Distinct modes of gene regulation by a cell-specific transcriptional activator

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The architectural layout of a eukaryotic RNA polymerase II core promoter plays a role in general transcriptional activation. However, its role in tissue-specific expression is not known. For example, differing modes of its recognition by general transcription machinery can provide an additional layer of control within which a single tissue-restricted transcription factor may operate. Erythroid Kruppel-like factor (EKLF) is a hematopoietic-specific transcription factor that is critical for the activation of subset of erythroid genes. We find that EKLF interacts with TATA binding protein-associated factor 9 (TAF9), which leads to important consequences for expression of adult β-globin. First, TAF9 functionally supports EKLF activity by enhancing its ability to activate the β-globin gene. Second, TAF9 interacts with a conserved β-globin downstream promoter element, and ablation of this interaction by β-thalassemia-causing mutations decreases its promoter activity and disables superactivation. Third, depletion of EKLF prevents recruitment of TAF9 to the β-globin promoter, whereas depletion of TAF9 drastically impairs β-promoter activity. However, a TAF9-independent mode of EKLF transcriptional activation is exhibited by the α-hemoglobin-stabilizing protein (*AHSP*) gene, which does not contain a discernable downstream promoter element. In this case, TAF9 does not enhance EKLF activity and depletion of TAF9 has no effect on *AHSP* promoter activation. These studies demonstrate that EKLF directs different modes of tissue-specific transcriptional activation depending on the architecture of its target core promoter.

The recruitment of transcription factor IID (TFIID) to the promoter is the first step in the assembly of a preinitiation complex. At promoters that contain a TATA binding protein (TBP) binding site, the mode of recruitment is via TBP binding to the TATA element. However, many promoters lack a consensus TATA element, and under these circumstances TATA binding protein-associated factors (TAFs) may be more explicitly involved in promoter selective recruitment of TFIID (1, 2). Although essential, each TAF is not universally required at all promoters (3–5), raising the possibility that certain TAFs may be discerning about which promoters they are associated with. Of particular relevance are promoters that contain important sequence elements that lie downstream of the transcription initiation site (1, 2, 6). Mammalian TAF1, TAF9, TAF6, TAF4b, and TAF12 all contact DNA (7). But more specifically, TAF9 is thought to play a role in transcriptional initiation at promoters that contain an imprecisely characterized sequence called the downstream promoter element (DPE), located at around +30 (defining transcription initiation as +1) (1). The DPE consensus sequence (from least to most stringently defined) is A/G/T-C/G-A/T-C/T-A/C/G-C/T, A/G-G-A/T-C/T-G/A/C, A/G-G-A/T-C/T-G-T, or A/G-G-A/T-CGTG (8, 9), and it is usually found in TATA-less promoters but with the initiator element (INI) that surrounds an A at +1, whose consensus is Py-Py-A(+1)-N-T/A-Py-Py (6). Allthough DPE elements are found more widely than the TATA box (10, 11), they have been characterized only within a very limited number of native mammalian promoters (2, 8, 12).

The basal promoter of human adult β-globin gene is composed of a noncanonical TATA box (CATAAA) located 25–30 bp upstream of the transcription start site (13). Deviation from the consensus TATA box often weakens the promoter and leads to the requirement of additional elements for the stabilization of transcription complexes. In this context, one element that contributes to high-level β-globin gene transcription is an INI located at the transcription start site (14). In addition to the INI, Lewis et al. (15) have demonstrated the presence of a promoter element that extends downstream from +10 to +40 and functions in the context of the β-globin TATA box in a heterologous TATA-less context. Notably, downstream β-thalassemia mutations lie at positions +1, +22, and +33 (16, 17).

Erythroid Kruppel-like factor (EKLF) is a zinc finger transcription factor that activates adult β-globin promoter by means of its high-affinity binding to the CACCC element located at −90 (18). Its preferential binding to adult β-globin CACCC element rather than those at the embryonic and fetal globin genes raised the possibility that EKLF is involved in the developmental switch from embryonic/fetal to adult globin expression (19), a suggestion that was verified by the embryonic lethality seen after its genetic ablation (20, 21). Recent studies show that EKLF also plays critical roles in transcriptional activation of a variety of erythroid genes (22, 23).

Molecular studies have shown that EKLF integration of post-translational modifications and specific protein interactions with coactivators and chromatin remodelers are critical for optimal activity (24–28). However, how these interactions impinge on the basic transcriptional machinery and lead to specific downstream effects directly at the promoter are not known.

Here, we report a study investigating the recruitment mechanism of TFIID complex to the β-globin locus during erythroid differentiation. We show that the TAF9 component of TFIID associates with EKLF and is recruited to the adult β-globin locus in an EKLF-dependent manner. We also demonstrate that TAF9 is required for optimal β-globin expression and show that it binds directly to the downstream element of the β-globin promoter, providing evidence for TAF9 binding to and involvement in tissue-specific mammalian promoter activation. Compared with α-hemoglobin-stabilizing protein (AHSP), a gene also regulated by EKLF but harboring a different promoter architecture, we unexpectedly find that despite their common erythroid-specific production, AHSP is not activated by an equivalent mechanism to that of β-globin.

Results

Specific Interaction Between EKLF and TAF9. In vitro pull-down assays were used to determine whether EKLF interacts with any of the TAFs that contain a histone fold domain (HFD). These TAFs (TAF4, TAF12, TAF6, and TAF9) have been implicated in interactions with other activators and in being recruited to promoters

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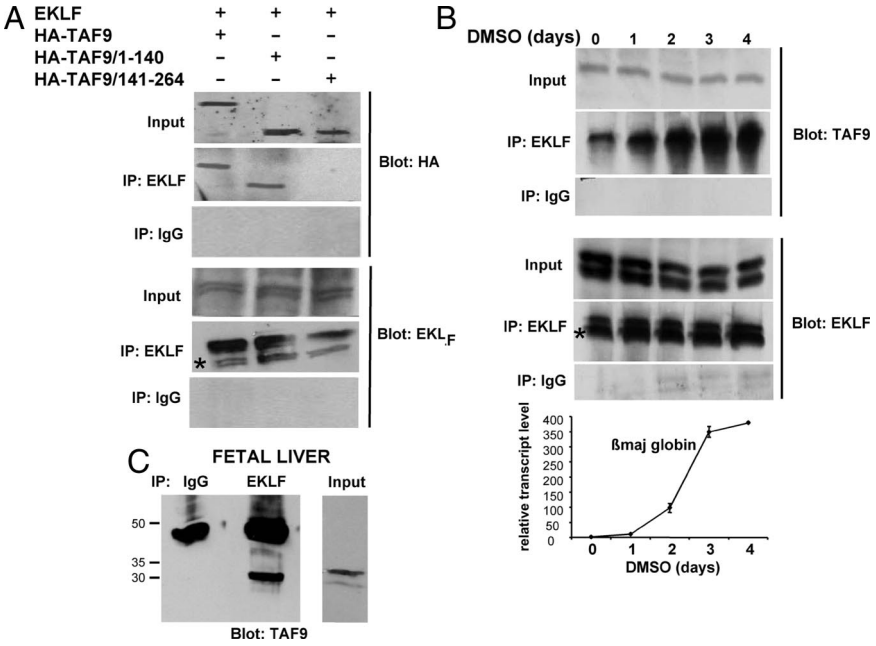

Fig. 1. EKLF interacts with TAF9 in vivo. (*A*) Full-length or various deletion constructs (as indicated) of HA-TAF9 were cotransfected with full-length EKLF into 293T cells. Whole cell lysates were prepared and immunoprecipitation was performed with anti-EKLF mAb (7B2) or control isotype IgG as indicated on the left. The immunoprecipitates were subjected to immunoblot analysis with anti-HA antibody to detect TAF9 and reprobed with anti-EKLF antibody to confirm successful immunoprecipitation (indicated on the right). Input represents 5% of the whole cell lysate used for immunoprecipitation. The asterisk denotes the specific EKLF band. (*B*) MEL cells were grown in the presence of 1.5% DMSO for varying lengths of time (days) to induce hemoglobin expression as confirmed by real-time RT-PCR of adult β-globin RNA. Extracts were immunoprecipitated (IP) with 7B2 EKLF antibody or control IgG and analyzed by Western blotting using anti-TAF9 or anti-EKLF antibody. Input represents 10% of the lysates used for immunoprecipitation. The asterisk denotes the specific EKLF band. (*C*) Extracts from E13.5 murine fetal livers were immunoprecipitated with control IgG or anti-EKLF mAb (7B2) as indicated and probed by Western blot analysis using anti-TAF9. Input represents 5% of extract used for immunoprecipitation; molecular mass markers (in kDa) are on the left.

(29–33). We find that full-length EKLF only interacts with TAF9 (Fig. S1*A*). This finding is of interest because TAF9 interacts with a number of transcription activators including p53, VP16, and NF-κB (34).

EKLF is a modular protein containing a proline-rich transactivation domain and 3 C2H2 zinc finger DNA-binding motifs (18). To identify a minimal binding site between EKLF and TAF9, proteins containing either full-length EKLF, its proline domain (amino acids 20–291), or zinc-finger domain (amino acids 287–376) were expressed in bacteria, and their in vitro interaction with full-length TAF9 was examined. TAF9 interacts with full-length EKLF or its zinc finger domain, but not with its proline domain (Fig. S1*B*).

To determine which domain of TAF9 was involved in interaction with EKLF, either full-length TAF9 (7) (amino acids 1–264), the conserved region (CR) domain alone (amino acids 75–147), or a domain lacking the HFD (amino acids 75–264) were expressed and analyzed for in vitro binding to the full-length or zinc finger domain of EKLF. We find that both TAF9 fragments (amino acids 75–147 and 75–264) containing the CR domain but lacking the histone fold motif interact with full-length EKLF (Fig. S1*C*) or with the zinc finger alone (Fig. S1*D*). Together, these results show that the interaction between EKLF and TAF9 is mediated via the zinc finger domain of EKLF and the CR domain of TAF9.

EKLF and TAF9 Interact in Vivo. To confirm the results from in vitro pull-down assays, full-length EKLF was transfected either alone or in combination with full-length HA-tagged TAF9, TAF9/1–140 or TAF9/141–264 in 293T cells. Whole-cell extracts were immunoprecipitated with anti-EKLF mAb or mouse control IgG, and the precipitates were analyzed by immunoblotting with anti-HA antibody. EKLF interacts either with full-length or the N-terminal fragment of TAF9 but not with the C-terminal fragment (Fig. 1*A*). These data corroborate our in vitro results and show that the in vivo interaction between EKLF and TAF9 is mediated via the CR domain of TAF9.

We next examined in 2 ways whether endogenous EKLF interacts with TAF9. First, we used the murine erythroleukemia (MEL) cell line. Terminal differentiation of MEL cell in culture was achieved by treating with DMSO and confirmed by quantitative RT-PCR using β-globin as a marker for erythroid differentiation (Fig. 1*B*). Total protein extract from MEL cells was immunoprecipitated with EKLF mAb, then analyzed by immunoblotting with TAF9 antibody. We find that, although there is a significant level of endogenous TAF9 interaction with EKLF in the uninduced cell, this level increases dramatically over the course of MEL cell differentiation (Fig. 1*F*). Second, we used embryonic day (E) 13.5 fetal livers and performed endogenous coimmunoprecipitations with EKLF mAb or control IgG. These results show that EKLF also interacts with TAF9 in primary erythroid cells (Fig. 1*C*).

Enhancement of EKLF-Mediated Transactivation by TAF9 at the β-Globin Promoter. To elucidate the functional consequence of the interaction between EKLF and TAF9, we analyzed EKLF transcriptional activity by cotransfection with a human β-globin gene promoter/luciferase reporter into K562 erythroleukemic cells. As expected, EKLF activates the reporter (Fig. 2*A*). Although transfection of TAF9 alone had no effect, it superactivated EKLF in a dose-dependent manner (Fig. 2*A*). TAF9 deletion mutants were also tested in similar assay. In contrast to the WT protein, both N-terminal and C-terminal mutants did not superactivate, but rather inhibited EKLF-mediated transcriptional activity (Fig. S2). In this case, it is possible that the expression of the TAF mutants disrupts formation of the proper transcription complex and thus generally inhibits optimal transcription activity [similar to their effect on p53 transcriptional activation (29)].

We next tested whether DNA binding by EKLF, along with its zinc finger structure, was required for the TAF9 effect by using 2 deletion variants of EKLF in the β-globin transactivation assay (35). The Δ60-310 variant, which lacks most of the transactivation domain and part of zinc finger 1, is not affected by inclusion of TAF9 (Fig. 2*B*). However, the Δ60-195 variant, which retains all 3 zinc fingers and is attenuated in its activity caused by the partial deletion within the proline domain, nonetheless is superactivated by TAF9 (Fig. 2*B*), providing results consistent with the TAF9/EKLF interaction that was mapped in Fig. 1.

TAF9 Directly Binds to the β-Globin Promoter. The nucleosome-like interaction of TFIID with DNA and the presence of histone-like folds in components of TFIID have led to the speculation that TFIID may be involved in direct interaction with DNA. In support of this idea, some of the TAFs within TFIID contact DNA (7), which is of potential relevance to β-globin regulation for a number of reasons. One is that the adult β-globin promoter contains conserved sequences that fit both the INI and DPE consensus (Fig.

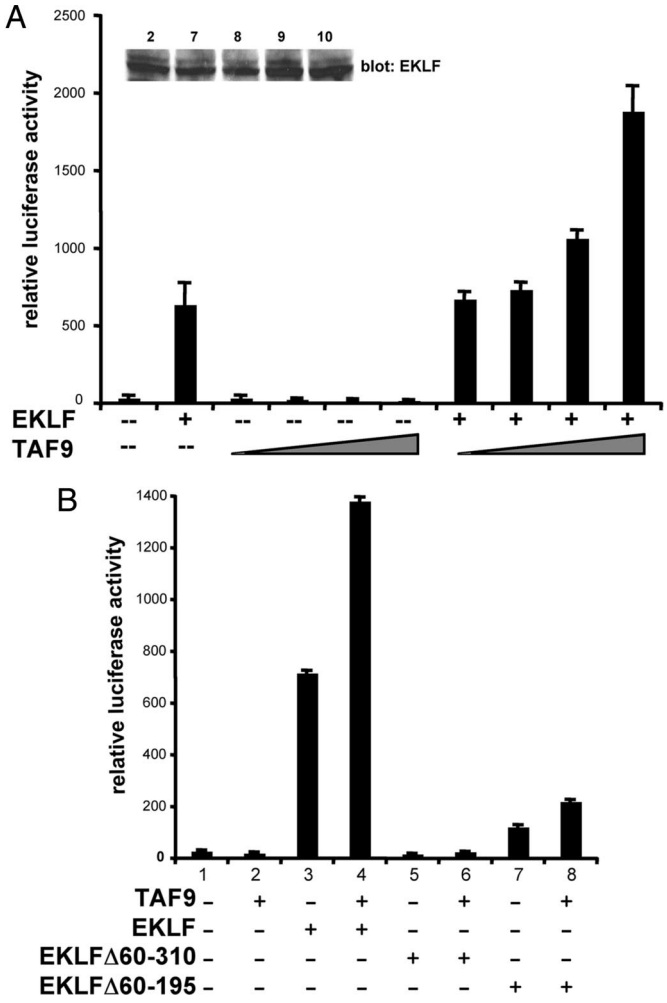

Fig. 2. TAF9 superactivates EKLF-mediated transcriptional activity of the adult β-globin promoter. (*A*) K562 cells were transfected with a luciferase reporter containing the adult β-globin promoter together with expression plasmids for EKLF and TAF9; TAF9 levels were titrated up as indicated. (*Inset*) An anti-EKLF blot from the indicated samples to demonstrate its constant expression level. (*B*) K562 cells were transfected with the β-globin/luciferase reporter along with expression plasmids for TAF9 and where indicated with full-length and various deletion constructs of EKLF. In all cases, luciferase activity was normalized against Renilla activity from a cotransfected control vector. The relative luciferase activity reflects the values obtained in triplicates.

3*A*). Second is that both are sites of known β-thalassemia point mutations (16, 17, 36). Third is that TAF9, in combination with TAF6, is thought to interact with promoters that contain these elements (7, 8). As a result, we used gel-shift assays to directly test whether TAF9 can interact with any of these regions. We tested binding of bacterially expressed (Fig. 3*B*) or affinity-purified (Fig. S3*A*), GST-TAF9 to oligos spanning 3 regions of the mouse β-promoter: INI, DPE (+20), and a conserved E-box site (+60) (13). TAF9 bound only to the DPE (+20) element (Fig. 3*B* and Fig. S3*A*). We also found that TAF9 will not bind to either of 2 thalassemia mutants located within or near the site (mutation at +22 or +33) (Fig. 3*B*). Interaction with this element is specific, because it is blocked by itself, but not by either of the point mutant variants (Fig. 3*B*). Next, we used MEL extracts to confirm the binding of a cellular activity to the DPE element that exhibited the same specificity as that seen with purified TAF9 (Fig. 3*C*). We conclude that TAF9 can interact with the β-globin promoter in a region centered at the postulated DPE element.

A prediction from these data is that the activity of the promoter should be adversely affected if it contains a point mutant at +22 and +33, and that the residual activity should not be superactivated by additional TAF9. We tested this with the β-globin cotransfection assay and found each of these predictions to be true (Fig. 3 *D* and *E*). These results indicate the DPE element is an important component in the design of an optimal β-globin promoter. These results further substantiate the previous studies by Lewis et al. (15) that mutations +22/24 and +31/33 severely affected rTFIIA/TFIID binding relative to the probe. Our results provide further insight by showing that TAF9 protein of the TFIID complex is responsible for the DNA protein interactions at this region.

A TAF9-Independent Transcriptional Activation Mechanism for EKLF. The *AHSP* promoter has recently been characterized as another highly EKLF-dependent transcriptional target from array data and direct analysis (22, 37). Inspection of the promoter sequence shows that it contains neither an INI nor a DPE, raising the question of whether TAF9 plays a role in its transcriptional activation. We checked by gel-shift assays if TAF9 binds to AHSP promoter region by using bacterially expressed and affinity-purified GST-TAF9 along with 2 regions of *AHSP* promoter (+1–+20 and +21–+40) and found that TAF9 does not bind to either of these AHSP sequences (Fig. S3*B*). Next, to test whether TAF9 nonetheless plays any role in AHSP activation, we used *AHSP* promoter coupled to the luciferase reporter and tested its activity in vivo with the K562 cell transactivation assay. We found that, although stimulated by EKLF, it is not superactivated by TAF9 (Fig. 4). These data suggest that the *AHSP* promoter, although dependent on EKLF for activity, does not require TAF9 as the β-globin promoter does.

TAF9 Recruitment to β-Globin Promoter Is EKLF Dependent. Our studies suggest that EKLF and TAF9 form critical interactions with specific regions of the β-globin promoter. This finding is not unexpected, because promoter regions and other regulatory sequences contain binding sites for numerous protein factors. However, these interactions can be interdependent and contingent on active recruitment by one of the component proteins, particularly if tissue specificity is involved. To address this issue within the EKLF/TAF9/β-globin system, we first studied TAF9 occupancy at the β-globin promoter by ChIP of MEL cells. As shown in Fig. 5*A*, TAF9 is present and its occupancy increases after differentiation at the adult β-globin promoter (encompassing the TAF9 binding region detected in Fig. 3) but not at a region located 3 kB upstream. This corresponds to a time when acetylated histone H3 occupancy increases generally across the locus (Fig. S4) after induction of differentiation.

We next addressed whether the presence of TAF9 on the β-major promoter is EKLF dependent by using RNA interference to knock down EKLF levels. A stable MEL cell clone expressing a doxycycline (Dox)-inducible anti-EKLF short hairpin RNA (shEKLF) was used (38), and initially the knockdown of EKLF protein (Fig. 5*B*) and message (Fig. 5*C*) were monitored after treatment with Dox for 2 days. As a further test, after the initial 2-day exposure to Dox, cells were treated with DMSO to induce terminal differentiation and expression of β-major. Four days later, real-time RT-PCR was performed to monitor the effects of Dox induction (EKLF knockdown) on EKLF and β-major transcripts. Although the EKLF knockdown has no effect on cell viability or other erythroid genes that are not targeted by EKLF such as GATA1 (38), an expected decrease is observed in the production of EKLF and β-major transcripts (Fig. 5*C*) after erythroid differentiation only if these cells have been treated with Dox. At the same time, the Dox treatment itself has no effect on any of these parameters in the parental (control) cell line (Fig. 5 *B* and *C*) and in benzidine staining for cellular hemoglobin (Fig. S5). Using these cells we find that TAF9 occupancy at the β-globin promoter, which normally increases during differentiation in the parental cell line irrespective of Dox, is prevented from doing so by the knockdown of EKLF (Fig. 5*D*).

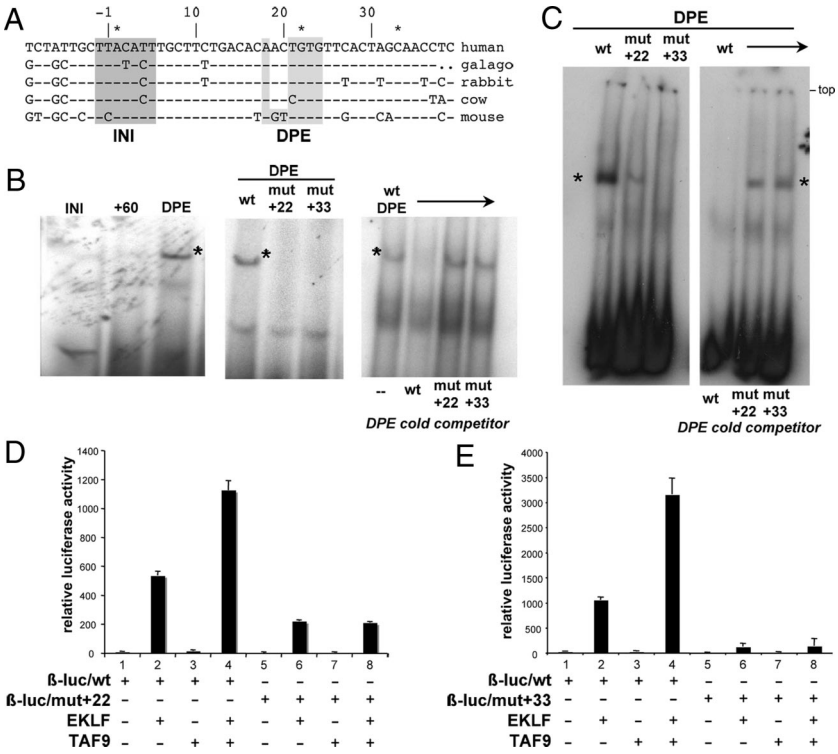

Fig. 3. Interaction of TAF9 with β-globin promoter. (*A*) Alignment of β-globin gene sequences from different species. Conserved INI and DPE elements are boxed. Asterisks indicate the position of known β-thalassemic mutations (at +1, +22, and +33). (*B*) (*Left*) EMSA was carried out by using radiolabeled DNA oligonucleotides encompassing the initiator (INI), the DPE box (+20), or a downstream E-box (+60) after incubation with bacterial extracts containing overexpressed TAF9 protein. (*Center*) EMSA with TAF9 was carried out with a radiolabeled oligonucleotide encompassing the WT DPE box (+20) or the same oligonucleotide with a mutation at +22 or +33 as indicated. (*Right*) EMSA with TAF9 was carried out by using a radiolabeled fragment encompassing the WT DPE box (+20) after preincubation of the TAF9 extract with nonradioactive (cold) WT (+20) or β-thalassemic oligonucleotide (+22 or + 33) as indicated below. Asterisks indicate the specific TAF9/DNA complexes. (*C*) EMSA was performed with WT or mutant radiolabeled DPE oligonucleotides (as described in *B*) after incubation with an extract from MEL cells. Competition with cold oligonucleotides (shown below) was performed as for *B*. (*D* and *E*) K562 cells were transfected with the β-globin luciferase reporter containing WT or point mutated (+22 in *D*; +33 in *E*) promoters along with expression plasmids for EKLF and TAF9. Luciferase activity was normalized against Renilla activity from a cotransfected control vector. The relative luciferase activity reflects the values obtained in triplicates.

These results, in combination with our interaction assays, suggest that EKLF actively recruits TAF9 to the β-globin promoter.

TAF9 Is Required for β-globin, but Not AHSP, Transcription. To confirm that TAF9 recruitment to the β-globin promoter is essential for β-globin transcription, we transfected MEL cells with TAF9-specific siRNA oligonucleotides. Four different siRNAs directed against different regions of TAF9 were tested. Cells were transfected with 25 nM of each of the TAF9 siRNAs (siRNA 1, 2, 3, or 4) or a scrambled siRNA (control), and 16 h posttransfection cells were induced to differentiate by the addition of DMSO. Induction was carried out for 72 h to be able to analyze the expression of genes that are primarily the effect of the knockdown and not of cell death.

Of the 4 siRNAs tested, siRNA 1 does not show any reduction in TAF9 transcript or protein level (Fig. 6*A*), so it was not used further. Although siRNA 3 causes the most significant reduction in the levels of TAF9 transcript and protein as compared with a control scrambled siRNA, siRNAs 2 and 4 also show a significant reduction in TAF9 (Fig. 6 *A* and *B*). The decrease in levels of EDEM2, a known TAF9 target (39) used as a control, further supports the conclusion that the TAF9 knockdown by any of the 3 siRNA is successful in achieving a downstream effect (Fig. 6*B*). Using this system we find that TAF9 reduction leads to a significant drop in the levels of β-major transcript, with no effect on EKLF or GATA1 expression (Fig. 6*B*).

We also tested whether TAF9 depletion has any effect on AHSP expression by including it in this experiment. We find that its expression level is not affected by TAF9 depletion in all 3 cases where TAF9 siRNA has an effect on ß-globin levels (Fig. 6*B*).

In total, these data demonstrate that TAF9 recruitment by EKLF to the β-globin promoter is critical for initiating expression of this gene. However, they also suggest that the AHSP promoter, although dependent on EKLF for activity, does not require TAF9. Based on their significantly different promoter architecture, in the larger context these results enable us to postulate that EKLF/TAF9 interactions are most critical at those promoters that contain INI/DPE elements and suggest that EKLF has 2 distinct ways in which it can optimally transactivate its target promoter, as exemplified by the β-globin and AHSP genes.

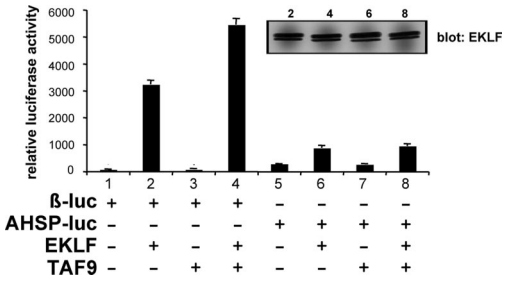

Fig. 4. TAF9 does not superactivate EKLF activity on the AHSP promoter. K562 cells were transfected with either a β-globin or an AHSP luciferase reporter along with EKLF and TAF9 as indicated. (*Inset*) An anti-EKLF blot from the indicated samples demonstrates its constant expression level. Luciferase activity was normalized against Renilla activity from a cotransfected control vector. The relative luciferase activity reflects the values obtained in triplicates.

Discussion

EKLF plays critical roles in the transcriptional activation of a variety of erythroid genes. How EKLF integrates its posttranslational modifications and coactivator interactions with the basic transcriptional machinery is not known. Our studies demonstrate that EKLF interacts with the TAF9 protein of the TFIID complex, suggesting one mechanism by which this can be accomplished. Most intriguing is that the data suggest this interaction is not required at all target promoters, but rather depends on the particular promoter architecture that resides at the target location. In the absence of EKLF, recruitment of CBP, BRG1, TBP, and polymerase (Pol) II to the β-globin promoter are negatively affected (28). In the present study we report a unique interaction of EKLF with a TAF, TAF9, thus providing direct evidence that a tissue-specific, DNA-bound transcriptional activator is required for initial targeting of the TFIID activation complex to the β-globin locus. Furthermore, we show

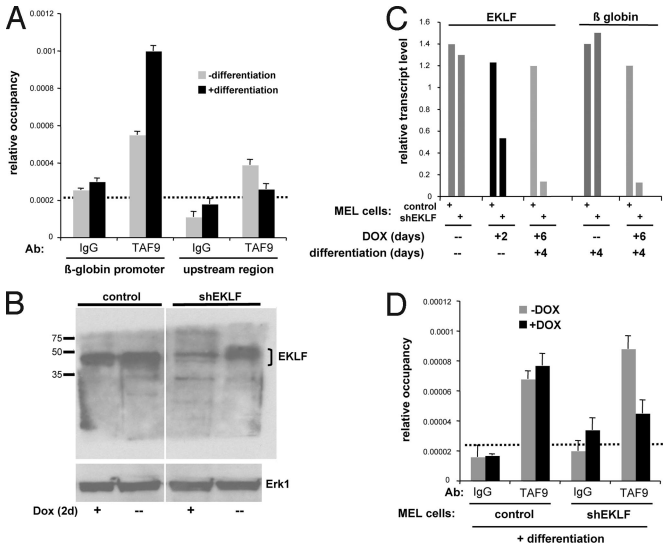

Fig. 5. Binding of TAF9 to the endogenous β-globin promoter is mediated by EKLF. (*A*) Quantitative ChIP analysis of TAF9 occupancy on β-major globin promoter or a region 3 kb upstream was performed with anti-TAF9 antibody or control IgG in MEL cells without differentiation (day 0) or after DMSO-induced differentiation (day 4) as indicated. Standard error reflects values from triplicate experiments. (*B*) Western blot analysis of Dox-inducible knockdown of EKLF in a derivative of MEL cells. shEKLF represents an independent clone of MEL cells that expresses a hairpin against EKLF (38). Control (parental) MEL cells express only the TetR. Extracts were prepared from the indicated MEL cell lines before or after treatment with Dox for 2 days, and extracts were probed with anti-EKLF or anti–Erk1 (control) antibodies. (*C*) Total RNA was prepared from control (parental) or shEKLF MEL cell lines (as indicated) grown in the absence of Dox (−) or differentiation (−); after Dox treatment for 2 days (+2) as in *B*; after Dox treatment for 2 days followed by differentiation for 4 days with 1.5% DMSO (+6); or after differentiation alone (+4). Real-time RT-PCR was performed by using primers for EKLF and β-major globin. The average of duplicates from a single experiment that is representative of 2 is shown. (*D*) TAF9 occupancy at the β-major promoter was measured by quantitative ChIP using TAF9 or IgG antibodies on untreated (−) or Dox-treated (+) control (parental) or shEKLF MEL cells that were also differentiated for 4 days with DMSO. An average of 2 independent experiments each performed in triplicate are shown, with error bars representing standard deviation.

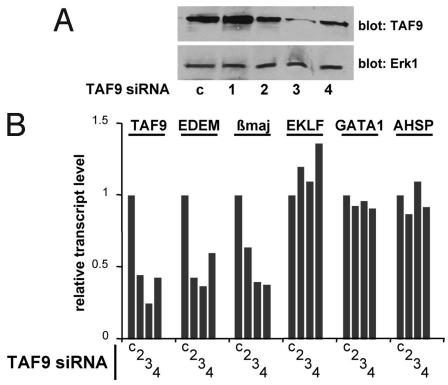

Fig. 6. TAF9 is required for β-major globin, but not AHSP, promoter activity in MEL cells. MEL cells were transfected with TAF9 specific siRNAs (lanes 1–4) or scrambled siRNA (c, control). Cells were differentiated with 1.5% DMSO starting from 18 h posttransfection. Total RNA or cell extracts were prepared from cells collected after 3 days of differentiation. TAF9 and Erk1 protein levels were monitored by Western blot (*A*), and RNA was analyzed by quantitative RT-PCR for TAF9, EDEM, β-major globin, EKLF, GATA1, or AHSP expression using the indicated detection primers (*B*). The average of duplicates from a single experiment that is representative of 2 is shown.

that the zinc finger domain of EKLF is the site for direct interaction with TAF9. This domain is acetylated at lysines 288 and 302 by CBP. Acetylation of EKLF at lysine 288 is essential for its optimal function as activator (26, 40), a result that may be partly explained by speculating that acetylated lysine 288 yields a more efficient interaction surface between EKLF and TAF9. This line of thought is supported by a recent finding that p53 is diacetylated at lysines 373 and 382 in response to DNA damage, which in turn recruits TAF1 to p53 binding sites on the p21 promoter (41).

Role of TAFs in Transcriptional Regulation. Similar activator-mediated recruitments of TFIID to promoters have been documented in yeast, suggesting that direct activator–TAF interactions play important roles in response to growth stimulation (42). It was concluded from a genomewide expression profile study that 84% of yeast Pol II genes are TAF dependent (43). In *Cenorhabditis elegans*, individual TAF knockdowns by RNA interference affect embryo development and gene expression to different degrees (44, 45). In *Drosophila*, TAF1 regulates several differentiation processes (46), and dTAF4 and dTAF6 affect dorsal-mediated transcription (47) whereas dTAF6 seems to be broadly required for somatic and germ cell development (48). On the one hand, these studies provide compelling evidence supporting the involvement of TAFs in gene transcription; however, they also show that not all genes equally depend on them. Taken together, these previous reports and our current study demonstrate that involvement of a specific TAF in transcription of a particular gene depends largely on the promoter structure of the gene in question.

In *Saccharomyces cerevisiae*, which has only 1 TAF9 gene, TAF9 depletion causes a decrease in 60–65% of the genes (43). Thus, our study showing that TAF9 accentuates EKLF-mediated transcriptional activation of β-globin and that its knockdown reduces β-globin expression places it within a tissue-specific transcriptional activation network and extends these previous findings.

Dynamic Interaction of TAF9 with the β-Globin Promoter. Components of the TFIID family have been shown to interact with core promoter elements. Well documented is the interaction of the TATA box with TBP. Two members of the TFIID complex, TAF1 and TAF4, have been shown to interact with initiator and downstream elements (49, 50). *Drosophila* and mouse TFIID was found to footprint the DPE (15, 51), and mutations in the DPE correlate with reduced TFIID binding. Furthermore, the DPE can be cross-linked to 2 components of the *Drosophila* TFIID complex, dTAF9 and dTAF6 (8, 48). We have shown direct binding of TAF9 to an endogenous mammalian promoter. TAF9 recruitment to the β-globin promoter critically depends on EKLF as there is a marked reduction in the occupancy of TAF9 in its absence. The promoter of the β-globin gene contains a noncanonical TATA box, and deviation from such a consensus often leads to the incorporation of additional elements for the efficient recruitment or stabilization of transcription initiation complexes.

The importance of the DPE element, and its interaction with TAF9, is particularly intriguing because of β-thalassemia point mutations that map to the region. Mutation at +22 and +33 negatively affects TAF9 binding in vitro and leads to a decreased level of promoter activity in vivo that can no longer be superactivated by TAF9. Lewis et al. (15) have also shown that TFIID interacts with this region of the β-globin promoter in vitro and that a number of mutations across the region reduce transcription efficiency.

Array data and direct analysis have shown that the *AHSP* promoter is another EKLF-dependent transcriptional target that is highly expressed in erythroid cells (22, 37, 52). However, its promoter contains neither an INI nor a DPE. We found that although highly stimulated by EKLF, it could not be superactivated by TAF9, much like the +22 or +33 β-thalassemia mutant β-globin promoter. In addition, depletion of TAF9 had no effect on its

expression. These unexpected observations demonstrate that a tissue-specific activator can rely on more than one mechanism to optimally interact with the basal transcription machinery and transactivate its target genes. In the present case, a promoter architecture that, unlike that of β-globin, does not contain INI/DPE elements can still be strongly activated, bypassing TAF9. Even though the EKLF knockdown does not have a general effect on non-EKLF erythroid targets, it remains possible that the decreased association of TAF9 with the adult β-globin promoter results from a general loss of general transcription factor association after EKLF knockdown. However, our observations are in concert with previous reports that although TAF9 is part of a stable core subcomplex of TFIID (49), assembly of the preinitiation complex varies from gene to gene (2).

Our data led us to formulate a hypothesis on the functional importance of proteins that interact at the β-globin promoter and provide a deeper insight into the mode of action of EKLF as an activator. EKLF interacts with TAF9 in adult erythroid cells, and we suggest that this interaction provides a platform for the efficient recruitment of transcription complexes, particularly at promoters that contain INI+DPE+ sequences. Consistent with this idea are reports showing that EKLF−/− cells have reduced TBP and Pol II occupancy at the β-globin promoter (28). However, also coupled with this idea are the observations that AHSP, although still critically dependent on EKLF, is activated by a TAF9-independent mechanism. Additional details of how EKLF selectively generates 2 differing modes of downstream activation, and whether its post-translational modification signature plays any role in this determination, require further investigation.

Materials and Methods

MEL745A, K562, and 293T cells were maintained as described (40). Differentiation of MEL cells was carried out by using 1.5% DMSO for 72–96 h. The shEKLF MEL lines were established and maintained as described (38). Transfection of 293T cells, use of EKLF constructs, preparation of nuclear extracts, immunoprecipitations, and transactivation assays followed published protocols (26, 27, 40, 53). +22 or +33 mutants were derived by using the QuikChange mutagenesis kit (Stratagene). The ChIP analysis followed published procedures (54, 55). Additional details are included in *SI Text*.

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