in the carboxyl terminus of ELL. The deletion mutant containing amino acids 1–379 produced a
weak increase in luciferase activity (1.6-fold). On the other hand, the deletion mutant containing amino acids 46–621 had a decrease in luciferase activity, indicating that amino acids 46–446 likely contain a repressive domain. It appears that amino acids 1–45 may, at least in part, neutralize the repressive ability of amino acids 46–446, leading to the increase in transcriptional activity seen with full-length ELL.

We next analyzed whether Eaf1, U19/Eaf2, or p53, three known binding partners of ELL, could affect the ability of ELL to promote transcription. We used the same GAL4 system described previously to measure ELL transactivation activity in the presence of Myc-tagged Eaf1, U19/Eaf2, and p53. U19/Eaf2 synergistically enhanced the transactivation activity of ELL (p < 0.05), whereas p53 and Eaf1 abrogated ELL-mediated transactivation of the reporter in a statistically significant manner (p < 0.05 and p < 0.001, respectively) (Fig. 1B, panel a). A Western blot using anti-Myc polyclonal antibody demonstrated that the HA1E cells expressed each gene efficiently (Fig. 1B, panel b).

ELL Acts as a Transcription Factor for Direct TSP-1 Regulation

To identify the regions of ELL responsible for TSP-1 up-regulation, we cloned five of the ELL deletion mutants used previously into a Myc-tagged expression vector (Fig. 2B). Western analysis using an anti-ELL polyclonal antibody showed that the mutants were all expressed efficiently at a similar level (Fig. 2C). Only the full-length ELL (amino acids 1–621) stimulated TSP-1 promoter activity above that of the empty vector control (Fig. 2C). Previous experiments indicated that the transactivation domain of ELL resides in the carboxyl terminus (Fig. 1A), so it was not surprising that the 1–379 and 1–508 deletion mutants, which lack the carboxyl terminus, had no obvious transcriptional activity. The amino-terminal deletion mutants 46–621, 447–621, and 509–621 had relative luciferase activity levels similar to that of the empty vector control (Fig. 2C). Because these mutants contained the putative transactivation domain, these results suggested
that the DNA binding domain might reside in the first 45 amino acids.

To further test whether the DNA binding domain resides in the first 45 amino acids, we fused 1–45 with VP16 activation domain using pVPI6 vector (Clontech). Subsequently, we co-transfected VP16-ELL-(1–45) along with TSP-1 promoter luciferase reporters TSP-1 (−2033 to +750) or TSP-1 (−2033 to 0), respectively. The luciferase assays showed that VP-ELL-(1–45) could indeed transactivate the TSP-1 promoter reporters significantly ($p < 0.05$). The expression of VP16-ELL-(1–45) was verified by Western blot (Fig. 2D). These observations supported that the DNA binding domain resided in the first 45 amino acids of ELL. However, this conclusion still needs to be investigated thoroughly using some more direct approaches such as gel shift assays, etc.

**ELL Acts as Transcription Factor for Direct TSP-1 Regulation**

**ELL Response Elements in the TSP-1 Promoter**—We next wanted to map the ELL response region in the TSP-1 promoter by comparing reporter activities of full-length human TSP-1 promoter (−2033 to +750 bp) to various promoter deletion mutants in the presence or absence of ELL (Fig. 3A and B). Five different deletion mutants of the TSP-1 promoter were cloned into the pGL3-Basic vector and then co-transfected into 293 cells with the full-length ELL expression vector. We observed that the deletion of region −1608 to −1270 of the TSP-1 promoter abrogated the transactivation activity of ELL, indicating that the ELL response element might localize to this region. Interestingly, the deletion of −2033 to −1608 and 0 to +750 regions enhanced the transactivation of the TSP-1 promoter by ELL, which suggested that the TSP-1 promoter might have suppressive elements.

To further confirm whether the ELL response element localizes to region −1658 to −1270, we cloned this region into pGL2 promoter vector (Fig. 3C). The promoter luciferase assays indicated that the region −1658 to −1270 was indeed significantly responsible for ELL overexpression ($p < 0.05$) (Fig. 3D).

**FIGURE 2.** ELL serves as a transcription factor for the TSP-1 promoter. **A,** dose-dependent up-regulation of TSP-1 promoter activity by ELL. HA1E cells were used for assays. The expression of Myc-ELL was verified by Western blot using anti-ELL polyclonal antibody. IB, immunoblot. **B,** schematic depiction of ELL functional domains and ELL truncated mutants. C, domain mapping of ELL in up-regulation of TSP-1 promoter activity. The expression of Myc-tagged deletion mutants of ELL was verified by Western blot using anti-ELL polyclonal antibody. D, possible DNA binding domain localized in 1–45-amino acid region of ELL was tested by promoter assays. The 1–45-amino acid region of ELL fused with VP16 (VP) activation domain was co-transfected with pGGL3-Tsp1 promoter (−2033 to +750) or pGL3-Tsp1 promoter (−2033 to 0), respectively. The luciferase assays showed that VP-ELL-(1–45) could transactivate the Tsp-1 promoter significantly ($p < 0.05$ and $p < 0.001$, respectively). The expression of VP-ELL-(1–45) was verified by Western blot using anti-ELL polyclonal antibody.

To further define the core binding site of ELL in the TSP-1 promoter region −1658–1270, we did fine domain mapping. First, we made six series deletion mutation reporters for −1658–1270 region (Fig. 4A). As shown in Fig. 4B, the deletion...
ELL Acts as Transcription Factor for Direct TSP-1 Regulation

We found that ELL directly induces TSP-1 expression; however, studies from other groups present the possibility that ELL may indirectly decrease TSP-1 expression through inhibition of p53 (26, 39). To address this, 293 cells were co-transfected with vectors expressing Myc-p53, Myc-ELL, or a combination of both along with the TSP-1-luciferase reporter construct. Fig. 5A reveals that exogenous p53 did not induce transcription at the TSP-1 promoter. Indeed, the presence of Myc-p53 inhibited ELL-mediated transcription, similar to what we observed previously with the GAL4 promoter (Fig. 1B). As before, U19/Eaf2 enhanced ELL-mediated transcription at the TSP-1 promoter, whereas Eaf1 abrogated transcription (Fig. 5A). These results indicated that the effects of the binding partners of ELL, p53, Eaf1, and U19/Eaf2, on TSP-1 promoter activity were consistent with their roles in affecting transcriptional activity of ELL protein. Together, these observations further suggested that ELL could indeed serve as a transcriptional factor to regulate TSP-1 expression.

We also evaluated the ability of the oncogenic protein MLL-ELL to induce transcription at the TSP-1 promoter. The chimeric MLL-ELL lacks the first 45 amino acids of ELL. Not surprisingly, the empty vector and the vector expressing MLL-ELL produced approximately the same levels of relative luciferase activity (Fig. 5B). This is consistent with the results obtained with the 46–621 ELL deletion mutants that also lack the first 45 amino acids (Fig. 2C). Again, these results support the possibility of a DNA-binding site in this region of ELL.

ELL Regulates TSP-1 mRNA Expression—We next wanted to test if ELL could up-regulate expression of the endogenous TSP-1 gene. Either the Myc-ELL expression vector or an empty vector control was transfected into 293 cells. Total RNA was extracted from cells 24 h after transfection. We employed semi-quantitative RT-PCR to determine the mRNA levels of TSP-1. Fig. 6A shows that overexpression of ELL results in an increase of 25-fold of TSP-1 mRNA. The up-regulation of TSP-1 mRNA by ELL was further confirmed by Northern blot (Fig. 6B). These results suggested that ELL could indeed serve as a transcriptional factor to transactivate TSP-1 mRNA expression.

ELL Binds to the TSP-1 Promoter in Vivo—To obtain evidence that regulation of TSP-1 by ELL occurs in vivo, we immunoprecipitated cross-linked chromatin from 293 cells with an ELL-specific antibody. The precipitated chromatin was then

FIGURE 3. Mapping the ELL-response element in the TSP-1 promoter (+2033 to +750 bp). A, schematic of TSP-1 promoter deletion constructs. B, quantitation of ELL transcriptional activity on the different TSP-1 promoter deletion constructs. 293 cells were used for assays. C, schematic of TSP-1 promoter region −1658 to −1270 cloned into pGL2 promoter vector. D, Myc-ELL could transactivate pGL2-p-TSP-1(−1658 to −1270) significantly (p < 0.05). B and D, black bar represents relative reporter activity of mutated TSP-1 promoter co-transfected with the ELL expression vector, and the gray bar represents relative reporter activity of mutated TSP-1 promoter co-transfected with empty vector as a control.

of region −1658 to −1454 did not affect the promoter activity significantly induced by ELL overexpression. However, when we deleted the region −1454 to −1404, the promoter activity induced by ELL overexpression disappeared, which suggested the core-response element localized in the region −1454 to −1404. To further narrow down the core-response region, we made another reporter construct with region −1428 to −1270. As shown in Fig. 4, C and D, the deletion of the region −1454 to −1429 also did not affect the promoter activity significantly induced by ELL overexpression, narrowing down the core-response element to the region −1428 to −1403. Subsequently, we made homologous alignment for the TSP-1 promoter region from human, macaque, and mouse using MEGA 4.0 software. The region −1426 to −1418 corresponding to the positions of human TSP-1 promoter was evolutionarily conserved among human, macaque, and mouse (Fig. 4E), which implied that the conserved 9-bp region might be the core-response element in TSP-1 promoter for ELL up-regulation. To confirm this possibility, we made additional two site mutation constructs (−1427mut-1270 and −1427mut-0) (Fig. 4F), in which the nucleotide sequence AACACTCCC in TSP-1 promoter was mutated to CCACAGAAA. As expected, the ELL overexpression did not enhance the reporter activity of these two mutated promoter constructs significantly (Fig. 4G), indicating that the 9-bp region with nucleotide sequence AACACTCCC was the core-binding site of ELL in the TSP-1 promoter.

Of note, the magnitude of ELL transactivation on the TSP-1 promoter was generally higher in HA1E cells (Fig. 2, A and C) compared with that in 293 cells (Fig. 3, B and D; Fig. 4, B, D, and G). It is probably due to the difference between the HA1E cell line and the 293 cell line in promoter assays. Although the HA1E cell line was also a derivate of human embryonic kidney cells, similar to the 293 cell line, HA1E cells attached much better in a culture dish than 293 cells in general culture conditions. In addition, the transfection efficiency in HA1E cells is lower than that in 293 cells (data not shown). However, the real reason that caused the difference of ELL induction on the TSP-1 promoter between the HA1E cell line and the 293 cell line still needs to be further investigated.

Effect of ELL-binding Partners on TSP-1 Transactivation—We next wanted to test if ELL could up-regulate expression of the endogenous TSP-1 gene. Either the Myc-ELL expression vector or an empty vector control was transfected into 293 cells. Total RNA was extracted from cells 24 h after transfection. We employed semi-quantitative RT-PCR to determine the mRNA levels of TSP-1. Fig. 6A shows that overexpression of ELL results in an increase of 25-fold of TSP-1 mRNA. The up-regulation of TSP-1 mRNA by ELL was further confirmed by Northern blot (Fig. 6B). These results suggested that ELL could indeed serve as a transcriptional factor to transactivate TSP-1 mRNA expression.

ELL Binds to the TSP-1 Promoter in Vivo—To obtain evidence that regulation of TSP-1 by ELL occurs in vivo, we immunoprecipitated cross-linked chromatin from 293 cells with an ELL-specific antibody. The precipitated chromatin was then
analyzed using a primer pair that amplifies a segment of the TSP-1 promoter. A segment of the β-actin promoter amplified by a specific primer pair was used as control. The endogenous ELL protein bound to the TSP-1 promoter. In contrast, there was no ELL protein binding to the promoter region of β-actin (Fig. 7). Thus, these data suggest that TSP-1 can be regulated by ELL via direct binding to the TSP-1 promoter.

ELL Regulates TSP-1 mRNA Expression in Vivo—Given that ELL regulates TSP-1 expression in cell culture, we next asked whether ELL regulates TSP-1 mRNA expression in organisms. Thus, we turned to zebrafish, which, according to the zebrafish data base (ZFIN), possess homologs for both ELL and TSP-1. We analyzed the expression patterns of TSP-1 and ELL by in situ hybridization in whole-mount sections and found that spe-
ELL Acts as Transcription Factor for Direct TSP-1 Regulation

ELL mRNA injection resulted in TSP-1 up-regulation (Fig. 8C, panels c and g) compared with control GFP mRNA injection (Fig. 8C, panels b and f). Together, these findings further indicated that ELL regulated TSP-1 expression.

ELL Affects Zebrafish Vasculogenesis—Given TSP-1 was the first naturally occurring inhibitor of angiogenesis to be identified (38), we next asked whether ELL could affect vasculogenesis in a zebrafish model. After we checked zebrafish embryos injected with synthesized zebrafish ell mRNA at different time points post-fertilization, we found that the red blood cells were distributed abnormally in the body, exhibiting clumps in the trunks and tails, obviously distinguished from wild-type embryos (Fig. 9, A and A’, B and B’, C and C’, and D and D).

Subsequently, taking advantage of flk-GFP transgenic zebrafish, in which blood vessels were labeled by green fluorescent protein (Fig. 9E) (37), we observed that the blood vessels were completely disrupted by ell mRNA injection (Fig. 8F). These results suggest that ell influences zebrafish vasculogenesis, probably through up-regulating TSP-1.

DISCUSSION

The purpose of this study was to further define the physiological roles of ELL. Previous studies have shown that ELL regulates transcription indirectly. For example, ELL binds to and represses p53-mediated transcription (25–27) whole stimulating the transactivation activity of U19/Eaf2 (32). As well, ELL appears to act as a co-regulator for steroid receptor function (21). Some evidence suggests that ELL may also act as an independent transcription elongation factor in that ELL, along with its homologs ELL2 and ELL3 (15–17), can increase the rate of polymerase II transcriptional elongation by suppressing transient pausing in in vitro assays (15, 16). However, because of the lack of in vivo assays to measure elongation activity, the real function of ELL in transcriptional elongation requires further definition. Our in vitro and in vivo experimental evidence does indicate that ELL directly modulates transcription, specifically of the TSP-1 promoter. ELL contains a nuclear localization signal as well as a transactivation domain in the carboxyl terminus. Deletion assays suggest that the DNA binding domain of ELL localizes to the first 45 amino acids; however, mapping of this domain requires confirmation and further refinement.

As reported previously, ELL contains two major domains, including inhibitory domain (1–50 amino acids) and elongation activity domain (60–373 amino acids), which carry distinct functions (16). The inhibitory domain negatively regulates RNA polymerase II activity in promoter-specific transcriptional initiation in vitro, probably by physically blocking interaction of RNA polymerase II with TBP, TFIIH, or promoter...
DNA (16). The elongation activity domain accounts for increasing the rate of polymerase II transcriptional elongation (15, 28). However, in this study, we showed that the first 45 amino acids, the major part of the inhibitory domain, could bind to specific DNA sequences in the TSP-1 promoter required for ELL up-regulating TSP-1, and the elongation domain had no obvious transactivation activity. These results implied that the function of ELL acting as a transcription factor was distinct from its function acting as transcriptional elongation factor.

Human Eaf1 and U19/Eaf2 shared 52.6% identity in amino acids, and both of them were composed of six exons. Although Eaf1 and U19/Eaf2 have very similar structure and functional domains, some evidence showed that they might also behave differently. As reported previously, FB1 could inhibit transactivation of U19/Eaf2 but not Eaf1 (34). In addition, the ELL binding domain in Eaf1 was different from that in U19/Eaf2 (29, 30). Here we present another example to show that Eaf1 and U19/Eaf2 have opposite role in regulating the transcriptional activity of ELL.

Through promoter fine domain mapping, we localized the core binding site of ELL in the TSP-1 promoter to a conserved 9-bp region (AACACTCCC), implying that ELL could bind to a specific region of TSP-1 promoter. This observation further refined the result that ELL could specifically up-regulate TSP-1 expression through directly binding to the TSP-1 promoter. However, TSP-1, as the only target of ELL identified so far, we could not identify the consensus DNA binding sequence of ELL in this study. Because TSP-1 is unlikely the only target of ELL, to identify more ELL targets will help us to understand the general molecular mechanism of ELL serving as a transcription factor.

Evidence strongly suggested that ELL played an important role in carcinogenesis and progression through several different means. As a translocation partner for MLL in acute myeloid leukemia, ELL contributed to leukemogenesis through this association. Modulations of p53, U19/Eaf2, and steroid receptor activity as well as promotion of transcriptional elongation represented likely ways that ELL could contribute to cancer. At the same time, our finding that ELL, but not MLL-ELL, induced TSP-1 expression suggested another way that ELL might play a role in cancer.
ELL Acts as Transcription Factor for Direct TSP-1 Regulation

TSP-1 is an extracellular matrix glycoprotein that inhibits tumor growth and metastases (38). Targeted overexpression of TSP-1 in mice suppressed wound healing and tumorogenesis (40, 41), whereas the lack of functional TSP-1 resulted in increased vascularization of selected tissues (42). In addition, several oncogenes repress TSP-1 expression, including oncogenic ras, c-src, v-src, and c-jun, thus providing a potential mechanism for activation of the angiogenic switches in tumors (43). On the other hand, tumor suppressor proteins, such as p53 and PTEN, maintain high anti-angiogenic levels of TSP-1, such that the loss of functional wild-type p53 results in an angiogenic switch, in vitro, because of the transcriptional inactivation of TSP-1 expression (39, 44–46). Here we present data showing that p53 has no effect on TSP-1 promoter activity. After careful reevaluation of the first paper to show TSP-1 regulation by p53 (39), we found that they had used the classic chloramphenicol acetyltransferase assay to measure promoter activity. However, they lacked the appropriate internal control for transfection efficiency. We have found that co-transfection with p53 expression vector dramatically enhanced transfection efficiency and thus could skew the results (data not shown). As such, the lack of normalization might account for the discrepancy between our results and those in the literature (39). Indeed, subsequent reports did not support a relationship between TSP-1 expression levels and with p53 status (47, 48).

Although the function of TSP-1 is still debatable, its role in anti-angiogenesis is well defined so far (38, 40, 43). Using the zebrafish model, the biological effects of ELL on vasculogenesis were evaluated in this study. We found that ELL could inhibit zebrafish vasculogenesis, which appeared to be consistent with its function in up-regulating TSP-1. These observations might suggest that ELL influences vasculogenesis, at least in part, through up-regulating TSP-1. However, whether the inhibition of ELL on vasculogenesis is directly mediated by TSP-1 needs to be further defined by knockdown/overexpression assays. In sum, our findings highlight that ELL can directly serve as a transcriptional factor to regulate transcription of TSP-1 on several levels, warranting further investigation of this protein.

Acknowledgments—We thank Drs. Ali Shilatifard, Randolph S. Waternick, Olga Volpert, Jianfang Gui for the generous gift of various reagents. We also thank Moira Hitchens for editing.

REFERENCES

1. Thrisman, M. J., Levitan, D. A., Kobayashi, H., Simon, M. C., and Rowley, J. D. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 12110–12114
2. Yu, B. D., Hanson, R. D., Hess, J. L., Hornung, S. E., and Korsmeyer, S. J. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 10632–10636
3. Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002) Mol. Cell. 10, 1107–1117
4. Dou, Y., and Hess, J. L. (2008) Int. J. Hematol. 87, 10–18
5. Arigopoulous, B., and Humphries, R. K. (2007) Oncogene 26, 6766–6776
6. Armstrong, S. A., Staunton, J. E., Silverman, L. B., Pieters, R., den Boer, M. L., Minden, D. M., Sallan, S. E., Lander, E. S., Golub, T. R., and Korsmeyer, S. J. (2002) Nat. Genet. 30, 41–47
7. Aytton, P. M., and Cleary, M. L. (2003) Genes Dev. 17, 2298–2307
8. Eguchi, M., Eguchi-Ishimae, M., and Greaves, M. (2005) Int. J. Hematol. 82, 9–20
9. Ernst, P., Mabon, M. D., Davidson, A. J., Zon, L. I., and Korsmeyer, S. J. (2004) Curr. Biol. 14, 2063–2069
10. Hess, J. L. (2004) Crit. Rev. Eukaryot. Gene Expr. 14, 235–254
11. Kong, C. T., Sham, M. H., So, C. W., Cheah, K. S., Chen, S. J., and Chan, L. C. (2006) Leukemia 20, 1829–1839
12. Krivtsov, A. V., and Armstrong, S. A. (2007) Nat. Rev. Cancer 7, 823–833
13. Liu, H., Takeda, S., Cheng, E. H., and Hsieh, J. C. (2008) Cell Cycle 7, 428–435
14. Somervaille, T. C., and Cleary, M. L. (2006) Cancer Cell 10, 257–268
15. Shilatifard, A., Lane, W. S., Jackson, K. W., Conaway, R. C., and Conaway, J. W. (1996) Science 271, 1873–1876
16. Shilatifard, A., Haque, D., Conaway, R. C., and Conaway, J. W. (1997) J. Biol. Chem. 272, 22355–22363
17. Shilatifard, A., Duan, D. R., Haque, D., Florence, C., Schubach, W. H., Conaway, J. W., and Conaway, R. C. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 3639–3643
18. Miller, T., Williams, K., Johnstone, R. W., and Shilatifard, A. (2000) J. Biol. Chem. 275, 32052–32056
19. Mitani, K., Yamagata, T., Iida, C., Oda, H., Maki, K., Ichikawa, M., Asai, T., Honda, H., Kurokawa, M., and Hirai, H. (2001) Biochem. Biophys. Res. Commun. 279, 563–567
20. Johnstone, R. W., Gerber, M., Landewe, T., Tollefson, A., Wold, W. S., and Shilatifard, A. (2001) Mol. Cell. Biol. 21, 1672–1681
21. Pascual-Le Tallon, L., Simone, F., Viengchareun, S., Meduri, G., Thirman, M. J., and Lombre, M. (2005) Mol. Endocrinol. 19, 1158–1169
22. DiMartino, J. F., Miller, T., Aytton, P. M., Landewe, T., Hess, J. L., Cleary, M. L., and Shilatifard, A. (2000) Blood 96, 3887–3893
23. Luo, R. T., Lavau, C., Du, C., Simone, F., Polak, P. E., Kawamata, S., and Thirman, M. J. (2001) Mol. Cell. Biol. 21, 5678–5687
24. Lavau, C., Luo, R. T., Du, C., and Thirman, M. J. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 10984–10989
25. Maki, K., Mitani, K., Yamagata, T., Kurokawa, M., Kanda, Y., Yazaki, Y., and Hirai, H. (1999) Blood 93, 3216–3224
26. Wiederschain, D., Kawai, H., Gu, J., Shilatifard, A., and Yuan, Z. M. (2003) Mol. Cell. Biol. 23, 4230–4246
27. Wiederschain, D., Kawai, H., Shilatifard, A., and Yuan, Z. M. (2005) J. Biol. Chem. 280, 24315–24321
28. Shimobu, N., Maeda, T., Aso, T., Ito, T., Kondo, T., Kiike, K., and...
ELL Acts as Transcription Factor for Direct TSP-1 Regulation

Hatakeyama, M. (1999) J. Biol. Chem. 274, 17003–17010
29. Simone, F., Polak, P. E., Kaberlein, J. J., Luo, R. T., Levitan, D. A., and Thirman, M. J. (2001) Blood 98, 201–209
30. Simone, F., Luo, R. T., Polak, P. E., Kaberlein, J. J., and Thirman, M. J. (2003) Blood 101, 2355–2362
31. Xiao, W., Zhang, Q., Jiang, F., Pins, M., Kozlowski, J. M., and Wang, Z. (2003) Cancer Res. 63, 4698–4704
32. Xiao, W., Jiang, F., and Wang, Z. (2006) Prostate 66, 1–12
33. Hahn, J., Xiao, W., Jiang, F., Simone, F., Thirman, M. J., and Wang, Z. (2007) Prostate 67, 146–153
34. Jiang, F., Ai, J., Xiao, W., and Wang, Z. (2007) Cancer Lett. 253, 265–272
35. Xiao, W., Zhang, Q., Habermacher, G., Yang, X., Zhang, A. Y., Cai, X., Hahn, J., Liu, J., Pins, M., Doglio, L., Dhir, R., Gingrich, J., and Wang, Z. (2008) Oncogene 27, 1536–1544
36. Yang, Z., Liu, N., and Lin, S. (2001) Dev. Biol. 231, 138–148
37. Zhong, H., Wu, X., Huang, H., Fan, Q., Zhu, Z., and Lin, S. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 16800–16805
38. Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 6624–6628
39. Dameron, K. M., Volpert, O. V., Tainsky, M. A., and Bouck, N. (1994) Science 265, 1582–1584
40. Rodriguez-Manzaneque, J. C., Lane, T. F., Ortega, M. A., Hynes, R. O., Lawler, J., and Iruela-Arispe, M. L. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 12485–12490
41. Lawler, J., Miao, W. M., Duquette, M., Bouck, N., Bronson, R. T., and Hynes, R. O. (2001) Am. J. Pathol. 159, 1949–1956
42. Agah, A., Kyriakides, T. R., Lawler, J., and Bornstein, P. (2002) Am. J. Pathol. 161, 831–839
43. Watnick, R. S., Cheng, Y. N., Rangarajan, A., Ince, T. A., and Weinberg, R. A. (2003) Cancer Cell 3, 219–231
44. Wen, S., Stolarov, J., Myers, M. P., Su, J. D., Wigler, M. H., Tonks, N. K., and Durden, D. L. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 4622–4627
45. Sheibani, N., and Fraizer, W. A. (1996) Cancer Lett. 107, 45–52
46. Kranenburg, O., Gebbink, M. F., and Voest, E. E. (2004) Biochim. Biophys. Acta 1654, 23–37
47. Reiber, F. K., Ivanovich, M., Huang, H., Smith, N. D., Bouck, N. P., and Campbell, S. C. (2001) J. Urol. 165, 2075–2081
48. Cinatl, J., Kotchetkov, R., Scholz, M., Cinatl, J., Vogel, J. U., Driever, P. H., and Doerr, H. W. (1999) Am. J. Pathol. 155, 285–292