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Lyl1 interacts with CREB1 and alters expression of CREB1 target genes ☆

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Abstract

The basic helix-loop-helix (bHLH) transcription factor family contains key regulators of cellular proliferation and differentiation as well as the suspected oncoproteins Tal1 and Lyl1. Tal1 and Lyl1 are aberrantly over-expressed in leukemia as a result of chromosomal translocations, or other genetic or epigenetic events. Protein-protein and protein-DNA interactions described so far are mediated by their highly homologous bHLH domains, while little is known about the function of other protein domains. Hetero-dimers of Tal1 and Lyl1 with E2A or HEB, decrease the rate of E2A or HEB homo-dimer formation and are poor activators of transcription. In vitro, these hetero-dimers also recognize different binding sites from homo-dimer complexes, which may also lead to inappropriate activation or repression of promoters in vivo. Both mechanisms are thought to contribute to the oncogenic potential of Tal1 and Lyl1. Despite their bHLH structural similarity, accumulating evidence suggests that Tal1 and Lyl1 target different genes. This raises the possibility that domains flanking the bHLH region, which are distinct in the two proteins, may participate in target recognition. Here we report that CREB1, a widely-expressed transcription factor and a suspected oncogene in acute myelogenous leukemia (AML) was identified as a binding partner for Lyl1 but not for Tal1. The interaction between Lyl1 and CREB1 involves the N terminal domain of Lyl1 and the Q2 and KID domains of CREB1. The histone acetyl-transferases p300 and CBP are recruited to these complexes in the absence of CREB1 Ser 133 phosphorylation. In the Id1 promoter, Lyl1 complexes direct transcriptional activation. We also found that in addition to Id1, over-expressed Lyl1 can activate other CREB1 target promoters such as Id3, cyclin D3, Brca1, Btg2 and Egr1. Moreover, approximately 50% of all gene promoters identified by ChIP-chip experiments were jointly occupied by CREB1 and Lyl1, further strengthening the association of Lyl1 with Cre binding sites. Given the newly recognized importance of CREB1 in AML, the ability of Lyl1 to modulate promoter responses to CREB1 suggests that it plays a role in the malignant phenotype by occupying different promoters than Tal1.

Keywords: Acute myelogenous leukemia; Gene expression; Helix-loop-helix transcription factor; Lyl1; Tal1; CREB1

1. Introduction

The family of helix-loop-helix (HLH) transcription factors (TFs) contains key regulators of lymphocyte development and maturation such as E2A (TCF3), Id1, and Tal1/SCL /TCL5 [1–4]. Two other basic HLH (bHLH) TFs, Lyl1 and Tal2, are structurally closely related to Tal1 but their functions are largely unknown. Tal1 and Lyl1 were initially identified as over-expressed proteins arising from chromosomal translocations in T-cell acute lymphoblastic leukemia (T-ALL). This suggested their possible involvement in the malignant phenotype [5–12]. Tal1 and Lyl1 are also expressed at lower levels in normal tissues, such as CD34+ progenitor cells, fetal liver and HUVEC cells, consistent with their contribution to hematopoiesis. Lyl1 is highly expressed in heart, lung, testis and placenta, where Tal1 expression is low or absent. Tal1 is required for embryonic hematopoiesis as its ablation leads to failure of yolk sac formation and early lethality [13]. In addition, Tal1 is needed for the establishment of definitive hematopoiesis, megakaryopoiesis and short-term repopulating capacity of hematopoetic stem cells (HSC), but not for HSC self-renewal. [13–16]. By contrast, Lyl1 knockout

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animals are viable but display reduced B-cell counts and as well as severe deficits in competitive repopulation assays [17]. The absolute requirement for Tal1 but not for Lyl1 in early hematopoiesis indicates that the two molecules have distinct, rather than redundant cellular functions. This view is also supported by enforced expression studies. Cord blood cells with enforced Tal1 expression are induced to differentiate towards the erythroid lineage while neutrophil and B-cell differentiation are blocked [18]. By contrast, enforced Lyl1 expression promotes differentiation towards both pro-erythrocytic and pro-megakaryocytic paths suggesting effects on earlier progenitors [19].

Differences between Tal1 and Lyl1 phenotypes caused by either over-expression or ablation of the genes cannot be readily explained at the molecular level. The bHLH domains of the two TFs are functionally interchangeable [20] while the remaining, albeit distinct amino-acid sequences, have no ascribed functions. Nevertheless, Tal1 and Lyl1 have distinct *in vitro* binding signatures [21]. By extension, this raises the possibility that different promoters are regulated *in vivo*. Microarray data in T-ALL showed that leukemias with high Tal1 expression have distinct gene expression patterns compared to those with high Lyl1 expression [22].

The cyclic AMP receptor element binding protein 1(CREB1) is a ubiquitously-expressed 43 kDa basic/leucine zipper TF that is critical to a range of physiological processes such as memory, factor-dependent cell survival, and glucose metabolism. CREB1 binds to an eight base pair consensus sequence in the promoters of target genes, by association with itself or with other members of the CREB/ATF family[23–25]. Recruitment of the transcriptional co-activators p300 and CBP to CREB1, requires phoshorylation of CREB1 serine 133 [26]. Over-expression of this phosphorylated CREB1 has been associated with increased growth and survival of leukemic cells, myeloproliferative syndrome with splenomegaly in transgenic mice and increased risk of post-chemotherapy relapse in AML patients [27,28]. Among the identified CREB1 target genes are Id1 and Id3, inhibitors of differentiation that can also promote cellular immortalization, cyclin D3 and cyclin A1 implicated in cellular proliferation, and the anti-apoptotic protein Bcl2, to name a few.

While investigating the factors involved in Id1 promoter regulation in leukemic cells we observed the loss of Lyl1-dependent transcriptional activation caused by mutation of a critical Cre site. This suggested a possible interaction between Lyl1 and CREB1. Since the bHLH motif of Lyl1 regulates interactions with E12/E47 and we observed no direct interaction between this domain and CREB1, we hypothesized that other Lyl1 domains may interact with CREB1. Upon dissection of the Lyl1 molecule we found that the N terminal 140 aa domain participates in protein-protein interactions, in yeast two-hybrid assays (unpublished observations). These observations prompted the present study in which we describe the molecular mechanism of the interaction between Lyl1 and CREB1 and further show that Lyl1 can up-regulate several previously identified CREB1 targets. We find that through its N terminal domain Lyl1 interacts with both Q2 and KID domains of CREB1. The transcriptional co-activators p300 and CBP are recruited to the complex and do not require CREB serine 133 phosphorylation. Unexpectedly, ChIP- chip experiments using a CpG island representation of the human genome revealed that co-regulation by Lyl1 and CREB1 may be widespread, as ~ 50% of all genomic targets identified were common between CREB1 and Lyl1 chips. Since precise timing of CREB1 phosphorylation is essential for the proper execution of cellular programs, part of the oncogenic potential of Lyl1 may involve its ability to untimely recruit p300/CBP co-activators to un-phosphorylated CREB1. Furthermore, since Tal1 does not interact with CREB1, these observations may explain some of the differences between $Tal1^{-/-}$ and $Lyl1^{-/-}$ phenotypes.

2. Materials and methods

2.1. Cell culture and transient transfections

All cell lines were grown in RPMI 1640 media supplemented with 10% FCS and antibiotics. For stable expression, pMIK-HA-Lyl1 or pMIK-HA empty vector was electroporated into K562 cells and antibiotic resistant clones were selected in 0.8 mg/mL G418. DMRIE-C or Lipofectamine and Optimem media (Invitrogen) were used for transient transfection of 1.5 X 10^6 suspension cells or 0.5 X 10^6 adherent cells.

2.2. Plasmids

The pMIK-HA plasmid was supplied by O. Yoshie (Shionogi Institute for Medical Science, Settsu-shi, Osaka 566-0022, Japan) and was previously described [29]. To generate pMIK-HA-Lyl1, full-length human Lyl1 was amplified

Table 1
Primers used for PCR amplification

Procedure	Primers
ChIP	Id1F 5′-CCAGTTTGTCGTCTCCATGG-3′
	Id1R 5′-CTCAGACCGTTAGACGCCAGG-3′
	Id2F 5′-GCCCACACTAAGCCTGTCGT-3′
	Id2R 5′-CAGTTCACTGCAACCCATCG-3′
	Id3F 5′-AACCTCAGCTTCACCGCAAT-3′
	Id3R 5′-CACAGCTCCTCCGAGGTCAT-3′
	Id4F 5′-GCAGGAACTTCGCTCTCTCTTT-3′
	Id4R 5′-ATGGGAAGGGCACTCCATT-3′
	CycD3F 5′-TACATCGTGAGGCTTTCGAG-3′
	CycD3R 5′-CTCTAGTCACCCAGGAAACAATTT-3′
	Egr1F 5′-CTAGGGTGCAGGATGGAGGT-3′
	Egr1R 5′-GCCTCTATTTGAAGGGTCTGG-3′
	Brca1F5′-GCCGCAACTGGAAGAGTAGA-3′
	Brca1R5′-ACAGAAAGAGCCAAGCGTCT-3′
	Btg2F5′-CCTCCTTTCAGAGCTCTCAGTC-3′
	Btg2R5′-CTCGCTGTCGTCAGTGCTA-3′
	BambiF5′-GGGGCACCCAAAAGAAGC-3′
	BambiR5′-CTGCGCGTCAGTGACCTTC-3′
mRNA	Id1F 5′-TCAAGGAGCTGGTGGCCA-3′
	Id1R 5′-GACGTGCTGGAGAATCTCCAC-3′
	Id2F 5′-TGATCATCTTCCCAGGGTGT-3′
	Id2R 5′-AGATTGGGCAATTCCTGGTG-3′
	Id3F 5′-CTTCACCAAATCCCTTCCTG-3′
	Id3R 5′-GCCTTGGCATAGTTTGGAGA-3′
	Id4F 5′-GGATGAGGAAATGCTTGGAT-3′
	Id4R 5′-TCTTTGGAGGAAGGAAAGCA-3′
	Lyl-1 F5′-CCGAAGAAGGACCAGTGAAG-3′
	Lyl-1 R5′-ATGACTGCTCTGCCATGTTG-3′
	GAPDH F 5′-ATCATCAGCGCCTCCTG-3′
	GAPDH R 5′-CTGCTTCACCACCTTGA-3′

from cDNA and inserted into the EcoRI-BglII sites of pMIK-HA vector. VP16-Lyl1-FL contained full-length Lyl1 cloned at 5′ Eco RI and 3′Hind III sites in the pVP16 vector (MATCHMAKER Two-Hybrid Assay Kit from Clontech). Lyl1 deletion mutants were PCR amplified with Pfu (Stratagene) from the full-length plasmid, with primers:

P1 (5-'CCGGAATTCATGACTGAGAAGGCAGAG-3′) and

P2 (5′-CCCAAGCTTCACGCGCCGGGCCACCTTCTG-3′) for the N-terminal 140 aa (Lyl1 NT); P3 (5′-CCGGAATTCCTGGACCTGGCTGAGGGGCAC-3′) and

P4 (5′-CCCAAGCTTCCTGCGTCCGGATCCCCG-3′) for aa 125 to 267 (Lyl1 ΔN). Constructs were verified by sequencing and cloned EcoRI-BamH3 in the VP16 vector. Id1 promoter luciferase constructs were generously provided by Robert Benezra (Memorial Sloan-Kettering, New York). Gal4-CREB, Gal4-CREBS133A were obtained from William Roesler (University of Saskatchewan). Gal4-p300 and Gal4-CBP were obtained from Neil Perkins (University of Dundee, Scotland). The genomic E1A expression plasmid was a gift from E Zacksenhaus (University of Toronto). The E47 expression plasmid pSV2B E2-5 was obtained from J.C.Cross (University of Calgary). Truncated CREB constructs were generated by PCR with Pfu (Stratagene) confirmed by sequencing and cloned 5′EcoRI and 3′ HindIII into the Gal4 vector. For full length CREB the primers were FL-sense(s): 5′-CCGGAATTCGGATCCATGACCATGGAATCTGGA-3′and FL-antisense (as): 5′-CCCAAGCTTGCGGCCGCATCTGATTTGTGGGAGTA-3′; for Q1, KID, and Q2 domains FL-s and Q2-as: 5′-CCCAAGCTTGCGGCCGCTGCTGCT-TCTTCAGCAGG-3′; for Q1 and KID domains FL-s and Kid-as: 5′-CCCAA-GCTTGCGGCCGCAGTCTCCTCTTCAGACTT-3′; for Q1 domain FL-s and Q1-as: 5′-CCCAAGCTTGCGGCCGCAGACTGAACTGTTTGGAC-3′; for Q2 domain Q2-s: 5′-CCGGAATTCGGATCCTCAGCACCTGCCATCACC-3′ and Q2-as; for KID domain Kid-s: 5′-CCGGAATTCGGATTTTAGATTTCAACTA-TTGCA-3′ and Kid-as; and for bZIP domain bZip-s: 5′-CCGGAATTCGGA-TCCAAGAGAGAGGTCCGTCTA-3′ and FL-as.

2.3. Antibodies

Rabbit polyclonal anti-CREB (cat#06-863), anti-CREB phosphoserine 133 (cat#06-519), and mouse monoclonal mixed anti p300/CBP (cat#05-267) were from Upstate, NY. Biotechnology (Lake Placid,NY). During the course of this work a commercial Lyl1 antibody preparation (sc-46158) was made available from Santa Cruz Biotechnology (Santa Cruz, California). A dilution of 1:200 of this antibody was used for ChIP experiments. Rabbit Gal4 DBD antibody (sc-577), Id1 antibody (sc-488), normal mouse serum (sc-2025) and normal rabbit serum (sc-2027), goat anti-rabbit HRP (sc-2004) and goat anti-mouse HRP (sc-2005) were also from Santa Cruz Biotech. Antibody to actin was from Sigma (A-2066). Anti hemagglutinin antibody (αHA, clone 12CA5) was obtained from Roche.

2.4. Electrophoretic mobility shift (EMSA)

Nuclear extracts were prepared as previously described [30]. Briefly, the cells were washed twice with ice-cold PBS, re-suspended in hypotonic lysis buffer [12.0 mM Hepes (pH 7.9), 4.0 mM Tris (pH 7.9), 0.6 mM EDTA, 10 mM KCl, 5.0 mM $MgCl_2$, 1 mM Na_3VO_4, 1.0 mM $Na_4P_2O_7$, 1.0 mM NaF, 0.6 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, and 2 mg/ml pepstatin A] and lysed on ice by repeated passage through a 25G 11/2'' needle. The nuclear fraction obtained by centrifugation at 12,000 g for 20 s was layered on top of a 30% sucrose solution of the above buffer and sedimented by centrifugation at 15,000 rpm for 30 min at 4 °C. Nuclei were lysed on ice in the above buffer supplemented with 300 mM KCl and 20% glycerol with occasional vortexing for 30 min. Lysates were stored at −70 °C. Complementary double stranded oligonucleotides were annealed and labeled with [^{32}P] γATP in the following reaction: 50 ng DNA, 5 μL polynucleotide kinase buffer, 150 μCi [^{32}P] γATP, 1.5 μL T4 PNK (New England), and H_2O to 50 μL. Labeled probes were purified on G25 nick columns (Pharmacia). Typically, probes with specific activities between 5-10 X 10^4 cpm per 0.25 ng of DNA were used. For the binding reaction, nuclear lysates (15-30 μg of protein) were pre-incubated on ice for 30 min with 3 μg of poly (dI-dC)-poly (dI-dC), competing oligonucleotide probes, normal serum or antibody, in 20 mM HEPES (pH 7.9), 50 mM KCl, 1.0 mM EDTA, 5% glycerol and a reaction volume of 30 μL. This was followed by 20 min incubation at room temperature with 0.25 ng of ^{32}P-labelled oligonucleotide probe. Nondenaturing 5% gels were run in 0.5 X TBE at 10 V/cm for 4.0 hrs. Gels were dried without fixing and processed for autoradiography.

The double-stranded oligonucleotide probes used were:

wt CRE: 5′-GAATGGGTGACGTCACGGGCC-3′
mCRE: 5′-GAATGGGTG*GTAGTA*CGGGCC-3′

(mutated sequences underlined) annealed to their complementary counterparts.

2.5. RT-PCR analysis

For real-time reverse-transcriptase PCR (qRT-PCR), RNA was prepared from cell lines and patient samples with an RNeasy kit (Qiagen) and converted to cDNA using TaqMan RT reagents (Applied Biosystems). qRT-PCR was performed with a PE Prism 7700 light thermocycler on 20-40 ng using SYBR Green PCR Master Mix (Applied Biosystems). Id1 mRNA copy number was calculated using the ΔΔCt method (ABI Prism 7700 User Bulletin #2). Amplification of endogenous promoters immunoprecipitated by ChIP was performed using PlatinumTaq and 4.0 μl of immunoprecipitated template (1:10 of total yield), as follows: 4 min at 94 °C (denaturation), 30 cycles at 94 °C for 30 s, 59 °C for 45 s and 72 °C for 45 s (amplification), and a final extension at 72 °C for 10 min. Primers used for qRT-PCR and PCR amplification of ChIP promoter DNA are shown in Table 1.

2.6. Chromatin immunoprecipitation (ChIP)

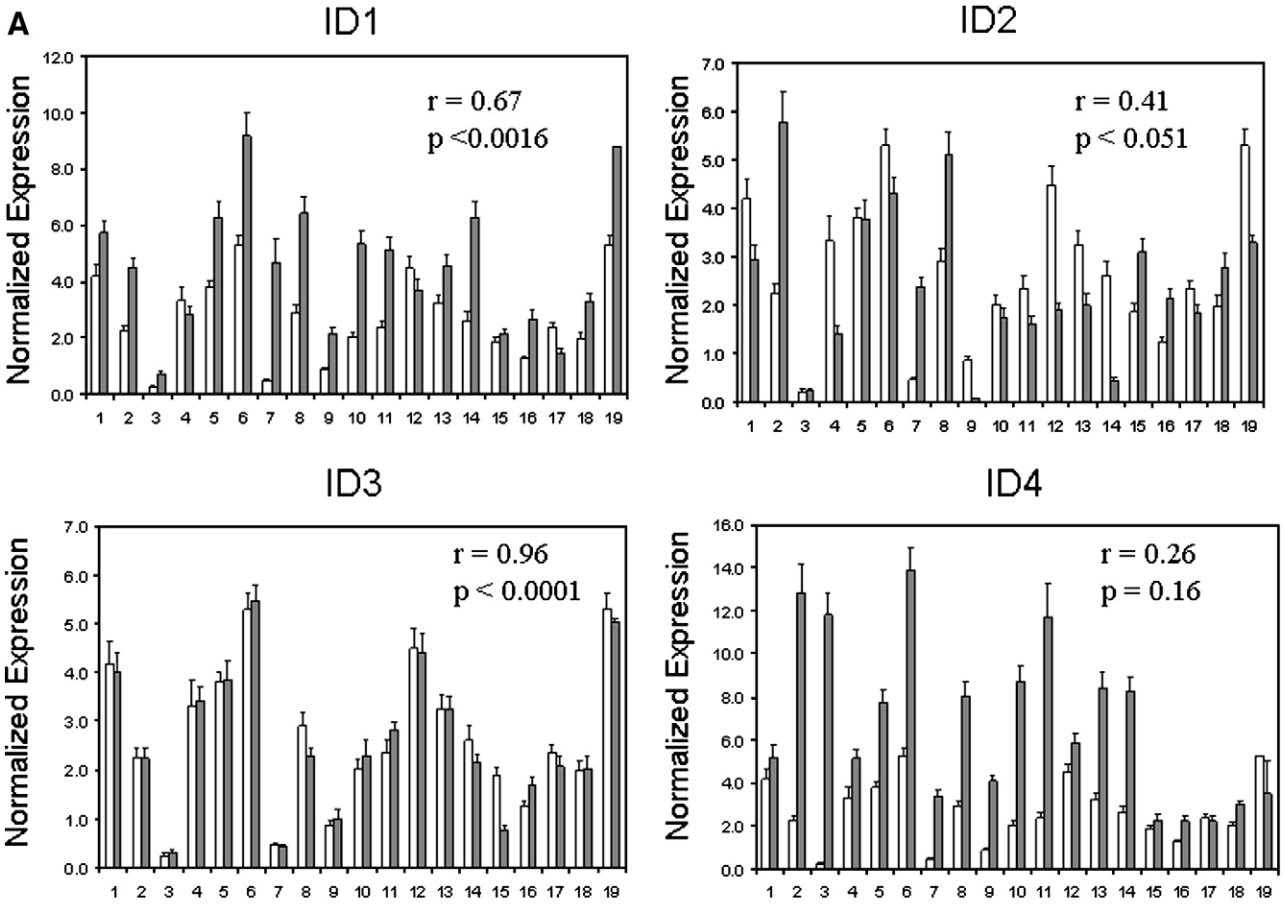
Immunoprecipitation of chromatin crosslinked to the endogenous promoters was performed as previously described [31]. The complete protocol followed is available at http://genomecenter.ucdavis.edu/farnham/farnham/.

2.7. Microarray hybridization and analysis

The complete protocol followed is available at www.microarrays.org. Briefly, immunoprecipitated and genomic DNA was dried and prepared for PCR amplification by annealing to degenerate primers with a constant 5′ sequence, followed by two cycles of primer extension with Sequenase (Perkin Elmer) (round A). Amplification of this DNA was performed with primers complementary to the fixed sequence in two rounds of PCR (B1 and B2) with 25 cycles per round. Amino-allyl dUTP was incorporated into templates in round B2. Templates were labeled with Alexa dyes A555 (genomic DNA) and A647 (ChIP DNA) according to the manufacturer's protocol (Molecular Probes). After washing to remove un-incorporated dyes, 3 μg of total genomic and ChIP DNA were hybridizedfor 16 hr to CGI microarrays. Intra- and inter-array normalization was performed with TIGR software (The Institute for Genomic Research) Bad spots and spots with intensities less than 1500 in either channel (<15 times background intensity) were excluded from further analysis. Only spots present in at least 3 of 4 slides and with intensity greater than 2 fold for DNA from ChIPs with αHA or αCREB, relative to total genomic DNA, were further considered.

2.8. Immobilized template assay

Pull-down assays with biotinylated DNA were performed as described previously [32] Single-stranded oligonucleotides corresponding to the reverse strand of a 3XGal4 promoter were synthesized and biotinylated according to the protocol supplied by the manufacturer (Pierce, cat #89818). Following annealing with the complimentary strand, 200 ng of this template was bound to streptavidin beads, washed twice with binding buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 5.0 mM $MgCl_2$, 0.5 mM EDTA, 0.01% NP40, 10% glycerol) and blocked for 15 min at room temperature in 50 μL binding buffer containing 0.1 mg/mL BSA. The reaction was supplemented with 4.5 μg of poly (dI: dC), 4.5 μg sheared salmon sperm DNA, protease inhibitors (Roche) and incubated on ice for another 15 min. Nuclear extract protein (250 μg) was finally added and the incubation was continued at 4 °C for 4.0 hrs with mixing. The beads were washed three times with binding buffer supplemented with 1 mM DTT, 1 mg/mL BSA and protease inhibitors. Proteins were eluted from the beads in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p <0.0016
ID2
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p < 0.051
ID3
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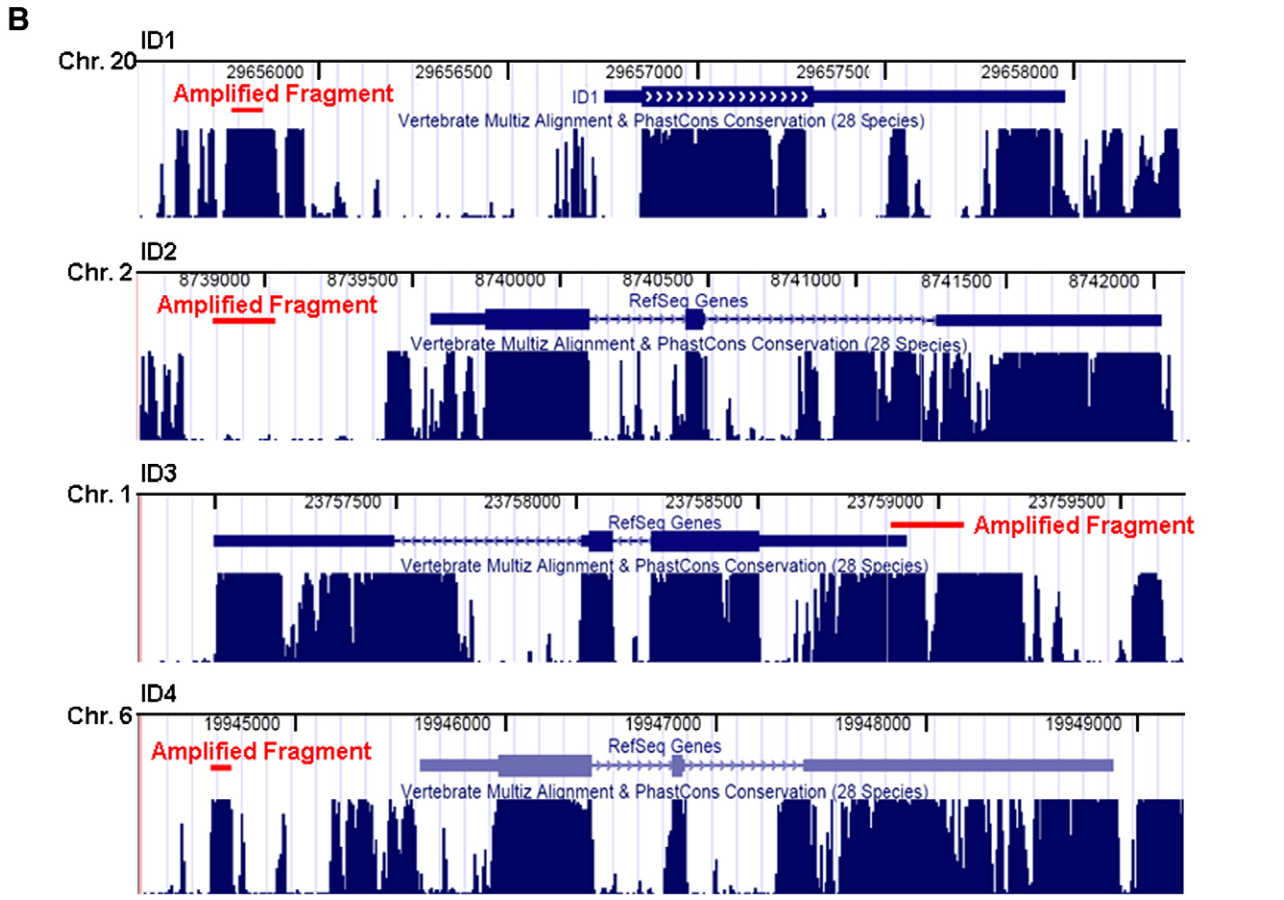
p < 0.0001
ID4
r = 0.26
p = 0.16
Normalized Expression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Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
ID2
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ID3
Chr. 1
RefSeq Genes
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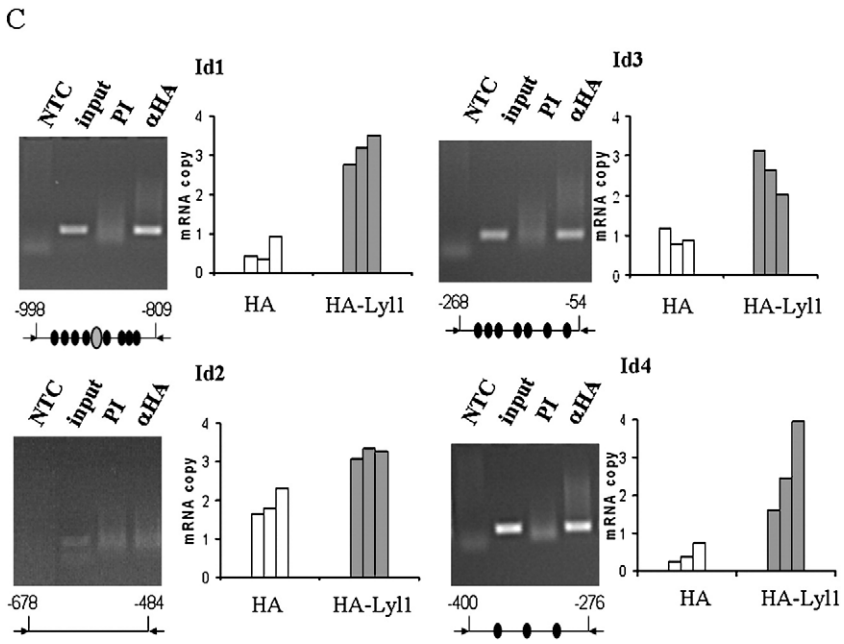


Fig. 1. Lyl1 and Id gene expression. (A) Real time PCR analysis of Lyl1 and Id genes expression in 19 AML patients after normalization to internal GAPDH levels. Empty bars show Lyl1 expression. Three replicate experiments were performed for each clinical sample. Results are shown as means and standard errors. (B) Diagram of Id promoters showing the location of fragments amplified by conventional ChIP experiments ("Amplified Fragment") relative to the location of the proximal promoters for each gene. For Id1, Id3 and Id4 CREB1 clusters were found located in regions with high conservation in mammals. Illustrations were produced using the Genome Browser at UCSC (www.genome.ucsc.edu). (C) Validation of Id targets. Conventional ChIPs with pre-immune (PI) serum or HA antibody (αHA) were performed using cells expressing HA-Lyl1. For comparison, the PCR amplification obtained in the absence of template (NTC) or with 10% of the total chromatin input (input) is shown. Cre sites are indicated by filled ovals. For Id1 the Cre site at position -927 is depicted by a larger symbol; this site together with flanking sequences (depicted in Fig. 2A) constituted the double-stranded (ds) oligonucleotide sequence used in the EMSA experiments. Arrows indicate the position of PCR primers relative to transcription start sites. Graphs show expression levels by real time PCR for each Id gene in HA and HA-Lyl1 expressing cells after normalization to internal GAPDH levels. Results of triplicate experiments are shown.

SDS buffer, resolved by gel electrophoresis and blotted with αFLAG, αHA, or αGal4 antibody.

3. Results

3.1. Lyl1 up-regulates endogenous Id1 and Id3 expression

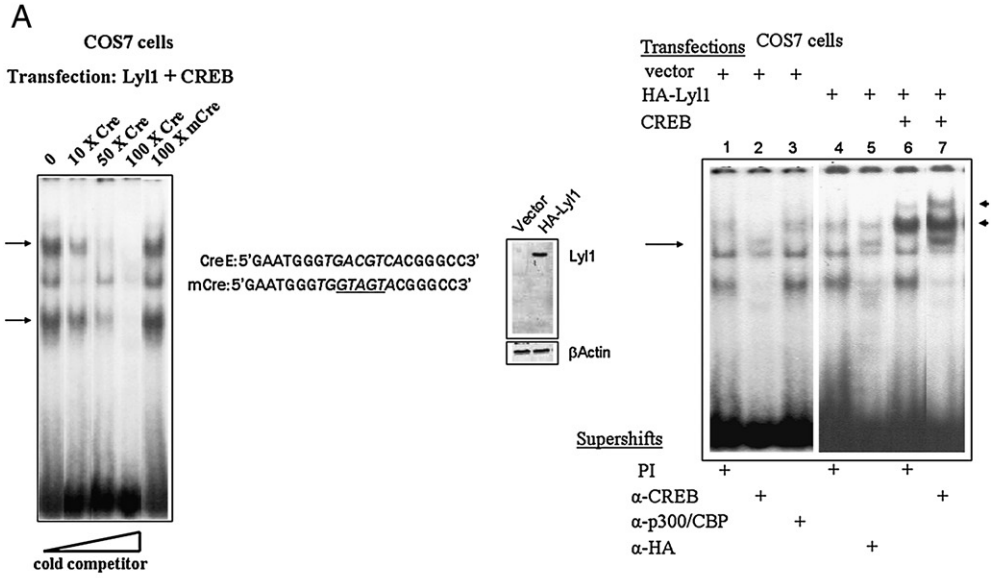
The bHLH domains of Lyl1 and Tal1 were found to be interchangeable in domain-swapping experiments [20]. This contrasts with the observations that their knock-out phenotypes are distinct [17]. Thus, we were motivated to investigate if other Lyl1 domains are involved in protein-protein interactions that confer transcription specificity. We used the longer, 140 aa N-terminal domain of Lyl1 as bait in a yeast two-hybrid screen of a K562 mRNA expression library and identified several interacting proteins (not shown). In this initial screen CREB1 was not identified as a binding partner. However, in leukemic cell lines we observed abrogation of Lyl1-dependent transcriptional activation caused by mutation of a critical CREB1 binding site in the murine Id1 promoter. Mutation of the nearby E2A binding site, where Lyl1-E12/E47 complexes would be expected to form, did not significantly alter the rate of transcription (see Supplementary Information). This suggested that Lyl1-dependent transcriptional activation was driven from the Cre and not the Ephrussi box (E box) site. Binding of Lyl1 to the Cre but not the E box site was verified by EMSA experiments (see Supplementary Information).

From the outset we narrowed down the investigation to previously-identified CREB1 gene targets and focused on the Id family of proteins because of their well-documented roles in hematopoiesis and because of the presence of conserved regulatory Cre sequences in their promoters. Ids inhibit cellular differentiation by preventing bHLH complexes from binding to promoter DNA. We measured the expression of Lyl1, Id1, Id2, Id3, and Id4 mRNA 19 AML samples using real-time PCR with correction for GAPDH expression. Significant correlations were found between the expression of Lyl1 and those of Id1 or Id3 (Fig. 1A). Id1 and Id3 (as well as Id2 whose expression in these patient samples did not correlate to that of Lyl1), were previously recognized as CREB1 targets. To further investigate a functional link between Lyl1 and Id's, we enforced expression of HA-tagged Lyl1 or empty HA vector in K562 cells and determined the

Table 2
Co-regulated genes in AML

	CREB1	Lyl1	Tal1	E47	CREB1&Lyl1	CREB1&Tal1	E47&Lyl1
ALL	827	515	400	391	48 (7.1%)	8 (1.3%)	17 (3.7%)
AML	390	283	375	437	68 (21.7%)	4 (0.5%)	4 (0.6%)

A published gene profile Affymetrix data set for ALL and AML patients [35] was analyzed for genes whose expression correlate ($r > 0.67$) with those of CREB1, Lyl1, Tal1, or E47 (TCF3). Total numbers (and %) of genes in common for the indicated combination of TFs is shown.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50 X Cre
100 X Cre
100 X mCre
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mCre:5'GAATGGG7GGTAGTACGGGCC3'
Vector
HA-Lyl1
Lyl1
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vector + + +
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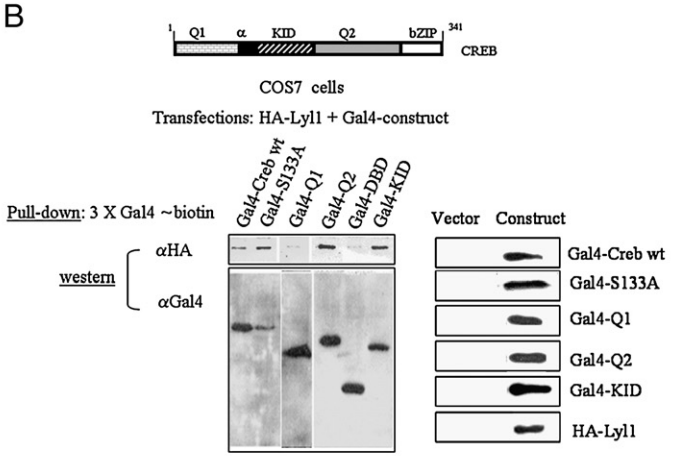
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Gal4-S133A
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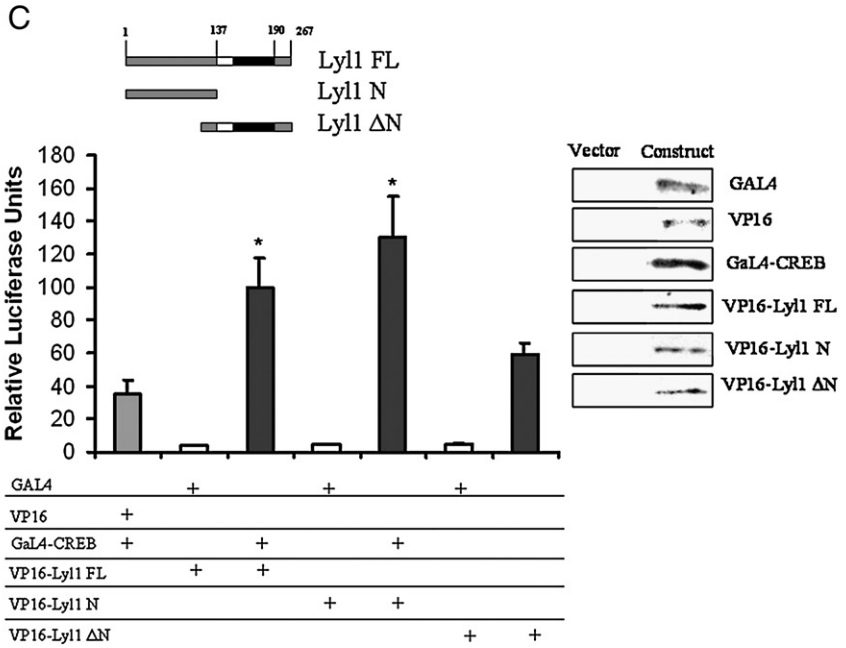
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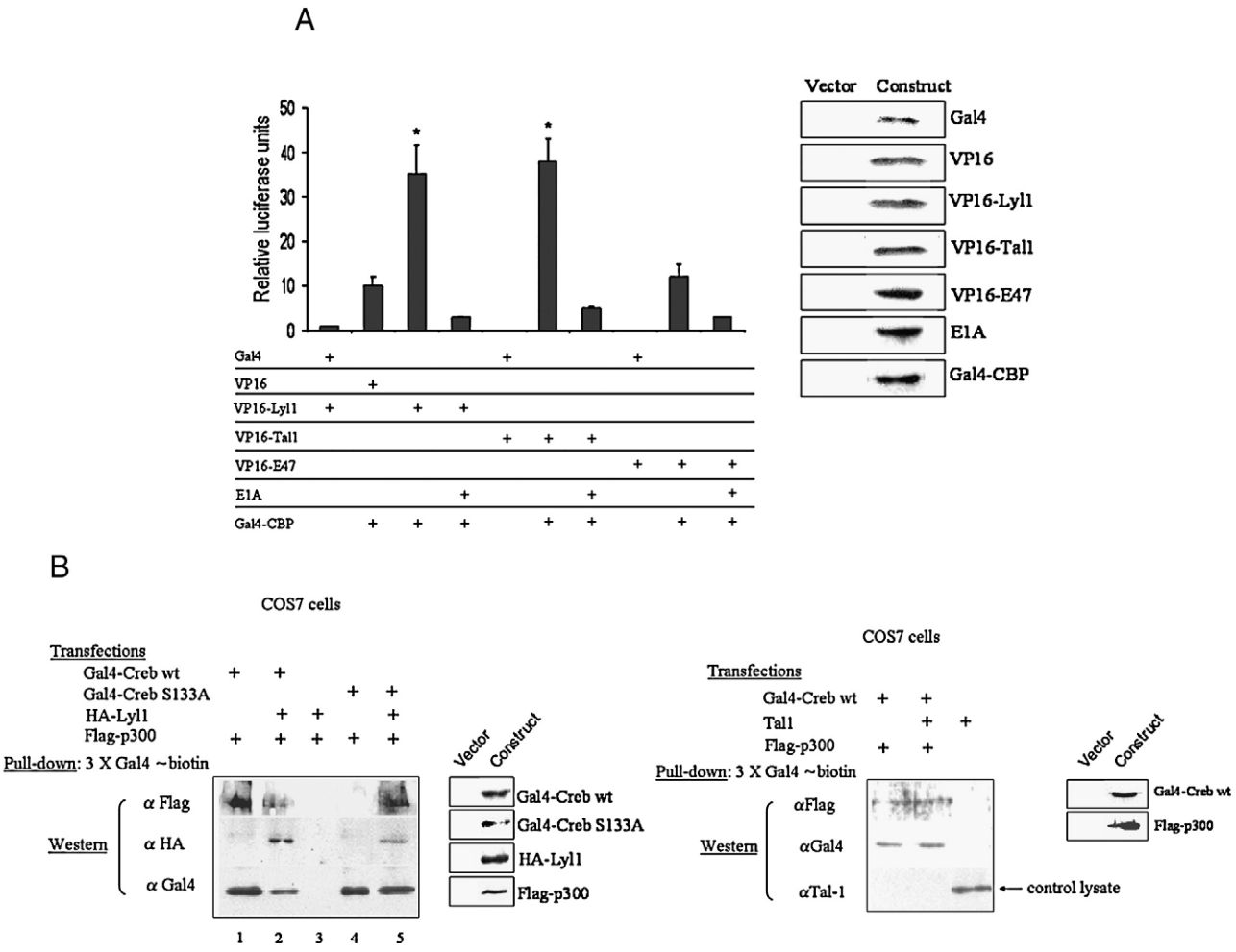

Fig. 3. Formation of a three-way complex containing Lyl1, CREB1, and p300. (A) Left: Lyl1 interacts with CBP and E1A. K562 cells co-transduced with 0.5 μg of the indicated plasmids, a luciferase reporter and a β-gal expression plasmid for data normalization. Empty pcDNA3 vector was added to maintain a constant amount of DNA in the transfection reaction. Right: protein expression of the various constructs compared to empty vector in crude lysates. (B) Lyl1 but not Tal1 forms tri-partite complexes with CREB1 and p300. *Left panel*. Pull-down assays from COS7 cells co-transduced with tagged Lyl1, CREB1 and p300 were performed with a biotinylated oligonucleotide, as described above. *Right panel*. A similar experiment in which Flag-Tal1 was expressed and its association with CREB1 was monitored. Representative western blots showing protein expression in 50 μg of crude lysates is shown for each experiment.

location of Lyl1 binding in the Id promoters as well as the levels of Id1 through Id4. Evolutionarily conserved clusters of CREB1 binding sites were identified and primers were designed to amplify DNA templates obtained by chromatin immuno-precipitation (ChIP) (Fig. 1B). Following pull-downs with HA antibody or pre-immune serum we observed the association of HA-Lyl1 with Id1, Id3, and Id4 promoters. Furthermore, we noted the up-regulation of Id1, Id3 and Id4 mRNA expression in these same cells (Fig. 1C). Interestingly, we did not observe a correlation in clinical samples between the expression of Lyl1 and that of Id4. Id4 expression was shown to vary greatly according to the methylation status of its promoter in different cell lines [33]. Thus,

Fig. 2. Lyl1-CREB1 interactions involve a conserved Cre site of the Id1 promoter. (A) *Left panel*. Specific complexes (arrows) form when nuclear lysates from COS7 cells, expressing transfected HA-Lyl1 and CREB1, are incubated with a ^{32}P-labelled oligonucleotide probe, encoding the Cre site at position -927 in the Id1 promoter. Complexes are competed by excess cold wild type Cre but not by excess mutant Cre (mCre) oligonucleotides. The 6 bp Cre site is boxed and mutations are shown in italics. Expression of HA-Lyl1 in K562 cells transduced with this construct or with empty vector in shown in the left panel. *Right panel*. Lyl1 forms CRE-bound complexes with endogenous and exogenous CREB1. Nuclear extracts from COS7 cells were harvested 48 h after transfection with the indicated plasmids, pre-incubated with pre-immune serum (PI) or HA antibody (αHA) and then incubated with the probe. The arrow identifies the position of a supershifted complex. The arrowhead shows a slower-migrating complex that was observed with nuclear extracts from cells co-expressing HA-Lyl1 and CREB1. (B) Top: a diagram of CREB1 domains that were fused to Gal4DBD. Bottom left: results of pull-down experiments. COS7 nuclear extracts expressing transfected HA-Lyl1 and Gal4DBD-CREB1 constructs were incubated with a biotinylated ds oligonucleotide encoding three tandem Gal4 binding sites. Bound proteins were pulled-down with streptavidin-agarose, resolved by SDS-PAGE, blotted with αHA. The same blot was stripped and re-probed with αGal4. Bottom right: protein expression in crude lysates compared to empty vector (50 μg total protein/lane). (C) Identification of the Lyl1 domain that interacts with CREB1. Full-length or truncated Lyl1 was fused downstream of the VP16 transcriptional activator and the construct's ability to interact with Gal4DBD-CREB1 was determined by means of two-hybrid assays in COS7 cells. Right panel: protein expression in crude lysates.

epigenetic regulation of Id4 expression may be different in clinical samples and in the K562 erythroleukemia cell line and these differences may account for the observed results. Due to the predicted presence of nearby E2A binding sites (see Fig. 4A) the observed effects cannot be solely attributed to Lyl1-CREB1 interactions but taken together, the data are suggestive of a link between the expression of Lyl1 and those of Id1 and Id3, two previously identified CREB1 targets.

3.2. A three-way complex containing Lyl1, CREB1 and p300 binds DNA

To investigate the possibility that CREB1 and Lyl1 co-regulate gene expression, we performed template matching analysis [34] using ALL and AML gene expression data from a published report [35]. First, we identified genes whose expression patterns matched the expression of CREB1, Lyl1, Tal1, and E2A, ninety-nine times out of a hundred ($p<0.01$). Then, common genes were identified in CREB1 and Lyl1, CREB1 and Tal1, and E2A and Lyl1 groups, for both ALL and AML. For the AML data, 1 in 5 genes were common between CREB1 and Lyl1. By comparison, approximately 1 in 100 genes were common between CREB1 and Tal1 or between Lyl1 and E2A (Table 2).

The above results suggest that Lyl1 and CREB1 may co-regulate a number of genes in leukemia. Since ChIP cannot specify where a protein binds in a sequence of immunoprecipitated DNA, we used EMSA to determine if the two TFs interact at the molecular level. Fig. 2A (left panel) shows the results of experiments in which COS7 nuclear extracts expressing exogenous CREB1 and HA-Lyl1 retard migration of a ^{32}P-labelled oligonucleotide probe containing a Cre site conserved in the human and murine Id1 promoters. The probe sequence corresponds to nucleotides -926 to -913 in the human Id1 promoter. In the absence of cold competitor, two prominent complexes (arrows) were identified following a relatively brief period of exposure to autoradiography (~4 hours). These complexes became fainter when lysates were pre-incubated with increasing amounts of cold competitor and eventually disappeared at 100 fold molar excess of the cold competitor. A cold competitor containing a mutation in the Cre site but retaining the wild type flanking sequences had no effect, thereby showing that protein complexes bound to the Cre site (Fig. 2A, 100XmCRE).

To verify that the observed complexes contained CREB1 and Lyl1 we performed antibody supershifting experiments (Fig. 2A right panel). Nuclear extracts from COS7 cells transduced with empty pcDNA3 vector were pre-incubated with pre-immune

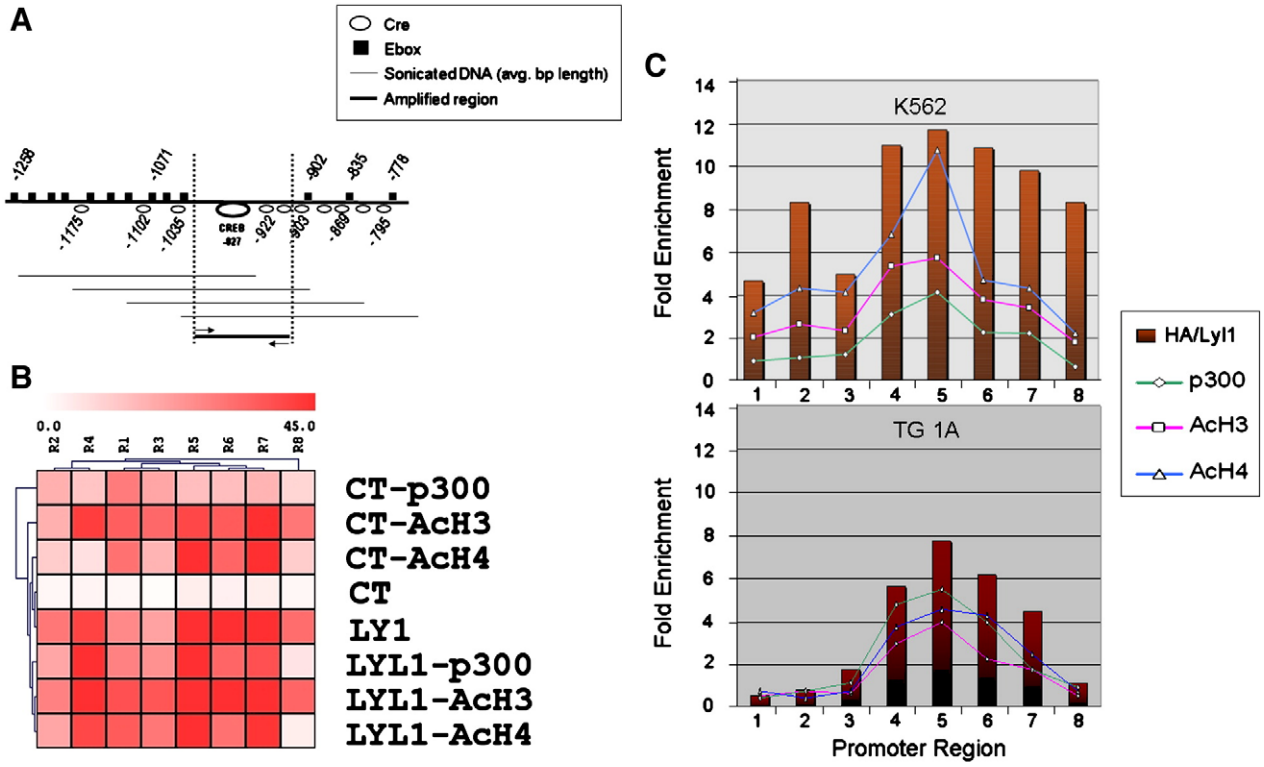

Fig. 4. Lyl1 binds, acetylates Id1 promoter in vivo. (A) Diagram of an upstream fragment of the human Id1 promoter centered on a 189 bp region of conserved DNA (thick line between amplifying primers). Boxes and ovals represent E box and Cre binding sites, respectively. The Cre sequence at -927 was used in EMSA and the site is enlarged for orientation. Thin lines represent the average length of sonicated chromatin (~300 bp). (B) ChIP-qPCR analysis of the Id1 promoter depicted in A. 1.5 X 10^7 K562 cell nuclei expressing empty vector (CT) or enforced HA-Lyl1 (Lyl1) were used for ChIP with the indicated antibodies in order to determine the relative enrichment of the promoter due to Lyl1 expression. QPCR results were corrected for primer amplification efficiency and results were expressed as ng of starting DNA by interpolation to standard curves determined for each primer set. HCL normalization and clustering was done using TIGR software. (C) Binding of HA-Lyl1 complexes along the Id1 promoter in two cell lines. Top: the results depicted in (B) for K562 cells expressing HA-Lyl1 are shown as a ratio between HA-Lyl1 and CT groups for each set of ChIP-qPCR data. Bottom: similar experiments with the TG 1A cell line that expresses high endogenous Lyl1 levels; ChIP was performed using a commercial antibody to Lyl1 (see Methods). Mock assays, i.e. no chromatin input were negative (not shown).

serum or CREB1 antibody and then with the Cre probe. A super-shifted complex containing endogenous CREB1 is clearly visible (compare lanes 1 and 2). This super-shifted complex is also visible with αHA in cells that were transduced with HA-Lyl1 (lane 5), an indication that transfected Lyl1 binds to the pre-existing CREB1 complex. In cells co-transduced with HA-Lyl1 and CREB1 two additional, probably non-specific complexes appear (arrowheads).

To identify the interacting CREB1 domains we co-expressed Gal4-fused CREB1 constructs together with HA-Lyl1 in COS7 cells and performed pull-down assays with a 3′ biotinylated oligonucleotide containing three tandem Gal4 binding sites. CREB1 constructs included a full-length wild type plasmid, a S133A mutant construct that is unable to recruit p300/CBP [36,37] and domain deletion mutants. Precipitated proteins were separated on western blots and probed with αHA or αGal4. The results are shown in Fig. 2B. Importantly, Lyl1 interacted with the CREB1 S133A mutant that is unable to recruit p300/CBP, thereby showing that CREB1-Lyl1 interactions do not require Ser133 phosphorylation. Furthermore, experiments with deletion mutants show that Lyl1 interacts separately with Q2 and with KID domains of CREB1.

To identify Lyl1 domains that interact with CREB1, we used a two-hybrid system strategy. Lyl1 domains cloned downstream of the yeast VP16 transactivator were co-transfected in COS7 cells together with Gal4-CREB1 and a reporter vector expressing luciferase driven from tandem Gal4 binding sites (Fig. 2C). An interaction recognized by a 2.5 fold increased in luciferase activity, was observed between CREB1 and the N-terminal domain of Lyl1 (Lyl1N). The interaction was abrogated upon deletion of this domain in the Lyl1 ΔN construct, which demonstrated that the rest of the Lyl1 molecule was not necessary.

The bHLH domains of Tal1, E47, and Lyl1 are structurally similar. In Tal1 and E47, they mediate interactions with p300 and the CREB-binding protein (CBP), two homologous transcriptional co-activators. Interaction between Lyl1 and these proteins was not previously shown but was expected to occur, and is shown in Fig. 3A. Enforcing the expression of E1A abolishes all interactions with CBP, including that of Lyl1, in agreement with previous results.

Having observed separate interactions between Lyl1 and CREB1 and Lyl1 and CBP, we next investigated the formation of a tripartite Lyl1, CREB1 and p300 complex with DNA, by co-transfecting expression plasmids into COS7 cells. Protein complexes assembled on a biotinylated oligonucleotide containing 4 tandem GAL4 binding sites were pulled down with streptavidin-agarose and identified by Western blot analysis (Fig. 3B left panel). Flag-p300 was pulled down with Gal4-CREB1 but not with Gal4-CREB1 S133A (compare lanes 1 and 4), in keeping with previous observations that p300 and CBP do not interact with this CREB1 phosphorylation mutant. Co-expression of HA-Lyl1 with p300 and CREB1 resulted in a complex of all three proteins that was pulled down (lane 2) but in the absence of CREB1, Lyl1 and p300 did not bind to the Gal4 promoter (compare lanes 2 and 3). Previously we showed that Lyl1 establishes independent interactions with the Q2 and KID domains of CREB (see Fig. 2B) and that it interacts with CREB S133A. P300 is recruited to the complex, presumably by Lyl1 (compare lanes 4 and 5). In similar pull down experiments performed with Tal1 we did not observe a Tal1-CREB1 interaction (Fig. 3B, right panel).

To summarize, using EMSA, promoter pull downs and two-hybrid assays we observed that Lyl1 interacts with CREB1 and that a three-way complex of these proteins with p300 can assemble.

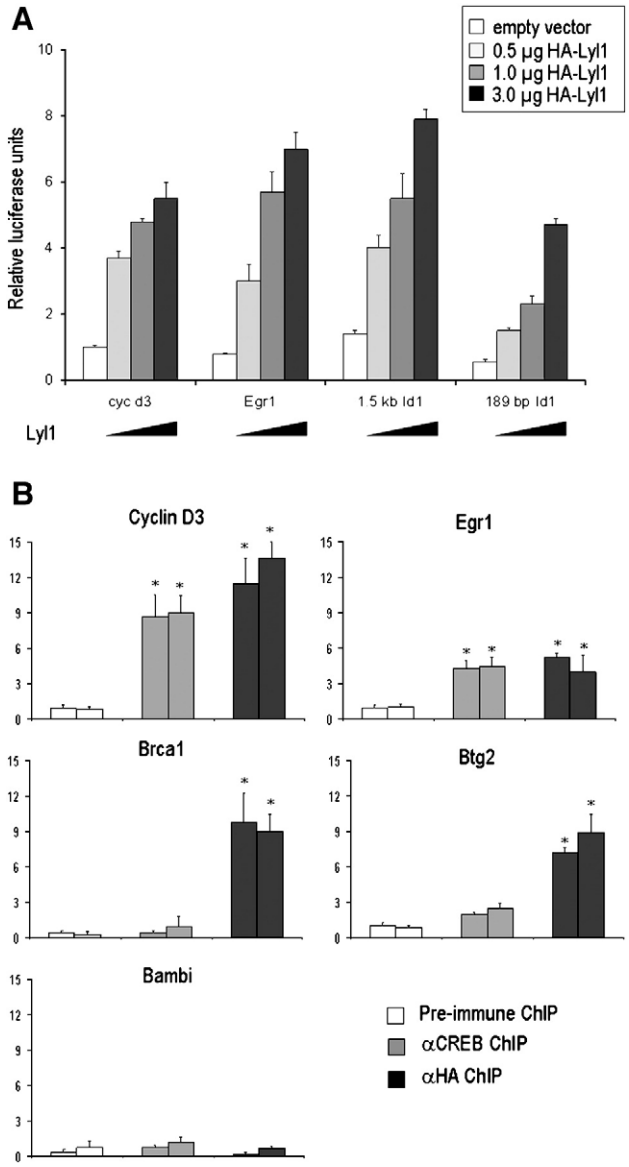

Fig. 5. Effect of Lyl1 on CREB1-driven promoters. (A) Transcriptional activation of CREB1-driven promoters. Promoter-luciferase constructs were co-transfected with empty vector or HA-Lyl1 (0.5, 1.0, or 2.0 μg of plasmid) in K562 cells. Transfection efficiencies were similar between experiments, as determined by the amount of co-transfected β-gal. Id1 promoter constructs contained 1.5 kb or 210 bp of Id1 upstream sequence (see Supplementary Information S1) fused to a luciferase reporter. (B) Association of Lyl1 with known CREB1 targets. DNA from ChIPs performed with the indicated antibodies was amplified with PCR primers designed to span proximal CREB1 binding sites (MATCH similarity score relative to CREB1 matrix >0.85). The ordinate represents the amount of ChIP template (ng) that was calculated by interpolating the experimentally-determined Cts with calibration curves obtained from serial dilution experiments with total genomic DNA.

3.3. Lyl1, CREB1 and p300 assemble in vivo on the Id1 promoter

Recruitment of the transcriptional apparatus to promoter DNA is hindered by close interactions between positively charged histones and the negatively charged DNA backbone. Histone acetylation relieves the spatial constraints and facilitates the positioning of the transcriptional machinery at the start site. The histone acetyltransferase (HAT) activity of p300/CBP is known to be involved in many instances of gene up-regulation. Since we previously observed the formation of Lyl1-CREB1-p300 complexes in COS7 cells, we used ChIP to investigate if these complexes form *in vivo* and alter the acetylation status of core histones within the Id1 promoter. This could explain the up-regulation of Id1 noted in HA-Lyl1 transfected cells. Fig. 4A shows a diagram of the Id1 promoter with the distribution of predicted Cre and E box sites relative to the average fragment length of the sonicated chromatin input for ChIP (~300 bp). The region represented is centered on a 189 bp sequence that corresponds to the minimal Id1 promoter in mouse fibroblasts and is conserved in the human promoter. In transfection assays the 189 bp promoter is activated by Lyl1 (see further). Conventional PCR of ChIP DNA using primers to amplify Cre -927 showed that HA-Lyl1 cells contained higher levels of p300/CBP and acetylated histone H4 bound to the promoter (not shown). In order to determine if this effect is due to the presence of this conserved Cre site or, alternatively, whether it extends to up-stream or down-stream regions in association with other predicted Cre and E box sites, immunoprecipitaed chromatin was analyzed by quantitative RT-PCR with primers spanning the entire region shown in Fig. 4A. Results were corrected for primer efficiency and expressed as nanograms of DNA by reference to standard curves obtained for each primer set. When analyzed with TIGR software, we observed that empty vector and HA-Lyl1 expressing cells cluster separately. The ratio between the expression of HA-Lyl1, p300, acetylated H3 and acetylated H4 in the two cell populations is shown in Fig. 4B. HA-Lyl1 bound to multiple sites on the promoter, in keeping with the abundance of predicted Cre and E box sites. However, the distribution of p300, acetylated H3 and acetylated H4 was uneven and peaked in region 5 which contains Cre -927 (Fig. 4C, top panel). Similar results were obtained by a ChIP experiment that targeted endogenous Lyl1 expression in the leukemia cell line KG 1A using a newly commercialized human Lyl1 antibody preparation (see Methods) (Fig. 4C, bottom panel).

In summary, enforced HA-Lyl1 expression in K562 cells increased Id1 promoter acetylation, consistent with increased recruitment of p300/CBP acetyltransferases to Lyl1 complexes with CREB1. Lyl1-E2A complexes are predicted to form and may also contribute to this effect.

3.4. Lyl1 transactivates CREB1 promoters

Next, we examined the effect of Lyl1 on transcriptional activation of several previously-described CREB1-driven human promoters [25]. Lyl1 increased cyclin D3, Egr1, and Id1 transcriptional activation (Fig. 5A) but had no effect on Bcl2 and Bcl-xL promoters (not shown). ChIPs followed by qRT-PCR amplification showed that both CREB1 and HA-Lyl1 bind to endogenous cyclin D3 and Egr1 promoters (Fig. 5B). We also tested the association of Lyl1 with three CREB1 promoter targets, identified previously in PC12 cells: Btg2, Brca1, and Bambi. We were unable to confirm association of CREB1 with any of these targets in K562 cells, however, Lyl1 did associate with Brca1 and Btg2. Neither TF associated with Bambi.

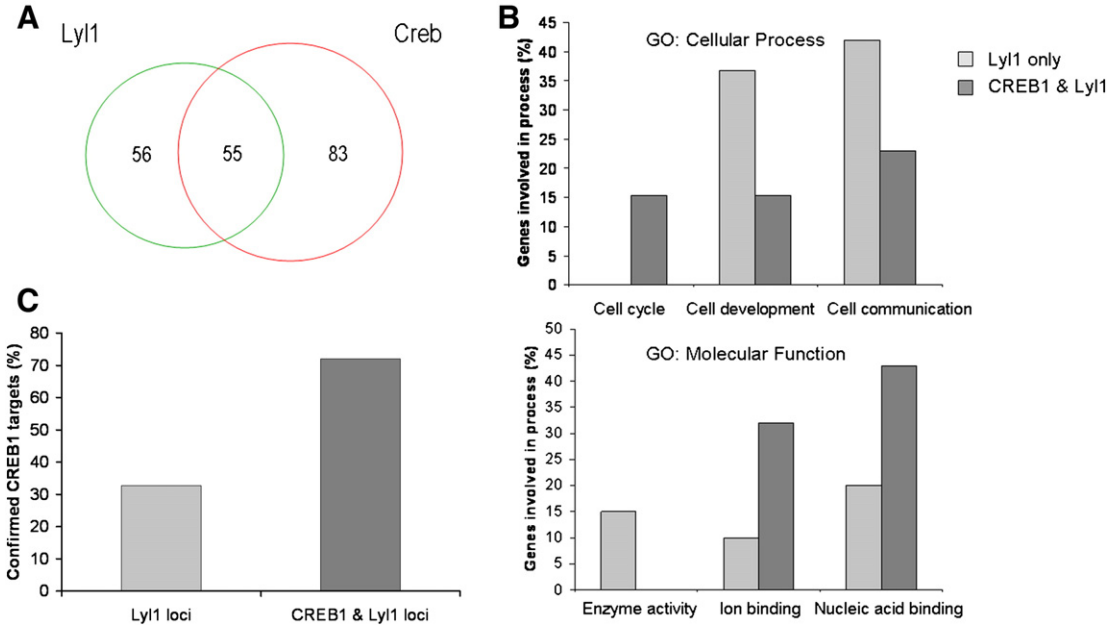

Fig. 6. Overlap between Lyl1 and CREB1 genomic targets in ChIP-chip experiments. (A) Venn diagram depicting common clones identified in the HA-Lyl1 and CREB1 pull-downs. DNA was hybridized to 12 K CGI arrays. (B) Gene Ontology features associated with unique Lyl1 loci compared to genomic loci occupied by both Lyl1 and CREB1. (C) Common CREB1 and Lyl1 targets are enriched in genes previously identified as CREB1 targets [39].

Table 3
Lyl1 and CREB1 genomic targets identified by ChIP-chip using CGI microarrays

Common for CREB1 & Lyl1		
Distance from known gene (bp)	Gene Name	Description
0	AK125187	Hypothetical protein FLJ43197.
0	APP	amyloid beta A4 protein precursor, isoform a
0	ASCC3	activating signal cointegrator 1 complex subunit
0	BC006438	Hypothetical protein.
0	CACNA2D3	calcium channel, voltage-dependent, alpha
0	COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2
0	DBR1	debranching enzyme homolog 1
0	DCC	deleted in colorectal carcinoma
0	FAM19A5	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5
0	FLJ10726	FLJ10726 protein.
0	GMFB	glia maturation factor, beta
0	LIAS	lipoic acid synthetase isoform 1 precursor
0	M6PRBP1	mannose-6-phosphate receptor binding protein 1
0	MKL1	megakaryoblastic leukemia 1 protein
0	MRPL27	mitochondrial ribosomal protein L27 isoform a
0	PCSK1N	proprotein convertase subtilisin/kexin type 1
0	PLCB1	phosphoinositide-specific phospholipase C beta 1
0	PRDM8	PR domain containing 8
0	PTTG1	pituitary tumor-transforming protein 1
0	RFX2	regulatory factor X2 isoform a
0	SNAP91	synaptosomal-associated protein, 91 kDa homolog
0	ZNF665	zinc finger protein 665
27	HIST1H4H	H4 histone family, member H
79	AK126015	Hypothetical protein FLJ44027.
262	MYST1	MYST histone acetyltransferase 1
308	TRH	thyrotropin-releasing hormone
474	ME3	Hypothetical protein FLJ34862.
686	LOC120379	hypothetical protein LOC120379
1211	RPL9	ribosomal protein L9
1612	EME1	essential meiotic endonuclease 1 homolog 1 (S. pombe)
2794	SLU7	step II splicing factor SLU7
2858	AK127974	Hypothetical protein FLJ46089.
3102	WDR5	WD repeat domain 5
4277	HOXD4	homeobox D4
4476	BCKDK	branched chain ketoacid dehydrogenase kinase
4671	ERAS	small GTPase protein E-Ras
6149	HOXD3	homeobox D3
7790	HOXD13	homeobox D13
9093	TMEM30A	transmembrane protein 30A
9921	DBR1	Hypothetical protein FLJ20109.
Specific for CREB1 Only		
0	ALS2CR11	amyotrophic lateral sclerosis 2 (juvenile)
0	EMX2	empty spiracles homolog 2
0	IL1RAPL1	interleukin 1 receptor accessory protein-like 1

Table 3 (*continued*)

Common for CREB1 & Lyl1		
Distance from known gene (bp)	Gene Name	Description
Specific for CREB1 Only		
0	BMS1L	BMS1-like, ribosome assembly protein
0	MSL3L1	male-specific lethal 3-like 1 isoform a
0	ZNF677	zinc finger protein 677
0	CSMD3	CUB and Sushi multiple domains 3
0	LRPAP1	low density lipoprotein receptor-related protein
0	COL14A1	COL14A1 protein.
0	TPR	translocated promoter region (to activated MET)
0	BRIP1	BRCA1 interacting protein C-terminal helicase 1
0	MGC33648	hypothetical protein LOC133383
0	PCAF	p300/CBP-associated factor
0	CRTC3	CREB1 regulated transcription coactivator 3
0	FAM19A5	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5
0	DLX6	distal-less homeobox 6
0	ETS1	ETS1 protein.
0	HD	huntingtin
0	C15orf33	hypothetical protein LOC196951
0	FANCD2	Fanconi anemia complementation group D2 isoform
0	PLA2G4B	phospholipase A2, group IVB
0	DLD	dihydrolipoamide dehydrogenase precursor
0	DHTKD1	dehydrogenase E1 and transketolase domain
0	RAE1	Hypothetical protein FLJ44036.
0	IGSF4B	immunoglobulin superfamily, member 4B
0	HIST1H2BC	Hypothetical protein.
0	MYRIP	myosin VIIA and Rab interacting protein
0	ADAMTS10	ADAM metallopeptidase with thrombospondin type 1
0	RNASET2	ribonuclease T2
0	WTAP	Wilms' tumour 1-associating protein isoform 1
0	KIAA1772	hypothetical protein LOC80000
0	ZRANB3	zinc finger, RAN-binding domain containing 3
1	MAPKBP1	mitogen-activated protein kinase binding protein
18	WTAP	PNAS-132.
39	ZRANB3	Hypothetical protein FLJ38043.
109	DTWD1	DTWD1 protein.
158	LGI2	leucine-rich repeat LGI family, member 2
186	HIST1H2BC	H2B histone family, member L
341	EPHA10	EPH receptor A10 isoform 2
453	R3HDM1	R3HDM1 protein.
746	MMP25	matrix metalloproteinase 25 preproprotein
1123	ZNF425	zinc finger protein 425
1470	ZNF524	zinc finger protein 524
1635	KIAA1287	integrator complex subunit 2
2687	MEIS1	Meis1 homolog
4007	AKAP2	A kinase (PRKA) anchor protein 2
4836	CIDEC	CIDEC protein.
5179	CDKN1B	cyclin-dependent kinase inhibitor 1B

(continued on next page)

Table 3 (*continued*)

Common for CREB1 & Lyl1		
Distance from known gene (bp)	Gene Name	Description
Specific for CREB1 Only		
5484	IRX1	iroquois homeobox protein 1
6581	AK128093	Hypothetical protein FLJ46214.
7568	SEC61A2	SEC61A2 protein.
7889	BC047593	LOC440508 protein.
7911	AK096426	Hypothetical protein FLJ39107.
8050	ZIC5	zinc finger protein of the cerebellum 5
8186	AF271776	ATP synthase a chain (EC 3.6.3.14) (ATPase protein 6).
8864	DKFZP781I1119	Hypothetical protein DKFZp686J16125.
9890	DLX5	distal-less homeobox 5
Specific for Lyl1 only		
0	ADRB3	adrenergic, beta-3-, receptor
0	AKT1S1	AKT1S1 protein.
0	ASAH3L	ASAH3L protein.
0	ATOH8	atonal homolog 8
0	C13orf24	C13orf24 protein.
0	C18orf54	hypothetical protein LOC162681
0	C3orf26	hypothetical protein LOC84319
0	C6orf173	hypothetical protein LOC387103
0	CRELD2	hypothetical protein LOC79174
0	DCDC2	doublecortin domain containing 2
0	DKFZp761B107	hypothetical protein LOC91050
0	DLG2	discs, large homolog 2, chapsyn-110 (Drosophila)
0	ELOVL5	homolog of yeast long chain polyunsaturated
0	FBXO4	F-box only protein 4 isoform 1
0	LOC390927	hypothetical protein LOC390927
0	LOC442578	Hypothetical protein LOC442578.
0	MEIS1	Meis1 homolog
0	NTRK3	neurotrophic tyrosine kinase, receptor, type 3
0	PDCD6IP	programmed cell death 6 interacting protein
0	PIAS1	protein inhibitor of activated STAT, 1
0	RPS25	ribosomal protein S25
0	SERPINB6	MSTP057.
49	PDCD6IP	programmed cell death 6 interacting protein
114	AJ968414	Cysteine-rich with EGF-like Domains 2 (CRELD2) beta isoform.
241	PHACTR3	OTTHUMP00000031426.
286	ASAH3L	N-acylsphingosine amidohydrolase 3-like
613	TBC1D17	TBC1 domain family, member 17
742	EFNA5	ephrin-A5
1378	ARL1	ADP-ribosylation factor-like 1
1403	MXRA5	adlican
1779	NKX2-8	NK2 transcription factor related, locus 8
1996	DLNB14	hypothetical protein LOC338657
4691	DKFZP434A0131	Hypothetical protein DKFZP434A0131.
6979	ALS2	alsin
7291	ARHGEF10	Rho guanine nucleotide exchange factor (GEF) 10
7310	KBTBD11	hypothetical protein LOC9920
8359	BC069659	PRO0641.
9021	PNKP	polynucleotide kinase 3′-phosphatase
9113	KAAG1	hypothetical protein LOC353219
9650	THAP5	THAP domain containing 5

3.5. ChIP-chip data reveal large overlap between Lyl1 and CREB targets

Results show that enforced HA-Lyl1 can associate with the promoters for several CREB1 target genes. To identify genome-wide candidate targets for Lyl1 and CREB1 co-regulation we used ChIP followed by DNA hybridization to a cDNA microarray (ChIP-chip). ChIP-chip has been used extensively to map the location of TF binding sites (TFBS) in the genomes of man, mouse and yeast. In order to focus on promoter targets with highly probable regulatory potential, we used a 12K microarray representation of CpG islands (CGI) in the human genome, that was previously described [38]. We found an overlap of ~50% between Lyl1 and CREB1 genomic targets in HA-Lyl1-K562 cells (Fig. 6A). This result is surprisingly high but similar to our analysis of the AML data in which one out of every five genes was co-regulated by Lyl1 and CREB1. The genes identified by ChIP-chip are shown in Table 3. The principal gene ontology categories that showed enrichment by this analysis are shown in Fig. 6B. Genes associated with loci specific for Lyl1 are associated with cellular development and cell communication processes, while the set of genes common to Lyl1 and CREB1 regulates cell cycle, as well as ion and nucleic acid binding processes. This latter set of genes was enriched in CREB1 targets that had been previously identified by ChIP-chip analysis of three different cell lines [39]. Notably, 73% of the gene targets common for both CREB1 and Lyl1 had been previously identified as CREB1 target genes (Fig. 6C).

4. Discussion

High levels or mistimed expression of bHLH TFs can initiate malignant transformation [40–46]. We previously found that Lyl1 is over-expressed in the majority of AML clinical samples relative to its expression in normal bone marrows [19]. This event was shown to occur as a consequence of chromosomal translocations or bi-allelic gene expression [9,12,47]. However, epigenetic factors, such as changes in Lyl1 promoter methylation may also be implicated, given the wide-spread hypomethylation of the AML genome. The role of Lyl1 in leukemia is unknown. By analogy with Tal1 it was suggested that Lyl1 may also compete with E2A/HEB protein complexes [21] and reduce the formation of homodimers of these TFs, as well as generate hetero-dimers with abnormal binding activity [48,49]. The change from E2A homodimers to E2A-Tal1 hetero-dimers blocks differentiation [50], but similar experiments have not been conducted for Lyl1. Overexpression of Lyl1 reportedly decreased both erythrocytic and megakaryocytic differentiation but the underlying mechanism was not investigated [19]. However, in vitro experiments demonstrated that Lyl1-E2A complexes recognize E boxes with different flanking regions, compared to Tal1-E2A complexes and thereby suggested that the distribution of binding sites in vivo may be different for these two TFs. The bHLH domains of Tal1 and Lyl1 mediate molecular interactions with a constant set of factors but the observation that Lyl1 cannot rescue a Tal1 knockout phenotype [51]suggests that domains other than the bHLH are required for full functional activity. Furthermore, the fact that Tal1

has binding-independent functions that do not require an association with E box-containing promoters demonstrates the relevance of non-bHLH domains [52]. The results presented here further illustrate the importance of a non-bHLH domain, namely the N terminal 140 amino acids of Lyl1. It mediates Lyl1-CREB1 interactions and is not present in Tal1. Expectedly, we did not observe Tal1-CREB1 interactions. Using expressed CREB1 domains we found that Lyl1 interacts with domains Q2 and KID. The KID domain is essential for recruiting the transcriptional co-activators p300 and CBP, upon phosphorylation of Ser 133. Experiments with the CREB1 substitution mutant S133A demonstrated that this phosphorylation event is not required for the CREB1-Lyl1 interaction. The tripartite interaction Lyl1-CREB1-p300 occurs in the presence of phosphorylation deficient CREB1 A133S mutant. This may explain how over-expressed Lyl1 can interfere with CREB1-dependent gene regulation. Mitogenic signals trigger cellular proliferation and through the STAT and MAPK pathways lead to phopshorylation of Ser 133 and other critical CREB1 residues. This series of events ensures that CREB1 phosphorylation, histone acetlylation by the HAT activity of recruited p300/CBP and appropriate transcriptional events are all in step with the mitogenic stimulation. Aberrant expression of Lyl1 and the subsequent formation of Lyl1 complexes with un-phosphorylated CREB1 can lead to mistimed recruitment of p300/CBP and possibly to out-of step transcriptional activation events. In addition, formation of Lyl1-E2A complexes could further compromise physiologic gene regulation by one of the mechanisms previously discussed.

Id proteins lack the basic DNA binding domains but can associate with bHLH proteins into complexes devoid of DNA binding activity [53]. CREB1 was recently identified as a suspected oncoprotein in AML [27,28]. As Id's have been shown to play a role in malignant transformation through blocking differentiation [41], while at the same time Id promoters contain conserved CREB1 binding sites, we pursued the possibility that Lyl1 and CREB1 could regulate Id expression in AML. By real-time RT-PCR analysis of 19 AML samples we found a statistically significant correlation between the level of Lyl1 and Id1 and Id3 expression. Since in addition to Lyl1 expression many other factors may account for the observed in vivo correlation, we enforced the expression of a tagged Lyl1 construct in K562 cells and investigated the expression of Id genes in these cells relative to cells expressing the empty vector. We found increased levels of Id1, Id3 and Id4 in these experiments. The increase in Id4 expression was not observed in the clinical samples and may reflect the particular characteristics of the pro-erythrocytic leukemia line K562, as opposed to the promyelocytic primary cells as well as the heterogenous nature of primary samples in which variable sets of upstream regulators can activate the Id genes. In keeping with a direct activation effect of Lyl1 on Id genes, ChIP experiments showed that HA-Lyl1 was bound to the promoter regions of Id1, 3 and 4 but not Id2.

We found that HA-Lyl1 occupies an extended region of the Id1 promoter. This is in agreement with the presence of several predicted E box and CREB1 binding sites in this promoter region (Fig. 4) but raises questions about the specificity of the observed effect. A review of recent ChIP-chip studies revealed that for a given TF the number of binding sites outnumber that of functional sites that can be directly linked to the regulation of down-stream genes. Euskirchen et al identified thousands of CREB binding sites along chromosome 22 and estimated that genome-wide the number of CREB1 binding sites is in the tens of thousands[54]. A recent study extends that number to over 750,000 half and full-length CREB1 binding sites in the human genome, but with fewer that 2% being involved in gene regulation [39]. The issue of the functionality of docking sites was also raised by experiments with T bet, a T-box family TF that occupies genomic sites which are un-related to the location of the genes it regulates [55]. These studies and others suggest that out of many sites occupied by a given TF only a few are functional in any given promoter and/or cellular context. By extension, it is possible that Lyl1 directs Id1 expression from a limited number of sites but that the presence of other co-factors may activate Lyl1 bound at different sites. Since Lyl1 forms multimeric complexes with E2A, LMO2, Ldb1, and GATA [31,56–58], Lyl1-CREB1 complexes bound to Id1 may become activated by recruting these proteins and/or factors that interact directly with CREB1.

The presence of multiple E box sites in the Id1 promoter complicates the interpretation of the results. We cannot exclude the possibility that Lyl1-E2A interactions contribute to Id1 upregulation. However, using competition experiments with the minimal murine Id1 promoter identified by Tournay and Benezra [59] we found that complexes containing Lyl1 assemble only on the Cre sequence and not on the E box or other sequences of this promoter. Furthermore, the wt and mutated E box promoters are equally activated in K562 cells that overexpress untagged Lyl1 compared to the empty vector, but mutation of the Cre site abolishes this effect (see Supplementary Information). This demonstrates that Lyl1-CREB1 interactions activate the Id1 promoter, in this system. Furthermore, we explored the presence of an E box-dependent transcriptional activating component by performing similar experiments with Tal1 but did not observe an effect (not shown).

Until recently, only a handful of Tal1 promoters had been recognized, such as c-kit, retinal aldehyde dehydrogenase and AK14772, a gene of unknown function [29,60,61]. These genes all contain closely spaced E2A and GATA sites, or a critical Sp1 site (c-kit). Many new Tal1 promoters were recently identified [62], but there are still no known genes regulated by Lyl1. To begin the search for Lyl1 targets we performed ChIP-chip experiments. Because our supply of the polyclonal Lyl1 antibody [21] was insufficient to carry out these experiments while Lyl1 protein is poorly expressed in K562 cells, we over-expressed HA-Lyl1 and conducted ChIP-chip experiments using αHA and αCREB1. Surprisingly, one half of the sites occupied by Lyl1 were also occupied by CREB1. Of these common genes, 73% had been previously identified as CREB1 targets. These data, although limited to a 12K representation of the human genome associated with CpG regions, are suggestive of a widespread co-regulation of transcription by Lyl1 and CREB1. Nevertheless, enforced expression of HA-Lyl1 may have generated artifacts, such as displacement of endogenous complexes or the

generation of artificial ones. The extent of such artifacts is difficult to estimate. However, it is unlikely that artifacts can account for the approximately 50% overlap between the genomic Lyl1 and CREB1 sites identified in separate experiments. This is reinforced by the observation that ChIP-chip experiments performed with cells expressing Flag-Tal1 did not reveal a significant overlap between Tal1 and CREB1 sites (not shown). Furthermore, analysis of a landmark study showed that approximately 20% of the genes expressed in AML clinical samples correlate with the expression of both Lyl1 and CREB1, which may co-regulated their expression (Table 2). The 12K CpG microarray did not include the promoter regions for Id1, Id3 and Id4 that were amplified in the original ChIP assays (Fig. 1B) so we could not confirm Lyl1 and CREB1 binding to their promoters by ChIP-chip.

Recent reports have linked high levels of CREB1 expression with poor prognosis following chemotherapy in AML patients [27,28]. This is likely due to the regulation of specific targets by CREB1. It is recognized that many of the functions mediated by CREB1 require its activation through a specific phosphorylation event involving Ser 133 residue, but induction of Ser 133 accounts for the activation of only 2% of all CREB1 targets [54]. This suggests that additional factors are required for the expression of other CREB1 regulated genes. As Lyl1 and CREB1 are co-expressed in AML, and as the two proteins can interact in the absence of Ser133 phosphorylation CREB1-Lyl1 complexes may contribute to gene deregulation in AML. In keeping with this, we have shown that Lyl1 could increase the expression of CREB1 responsive genes such as cyclin D3, Egr1 and Id1, which are highly expressed in AML [25]. It is of note that Lyl1 did not alter the expression of Bcl2, Bcl-xL or Id2 genes that are also regulated by CREB1. This inconsistency may be due to the fact that the Bcl2 and Bcl-xL genes are already maximally stimulated in the absence of Lyl1, or that other factors are playing a role in the regulation of these genes in leukemic cells.

In conclusion, this is the first study to show that Lyl1 can potentially transactivate a different set of genes than Tal1, based upon the ability of Lyl1 to interact with CREB1. This may explain in part the difference in phenotypes in leukemias in which Lyl1 or Tal1 are the predominant bHLH oncogenes and may open a new avenue of research into the function of these two oncoproteins in AML.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.11.015.

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