

Functional redundancy between the transcriptional activation domains of E2A is mediated by binding to the KIX domain of CBP/p300

Christopher M. Denis^{1,*}, David N. Langelaan^{1,*}, Alyssa C. Kirlin¹, Seth Chitayat¹,
Kim Munro², Holly L. Spencer¹, David P. LeBrun^{2,3,4,†} and Steven P. Smith^{1,2,†}

¹Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario, K7L 3N6, Canada,

²Protein Function Discovery Group, Queen's University, Kingston, Ontario, K7L 3N6, Canada, ³Department of Pathology and Molecular Medicine, Queen's University, Kingston, Ontario, K7L 3N6, Canada and ⁴Division of Cancer Biology and Genetics, Cancer Research Institute, Queen's University, Kingston, Ontario, K7L 3N6, Canada

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ABSTRACT

The E-protein transcription factors play essential roles in lymphopoiesis, with E12 and E47 (hereafter called E2A) being particularly important in B cell specification and maturation. The *E2A* gene is also involved in a chromosomal translocation that results in the leukemogenic oncoprotein E2A-PBX1. The two activation domains of E2A, AD1 and AD2, display redundant, independent, and cooperative functions in a cell-dependent manner. AD1 of E2A functions by binding the transcriptional co-activator CBP/p300; this interaction is required in oncogenesis and occurs between the conserved ϕ -x-x- ϕ - ϕ motif in AD1 and the KIX domain of CBP/p300. However, co-activator recruitment by AD2 has not been characterized. Here, we demonstrate that the first of two conserved ϕ -x-x- ϕ - ϕ motifs within AD2 of E2A interacts at the same binding site on KIX as AD1. Mutagenesis uncovered a correspondence between the KIX-binding affinity of AD2 and transcriptional activation. Although AD2 is dispensable for oncogenesis, experimentally increasing the affinity of AD2 for KIX uncovered a latent potential to mediate immortalization of primary hematopoietic progenitors by E2A-PBX1. Our findings suggest that redundancy between the two E2A activation domains with respect to transcriptional activation and oncogenic function is mediated by binding to the same surface of the KIX domain of CBP/p300.

INTRODUCTION

Coordinated transcriptional regulation is a critical aspect of hematopoiesis, including the lineage specification of B- and T-lymphocytes, natural killer cells and plasmacytoid dendritic cells from pluripotent hematopoietic stem cells (1–7). The precise cellular programming of this process dictates progressive lineage-specific differentiation, which comprises several intermediates and ultimately the mature, functional cell, through specific gene expression systems that are tightly regulated by a series of transcription factors. The E-protein family of class I basic helix-loop-helix (bHLH) transcription factors represents one such set of transcription factors that play essential roles in the development and speciation of B- and T-lymphocytes (8–12).

The E-protein family comprises the proteins E12 and E47 (hereafter referred to as E2A), which are alternatively spliced products of the *E2A* gene (also called *TCF3*), as well as HEB and E2-2 (9,13–15). Each family member contains a C-terminal bHLH domain that mediates E-protein dimerization and binding to DNA at the E-box CANNTG consensus sequence in transcriptional promoters and enhancers (16–19). Two transcriptional activation domains (ADs), AD1 and AD2, are found, respectively, at the N-terminus and in the central region of the proteins (10,13,15) (Figure 1). AD1 and AD2 have been shown to display cell type-specific, redundant, cooperative or independent transcriptional regulatory functions (20–26). For example, AD1 or AD2 can induce transcription individually, their contributions to reporter gene induction are greater than additive, and deletion of either AD1 or AD2 abrogates the ability of E2A to induce B-lymphoid differentiation in a mouse-derived pre-B cell line, suggesting functionally redundant and cooperative roles in B cell development (20–

*These authors contributed equally to the work.

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

†To whom correspondence should be addressed. Tel: +1 613 533 3188; Fax: +1 613 533 2497; Email: steven.smith@queensu.ca

Correspondence may also be addressed to David P. LeBrun. Tel: +1 613 533 3209; Fax: +1 613 533 2907; Email: lebrun@cliff.queensu.ca

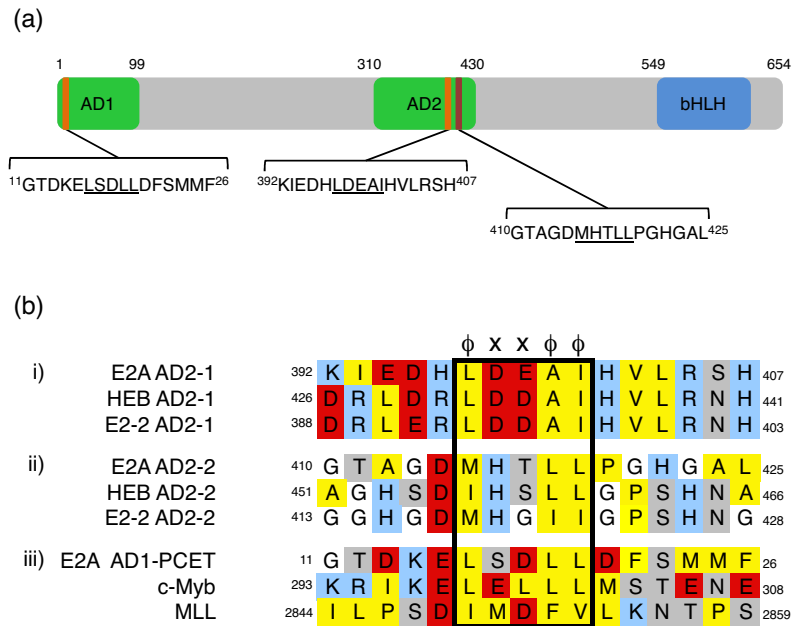


Figure 1. Conserved ϕ -x-x- ϕ - ϕ sequences in E2A. (a) Domain architecture of E2A illustrating the activation domains AD1 and AD2 as well as the C-terminal dimerization and DNA-binding bHLH domain. The ϕ -x-x- ϕ - ϕ containing sequences within these domains are illustrated. (b) Sequence alignment of the ϕ -x-x- ϕ - ϕ containing sequences of (i) AD2-1 and (ii) AD2-2 from E2A, HEB and E2-2 and (iii) the KIX-interacting E2A AD1-PCET motif (31,32) and the ADs of c-Myb and MLL (31). The ϕ -x-x- ϕ - ϕ sequence common to KIX-interacting ADs is indicated and boxed, with ϕ representing a hydrophobic amino acid and x any amino acid. Two sequences within AD2 of E2A, HEB and E2-2 possessing this common sequence are indicated as AD2-1 and AD2-2. Numbering is in accordance with the native protein sequence.

22,26). Their transcriptional function appears to involve the recruitment of transcriptional coactivators including CBP/p300, or corepressors including ETO (eight twenty one encoded by *RUNKIT1*) to transcriptional regulatory complexes associated with DNA (27). Indeed, CBP/p300 and ETO have been shown to compete for binding to a region of AD1 domain referred to as the ‘p300/CBP and ETO target in E-proteins’ or PCET motif, thereby providing a mechanism for E-protein mediated transcriptional silencing (28). A functional role for AD2 is supported by the existence of N-terminal truncated forms of HEB and E2-2 that lack AD1, termed HEBAIt and E2-2Alt, and result from the use of alternative transcription start sites (29). HEBAIt plays a prominent transcriptional regulatory role in the genesis of early T cell precursors.

The PCET motif of AD1 is highly conserved among E-proteins and comprises the overlapping LxxLL and LDFS sequences, which participate in transcriptional regulation by mediating protein-protein interactions (24,30). The LxxLL sequence conforms to the generic ϕ -x-x- ϕ - ϕ motif (where ϕ represents a bulky hydrophobic residue and x corresponds to any amino acid), which mediates numerous interactions between transcription factors and transcriptional co-regulators. For example, the KIX domain of CBP/p300 interacts directly with ϕ -x-x- ϕ - ϕ sequences within the PCET motif of E2A and in the ADs of p53, mixed lineage leukemia (MLL) and FOXO3a (28,31-35). Direct binding of E2A to the KIX domain of CBP/p300 results in acetylation of E2A and enhanced acetyltransferase activity of CBP/p300 (20,22,32,36-38).

The two ADs of E2A are also retained in the oncogenic transcription factor E2A-PBX1, which is produced as a consequence of the recurring chromosomal translocation t(1;19) in acute lymphoblastic leukemia. In generating E2A-PBX1, translocation t(1;19) effectively replaces the bHLH domain of E2A with the DNA-binding homeodomain of PBX1 (39-41). Despite considerable study, the mechanistic contribution of E2A-PBX1 to leukemic transformation has not been fully elucidated. However, as in B-lymphopoiesis, the recruitment of transcriptional coactivators by E2A-PBX1 appears essential. Our group and others have recently shown that the E2A PCET:KIX interaction is required in E2A-PBX1-mediated oncogenesis (32,36,42).

Similar to AD1, AD2 of E2A has been shown to interact with CBP/p300 either independently of, or cooperatively with, AD1 to promote transcriptional induction, E2A acetylation, E2A nuclear retention and lymphoid development (20-22). Although existing evidence implicates the KIX domain of CBP/p300 in mediating binding to AD2 (20), the structural and biophysical properties of E2A AD2:CBP/p300 binding have not been characterized.

In this study, we characterize a direct interaction between E2A AD2 and the KIX domain of CBP/p300, identify the structural determinants of this interaction using nuclear magnetic resonance (NMR) spectroscopy and demonstrate their functional importance for binding, transcriptional activation and E2A-PBX1-driven oncogenesis. Our findings help to explain previous functional observations, including the redundant functional roles of the two ADs of E2A and the other E-protein family members.

MATERIALS AND METHODS

Peptides and recombinant protein constructs

The following synthetic peptides, each with an N-terminal fluorescein isothiocyanate (FITC) tag, were generated by the Sheldon Biotechnology Centre or BioBasic Inc.: E2A AD1 (residues 11–24; Ac-GTDKELSDLLDFSM-NH₂), E2A AD2–1 (residues 394–407; Ac-EDHLDEAIHVLRSH-NH₂ and Ac-EDHLDEAIHVLRSHY-NH₂ in which a C-terminal tyrosine was incorporated for direct comparison with the synthetic E2A AD2–1 construct used in NMR studies), AD2–2 (residues 408–420; Ac-AVGTAGDMHTLLP-NH₂), E2A AD2–1/2 (residues 394–420 Ac-EDHLDEAIHVLRSHAVGTAGDMHTLLP-NH₂) and E2A AD2–1 Leu397Ala, Ala400Leu, Ile401Ala, His402Ala or Leu404Ala mutant peptides. FITC-labeled peptides were quantified by absorbance of the fluorescein moiety at 496 nm using a molar extinction coefficient of 68 000 l·mol⁻¹·cm⁻¹. A non-fluorescent E2A AD2–1 peptide containing an additional C-terminal tyrosine residue for quantification by absorbance at 280 nm was generated for NMR-based chemical shift mapping studies.

A pET21a(+) derived plasmid encoding the KIX domain from mouse CBP 586–672 was kindly provided by Dr Peter Wright (Scripps Research Institute, La Jolla, CA). A Phe612Ala/Asp622Ala/Arg624Ala/Lys667Glu quadruple KIX mutant (KIX^{ΔPCETsite}) and Tyr650Ala/Ala654Gln/Tyr658Val triple KIX mutant (KIX^{ΔMybsite}) were generated with the QuikChange site directed mutagenesis kit (Stratagene). Upon transformation of the KIX-encoding plasmids into *Escherichia coli* BL21 (DE3) cells, recombinant protein expression, uniform ¹³C- and ¹⁵N-isotopic labeling and purification of the KIX constructs were performed in a similar manner to that described previously (32), with the exception that the protein constructs were further purified by size exclusion chromatography on a S75 column in 20 mM MES pH 6.0, 1 mM β-mercaptoethanol. Pooled protein solutions were quantified by absorbance at 280 nm with molar extinction coefficients of 12 950 l·mol⁻¹·cm⁻¹ for KIX / KIX^{ΔPCETsite} and 9970 l·mol⁻¹·cm⁻¹ for KIX^{ΔMybsite}, concentrated to 2–3 mM and stored at –20°C.

DNA for the first 37 residues of E2A, which comprises the PCET KIX-interacting region of E2A AD1, and for E2A AD2–1/2 (E2A residues 394–420) was cloned into a pET21a(+) derived plasmid containing upstream sequences encoding a hexahistidine affinity tag, the B1 domain of protein G and a tobacco etch virus (TEV) protease recognition sequence. The plasmids coding for these His-GB1-E2A fusion proteins were electroporated into the BL21 (DE3) strain of *E. coli*. Cultures were grown in either LB or ¹⁵N- or ¹³C/¹⁵N-enriched M9 media and expression was induced at an optical density of 0.6 by addition of isopropyl β-D-1 thiogalactopyranoside (IPTG) to final concentration of 0.5 mM. Growth was continued for 4 h at 37°C or overnight at 23°C for the His-GB1-E2A AD1(1–37) and His-GB1-E2A AD2–1/2 constructs, respectively. Cell pellets from 1 l of cultures were resuspended in 20 ml of lysis buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl) and heated to 80°C for 15

min. The resulting lysate was clarified by centrifugation, purified by Ni²⁺-affinity chromatography and dialyzed extensively into 20 mM MES pH 6.0, 1 mM β-mercaptoethanol. To generate the unfused E2A AD2–1/2 construct, His-GB1-E2A AD2–1/2 was dialyzed into cleavage buffer (20 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol), cleaved with TEV protease (1:200 molar ratio with His-GB1-E2A AD2–1/2) overnight at 4°C, applied to Ni²⁺-charged resin to remove the His-GB1 fragment, and subsequently to a Vydac C18 reversed phase high performance liquid chromatographic column. Peptide-containing fractions were pooled, lyophilized and stored at –20°C.

Fluorescence anisotropy titration experiments

Fluorescence anisotropy titrations were performed in filtered 20 mM MES pH 6.0, 1 mM β-mercaptoethanol. Wild-type and mutant KIX solutions spiked with 100 nM FITC-labeled E2A peptide were titrated into the same FITC-labeled peptide. Fluorescence anisotropy readings were measured on a Spex Fluorolog Tau-3 spectrofluorometer (Horiba Jobin Yvon Inc.). The excitation (λ_{ex}) and emission (λ_{em}) wavelengths used were 492 nm (bandpass of 2 nm) and 523 nm (bandpass of 5 nm), respectively, with a photomultiplier voltage of 950 V and integration time of 10 s for each reading. Three readings were taken after each injection of titrant and averaged. The data were fitted to the quadratic solution to a one-site binding model with an additional linear term added to account for non-specific binding (34). Experiments were performed in duplicate with the mean ± standard error reported.

NMR spectroscopy

All NMR experiments were performed on a Varian INOVA 500 MHz NMR spectrometer equipped with triple resonance cryoprobe at 25°C on samples prepared in 20 mM MES pH 6.8, 1 mM β-mercaptoethanol, 90% H₂O/10% D₂O. A titration of 200 μM ¹³C/¹⁵N-labeled KIX with 0–5 equivalents of unlabeled E2A AD2–1 was monitored by collection of a 2D ¹H-¹⁵N HSQC spectrum after each incremental addition. NMR samples comprising 600 μM ¹³C/¹⁵N-labeled KIX in the absence and presence of 2 mM E2A AD2–1 were prepared and the following experiments collected to allow assignment of backbone chemical shifts of the free KIX and KIX in complex with E2A AD2–1: ¹H-¹⁵N HSQC, HNCACB, CBCA(CO)NH and ¹⁵N-NOESY-HSQC (100 ms mixing time). Backbone amide group chemical shift perturbations were calculated as previously described (43).

The NMR-based competition experiment assessing the displacement of E2A AD2–1/2 by E2A AD1-PCET involved a sample of 100 μM ¹⁵N-labeled E2A AD2–1/2 and 300 μM unlabeled KIX in 20 mM MES pH 6.8, 1 mM β-mercaptoethanol, 90% H₂O/10% D₂O. This sample was subsequently titrated with the PCET-containing unlabeled His-GB1-E2A AD1(1–37) construct to a final concentration of 1.3 mM. Backbone ¹H, ¹⁵N, C^α and C^β chemical shifts for recombinant E2A AD2–1/2 obtained by collection and analysis of ¹H-¹⁵N HSQC, HNCACB, CBCA(CO)NH, HNCACO and HNCO experiments. The

data was processed and analyzed with NMRPipe and NMRView, respectively (44,45).

Molecular modeling

A sequence alignment of ADs known to bind KIX, including E2A AD1-PCET, E2A AD2-1, MLL and p53, was performed using Praline (46). The alignment was used as input in Modeller 9.9 (47) along with the minimized average structure of the E2A AD1-PCET:KIX complex structure (PDB: 2KWF; (32)) to generate a E2A AD2-1:KIX complex structural model. Figures of the model were generated with PyMOL (48).

Transfections and transcriptional activity assays

Plasmids conferring mammalian expression of wild-type and mutant GAL4-E2A(1-483) fusion proteins were assembled as previously described (20). E2A mutations were generated using the QuikChange site directed mutagenesis kit (Stratagene). Transfections were performed by the calcium phosphate precipitation method using SV293T cells seeded at 8×10^4 cells/well in a 12-well tissue culture plate as previously described (36) except for the quantities of plasmid DNA used (0.2 μ g/well pCMV-GAL4 construct, 0.7 μ g/well p5xGAL luciferase reporter and 0.1 μ g/well pCMV-Renilla internal control). For each Gal4-E2A(1-483) fusion construct, at least three independent transfections were performed in 12-well tissue culture plates and the average of the *Renilla* normalized luminescence values was reported; error bars indicate the standard deviation. Statistical significance was measured using one-way ANOVA with Games-Howell post hoc test and a significance threshold of a one-tailed *P* value < 0.05.

Retroviral transduction and bone marrow immortalization assays

The cDNA encoding E2A-PBX1b was ligated into a GFP (green fluorescence protein)-expressing pMIEV retroviral backbone plasmid using *NotI* and *SalI* restriction sites. Generation of the E2A-PBX1b Leu20Ala, Leu20Ala/Ala400Leu and Leu20Ala/His402Ala mutants was performed using a polymerase chain reaction-based method with *Pfu* Ultra DNA polymerase from Stratagene, as described previously (49). Virus was generated as previously described (32) and the viral titre measured by infecting NIH 3T3 fibroblasts with serial dilutions of viral supernatant and assessing GFP and E2A-PBX1b expression by flow cytometry of live cells. Relative expression of recombinant proteins was determined by immunoblotting cell lysates using a 1/1000 dilution of anti-E2A mouse monoclonal antibody (Yae, Santa Cruz Biotechnology) and a 1/2000 dilution of mouse monoclonal anti-GFP (Roche). Immortalization assays were performed in a similar manner to that described previously (32). At 45 days post-transduction the cells were stained with anti-CD11b-PE, anti-F4/80-PECy7 and anti-Gr1-PECy5 (BioLegend) and analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter). Data analysis was performed with FlowJo 7 (Treestar).

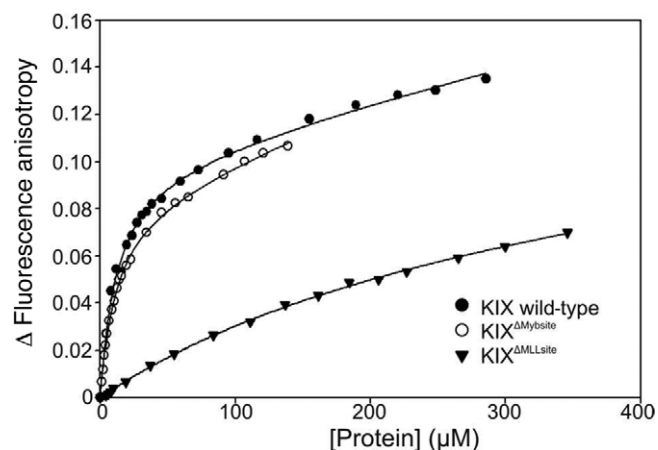


Figure 2. E2A AD2 binding to KIX is unaffected by disruption of the cMyb-binding site. Representative fluorescence anisotropy titration binding curves of FITC-labeled E2A AD2-1 peptides with wild-type KIX or the KIX Δ Myb site and KIX Δ PCET site mutants in which the c-Myb binding site (Tyr650Ala/Ala654Gln/Tyr658Val) or MLL binding site (Phe612Ala/Asp622Ala/Arg624Ala/Lys667Glu) are disrupted, respectively.

RESULTS

E2A AD2 binds KIX through a single ϕ -x-x- ϕ - ϕ sequence

Since AD2 can interact with the KIX domain of CBP, and KIX binding by several proteins is mediated by ϕ -x-x- ϕ - ϕ sequences (20,21,32,50), we inspected the peptide sequence of AD2 for ϕ -x-x- ϕ - ϕ sequences (20,21,32,50). Two such sequences were identified at positions 397-401 (Leu-Asp-Glu-Ala-Ile) and 415-419 (Met-His-Thr-Leu-Leu) of E2A; similar ϕ -x-x- ϕ - ϕ containing sequences were observed in AD2 of related HEB and E2-2 proteins (Figure 1). The presence of these sequences in tandem suggested that the corresponding regions in the E-proteins could bind to the KIX domain simultaneously and perhaps in a cooperative manner similar to that previously observed for p53 and FOXO3a (35,50).

Fluorescence anisotropy-based titrations indicated that synthetic peptides corresponding to residues 394-407 and 408-420 of E2A, termed AD2-1 and AD2-2, respectively, bound to the KIX domain with affinities of 17 μ M and 283 μ M (Figure 2; Table 1). A peptide encompassing both ϕ -x-x- ϕ - ϕ containing sequences from E2A AD2 (AD2-1/2; residues 394-420 of E2A) displayed an affinity for the KIX domain (21 μ M) very similar to that observed for E2A AD2-1 (Table 1), indicating that the E2A AD2-1 sequence was the region responsible for KIX recognition and that the adjacent ϕ -x-x- ϕ - ϕ containing E2A AD2-2 sequence did not contribute to a cooperative mode of binding to the KIX domain of CBP/p300. The presence of a C-terminal tyrosine residue on the E2A AD2-1 peptide for quantitation in the NMR studies described below had no significant effect on KIX-binding affinity ($21 \pm 5 \mu$ M; data not shown).

Table 1. Affinity of E2A AD1 and AD2 for KIX

Ligand	K_d (μ M)
(i) E2A peptides titrated with the KIX domain in 20 mM MES, pH 6.0. 1 mM β -mercaptoethanol	
E2A AD1 (residues 11–24)	5.2 \pm 0.9
E2A AD2–1 (residues 394–407)	17 \pm 4
E2A AD2–2 (residues 408–420)	283 \pm 5
E2A AD2–1/2 (residues 394–420)	21 \pm 3
E2A AD2–1 Leu397Ala	102 \pm 10
E2A AD2–1 Ala400Leu	7.2 \pm 0.8
E2A AD2–1 Ile401Ala	106 \pm 13
E2A AD2–1 His402Ala	13.5 \pm 0.1
E2A AD2–1 Leu404Ala	86 \pm 5
(ii) Titration of KIX ^{ΔMyb site} into E2A	
E2A AD1 (residues 11–24)	3.7 \pm 0.2
E2A AD2–1 (residues 394–407)	13.9 \pm 0.8
(iii) Titration of KIX ^{ΔPCET site} into E2A	
E2A AD1 (residues 11–24)	77 \pm 4
E2A AD2–1 (residues 394–407)	250 \pm 70

E2A AD2–1 binds KIX specifically at a site similar to E2A AD1-PCET and MLL

Two binding surfaces have been characterized on the KIX domain. Whereas most KIX-interactive partners, including AD1-PCET of E-proteins and the AD of MLL, recognize a deep hydrophobic cleft between helices H2 and H3 of KIX, the ADs of c-Myb and phospho-cAMP response element binding protein (CREB) interact with a shallow hydrophobic groove on the opposite face of KIX (31,32,51,52). Fluorescence anisotropy and NMR spectroscopy were employed to delineate whether E2A AD2–1 was engaging the E2A AD1-PCET/MLL site (31,32) or the c-Myb/CREB site (31,51,52) on the KIX domain. A KIX ^{Δ Myb site} mutant and a KIX ^{Δ PCET site} mutant were generated to disrupt binding selectively at the c-Myb and the E2A AD1-PCET/MLL binding sites, respectively (53,54). In fluorescence anisotropy titrations, the KIX ^{Δ Myb site} mutant bound E2A AD2–1 with the same affinity to that of the native KIX domain whereas the KIX ^{Δ PCET site} mutant displayed a 15-fold decrease in affinity for E2A AD2–1 (250 μ M versus 17 μ M; Figure 2, Supplementary Figure S1, Table 1).

NMR-based chemical shift perturbation mapping studies were performed to more clearly define the E2A AD2–1 binding site on the KIX domain and complement the KIX mutagenesis studies. Comparison of the backbone amide proton and ¹⁵N chemical shifts of the KIX domain in the absence and presence of E2A AD2–1 on a per-residue basis revealed those residues displaying significant chemical shift changes (greater than 1 standard deviation above the mean chemical shift change; Figure 3, Supplementary Figure S2).

Mapping of the significantly affected residues onto the KIX domain from our recently reported NMR solution structure of the E2A AD1-PCET:KIX complex (32) showed that the E2A AD2–1 binding surface localized to a hydrophobic cleft between helices H2 and H3 and buttressed by the intervening L₁₂ loop and G2₃₁₀-helix (Figure 4a and b). This region corresponded to the binding site of E2A AD1-PCET as well as to that of the ADs of MLL, p53, FOXO3a, c-Jun and HTLV-Tax (Figure 4d) (31–33,35,50,55,56). No significant chemical shift perturbations

were observed on the opposite face of the KIX domain corresponding to the c-Myb/CREB binding site (Figure 4c and e), even in the presence of a significant excess of the E2A AD2–1 peptide (7-fold greater than KIX).

To directly assess whether E2A AD1-PCET and E2A AD2–1 competed for the same binding on the KIX domain, an NMR-based *in vitro* displacement experiment was performed. Backbone ¹H-¹⁵N resonances corresponding to the AD2–1 region (residues 394–407) of the ¹⁵N-E2A AD2–2/1 construct displayed significant chemical shifts in the presence of unlabeled KIX domain consistent with complex formation (Figure 5a). Upon sequential additions of the unlabeled PCET-containing E2A AD1(1–37) fragment to this sample, those backbone ¹H-¹⁵N resonances corresponding to the AD2–1 region of the ¹⁵N-E2A AD2–2/1, which displayed KIX-induced changes, returned to chemical shift values consistent with the free form of the E2A AD2–2/1 peptide (Figure 5b).

Modeling of the E2A AD2:KIX complex

A structural model of the E2A AD2–1:KIX complex was generated using the E2A AD1-PCET:KIX complex structure (PDB: 2KWF; (32)) as a template in Modeller (47) to aid in deciphering the molecular basis of this interaction (Figure 6a and b). The model suggested that the E2A AD2–1:KIX interaction involved numerous hydrophobic interactions, particularly along one face of the helical E2A AD2–1 peptide that is buried in the hydrophobic cleft on the KIX domain (Figure 6b). The hydrophobic residues Leu397, Ala400 and Ile401 within the ϕ -x-x- ϕ - ϕ sequence of E2A AD2–1 were predicted to participate in extensive contacts with the KIX domain. These include interactions of Leu397 of E2A AD2–1 with non-polar side chains on the α 2 and α 3 helices of the KIX domain, Ala400 with non-polar side chains located on the α 1 and α 2 helices and Ile401 with non-polar side chains on the α 3 helix of the KIX domain. Additionally, Leu404 was positioned to form hydrophobic interactions with non-polar residues within the L₁₂ loop and the α 3 helix. Overall, these results are consistent with the fluorescence anisotropy data, in which the KIX ^{Δ Myb site} mu-

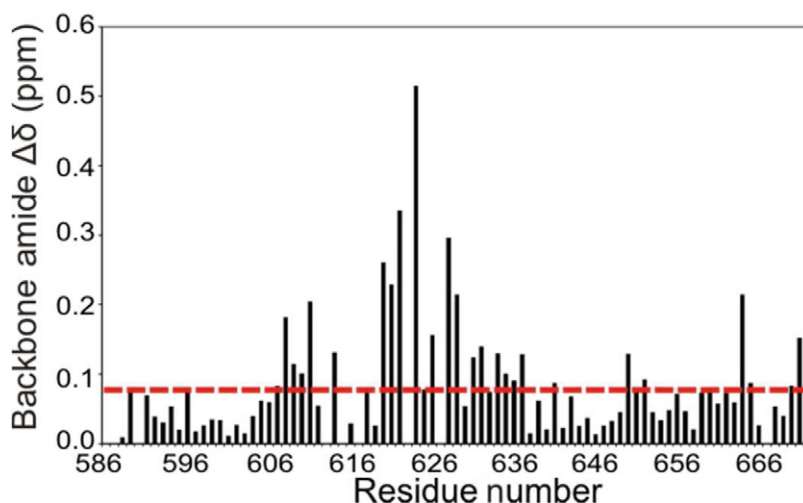


Figure 3. NMR analysis of the E2A AD2-1:KIX interaction. Plot of average backbone amide chemical shift changes ($\Delta\delta$) versus the KIX sequence induced by the binding of the E2A AD2-1 peptide. The backbone amide resonances for Arg623, Asn627 and Lys667 broadened out upon E2A AD2-1 binding and could not be assigned in the bound spectrum. Chemical shifts were calculated using the formula $\Delta\delta = [(0.17\Delta\delta_N)^2 + (\Delta\delta_{HN})^2]^{1/2}$, as previously described (43). The dashed line indicates the mean average chemical shift change.

tant bound E2A AD2-1 with wild-type affinity while the KIX ^{Δ PCETsite} mutant had a significantly lower affinity for E2A AD2-1, and indicate that E2A AD2-1 binds to the surface on the KIX domain that is also bound by E2A AD1-PCET.

Affinity of the E2A AD2-1:KIX interaction correlates with E2A transcriptional activity

To investigate the functional implications of the E2A AD2-1:KIX interaction, the effect of alanine substitution of Leu397 (Leu397Ala) and Ile401 (Ile401Ala) at the N- and C-terminus of the E2A AD2-1 ϕ -x-x- ϕ - ϕ sequence and His402 and Leu404, which lie C-terminal to this sequence, was tested in *in vitro* binding and transcriptional activation assays (Figure 6c and d). In fluorescence anisotropy titration experiments, the Leu397Ala and Ile401Ala E2A AD2-1 mutants each displayed a 6-fold decrease in affinity for the KIX domain while the Leu404Ala mutation decreased the affinity of E2A AD2-1 for the KIX domain by approximately 5-fold (Figure 6c, Table 1). The His402Ala E2A AD2-1 mutant, which was generated as a negative control based on its largely exposed position in the structural model, did not significantly alter the affinity of the E2A AD2-1:KIX interaction (Figure 6c, Table 1).

The presence of a small hydrophobic residue at the fourth position of the ϕ -x-x- ϕ - ϕ motif in E2A AD2-1 (Ala400), our previous observation that an alanine substitution at this position in the E2A AD1-PCET motif (Leu19Ala) impaired the ability of E2A (1-483) to pull down CBP/p300 (36), and the structural model of the E2A AD2-1:KIX complex together suggested that the affinity of this interaction could be enhanced by increasing the hydrophobicity at this position in E2A AD2-1. Consistent with this hypothesis, an Ala400Leu E2A AD2-1 mutant peptide bound to the KIX domain with an affinity greater than twice that of the native E2A AD2-1 sequence (7 μ M versus 17 μ M; Table 1).

The consequences of these amino acid substitutions on the transcriptional activation of E2A (1-483) were assessed in a cell-based reporter gene assay (Figure 6d). A mammalian expression plasmid was engineered to express E2A (1-483) fused to the DNA-binding domain of GAL4. This plasmid was transiently co-transfected into SV293T cells with a plasmid containing a firefly luciferase reporter gene regulated by multiple GAL4 binding sites. Deletion of the AD1-PCET motif (Δ 16-23) reduced E2A-driven expression to 22% of wild-type E2A (1-483) (Figure 6d). Mutations affecting E2A AD2-1 were evaluated in the context Δ 16-23 in order to determine their effects in an E2A construct whose transcriptional function relies predominantly on AD2. E2A AD2 Leu397Ala, Ile401Ala or Leu404Ala substitutions further reduced activity to less than 5% of that seen with wild-type E2A (1-483) (Figure 6d). In contrast, the Ala400Leu or His402Ala substitutions partially restored activity to 69% and 60%, respectively, of that observed with wild-type E2A(1-483). These substitutions largely compensated for the impairment of AD1 function caused by Δ 16-23, with the Ala400Leu mutation restoring activity to levels that were not significantly less than that observed for wild-type E2A(1-483) ($P = 0.09$) while the His402Ala mutation restored activity to levels only marginally less than wild-type E2A(1-483) ($P = 0.04$). The observed effects on E2A transcriptional activity are approximately commensurate with the effects on KIX-binding affinity produced by the same mutations, which supports the functional relevance of the contact points predicted by the E2A AD2-1:KIX model. These findings also suggest that affinity for the KIX domain is a major determinant of transcriptional activation by E2A.

Affinity of the E2A AD2-1:KIX interaction correlates with immortalization of primary bone marrow cells by E2A-PBX1

Several studies have shown that E2A AD1 is required for E2A-PBX1-mediated oncogenesis whereas E2A AD2 is dis-

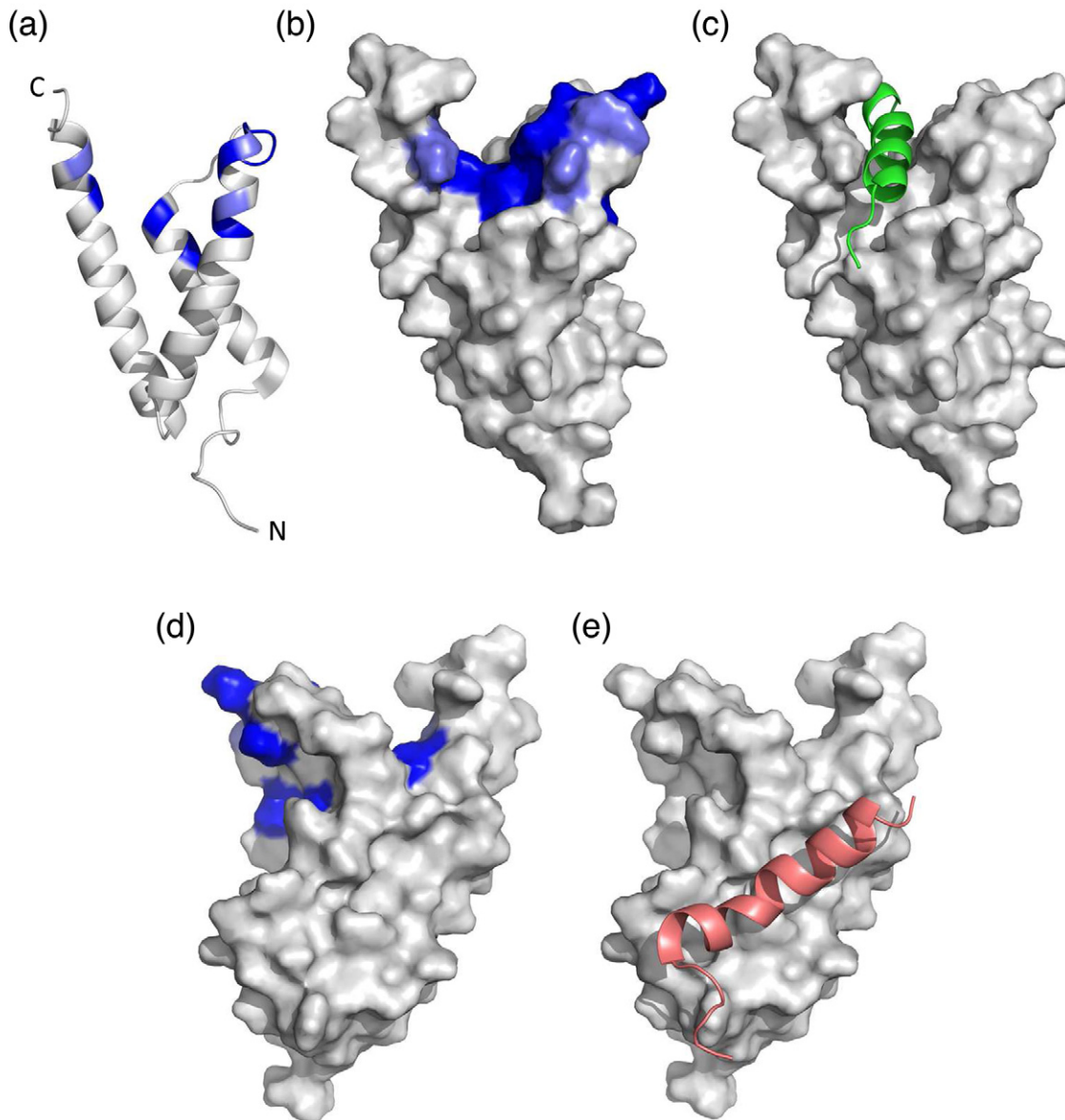


Figure 4. E2A AD2-1 binds to the KIX domain at the E2A PCET/MLL site. (a) Ribbon representation of the KIX domain from the E2A AD1-PCET:KIX complex (PDB: 2KWF; (32)) with those residues perturbed by more than 1 standard deviation above average chemical shift change upon saturation with E2A AD2-1 colored dark blue and resonances of residues that could not be assigned upon saturation due to peak broadening colored light purple. The E2A AD1-PCET peptide was removed for clarity. (b) Surface representation of the KIX domain illustrated in panel (a). (c) E2A AD1-PCET:KIX complex (PDB: 2KWF; (32)) in which the KIX surface and a cartoon representation of E2A AD1-PCET (in green) are shown to illustrate its location on the KIX domain. (d) Surface representation of the KIX domain depicted in panel (b) rotated 180°. (e) Cartoon representation of the c-Myb peptide (pink) depicted on the surface of the KIX domain from the MLL:KIX:c-Myb complex (PDB: 2AGH; (31)).

pensable (20,36,57). The differential contribution by these two ADs could be attributable to uncharacterized qualitative differences in their functions, as reflected, for example, in their differential activity across different cell types (13,21). Alternatively, our current results raise the possibility that the lesser oncogenic role of E2A AD2 may be explained more simply by its lower affinity for the KIX domain in CBP/p300. To investigate the possibility of a latent relationship between E2A AD2-mediated recruitment of CBP/p300 and E2A-PBX1-driven oncogenesis, amino acid substitutions that increased the affinity of E2A AD2 for the

KIX domain were engineered into full-length E2A-PBX1 in the context of a substitution (namely Leu20Ala) that disrupts KIX binding to E2A AD1 and abrogates immortalization of primary bone marrow cells by E2A-PBX1 (36). Primary mouse bone marrow cells infected with a retrovirus that confers expression of E2A-PBX1 and then maintained *ex vivo* in the presence of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) proliferated exponentially and continuously after a brief latency period, whereas cells infected with either the vector control or the Leu20Ala-substituted E2A-PBX1 mutant de-

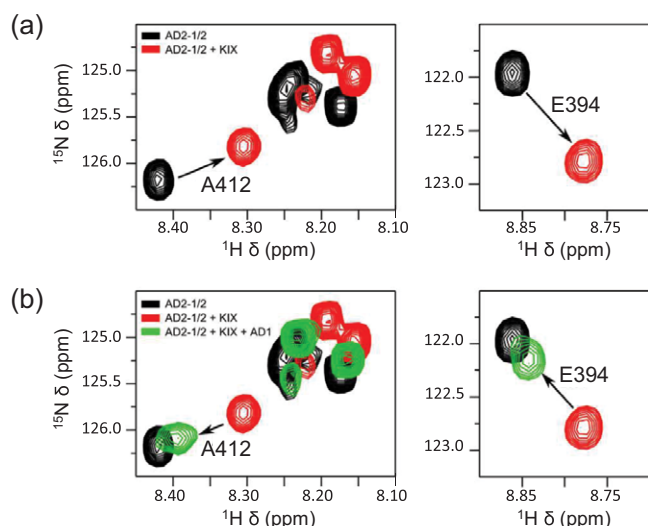


Figure 5. E2A AD1-PCET and E2A AD2-1 compete for binding to the same site on the KIX domain *in vitro*. (a) Overlay of two selected regions from 2D ^1H - ^{15}N HSQC spectra of 100 μM ^{15}N -labeled E2A AD2-1/2 in the absence (black) and presence of 300 μM unlabeled KIX (red). (b) Overlay of same two selected regions depicted in (a) from 2D ^1H - ^{15}N HSQC spectra of 100 μM ^{15}N -labeled E2A AD2-1/2 in the absence (black) and presence of 300 μM unlabeled KIX (red) and in the presence of 300 μM unlabeled KIX after addition of 1.3 mM E2A AD1(1-37) (green). Some residues of E2A AD2-1/2 whose backbone amide resonances undergo significant ^1H and ^{15}N chemical shifts in the presence of KIX and are subsequently displaced by the addition of E2A AD1(1-37) are indicated by black arrows, single-letter amino acid code and their respective position in full-length E2A, respectively.

clined rapidly after 2 weeks (Figure 7a). Cells infected with the Leu20Ala/Ala400Leu E2A-PBX1 mutant proliferated persistently for over 5 weeks, albeit at a lower rate than those infected with wild-type E2A-PBX1, and appeared established in culture (Figure 7a). Unlike cells that had been immortalized with wild-type E2A-PBX1, which were predominantly non-adherent, the cells expressing the Leu20Ala/Ala400Leu E2A-PBX1 mutant rapidly segregated after each passage into non-adherent and adherent sub-populations with the latter forming a confluent monolayer of fusiform cells. Characterization by flow cytometry indicated that the cells immortalized with wild-type E2A-PBX1 manifested an immunophenotype (CD11b^+ F4/80^+ Gr-1^-) characteristic of granulocyte/macrophage progenitors (Figure 7b). The immunophenotype of cells expressing the Leu20Ala/Ala400Leu E2A-PBX1 mutant depended on their adherence status. Whereas the non-adherent population included many cells with evidence of granulocytic differentiation (F4/80^- Gr-1^+), the adherent cells included a distinct and prevalent population of cells with macrophage features (F4/80^+ $\text{Gr-1}^{+/-}$). This experiment was carried out twice with equivalent results. These results suggest that Leu20Ala/Ala400Leu E2A-PBX1 mutant is less effective than the wild-type oncoprotein in blocking GM-CSF-induced differentiation of hematopoietic progenitors to granulocytes and macrophages. More generally, these functional results based on primary mouse bone marrow cells show that increasing the affinity of E2A AD2 for the KIX domain of CBP/p300 to one approaching the affin-

ity of the E2A AD1-PCET:KIX interaction uncovered a latent potential of E2A AD2 to mediate potentially oncogenic transcriptional effects of E2A-PBX1.

DISCUSSION

The second AD of E2A, AD2, has previously been implicated in the regulation E2A-mediated transcription induction and lymphoid development; roles consistent with its ability to interact with CBP/p300 (20–22). The presence of two tandem ϕ -x-x- ϕ motifs within AD2 of E2A, as well as HEB and E2-2, suggested the hypothetical possibility of cooperative binding to the two well-characterized binding surfaces on the KIX domain. However, our findings demonstrate that a region comprising only the more N-terminal of these motifs in E2A AD2 (397-Leu-Asp-Glu-Ala-Ile-401) binds KIX with micromolar affinity (Table 1), and that this motif binds to the same hydrophobic cleft on the KIX domain as that targeted by the ϕ -x-x- ϕ containing PCET motif of E2A AD1 (Figure 4). Furthermore, the observation that disruption of the c-Myb/CREB binding site did not impair E2A binding and that this surface is on the opposite face of the KIX domain relative to that bound by the E2A domains (Figure 2; (51,52)) and AD1 and AD2 directly compete for the same binding site on the KIX domain (Figure 5) rules out any potential participation of the c-Myb/CREB site in simultaneous binding to KIX by multiple ϕ -x-x- ϕ motifs on E2A. Finally, structures of the AD1-PCET:KIX (32), MLL:KIX:c-Myb (31), FOXO3a:KIX (35) and MLL:KIX:pKID (58) complexes have a deep binding cleft, which we have shown here to recognize E2A AD2, with a contiguous hydrophobic surface and narrow apex formed by helices 2 and 3 and intervening L_{12} loop and G2 3_{10} -helix of the KIX domain. The extensive contacts between the KIX domain and helical ADs at this site in the complex structures suggest that, without a significant conformation change yet to be observed for the KIX domain, this binding site could only occupy a single binding ϕ -x-x- ϕ motif thereby ruling out cooperative binding of the E2A ADs at the same site; a hypothesis support by our NMR-based competition experiments (Figure 5). Thus, our findings provide support and a structural basis for the functional redundancy observed for E2A AD1 and AD2 (20–22).

Our findings are inconsistent with demonstrated ability of E2A AD1 and AD2-1 to induce reporter gene expression and bind to CBP/p300 in a greater-than-additive manner (20,21). CBP/p300 comprises several domains in addition to KIX, including the TAZ1, TAZ2/CH3 and NCBD/IBiD domains, which have been shown to participate protein-protein interactions, most notably with ADs of various transcription factors (27). For example, the two N-terminal transcriptional ADs of p53 can bind the KIX, TAZ1, TAZ2 and NCBD domains of CBP/p300 (34,50,59–61); interactions which are modulated phosphorylation (50,60,62–64). In the case of E2A, we have shown that the KIX domain is absolutely required for binding of full-length CBP to E2A 1–483, as determined using purified proteins in a pull-down experiment (37). However, it remains possible that elements within AD1 or AD2 of E2A can also interact with these other domains of CBP/p300 in conjunction with the

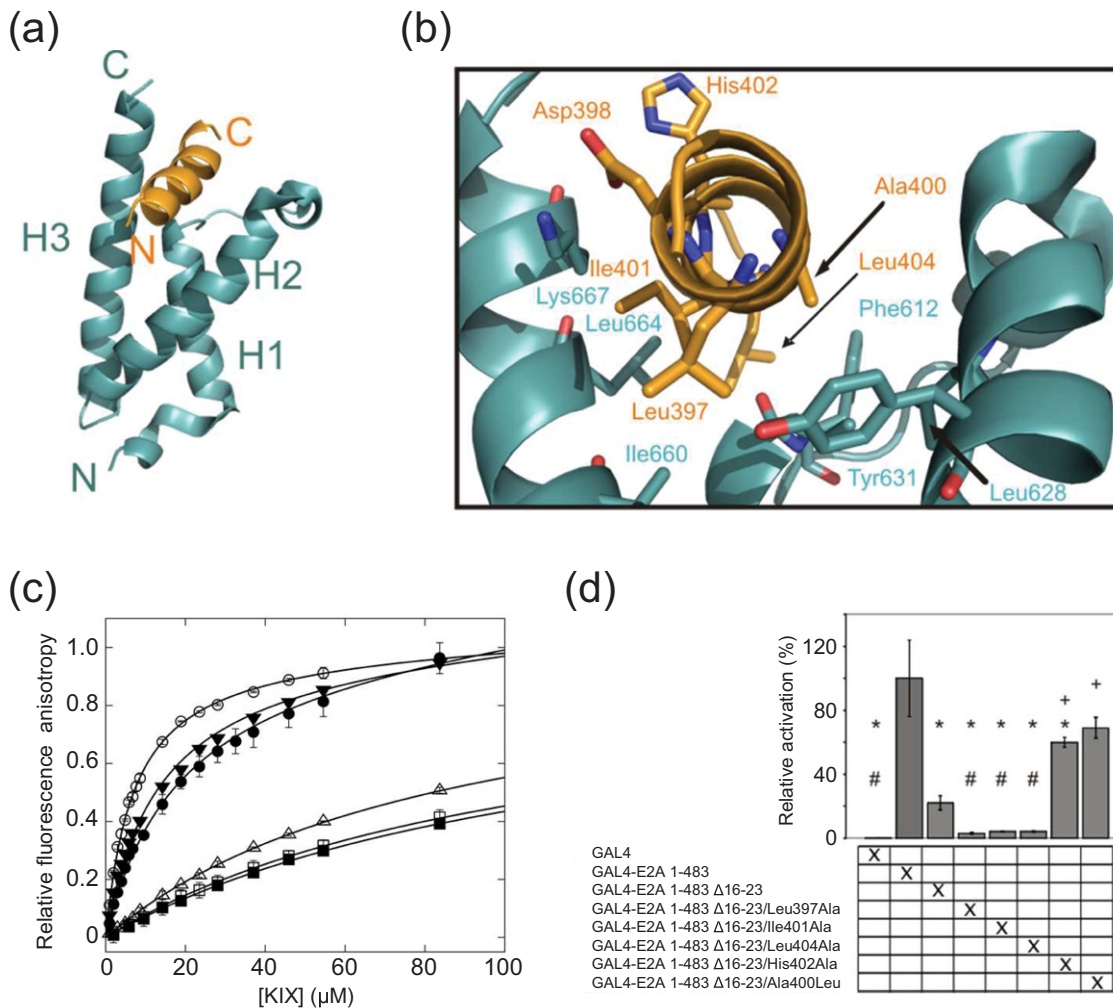


Figure 6. E2A AD2-1 residues critical to KIX domain recognition. **(a)** Backbone ribbon representation of a Modeller-generated structural model of the E2A AD2-1 peptide (orange) bound to the KIX domain (teal). The three α -helices of KIX are indicated, as are the N and C termini of both proteins. **(b)** The E2A AD2-1:KIX interaction surface from the model of the complex with stick representations of the side chains of residues that are potentially forming contacts. **(c)** Fluorescence anisotropy titrations of FITC-labelled E2A AD2-1 mutant synthetic peptides (●, AD2-1 wild-type; ○, AD2-1 Ala400Leu; ▼, AD2-1 His402Ala; △, AD2-1 Leu404Ala; ■, AD2-1 Leu397Ala; □, Ile401Ala) informed by the model in (b) with recombinant KIX domain, where the curves are fitted to the average values of each titration point from three replicate experiments and error bars represent the standard error of each titration point among the replicate experiments. **(d)** The impact of E2A AD2-1 residue-specific substitutions listed in (c) on transcriptional activation of a Gal4-responsive luciferase reporter gene in HEK293T cells was tested using wild-type or the abovementioned mutant Gal4-E2A 1-483 constructs.

KIX domain, which could account for the apparent cooperativity of AD1 and AD2 with respect to CBP/p300 recruitment. Given that E2A is a target of phosphorylation and acetylation (38,65–70), including at the N-terminus and central region where AD1 and AD2 are located, respectively, these post-translational modifications could alter the binding affinities and specificities for the various domains of CBP/p300.

CBP/p300 is typically recruited to enhancers or promoters through promiscuous multivalent interactions with multiple transcription factors that typically cluster on DNA due to the proximity of their binding sites (27). The ϕ -x-x- ϕ - ϕ motifs in AD1 and AD2 of E2A that we have shown to interact with the KIX domain could bind simultaneously to two molecules of CBP/p300 or bind KIX simultaneously and potentially cooperatively with different transcriptional partners that interact with the c-Myb/CREB binding sur-

face on the opposite face of the KIX domain. Furthermore, it remains possible that the ϕ -x-x- ϕ - ϕ motifs of E2A recognize other, as yet unidentified transcriptional co-regulators. Our observations also suggest that the transcriptional regulatory functions of isoforms of E-proteins that lack AD1, such as HEBAlt and E2-2Alt, likely involve the recruitment by AD2 of CBP/p300 through the KIX domain (8,10,29).

Recent genome-wide studies have identified thousands of E2A binding sites in B-lymphoid progenitor cells most of which appear to fall within transcriptional enhancers (17). Since enhancers may be operationally defined by their ability to recruit CBP/p300 (71), it is reasonable to surmise that E2A-mediated recruitment of CBP/p300 contributes to the cell type-specific activation of enhancers and promoters that underlie hematopoietic lineage specification. Consistent with this general concept in relation to normal hematopoiesis, we propose that leukemia induc-

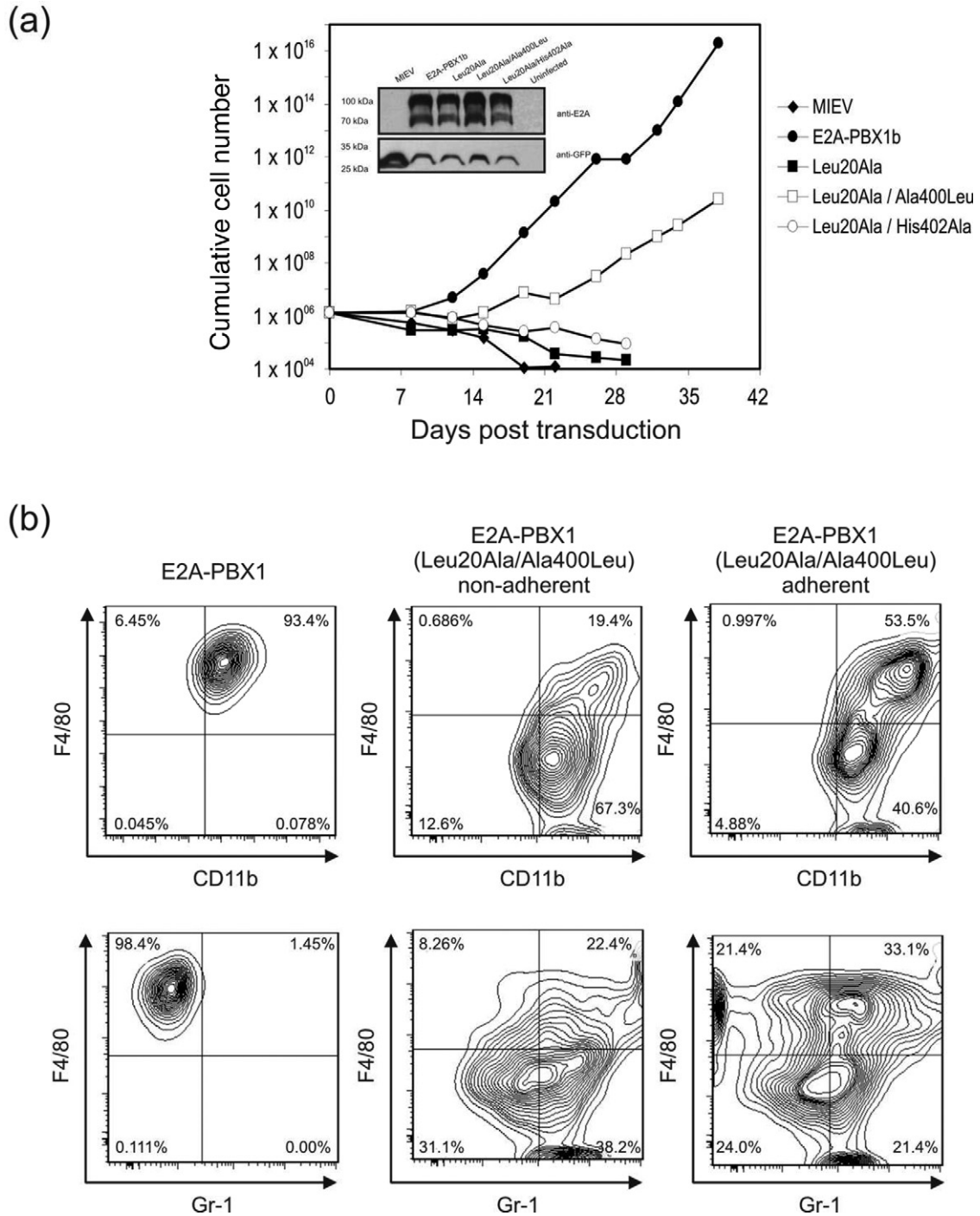


Figure 7. An E2A AD2 Ala400Leu substitution restores immortalization of primary bone marrow cells by AD1-defective E2A-PBX1. (a) Proliferation of hematopoietic progenitors expressing E2A-PBX1 or engineered variant. For each construct, 1.25×10^6 bone marrow cells were seeded initially in GM-CSF-containing medium immediately after retroviral infection and counted for 40 days. (Inset) Western blot of lysates from NIH 3T3 fibroblasts infected with retroviruses conferring expression of the indicated E2A-PBX1b constructs, confirming equivalent viral transduction and expression of recombinant proteins. (b) Flow cytometry-based immunophenotyping of myeloid cells expressing E2A-PBX1 (wild-type or Leu20Ala/Ala400Leu) after 40 days of culture. The cells immortalized with E2A-PBX1 (Leu20Ala/Ala400Leu) were separated into adherent and non-adherent layers for separate analysis and all samples were stained for CD11b, F4/80 and Gr-1.

tion by E2A-PBX1 involves the aberrant redistribution of CBP/p300 and other transcriptional co-regulators across the genome at one or more critical phases of hematopoietic development. This results in the establishment of an abnormal, self-sustaining transcriptional gene regulatory network and a leukemic cellular phenotype characterized by aberrant retention of cellular immaturity and self-renewal potential.

The oncogenic importance of aberrant CBP/p300 recruitment mediated by E2A-PBX1 is supported by our demonstration in the current and previous studies that the oncogenic potency of engineered E2A-PBX1 mutants is predictable based on their affinity for CBP/p300. Whereas we showed previously that amino acid substitutions within the E2A AD1-PCET motif that reduce its affinity for CBP/p300 impair oncogenesis (20,36), we show in the current study that a different substitution (namely Ala400Leu) that increases the affinity of E2A AD2 for CBP/p300 uncovers a latent ability of E2A AD2 to mediate E2A-PBX1 oncogenesis. Indeed, the ability of the individual E2A ADs to recruit CBP/p300 seems to be a better predictor of their oncogenic function than their transcriptional potencies as measured using reporter gene assays. In particular, while deletions within E2A AD2 are more deleterious for trans-activation than deletions within E2A AD1, they have no measurable effect on E2A-PBX1-mediated oncogenesis (20,21,36). We speculate that this discordance reflects the failure of simple reporter assays to accurately model the potentially complex and subtle gene regulatory consequences of aberrantly recruiting CBP/p300 to potentially numerous *cis* regulatory elements and perhaps away from others across the genome.

The binding affinities of the E2A AD1 (K_d 5.2 μ M), Ala400Leu-substituted E2A AD2 (K_d 7.2 μ M) and His402Ala-substituted E2A AD2 (K_d 13.5 μ M) for the KIX domain correlate well with the ability of full-length E2A-PBX1 to immortalize primary myeloid cells (Table 1 and Figure 6). The relatively modest growth rate, the emergence of adherent macrophages and the presence of immunophenotypic features indicating more advanced granulocytic and macrophage differentiation manifested by cells expressing Ala400Leu-substituted E2A-PBX1 suggest attenuated oncogenic potency attributable to the marginal affinity of this mutant for the KIX domain and CBP/p300. These results also tend to validate the use of biophysical studies with E2A fragments to model and predict functionally important interactions between full-length E2A-PBX1 and CBP/p300.

The data presented here confirm that AD1 and AD2 of E2A both bind the KIX domain at the same site, a relatively small hydrophobic cleft distinct from where transcription factors including c-Myb and CREB bind (51,52). Given the importance of this interaction in oncogenesis by E2A-PBX1, inhibition binding to this cleft on the KIX domain could prove a useful therapeutic approach in E2A-PBX1-associated leukemia.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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