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Differential involvement of E2A-corepressor interactions in distinct leukemogenic pathways

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ABSTRACT

E2A is a member of the E-protein family of transcription factors. Previous studies have reported context-dependent regulation of E2A-dependent transcription. For example, whereas the E2A portion of the E2A-Pbx1 leukemia fusion protein mediates robust transcriptional activation in t(1;19) acute lymphoblastic leukemia, the transcriptional activity of wild-type E2A is silenced by high levels of corepressors, such as the AML1-ETO fusion protein in t(8;21) acute myeloid leukemia and ETO-2 in hematopoietic cells. Here, we show that, unlike the HEB E-protein, the activation domain 1 (AD1) of E2A has specifically reduced corepressor interaction due to E2A-specific amino acid changes in the p300/CBP and ETO target motif. Replacing E2A-AD1 with HEB-AD1 abolished the ability of E2A-Pbx1 to activate target genes and to induce cell transformation. On the other hand, the weak E2A-AD1-corepressor interaction imposes a critical importance on another ETO-interacting domain, downstream ETO-interacting sequence (DES), for corepressor-mediated repression. Deletion of DES abrogates silencing of E2A activity by AML1-ETO in t(8;21) leukemia cells or by ETO-2 in normal hematopoietic cells. Our results reveal an E2A-specific mechanism important for its context-dependent activation and repression function, and provide the first evidence for the differential involvement of E2A-corepressor interactions in distinct leukemogenic pathways.

INTRODUCTION

E2A belongs to a family of basic helix-loop-helix (bHLH) transcription factors (1–3). These transcription factors can be divided into two classes based on their expression and function. Class I proteins, also called E-proteins, include E2A, HeLa E-box binding protein (HEB) and E2-2. Whereas E-proteins are ubiquitously expressed in different cell types, the expression of class II bHLH transcription factors is tissue-specific. Examples of class II bHLH transcription factors include MyoD and T-cell acute lymphocytic leukemia 1 (TAL1), which are important in myogenesis and hematopoiesis, respectively.

Both E-proteins and class II basic helix-loop-helix (bHLH) transcription factors target the E-box (CANNTG) sites located in the promoter or enhancer region of their target genes. E-proteins bind to DNA either as homodimers or as heterodimers with other E-protein members. In addition, E-proteins can also form heterodimers with and facilitate the DNA binding of class II bHLH transcription factors. By regulating the transcription of target genes, E-proteins and class II transcription factors play important roles in various cell differentiation pathways including lymphopoiesis, erythropoiesis and myogenesis (2,4–12). Underscoring its unique functions, E2A has also been shown to regulate cell cycle progression and apoptosis (13–15), and to function as a tumor suppressor as evidenced by the high frequency of tumor formation of E2A-deficient T-lymphocytes (16–18).

E-proteins activate or repress target gene expression by recruiting coactivators or corepressors in a mutually exclusive fashion. Two conserved activation domains, AD1 and AD2, are present in E-proteins (19–21). These domains cooperatively recruit p300/CBP and GCN5 histone acetyltransferases to facilitate the activation of target genes (8,22–25). The corepressors of E-proteins are eight twenty-one (ETO) family proteins (25,26), which include ETO/MTG8, ETO-2/MTG16 and MTGR1. Interestingly, both ETO and ETO-2 are implicated in leukemogenic chromosomal translocations. In ~15% of acute myeloid leukemias (AML), the t(8;21) chromosomal translocation combines the DNA-binding domain of the acute myeloid leukemia 1 (AML1)/runt-related transcription factor 1 (RUNX1) transcription factor with a nearly

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as Joint First Authors.

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full-length ETO to generate the AML1-ETO fusion protein. Similarly, ETO-2 is fused to AML1 in certain childhood or therapy-related AMLs (27–29).

ETO family corepressors contain four evolutionarily conserved domains termed Nervy homology region (NHR)1-4, all of which are present in the AML1-ETO fusion protein (25,26,30). NHR1 is also called the TAF4-homology (TAFH) domain owing to its similarity to a conserved region in the TATA box-binding protein-associated factor 4 (TAF4) protein, a subunit of the TFIID complex. NHR2 mediates the tetramerization of ETO and AML1-ETO. TAFH and NHR2 are the only ETO domains required for AML1-ETO to induce leukemogenesis in a mouse model (31,32). NHR4 contains two myeloid, Nervy, and DEAF-1 (MYND)-type zinc fingers that mediate interactions with nuclear receptor corepressors, which in turn interact with histone deacetylases to contribute to AML1-ETO- and ETO-mediated repression (26,30,33–35).

The domain interactions of E-proteins with corepressors and coactivators have been previously reported. AD1 can interact with both corepressors and coactivators (25,26,36). A conserved sequence called PCET (p300/CBP and ETO target) in AD1 has been shown to mediate mutually exclusive interactions with ETO family corepressors and p300/CREB-binding protein (CBP) coactivators. The consensus sequence of PCET (GTDKELSDLLDFS) combines LXXLL and LDFS motifs. The LDFS motif has been shown to mediate the interaction with the GCN5 coactivator (22,37,38). The AD1-corepressor interaction explains the previously reported repressive function of AD1 (39–41). Recently, another conserved ETO-interacting region called downstream ETO-interacting sequence (DES) downstream of AD1 has been identified (26). Whereas the AD1 interaction with ETO is mediated by the TAFH domain, the DES interaction with ETO is mediated by the NHR2 domain. Thus, in addition to sharing a common binding site (PCET), corepressors and coactivators also bind to distinct E-protein domains (DES and AD2, respectively).

In t(1;19) chromosomal translocation, the entire activation domain of E2A is fused to the DNA-binding domain of the Pbx1 transcription factor, resulting in the development of acute lymphoblastic leukemia (ALL) (42–47). Although the E2A-Pbx1 fusion protein contains both AD1 and DES, it shows constitutive transcriptional activation function. Similarly, wild-type E2A can act as a potent transcription activator in cell cycle progression, apoptosis and cell differentiation. On the other hand, the transcriptional activity of E2A can be silenced through its interaction with stoichiometric levels of corepressors, such as AML1-ETO in t(8;21) leukemia (25,26) and ETO-2 in hematopoietic progenitor cells (48–51).

In this study, by analyzing domain interactions between E2A and ETO, we unexpectedly found that the AD1 domain of E2A has intrinsically low binding affinity for the ETO family corepressors due to specific changes in three PCET C-terminal amino acids flanking the LXXLLDFS motif. Docking studies show that these E2A-specific amino acids are incompatible with the high-affinity corepressor binding conformation of PCET as existed in HEB-AD1. Replacing E2A-AD1 with HEB-AD1, or converting the three E2A-specific residues to those of HEB-AD1, reduces the ability of E2A-Pbx1 to activate target genes and to induce cell transformation. On the other hand, the weak binding affinity of E2A-AD1 for TAFH renders the DES domain essential for E2A to remain susceptible to repression by high levels of corepressors. Deletion of DES prevents the silencing of E2A activity by AML1-ETO in t(8;21) leukemia cells and confers resistance to ETO-2-mediated repression of target gene transcription. Our work thus shows that the reduced E2A-AD1-corepressor interaction confers E2A the ability to dynamically regulate transcription in response to variable intracellular corepressor levels, and reveals specific E2A determinants that opposingly link corepressor function to distinct leukemogenic pathways.

MATERIALS AND METHODS

Cell culture and luciferase assay

HEK293T and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS. Namalwa, Kasumi-1 and Jurkat cells were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS). For reporter assays, sub-confluent HEK293T cells grown in 24-well plates were transfected with equal total amounts of plasmids using FuGENE 6 transfection reagent (Roche). Luciferase assays were performed 36–48 h later. The amounts of plasmids used for transfection were as follows unless otherwise specified: ETO, VP16-ETO, ETO-K98E and VP16-ETO-K98E, 75 ng; AML1-ETO and its derivatives, 150 ng; Gal4-E2A-AD (amino acids 1–493) and its derivatives, 5 ng; HEB-AD1, 100 ng. The luciferase reporter contains five copies of a Gal4-binding site (Gal4-UAS). Luciferase units (Promega) were normalized to β-gal activity, which served as an internal control for transfection efficiency. Fold activation or repression was relative to the activity of Gal4-DNA-binding domain or other relevant empty vector control unless otherwise indicated. The results represent the average and standard error of duplicate samples in representative experiments.

Plasmids and protein expression

The mammalian expression vectors used for the expression of AML1-ETO, ETO, VP16-ETO, ETO-K98E, VP16-ETO-K98E, HEB-AD1 and E2A-AD1 have been previously described (25,26). The definition of the AD1 domain is based on a previous characterization of this domain (21). The E2A-AD1 (amino acids 1–90) and HEB-AD1 (amino acids 1–99) are derived from the homologous regions (Supplementary Figure S1). Mammalian expression vectors for other E2A derivatives were generated following standard molecular cloning/polymerase chain reaction (PCR) procedures and verified by DNA sequencing. Plasmids used for *in vitro* translation using the TNT® kit (Promega) were derived from a pCMX vector

containing a T7 promoter. The plasmids used for bacterial expression of Glutathione S-transferase (GST) fusion proteins have been described previously (25). Lentiviral expression vectors for HEB, E2A, ETO-2, E2A-Pbx1 and their derivatives were generated following standard molecular cloning/PCR procedures using pCDH Cloning and Expression Lentivectors (System Biosciences), and were verified by DNA sequencing. All shRNA constructs were from Sigma and were based on the pLKO.1 backbone. The control shRNA plasmid (Addgene plasmid 1864) contains a scrambled sequence (CCTAAGGTTAAGTCG CCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG). The human gene-specific shRNA constructs are listed below as sense sequences cloned in pLKO.1 (HEB, TRCN0000274222, CCGGCTTACGCGTGCGGGATA TTAACTCGAGTTAATATCCCGCACGCGTAAGTT TTTG; E2A, TRCN0000017534, CCGGCCCGGATCAC TCAAGCAATAACTCGAGTTATTGCTTGAGTGAT CCGGGTTTTT; ETO, TRCN0000013666, CCGGCCAT CTGTTAAACTGCATAATCTCGAGATTATGCAGT TTAACAGATGGTTTTT).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using SimpleChIP® Enzymatic Chromatin IP Kits (Cell Signaling Technology) following manufacturer's instruction. After reverse cross-linking, the samples were purified using QIAquick PCR Purification Kit (Qiagen). Quantitative PCR (qPCR) was performed using the SYBR® GreenER™ qPCR SuperMix Universal (Life Technologies). Chromatin binding was calculated as the percentage of immunoprecipitated DNA relative to the amount of input. Anti-GCN5 antibody was from Santa Cruz (SC-20698). It was also used for western blot analyses. Anti-E2A and anti-HEB antibodies were from Santa Cruz (SC-349 and SC-357). Anti-FLAG antibody was from Sigma. Anti-ETO antiserum was house made. The sequence of primers used in this study is provided in Supplementary Table S1.

Quantitative reverse transcription-PCR

RNA was extracted using The PureLink® RNA Mini Kit (Life Technologies). One microgram of total RNA was reverse-transcribed into cDNA by using the High Capacity cDNA Reverse Transcription kit (Life Technologies). qPCR was performed by using the SYBR® GreenER™ qPCR SuperMix Universal (Life Technologies). The primers used in this study are shown in Supplementary Table S2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control unless otherwise indicated.

Colony formation assay

NIH3T3 cells were transfected with plasmids encoding E2A-Pbx1, E2A(HEB^{AD1})-Pbx1, E2A(MPL-ASP)-Pbx1, HEB-Pbx1 or empty vector using TurboFect Transfection Reagent (Thermo Scientific). Transfected cells were grown for 24 h followed by subculture and puromycin selection for 2 weeks. The cells were then stained with crystal violet and the number of transformed foci counted.

Docking of HEB and E2A PCET to TAFH

HEB (I11-V31) and E2A (V10-V30) were used to generate homology models from the PCET portion of the PCET-KIX complex (2kwf) (52) using MODELLER (53,54). These models were then docked to the unbound structure of TAFH (2pp4) (55) through the HADDOCK web server configured with default parameters. Based on previous analyses (36,55) and our current mutation results, active residues for 2pp4 included 123,125,129,131,132,140,143, 173,178,180 and184, while passive residues included 119,120,121,124,128,135,136,138,139,141,142,143,176,177, 179,183 and187. Active residues for both HEB and E2A were identified as 7,9,10,11,12,13,15,16,17,18 and 19, while passive residues were marked for all residues that were not active (1–6,8,14,20,21). Thousand structures were generated during the rigid-body docking stage, after which the 200 lowest-intermolecular energy structures were subjected to semiflexible simulated annealing with the automatic setting of semiflexible fragments applied to TAFH, and with the entire chain allowed for PCET. The best structures of the lowest energy cluster were then taken and visualized using UCSF Chimera version 1.7.

GST pull-down, immunoprecipitation and western blot analyses

GST pull-down, immunoprecipitation (IP) and western blot analyses were performed as described previously (25,26). Input lanes show 1 (IP) or 5% (GST pull-down) of total. The relative binding to GST fusion proteins was quantified using Imagequant (Amersham Biosciences) following phosphorimager scanning, and calculated as the ratio of the signal in the pull-down lane to the signal in the input lane unless otherwise indicated. Anti-Gal4-DNA-binding domain (DBD) and anti-HA antibodies were from Santa Cruz. Anti-p300 antibody was from Santa Cruz (SC-584). Anti-FLAG M2-agarose and anti-FLAG antibody were from Sigma-Aldrich. Anti-actin antibody was from Millipore. Anti-tubulin antibody was from Active Motif. A pan-ETO family antibody used to detect ETO-2 has been previously described (25,26). Specific anti-ETO-2 antibody was from Santa Cruz (sc-9741).

RESULTS

E2A-AD1 has a low binding affinity for ETO

Previous studies have shown that E2A harbors two distinct domains (AD1 and DES) that interact with the TAFH and NHR2 domains of ETO, respectively (26). Here we first sought to explore the relative contributions of AD1 and DES to the overall E2A-ETO binding by comparing the abilities of different E2A derivatives to bind ETO (Figure 1A). The complete E2A activation domain (E2A-AD) contains the AD1, DES and AD2 domains that interact with corepressors (ETO family proteins) and coactivators (p300/CBP and GCN5). As

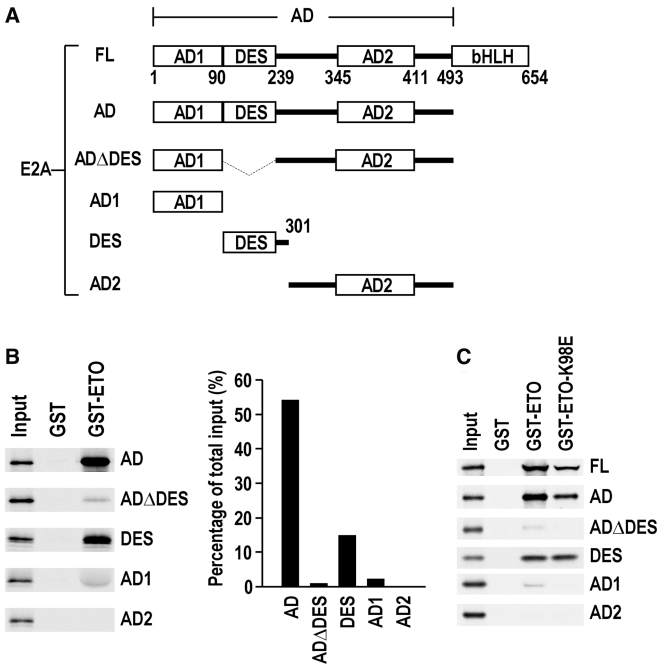

Figure 1. E2A-AD1 is a weak ETO-binding domain and requires DES for strong corepressor binding. (**A**) Schematic representations of E2A and its derivatives used in the experiment, indicating the regions of AD1, DES and AD2. (**B** and **C**) GST pull-down assays using the indicated GST fusion proteins and the *in vitro* translated and ^{35}S labeled E2A derivatives. These derivatives were expressed as fusion proteins with the Gal4-DNA-binding domain. The right panel of (B) shows quantification of the relative binding of each E2A fragment to GST-ETO as the percentage of total input.

expected, GST pull-down assays showed that E2A-AD strongly interacted with ETO (Figure 1B). The interaction was reduced by ~4-fold for the isolated DES domain alone, and by ~24-fold for the isolated AD1 domain alone (Figure 1B, right panel). The latter result revealed that the AD1 domain of E2A has a low binding affinity for ETO. Further supporting a weak E2A-AD1-ETO interaction, deleting DES from the complete E2A activation domain (generating E2A-ADΔDES) reduced the binding to ETO to a similar low level as observed with AD1 (Figure 1B). Despite its weak binding to ETO, AD1 was able to synergize with DES to mediate a strong ETO interaction as observed with E2A-AD or full-length E2A (E2A-FL) (Figure 1B and C).

The weak interaction between E2A-AD1 and ETO is specific, as E2A-AD1 minimally interacted with ETO-K98E (Figure 1C), which carries a mutation in the TAFH domain that abolishes the interaction between AD1 and ETO (25). Consistent with the lack of AD1-ETO-K98E interaction, no synergistic effect of AD1 and DES was observed in the interaction between E2A (either FL or AD) and ETO-K98E (Figure 1C).

Reduced binding of E2A-AD1 to ETO is due to E2A-specific changes in the PCET motif

Given that previous analyses of the HEB-ETO interaction have shown that the conserved PCET motif is a strong binding site for ETO (25,26,56), the finding of the weak binding between E2A-AD1 and ETO prompted us to directly compare ETO interactions with E2A-AD1 and HEB-AD1, which are derived from homologous regions of the corresponding proteins (Supplementary Figure S1A). GST pull-down experiments confirmed that the binding of E2A-AD1 to GST-ETO was indeed greatly reduced compared with that of HEB-AD1 (Figure 2A, left). Similar results were obtained with GST-TAFH (Figure 2A, middle). To extend these studies, full-length E2A and HEB proteins were mixed before the pull-down by GST-TAFH. The results showed that TAFH preferentially bound to HEB (Figure 2A, right). Similar preferential binding of full-length ETO to HEB was also observed *in vivo* by immunoprecipitating HA-ETO and probing for HEB and E2A from cells co-transfected with HA-ETO, FLAG-HEB and FLAG-E2A (Supplementary Figure S2A). To confirm that this binding difference produces a functional impact on transcription, the amounts of ETO and VP16-ETO were titrated in mammalian two-hybrid assays. The results showed that the *in vivo* association between HEB-AD1 and ETO was also much stronger than that between E2A-AD1 and ETO (Figure 2B, right). Consistently, HEB-AD1 was more sensitive to ETO-mediated repression than E2A-AD1 (Figure 2B, left).

Since AD1 also interacts with coactivators such as p300, we compared the binding of E2A-AD1 and HEB-AD1 to the KIX domain of p300, which mediates the binding between p300 and AD1 (24,52). Whereas GST-ETO showed a much weaker interaction with E2A-AD1, the interactions of GST-KIX with E2A-AD1 and HEB-AD1 were comparable (Figure 2C and D), consistent with their similar binding constants for KIX (52). These results demonstrated that E2A-AD1 has specifically reduced interaction with corepressors but not with coactivators.

Alignment of the PCET sequences of E-proteins showed that HEB and E2-2 have identical PCET sequences, whereas E2A has three amino acid changes (A25 to M, and S28P29 to PL) in the C-terminus of PCET flanking the LXXLLDFS motif (Figure 2E, Supplementary Figure S1A). These changes are conserved among E2A proteins from different species (Supplementary Figure S1A). Whereas mutation of A25 to M in HEB only modestly reduced the binding between HEB-AD1 and ETO (Figure 2F), mutating S28P29 to PL or mutating only S28 to P was able to strongly reduce the binding of HEB-AD1 to ETO to a similar low level as that observed with E2A-AD1 (Figure 2F and G). Conversely, the binding of E2A-AD1 to ETO was enhanced by converting E2A-specific residues to the corresponding HEB residues (M24P27L28 to ASP) (Figure 2F and G). These results demonstrated that the E2A-specific PCET residues are responsible for the reduced binding of the E2A-AD1 domain to ETO. The interaction between full-length E2A and ETO was also strongly enhanced by replacing the E2A-AD1 with HEB-AD1, or by mutating amino acids M24P27L28 to ASP (Supplementary Figure 2B). This result is consistent with the cooperativity observed between AD1 and DES in mediating E2A-ETO interaction, and indicates that the reduced corepressor interaction of AD1 has a dominant effect on the overall

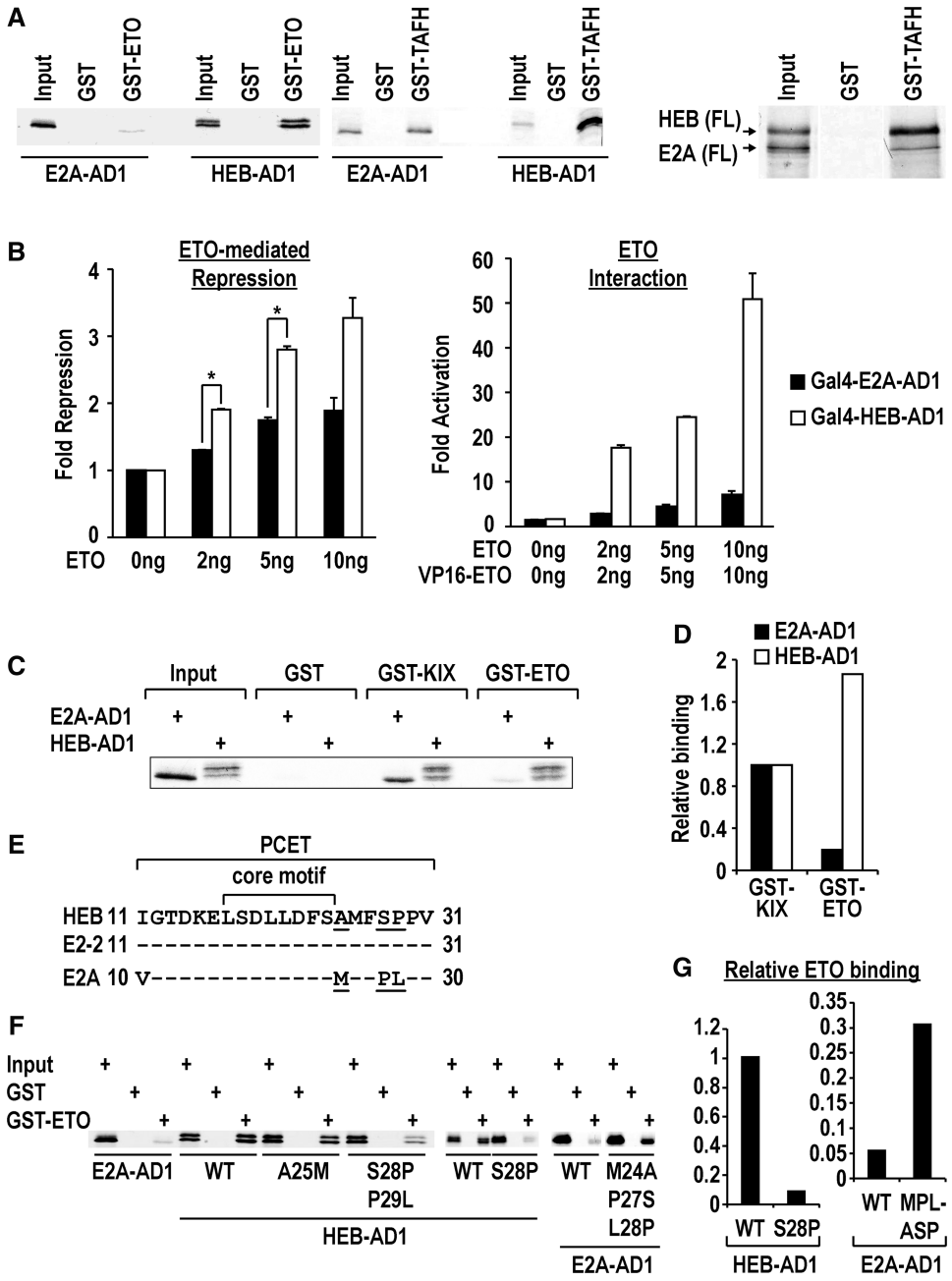

Figure 2. Mapping E2A-AD1 residues responsible for its reduced binding to the ETO corepressor. (**A**, **C** and **F**) GST pull-down assays comparing the interactions of wild-type and mutant E2A-AD1 and HEB-AD1 (A, C, F), or full-length HEB and E2A (A) with GST-ETO (A, C, F), GST-TAFH (A) or GST-KIX (C). In the right panel of (A), HEB and E2A were premixed before GST pull-down. (**B**) Luciferase reporter assays and mammalian two-hybrid assays comparing both the sensitivity of Gal4-E2A-AD1 and Gal4-HEB-AD1 to ETO-mediated repression, and their interaction with ETO. 'Fold Repression' (i.e. ETO-mediated repression) was calculated as the ratio of luciferase activities observed in the absence of ETO versus in the presence of ETO. 'Fold Activation' (i.e. ETO interaction) was calculated as the ratio of the luciferase activities observed in the presence of VP16-ETO versus in the presence of ETO. *P*-values were calculated from two-tailed *t*-test. $*P < 0.05$. (**D**) The quantification of the relative binding of E2A-AD1 and HEB-AD1 to GST-KIX and GST-ETO as shown in (C). The bindings of E2A-AD1 and HEB-AD1 to GST-KIX were similar and set to 1. (**E**) Comparison of E2A and HEB PCET sequences with E2A-specific residues at the C-terminal region underlined. (**G**) The quantification of the relative binding of wild-type and mutant E2A-AD1 and HEB-AD1 to GST-ETO as shown in (F).

binding affinity of full-length E2A. Interestingly, mutating S28 to P in full-length HEB did not significantly reduce its interaction with GST-ETO (data not shown), suggesting that the DES domain of HEB is able to compensate for the reduced AD1-TAFH interaction to maintain a strong HEB-corepressor interaction.

E2A-specific amino acids are incompatible with the high-affinity corepressor binding conformation of PCET

Our finding that the HEB-specific C-terminal PCET amino acids play a role in selectively enhancing the corepressor interaction suggests that these residues (or some of them) may directly bind to TAFH. This

possibility is also supported by a previous nuclear magnetic resonance (NMR) study showing that both A25 and S28 of HEB-PCET undergo significant chemical shift perturbations on binding to TAFH (36). The structures of HEB-PCET: TAFH have been previously reported (36,55,56). However, the three reported structures differ from each other. For example, the two docking studies show completely opposite N-C orientations of PCET (36,55). Although no direct contact between the PCET C-terminus and TAFH was shown in the NMR structure of the TAFH-PCET fusion protein, such contact may be bypassed by the covalent linkage between TAFH and PCET (56). One docking model did show a close proximity of the PCET C-terminus to TAFH (36). Since these docking studies examined only the HEB-derived PCET peptide, we sought to compare the ability of HEB and E2A PCET peptides to dock to TAFH to further understand their differential binding to TAFH.

All previous studies have used a short HEB-derived PCET peptide (Ile11-Ser28) that does not contain all E-protein-specific residues. Since our work has shown that the C-terminus of PCET modulates the binding affinity of ETO, we decided to use a longer HEB PCET peptide (Ile11-Val31) to dock to the unbound form of TAFH (55), using the same HADDOCK program (57,58) as used in the previous studies (36,55). Given the involvement of HEB-specific residues (A25, S28 and P29) in the binding, these residues were assigned as active residues in addition to previously defined active and passive residues (36). An Ile11-Val31 PCET model was generated by MODELLER based on the Ile11-Ser28 structure in the PCET-KIX complex (52). Two well-separated clusters were found from 200 water-refined structures (Supplementary Figure S1B). The best structure from the lowest energy cluster (192 structures) showed a hydrogen-bond interaction between S28 and R151 (Figure 3), consistent with our finding that the S28P mutation caused a strong reduction of the binding between AD1 and ETO. Furthermore, previous studies have shown that mutations of R151 to either Ala or Asp abolished TAFH-AD1 binding (36). Apart from the R151-S28 interaction, additional hydrogen-bond interactions also occurred between the two Asp residues (D19, D22) in the LSDLLDFS motif and the K98 residue of TAFH that we have shown to be required for the AD1-TAFH interaction. Similarly, both Asp residues have been previously shown to contribute to the interaction between AD1 and TAFH (25). When a similar docking was conducted using the E2A-derived PCET peptide (Val10-Val30), four poorly resolved clusters were generated from 189 water-refined structures (Supplementary Figure S1B). The best structure of the lowest energy cluster (136 structures) did not show direct interactions between R151 and PCET residues (Figure 3). Additionally, K98 failed to form hydrogen bond with the second Asp residue (D21) in the LSDLLDFS motif. We propose that HEB-specific PCET C-terminal residues such as S28 facilitate complex formation through direct interactions with TAFH residues such as R151. Changes of the HEB-specific residues in E2A affect the binding owing to the failure of E2A-specific residues to engage interactions with TAFH and/or their steric incompatibility with the high-affinity binding conformation of PCET for corepressors.

The reduced AD1-corepressor binding is important for the gene activation and oncogenic functions of E2A-Pbx1

Given the mutually exclusive interactions of AD1 with corepressors and coactivators, we reasoned that the reduced corepressor interaction of E2A-AD1 may allow AD1 to manifest a favorable interaction with coactivators. Given that coactivator interactions are crucial for the leukemogenic function of E2A-Pbx1 (37,38,43,52), along with our data showing that replacing E2A-AD1 with HEB-AD1 increased E2A-ETO interaction (Supplementary Figure S2B), we asked whether replacing E2A-AD1 with HEB-AD1 would compromise the ability of E2A-Pbx1 to activate gene transcription and to transform cells. An initial reporter assay showed that the AD1 domain of HEB was sufficient to effect a strong corepressor-mediated repression despite the activity of the E2A-AD2 domain (Supplementary Figure S2C). Since ETO-2 is the predominant form of the ETO family proteins expressed in hematopoietic cells (48,49,51,59) (Supplementary Figure S2E), we compared the binding of ETO-2 with E2A-AD1 and HEB-AD1. The results confirmed that ETO-2 also bound much more strongly to HEB-AD1 than to E2A-AD1 (Supplementary Figure S2D). These studies paved the way for functional assays to compare E2A-Pbx1 with the chimeric E2A(HEB^{AD1})-Pbx1, in which the AD1 of E2A was replaced by the AD1 of HEB (Figure 4A).

We first analyzed their abilities to activate the transcription of endogenous target genes. A recent work has identified three direct target genes of E2A-Pbx1 (60). These genes, CALD1, ARL4C and ST6GALNAC3, are strongly induced by E2A-Pbx1 in 697 cells derived from human t(1;19) ALL (60). We recapitulated the activation of these genes by a reconstituted E2A-Pbx1 in Namalwa cells, a human B lymphoblastoid cell line (Figure 4B). Remarkably, the chimeric E2A(HEB^{AD1})-Pbx1 protein failed to show any significant activation of these genes (Figure 4B). Western blot analysis confirmed that both proteins were expressed at similar levels (Figure 4C), and coimmunoprecipitation assays showed that only E2A(HEB^{AD1})-Pbx1, but not E2A-Pbx1, associated with endogenous ETO-2 (Figure 4C), the most abundant ETO family member expressed in Namalwa cells (Supplementary Figure S2E). These results indicate that the reduced corepressor binding of E2A-AD1 is an important determinant of the transcriptional activity of E2A-Pbx1.

To assess the impact of the increased AD1-corepressor interaction on the biological activity of E2A-Pbx1, we performed colony formation assay to measure the oncogenic activity of E2A-Pbx1 using NIH3T3 cells (42–47), which express ETO and its related protein MTGR1 (Supplementary Figure S3). Fourteen days after transfection of NIH3T3 cells with E2A-Pbx1 and E2A(HEB^{AD1})-Pbx1, the transformed colonies were counted. The results showed that the ability of E2A-Pbx1 to transform

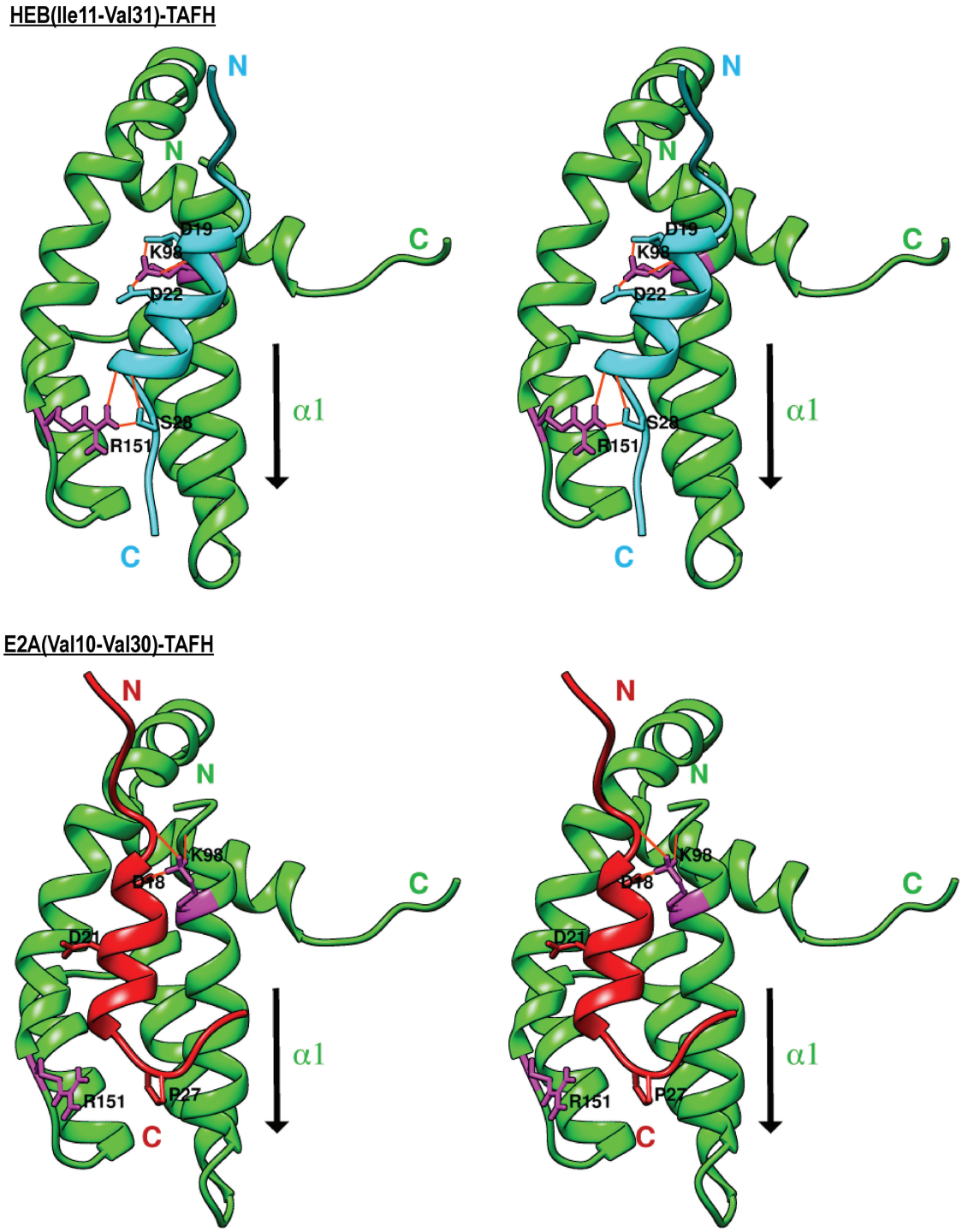

Figure 3. Modeling of TAFH interactions with HEB-PCET and E2A-PCET reveals direct involvement of HEB-specific C-terminal residues of PCET in complex formation with TAFH. Shown are stereo views of the modeled structures of HEB-PCET-TAFH (top) and E2A-PCET-TAFH (bottom). The final HEB (cyan) and E2A (red) docking structures from results involving semiflexible refinement of the PCET structure reveal differential intermolecular interactions of TAFH with HEB versus E2A in two areas: one area involving R151 and its interactions with S28 as well as the base of the HEB-PCET helix, and another area involving D19 and D22 of the HEB-PCET and their interactions with K98. In comparison, E2A only shows the interaction among the top portion of the helix involving D18. This, in turn, may explain the difference in binding affinities between HEB and E2A. Hydrogen-bond interactions are depicted by thin red lines.

NIH3T3 cells was essentially lost in E2A(HEB^{AD1})-Pbx1 (Figure 4D). As expected, replacing the entire E2A domain (containing AD1, DES, AD2) with the homologous sequence of HEB in E2A-Pbx1 also abolished the activity of E2A-Pbx1 to activate transcription and to transform NIH3T3 cells (Supplementary Figure S4). We also mutated the three E2A-specific PCET residues to the corresponding residues of HEB in E2A-Pbx1 (Figure 4A).

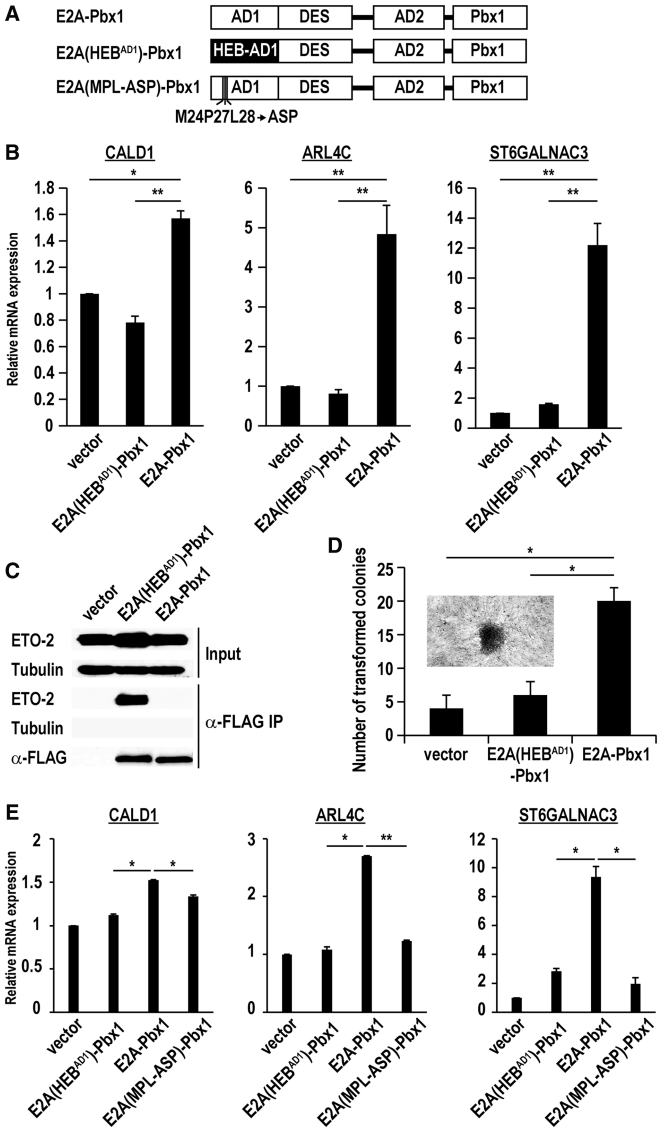

Figure 4. The weak corepressor binding of E2A-AD1 allows E2A-Pbx1 to bypass corepressor-mediated repression to facilitate its gene activation and oncogenic activities. (**A**) Schematic representation of wild-type E2A-Pbx1, E2A(HEB^{AD1})-Pbx1 that has E2A-AD1 replaced by HEB-AD1, and E2A(MPL-ASP)-Pbx1 that has residues M24, P27 and L28 mutated to A, S and P, respectively. (**B**) RT-qPCR showing that replacing E2A-AD1 by HEB-AD1 abolished the ability of E2A-Pbx1 to activate its endogenous target genes in transduced Namalwa cells. (**C**) Co-IP assays showing enhanced ETO-2 corepressor binding by E2A(HEB^{AD1})-Pbx1 in transduced Namalwa cells. (**D**) Colony formation assays performed in NIH3T3 cells showing dramatically reduced transforming capacity of E2A(HEB^{AD1})-Pbx1. The inset shows a typical transformed colony. (**E**) Effect of converting E2A-specific PCET residues to those of HEB on the ability of E2A-Pbx1 to activate target genes in transduced Namalwa cells. *P* values were calculated from two-tailed *t*-test. $*P < 0.05$. $**P < 0.01$.

The resulting E2A(MPL-ASP)-Pbx1 mutant was expressed at a level comparable with that of E2A-Pbx1 in transduced Namalwa cells (Supplementary Figure S5). Further confirming the importance of the defective corepressor binding of AD1 for the biological function of E2A-Pbx1, E2A(MPL-ASP)-Pbx1 failed to activate E2A-Pbx1 target genes (Figure 4E), similarly to the results observed with E2A(HEB^{AD1})-Pbx1. In transformation assays, E2A(MPL-ASP)-Pbx1 also showed significantly reduced transforming capacity compared with E2A-Pbx1 (Supplementary Figure S5). These results demonstrated that the reduced corepressor binding of E2A-AD1 as a result of changes in the PCET motif is necessary for E2A-Pbx1 to bypass corepressor-mediated repression to ensure its gene activation and oncogenic functions.

E2A requires the DES domain to maintain the sensitivity to corepressor-mediated repression

Previous studies have shown that ETO family corepressors and the AML1-ETO leukemia fusion protein can repress E2A-dependent transcription. The repression is thought to be important for the normal hematopoiesis and for the leukemogenic function of AML1-ETO (25,49–51). However, we found that, unlike HEB-AD1, the binding of E2A-AD1 to ETO is intrinsically weak. These findings prompted us to further study how the sensitivity of E2A to corepressor-mediated repression is regulated.

First, given our GST pull-down results showing that AD1 can cooperate with DES to elicit a strong ETO interaction with E2A, we asked whether AD1 and DES also cooperatively regulate the sensitivity of E2A to ETO-mediated repression. Figure 5A shows that the maximal level of ETO-mediated repression requires both the DES domain of E2A and the ability of ETO to bind to AD1. Deleting DES or preventing AD1 interaction by the K98E mutation similarly reduced ETO-mediated repression of E2A. Western blot results showed that the relative expression of E2A and E2AΔDES was not affected by the expression of ETO or ETO-K98E (Supplementary Figure S6). The contribution of AD1 to ETO-mediated repression was also evident by the reduced ability of ETO to repress the L20A mutant of E2A (Figure 5B), which carries a mutation in the core PCET motif (LXXLL) that abolishes its binding to TAFH (25). Similar results were obtained with AML1-ETO (Supplementary Figure S6A and B), consistent with the proposed corepressor function of AML1-ETO for E2A.

Next, the roles of AD1 and DES in regulating the corepressor sensitivity of E2A were further examined by correlating ETO-mediated repression with its interaction with E2A *in vivo* (Figure 5C). The results clearly demonstrated strong synergy between AD1 and DES for the ability of ETO to repress E2A-dependent transcription and to interact with E2A *in vivo* (Figure 5C). The various E2A derivatives shown in Figure 5C were readily expressed in transfected cells and their relative levels were not affected by ETO expression (Supplementary Figure S6). Notably, the strong synergistic effect between AD1 and DES was lost when the AD1-binding-defective ETO-K98E mutant was used in place of wild-type ETO (Figure 5C), demonstrating the specificity of the observed synergy between AD1 and DES in the binding of wild-type ETO and the consequent ETO-mediated repression.

We also examined the impact of AD2 on the corepressor sensitivity of E2A. The activity of AD2 has been mapped to a conserved LDEAIHVLR motif

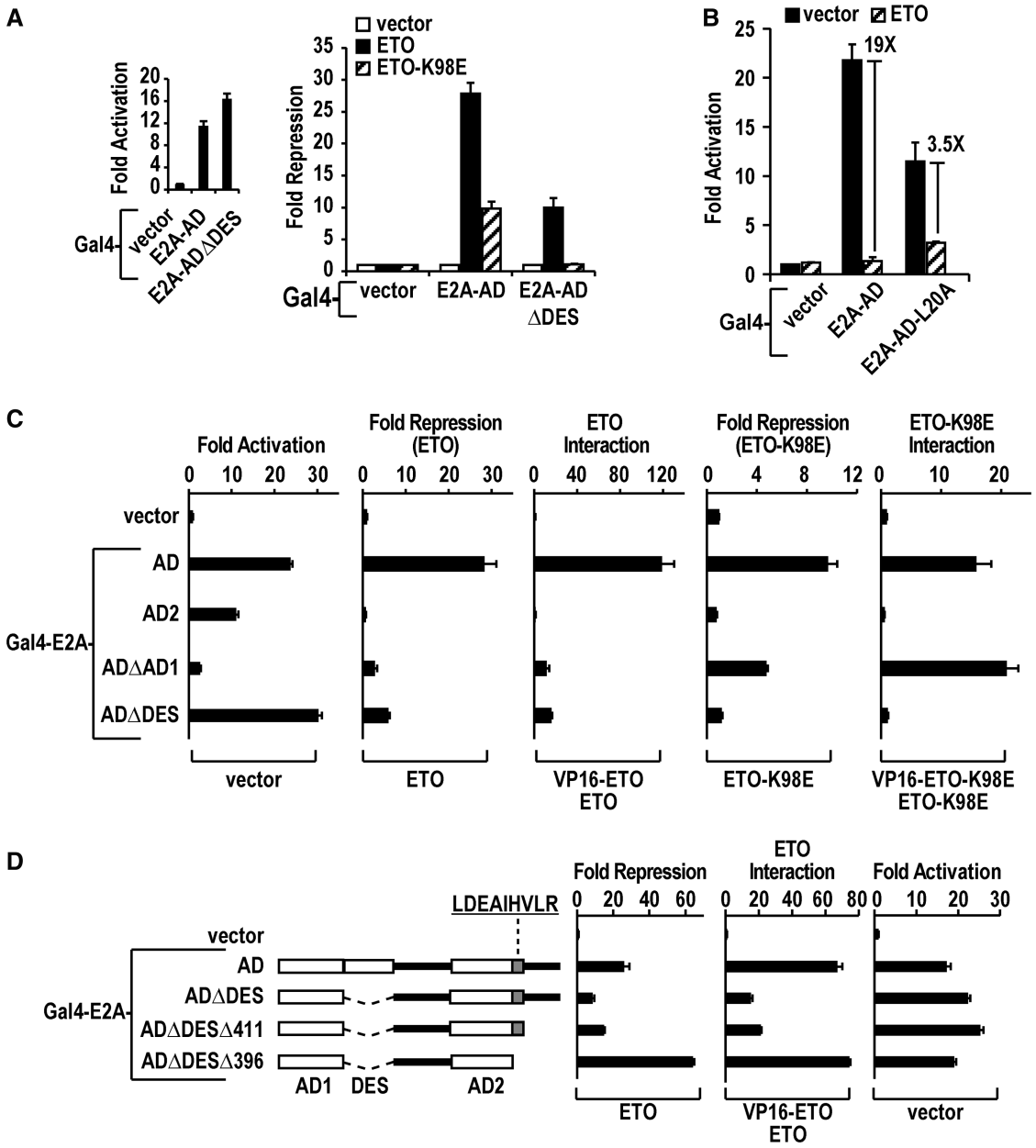

Figure 5. Both corepressor-binding domains (AD1 and DES) are important for the corepressor sensitivity of E2A. (**A** and **B**) Reporter assays showing that both DES (A) and AD1 (B) were required for the optimal E2A sensitivity to ETO-mediated repression. 'Fold Activation' was calculated as the ratio of the luciferase activity observed with each E2A derivative to the luciferase activity observed with Gal4-DBD. 'Fold Repression' was calculated as the ratio of the luciferase activities observed with each E2A derivative in the absence versus presence of ETO. (**C**) Mammalian two-hybrid assays showing that AD1 and DES cooperatively regulated the binding of E2A to ETO and the repression of E2A-dependent transcription. 'ETO/ETO-K98E interaction' was calculated as the ratio of the luciferase activities observed with each E2A derivative in the presence of VP16-ETO (or VP16-ETO-K98E) versus in the presence of ETO (or ETO-K98E). (**D**) Similar assays as in (C) showing that AD2 and DES opposingly regulated the corepressor sensitivity of E2A.

(Figure 5D) (61). As previously shown, deletion of DES reduced ETO-mediated repression and its interaction with E2A *in vivo* (Figure 5D). The reduced repression and interaction, however, were restored by further deletion of the AD2 motif (Figure 5D). This effect cannot be attributed to differences in the expression level of E2A derivatives (Supplementary Figure S6). These results are consistent with a functional antagonism between DES and AD2 in regulating the corepressor sensitivity of E2A. Since AD1 is cooperatively involved in both coactivator (along with AD2) and corepressor (along with DES) interactions, DES and AD2 may exert their effects by regulating the competitive AD1 interactions with corepressors and coactivators.

DES is functionally important for corepressor-mediated repression of physiological target genes of E2A

Given the ubiquitous expression of E2A coactivators such as p300/CBP and GCN5 in the nucleus, these

coactivators, presumably by enhancing the AD1-AD2 cross talk, should impose a constant inhibition on the interaction between E2A and corepressors. We therefore reasoned that DES should be critically important for the ability of E2A to sensitize to corepressor-mediated repression of endogenous target genes. To test this, we used two model systems where corepressor-mediated regulation of E2A has been previously documented. We first explored the importance of DES for the silencing of E2A activity by AML1-ETO in Kasumi-1 cells, a patient-derived t(8;21) leukemia cell line. An initial study carried out in transfected HEK293T cells showed that DES was important for AML1-ETO to interact with E2A and to repress E2A-dependent transcription from a reporter gene (Supplementary Figure S7). Similarly, when expressed in Kasumi-1 cells, E2A, but not E2AΔDES, showed a strong interaction with endogenous AML1-ETO fusion protein (Figure 6A). Consistent with the observed weak association between E2AΔDES and AML1-ETO, GST-E2A-AD1 failed to pull down AML1-ETO from Kausmi-1 extracts, whereas a strong interaction was evident for GST-HEB-AD1 (Figure 6B). This was not due to a defective E2A-AD1 protein used in the assay, as it showed stronger interactions with p300 and GCN5 compared with HEB-AD1 in the same assay (Figure 6B). These results are consistent with the competitive nature of AD1 interactions with coactivators and corepressors and the above-demonstrated reduced ability of E2A-AD1 to bind to corepressors.

These results prompted us to test whether deletion of DES could allow E2A to escape the silencing of its activity by AML1-ETO in Kasumi-1 cells. By analyzing previous ChIP-Seq data sets (GSE29225, GSE23730) and our own RNA-Seq results (data not shown), we identified Src-like-adaptor (SLA) as a direct target gene of AML1-ETO, E2A and HEB (Supplementary Figure S8). SLA is predominantly expressed in lymphocytes where it regulates TCR and BCR signaling (62,63). Its expression, however, is silenced by AML1-ETO in Kasumi-1 cells (Figure 6C) and in patients with t(8;21) AML (64). Among the three isoforms of SLA, NM_006748 is the predominant form in Kasumi-1 cells (RNA-Seq data not shown). AML1-ETO, E2A and HEB all bound strongly to an enhancer element located ~2-Kb upstream of the transcription start site of SLA NM_006748 (Supplementary Figure S8A, Figure 6D). Knockdown of E2A similarly increased the expression of SLA, although to a lesser extent than that observed with the AML1-ETO knockdown (Supplementary Figure S8B). These results confirmed that E2A is part of the AML1-ETO repression complex for SLA. We hypothesized that deletion of DES would affect the silencing of E2A's transcriptional activity by AML1-ETO, leading to increased expression of SLA. ChIP results confirmed that deletion of DES did not affect the binding of E2A to the enhancer element (Figure 6D). Confirming the hypothesis, quantitative reverse transcription-PCR (RT-qPCR) showed that whereas wild-type E2A reduced SLA expression, E2AΔDES significantly increased the expression of SLA (Figure 6E, left panel). Consistently, the binding of GCN5 was significantly increased, whereas AML1-ETO occupancy was reduced on the SLA enhancer in E2AΔDES-expressing cells compared with vector- or E2A-transduced cells (Figure 6E, right and middle panels). Interestingly, the occupancy of p300 did not appear to be significantly changed in these cells (data not shown). This may reflect a more dynamic property of p300 binding to chromatin during transcriptional activation. It has been shown that p300 needs to be released before transcription occurs (65). It also is possible that p300 may only transiently bind to chromatin and subsequently be replaced by GCN5. Similar results were obtained with another AML1-ETO target gene RASSF2 (Supplementary Figure S9). Together, these studies show that DES is important for AML1-ETO to prevent E2A-mediated coactivator recruitment and the consequent activation of AML1-ETO/E-protein target genes.

It has been recently shown that ETO-2 regulates E2A to dynamically control E2A target gene expression during hematopoiesis in a dose-dependent manner (48–51). Since ChIP-Seq has previously mapped the genomic binding sites of E2A and HEB in T-lymphocyte-derived Jurkat cells (66), we used this cell line to test the role of DES in ETO-2-mediated repression of E2A. Overlapping binding sites of E2A, HEB and TAL1 were mapped to their nearest genes using the HOMER program (67). A comparison of these genes with HEB-regulated genes in Jurkat cells based on our HEB knockdown and RNA-Seq results (data not shown) uncovered three high-confidence target genes of E2A (GADD45G, GATSL3 and SLAMF6), which were further confirmed by a knockdown experiment (Supplementary Figure S10). Among the target genes, GADD45G is particularly interesting, given its proposed tumor suppressor function (68). Although a link between GADD45G and E2A has not been reported, a previous work has reported the regulation of other GADD45 genes by E47 (69). The effects of E2A and E2AΔDES, with or without ETO-2, on the expression of these genes in Jurkat cells were determined by RT-qPCR. In all cases, a corepressor function of ETO-2 was evidenced by its ability to reduce the expression of these genes both in the absence and presence of wild-type E2A (Figure 6F). Remarkably, for all three genes, the expression of E2AΔDES in these cells completely abolished the corepressor function of ETO-2 (Figure 6F). ChIP assays confirmed similar binding of E2A and E2AΔDES to the nearest E2A binding sites of these genes as determined by ChIP-Seq, as well as the binding of ETO-2 to these sites (Supplementary Figure S10). Reporter assays confirmed that ETO-2 is a bona fide corepressor for E2A and its corepressor function is critically dependent on DES (Supplementary Figure S11). Of note, whereas E2A activated GADD45G and GATSL3 in Jurkat cells, it repressed SLAMF6. These effects, which match the E2A-knockdown effects (Supplementary Figure S10), highlight the context-dependent activation and repression function of E2A as previously reported (41). It is also interesting to note that deletion of DES compromised the E2A activation of both GADD45G and GATSL3 (Figure 6F). This is consistent with our previous finding that DES can function as an independent activation domain on a DNA template *in vitro* (26), suggesting that DES may

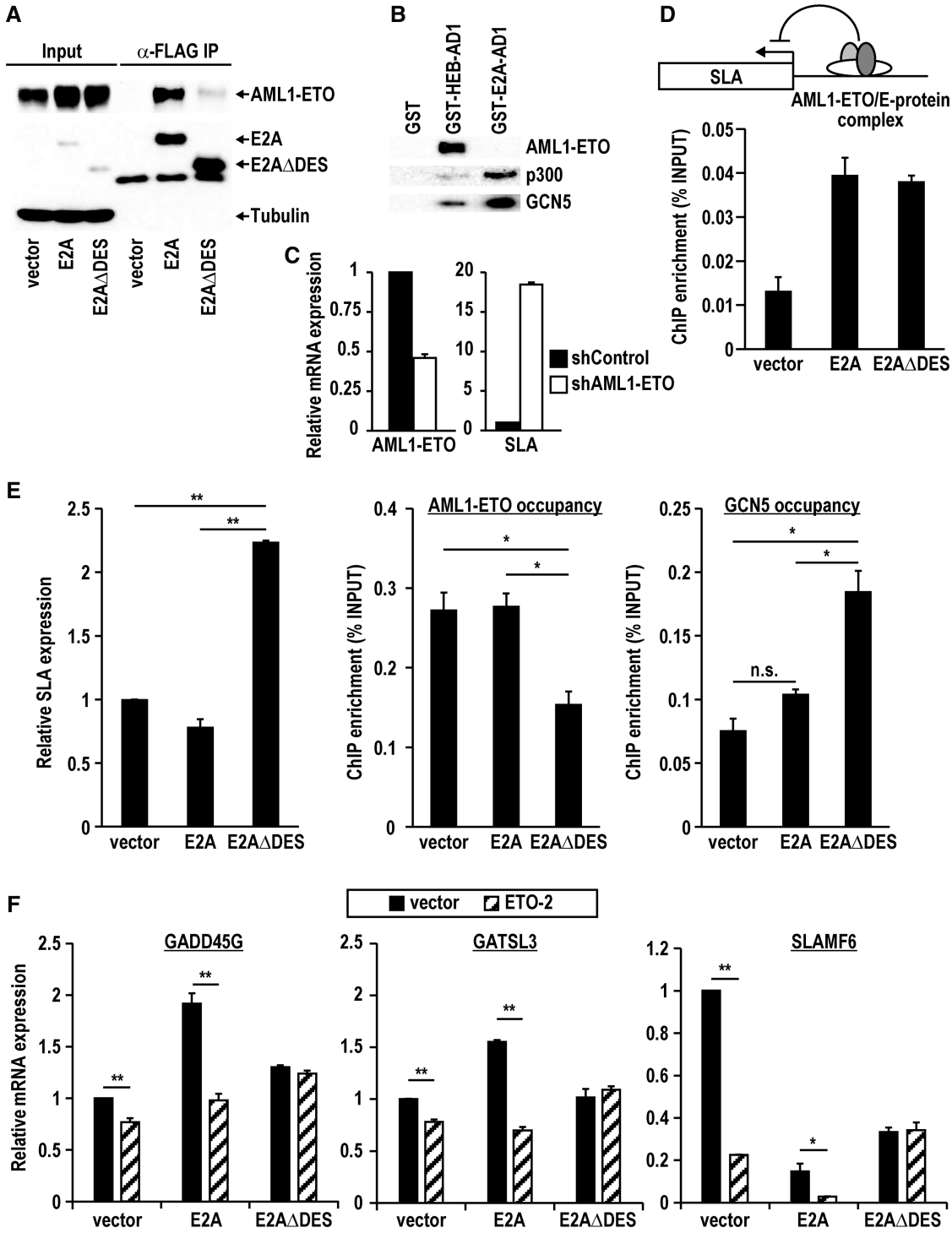

Figure 6. *In vivo* importance of DES for corepressor-mediated silencing of the transcriptional activity of E2A. (**A**) Co-IP showing that DES was critical for E2A to form complex with endogenous AML1-ETO in Kasumi-1 cells. (**B**) GST pull-down of Kasumi-1 whole cell lysate showing that E2A-AD1 had a much weaker interaction with AML1-ETO, but acquired increased binding to coactivators as compared with HEB-AD1. (**C**) Knockdown of AML1-ETO in Kasumi-1 cells increased the expression of SLA as assayed by RT-PCR. The 18S rRNA was used as the internal control. (**D**) FLAG-tagged E2A and E2AΔDES showed similar binding to an enhancer element of SLA in transduced Kasumi-1 cells. The anti-FLAG antibody was used. (**E**) DES was required for the silencing of E2A transcriptional activity by AML1-ETO in Kasumi-1 cells. (Left) RT-qPCR showing that ectopic expression of E2AΔDES, but not E2A, activated SLA expression. (Middle and Right) ChIP assays showing that ectopic expression of E2AΔDES, but not E2A, reduced the occupancy of AML1-ETO and enhanced GCN5 occupancy on SLA enhancer. (**F**) RT-qPCR assays showing that DES was required for ETO-2 to repress E2A-dependent activation or to potentiate E2A-dependent repression of distinct target genes in transduced Jurkat cells. *P*-values were calculated from two-tailed *t*-test. $*P < 0.05$. $**P < 0.01$. n.s., not significant.

play a role in facilitating RNA polymerase II function after chromatin remodeling. Furthermore, although E2AΔDES did not respond to ETO-2, it modestly repressed SLAMF6. One explanation is that E2AΔDES may displace other E-box-binding proteins from their binding sites, thereby reducing the basal level of SLAMF6. Taken together, these results confirmed that ETO-2 can modulate both context-dependent activation and repression function of E2A and that DES is critical for regulation of E2A by ETO-2.

DISCUSSION

The PCET sequence contains the invariant LSDLLDFS motif to allow its dual binding by corepressors and coactivators. The C-terminal flanking sequence of this motif contains three residues that are E-protein member-specific. Our results indicate that these residues selectively modulate corepressor but not coactivator interaction. The change of Ser28 in HEB to Pro in E2A is likely the most critical determinant that reduces corepressor binding affinity, as mutation of S28P alone is sufficient to reduce the corepressor binding affinity of HEB-AD1 to a low level similar to that observed with E2A-AD1 (Figure 2F and G). Whether changes of the other two residues in E2A-PCET have synergistic or redundant effects with S28P remains to be determined. We speculate that all three changes are important to ensure the low corepressor binding of E2A-AD1, which, in turn, is important for the biological function of E2A. In support of this idea, converting all three E2A-specific residues to those of HEB is necessary to restore a strong corepressor interaction both in the context of the AD1 domain and in the context of full-length E2A (Figure 2F, Supplementary Figure S2B, and data not shown).

Our results are consistent with the model that the E-protein member-specific residues act to facilitate or restrict corepressor interaction with the AD1 domain of HEB and E2A, respectively. Our docking study using a long PCET peptide comprising all three HEB-specific residues (A25, S28 and P29) showed that the C-terminus of HEB-PCET is in close proximity to residues of TAFH at its bottom PCET-binding pocket. One such residue is R151 in the loop of helix 3 and helix 4. Consistent with the importance of R151 (36) and S28 (this study) for the high-affinity binding between HEB-AD1 and TAFH, a direct hydrogen-bond interaction has been identified between R151 and S28. R151 also engages a hydrogen-bond interaction with another HEB-PCET residue (Figure 3), but does not interact with any E2A-PCET residues (Figure 3). Additionally, the dual hydrogen-bond interactions between K98 of TAFH with both D19 and D22 of PCET should also contribute to the high binding affinity of HEB-AD1, consistent with the importance of these amino acids in the interaction between TAFH and HEB-AD1 (25).

The selective involvement of HEB-specific PCET residues in the corepressor interaction is also evident by comparing our PCET-TAFH model with the previously reported structure of the PCET-KIX complex (52) (Supplementary Figure S12). These HEB-specific residues, such as S28, selectively contact TAFH but not KIX. Similarly, D19 and D22, whose mutations specifically affect repression but not activation, are engaged in interactions with TAFH, but not KIX (Supplementary Figure S12).

To obtain an unbiased comparison of docking simulation between HEB and E2A, our docking model used the unbound structure of TAFH (55). Consistent with our results, the previously generated model using the bound structure of TAFH in complex with HEB-PCET also showed a close proximity between the C-terminus of PCET and the binding surface of TAFH (36). However, a role for the PCET C-terminal residues in the interaction with TAFH was not revealed by the NMR structure of the TAFH-PCET fusion protein (56). This fusion approach is necessary to experimentally determine the atomic structure of PCET bound to TAFH due to exchange broadening of resonances from the complex assembled with separated PCET and TAFH fragments (36,55). However, the use of a short PCET peptide and the covalent linker between this peptide and TAFH may diminish the importance of the C-terminus-mediated interactions. Nevertheless, it is possible that the conformation of an initially formed PCET-TAFH complex differs from that of the final stable complex. Given that our docking has used the unbound TAFH structure, the results may reflect the role of the PCET C-terminus at the initial step(s) of the complex formation process, whereas such interactions could be dispensable in the final complex that may have a structure similar to that of the TAFH-PCET fusion protein.

The PCET motif of E-proteins is the overlapping binding surface for both coactivators and corepressors. Previous studies have shown that the PCET interactions with p300/CBP and with ETO family corepressors are mutually exclusive (25,26). Given the short length of the sequence, its binding by GCN5 is likely to also be competitive with the binding by ETO family corepressors. We propose that AD1 plays an important role in coordinating the exchange of coactivators and corepressors to dynamically switch transcription between active and repressive states (Figure 7A). Although changes in the relative corepressor and coactivator levels should passively affect their binding to transcription factors, additional mechanisms should also exist and play more active roles in the process of the exchange. For example, in the case of nuclear receptors, their ability to bind to corepressors and coactivators is strongly influenced by their ligand-binding state (70). We propose that a different mechanism is used by E-proteins, particularly E2A, to facilitate the exchange of corepressors and coactivators, which relies on the use of cofactor-specific interaction domains, i.e. DES and AD2 (Figure 7A). Thus, in the corepressor-bound state, coactivators may first bind to AD2 before displacing AD1-bound corepressors. Similarly, in the coactivator-bound state, the displacement of coactivators from AD1 may be facilitated by the initial binding of ETO family corepressors to DES. This may be particularly important for E2A, given the weak binding affinity between E2A-AD1 and TAFH.

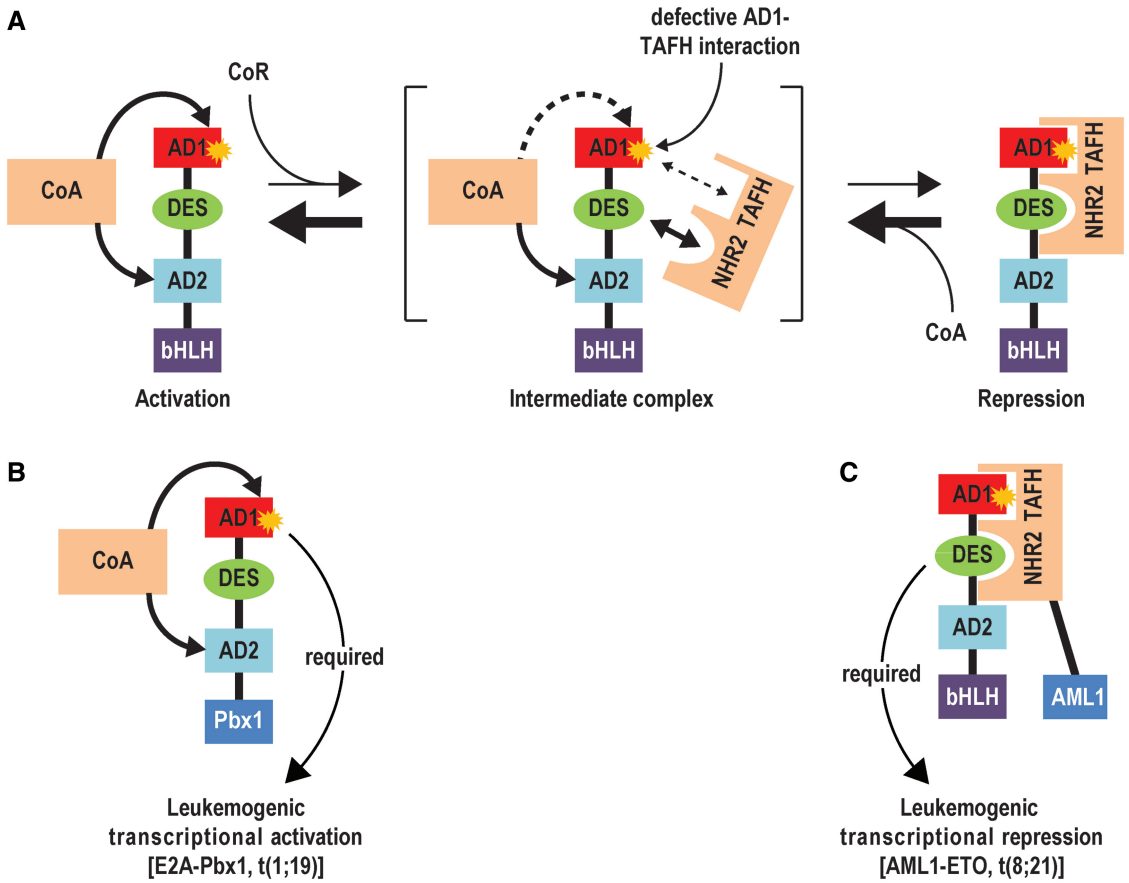

Figure 7. A mechanistic model depicting the role of E2A domains in the assembly of corepressor and coactivator complexes, and their importance for the transcriptional activity of E2A-Pbx1 and AML1-ETO leukemia fusion proteins. **(A)** We propose that the formation of stable E2A complexes with coactivators (CoAs) or corepressors (CoRs) requires cooperative interactions with two E2A domains. TAFH and NHR2 are the conserved domains of ETO family of CoRs that interact with AD1 and DES, respectively. At low CoR levels, due to the defective E2A-AD1-CoR interaction (shown by the yellow asterisk), the balance of CoR and CoA interactions favors the assembly of an E2A-CoA complex, explaining the high level of gene activation by E2A-Pbx1. At high CoR levels, CoRs such as AML1-ETO and ETO-2 drive the formation of their complexes with E2A through a stepwise mechanism that involves the formation of an intermediate complex containing both CoRs and CoAs. The formation of the intermediate complex before the formation of the stable E2A-CoR complex is facilitated by DES-dependent interactions with CoRs, explaining the importance of DES in CoR-mediated repression of E2A. The dashed lines denote competitive AD1 interactions with CoA and CoR, which dictate the formation of stable CoA or CoR complexes. (**B**) The E2A-specific changes in the PCET motif destabilize CoR binding, which is required for the ability of E2A-Pbx1 to drive gene activation in t(1;19) leukemia. (**C**) The DES domain is required for the repression of AML1-ETO/E2A target genes in AML1-ETO-dependent t(8;21) leukemia.

Owing to the reduced binding of corepressors to the AD1 domain of E2A, the physiological amount of corepressors may not be sufficient to compete with coactivators for binding to E2A. This explains the high level of transcriptional activity seen with E2A-Pbx1 and its critical dependency on the weak corepressor binding of AD1. Thus, E2A-Pbx1 exploits this specific feature of E2A to ensure leukemogenic activation by escaping corepressor-mediated repression (Figure 7B). Similarly to E2A-Pbx1, we found that E2A generally is more active than HEB in mediating transcriptional activation (C.G. and J.Z., unpublished data). It is possible that, among the three E-protein members, E2A favors its function as an activator, especially under low corepressor conditions. Other E-proteins, such as HEB, may require a lower threshold of corepressors to function as repressors as recently exemplified (71).

The reduced binding of E2A-AD1 to corepressors, however, does not completely eliminate the sensitivity of E2A to corepressor-mediated repression. Thus, under conditions of high corepressor levels, such as in t(8;21) leukemia cells, the corepressors drive the formation of repression complexes that contain E2A (Figure 7A and C). We propose that this process is facilitated by an initial binding of DES to these corepressors (AML1-ETO, ETO-2) to form an intermediate complex that contains both corepressors and coactivators before the ultimate displacement of coactivators from both AD1 and AD2 (Figure 7A). This explains the strong cooperativity observed between DES and AD1 in the E2A-ETO interaction *in vivo* and *in vitro*. Physiologically, consistent with this model, DES is critically important for AML1-ETO- and ETO-2-mediated repression of E2A target genes. These genes, such as GADD45G, RASSF2, SLA and SLAMF6, can function as tumor suppressors or facilitate

cell differentiation (69,72–79). Their regulation by E2A is consistent with the biological function of E2A, whose silencing by AML1-ETO is thus important for AML1-ETO-dependent leukemogenesis. Our finding that E2A-corepressor interactions are differentially involved in leukemogenic pathways also challenges the current dogma in the field that corepressors generally facilitate leukemia development, a model initially proposed given their common involvement in leukemias resulting from AML1- and RAR-related fusion proteins. Our finding of context-dependent function of corepressors highlights the importance of elucidating the specific mechanisms associated with individual leukemia fusion proteins. A further understanding of the structural and transcriptional mechanisms in the regulation of E2A by corepressors and coactivators carries the promise of developing targeted approaches to enhance or diminish the specific coactivator and corepressor interactions to fight leukemias resulting from E2A/ETO-related chromosome translocations.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including [80–82].

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