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CREBBP knockdown enhances RAS/RAF/MEK/ERK signaling in Ras pathway mutated acute lymphoblastic leukemia but does not modulate chemotherapeutic response

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ABSTRACT

Relapsed acute lymphoblastic leukemia is the most common cause of cancer-related mortality in young people and new therapeutic strategies are needed to improve outcome. Recent studies have shown that heterozygous inactivating mutations in the histone acetyl transferase, *CREBBP*, are particularly frequent in relapsed childhood acute lymphoblastic leukemia and associated with a hyperdiploid karyotype and *KRAS* mutations. To study the functional impact of *CREBBP* haploinsufficiency in acute lymphoblastic leukemia, RNA interference was used to knock down expression of CREBBP in acute lymphoblastic leukemia cell lines and various primagraft acute lymphoblastic leukemia cells. We demonstrate that attenuation of CREBBP results in reduced acetylation of histone 3 lysine 18, but has no significant impact on cAMP-dependent target gene expression. Impaired induction of glucocorticoid receptor targets was only seen in 1 of 4 CREBBP knockdown models, and there was no significant difference in glucocorticoid-induced apoptosis, sensitivity to other acute lymphoblastic leukemia chemotherapeutics or histone deacetylase inhibitors. Importantly, we show that CREBBP directly acetylates KRAS and that CREBBP knockdown enhances signaling of the RAS/RAF/MEK/ERK pathway in Ras pathway mutated acute lymphoblastic leukemia cells, which are still sensitive to MEK inhibitors. Thus, CREBBP mutations might assist in enhancing oncogenic RAS signaling in acute lymphoblastic leukemia but do not alter response to MEK inhibitors.

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Introduction

Childhood acute lymphoblastic leukemia (ALL) is the most common form of childhood malignancy and cause of cancer-related death.[1] Following many years of continually improving treatment protocols, incorporating risk stratification, the cure rate of children has reached excellent levels, with sustained remission approaching 90%.[2] Still, relapse following therapy remains a major clinical problem, with 5-year survival rates of only 25% for children classified as high-risk.[3,4] Understanding the mechanisms of relapse and targeting relapse-associated mutations may lead to improved therapies that are clearly necessary for these children.[5]

One gene implicated in ALL relapse encodes cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) binding protein (CREBBP/CBP), a member of the KAT3 family of histone acetyltransferases (HAT) along with its paralog, EP300. CREBBP is involved in a wide range of processes, including cAMP-dependent signaling, histone acetylation, acetylation-mediated activation or inactivation of non-histone proteins, Wnt signaling, cell cycle control, ubiquitination,

DNA damage repair and antigen presentation.[6-12] Germline mutations in *CREBBP* cause Rubinstein-Taybi Syndrome, which is characterized by developmental defects and an increased susceptibility to malignancies.[13,14] A study by Mullighan *et al.* identified that 18% of relapsed childhood ALL cases were *CREBBP* mutant,[15] and further studies showed enrichment in the high hyperdiploid (HHD) (51-68 chromosomes) and hypodiploid cytogenetic subgroups, seen in approximately 30% of cases.[16-18] *CREBBP* is most commonly affected by heterozygous alterations, mainly point mutations, and less frequently by deletions. *CREBBP* mutations affect primarily the HAT domain leading to attenuation or loss of function of the mutant protein, but without altering the activity of the remaining wild-type allele.[15] Thus, the ensuing functional outcome is haploinsufficiency. Biallelic alterations only occur in approximately 6% of cases.[15,16] In mouse embryonic fibroblast cell models, *CREBBP* mutations were shown to cause reduced acetylation of CREBBP target residues, as well as reduced expression of cAMP-dependent and glucocorticoid (GC) responsive genes.[15] These results, coupled with the observation that *CREBBP* mutations appear to be enriched at relapse, suggest that *CREBBP* mutations may be a determinant of drug resistance, increasing the risk of relapse. *CREBBP* mutations also frequently co-occur with Ras pathway activating mutations, particularly *KRAS*,[15,17-19] suggestive of a possible link between CREBBP attenuation and oncogenic signaling. From a therapeutic view point, the global reduction in HAT activity in *CREBBP* mutated cells may be reversed by the use of histone deacetylase (HDAC) inhibitors and sensitivity to the HDAC inhibitor (HDACi), vorinostat, has been previously shown.[15] Thus HDACi were proposed as potential therapies for CREBBP mutant ALL cases.

In this study, we are the first to assess the functional effects of *CREBBP* haploinsufficiency in ALL cell lines and primary-derived (primagraft) ALL cells. Our data do not support a role of *CREBBP* mutations in modulating response to GC, other ALL chemotherapeutic drugs or HDACi. We show, however, that KRAS is directly acetylated by CREBBP and that knockdown of CREBBP is associated with enhanced signaling of the RAS/RAF/MEK/ERK pathway in Ras pathway mutant ALL cells. Importantly, sensitivity to MEK inhibition was preserved.

Methods

Cell culture

Two B-cell precursor ALL (BCP-ALL) cell lines lacking CREBBP alterations (as determined by Sanger Sequencing and COSMIC database), derived from pediatric samples, were used in this study. PreB 697 (recently re-named EU-3 by the original author[20] and also referred to as "697" in cell line repositories) was a kind gift from Reinhard Kofler, Austria. These cells were cultured in RPMI-1640 (Sigma-Aldrich, Dorset, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rugby, UK). The near-haploid childhood BCP-ALL cell line, MHH-CALL-2,[21,22] was purchased from DMSZ (Braunschweig, Germany) and was maintained in RPMI-1640, supplemented with 20% FBS. All cell lines were cultured at 37°C in 5% (v/v) carbon dioxide and were routinely tested for mycoplasma contamination using MycoAlert® (Lonza, Basel, Switzerland). Primagraft ALL cells were maintained in short-term culture in RPMI-1640 supplemented with 10% FBS. To produce a maximal intracellular cAMP response, cells were treated with 100 μM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) and 10 μM forskolin (Sigma-Aldrich) for 90 minutes.

RNAi

PreB 697 cells were transduced with GFP-tagged pGIPZ lentiviral small hairpin RNA (shRNA) (Thermo Scientific, Rugby, UK) targeted to CREBBP (shCBP) or a vector containing a non-silencing sequence (shNEG) as control. ShCBP and shNEG cells were cultured in selection medium consisting of RF10 containing 10 μg/mL puromycin (Gibco) for three weeks and monitored by standard flow cytometry until 100% GFP positivity was attained. Small interfering RNA (siRNA) pool transfection targeting CREBBP (sc-29244) or non-targeting control (sc-37007) (Santa Cruz Biotechnology, Wembley, UK) was carried out, using concentrations between 120 nM and 500 nM of siRNA pool by electroporation using an EPI 2500 Elektroporation impulse generator at 350 volts.

Generation of ALL primagrafts

Primagrafts were originally created by intrafemoral injection of ALL cells into NOD SCID γ null mice and were characterized for Ras pathway mutation as previously described (*Online Supplementary Methods* and *Online Supplementary Table S1*).[23] CREBBP alterations were assessed by SNP 6.0 arrays and genotyping console software (Affymetrix, High Wycombe, UK) and/or Sanger sequencing (primer sequences available on request). All *in vivo* work was carried out in compliance with a Home Office licence (60/4552).

Real-time quantitative PCR

Real-time quantitative PCR (RQ-PCR) was carried out using the TaqMan® method on the 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK). TaqMan® Universal PCR MasterMix (Applied Biosystems) and TaqMan® assay probes (Applied Biosystems) were used (*Online Supplementary Table S2*). Data analysis was conducted using the Comparative Ct Threshold Method (ΔΔCT) with TBP, a universally expressed housekeeping gene, as the reference gene.

Western blotting

Membranes were probed with either anti-CBP (sc-369) (Santa Cruz), anti-AcH3K18 (ab1191) (Abcam, Cambridge, UK), anti-p-ERK (9101) (Cell Signalling, Hitchin, UK), anti-ERK2 (sc-153) (Santa Cruz), anti-KRAS (F234) (Santa Cruz), anti-acetyl lysine (New England Biolabs, Frankfurt am Main, Germany) or anti-α-tubulin (T6074) (Sigma-Aldrich) primary antibodies. Horseradish peroxidase (HRP) conjugated secondary anti-rabbit or anti-mouse antibodies (Dako, Glostrup, Denmark) were used and membranes were developed using an ECL prime detection kit (Amersham Biosciences, Buckinghamshire, UK) followed by exposure to X-ray film. Densitometry was carried out using AIDA image analysis software (Raytest, Straubenhardt, Germany).

In vitro acetylation assay

In vitro acetylation assays were performed to detect acetylation of KRAS by CREBBP using recombinant CREBBP and KRAS proteins (both Sigma-Aldrich, Vienna, Austria). Reaction was carried out for 1 hour at 30°C in non-denaturing lysis buffer with the addition of 10 μM sodium butyrate and 25 μM acetyl-CoA. Enzymatic reaction was terminated by addition of 6xSDS loading buffer and heating at 90°C for 10 minutes. Acetylation levels were assessed by Western blot analysis.

Alamar blue drug sensitivity assay

Cells were treated with dexamethasone (Sigma-Aldrich), vorinostat (suberanilohydroxamic acid) (Selleckchem, Suffolk, UK)

or U0126 (Calbiochem, Nottingham, UK), alone, or dexamethasone and vorinostat in combination, and incubated for 96 hours. Alamar blue reagent (Invitrogen, Rugby, UK) was added to each well and cells were incubated for an additional 4-8 hours. Cell viability was determined by fluorescence intensity output using a FLUOstar Omega plate reader and Omega data analysis software (BMG Labtech, Aylesbury, UK). Fluorescence readings were expressed as a percentage of the control vehicle (CV) and plotted as a survival curve using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Gene expression microarray

Gene expression profiling (GEP) was carried out using Affymetrix technology as detailed in the *Online Supplementary Methods*.

Statistical analysis

Statistical significance was determined using the unpaired Student *t*-test on GraphPad Prism software (GraphPad Software Inc.).

Results

Stable CREBBP knockdown in BCP-ALL cells is associated with reduced acetylation of H3K18 and an expression profile linked to the GR

The BCP-ALL cell line, PreB 697, was selected for lentiviral transduction as it was shown to have two *CREBBP* wild-type alleles (*data not shown*). It was also resolved at the correct protein size by western blotting (Figure 1A). Cells were transduced with a pGIPZ vector, targeting either CREBBP (shCBP) or a non-silencing control (shNEG). After selection, shCBP cells had an approximately 40% reduction in *CREBBP* mRNA transcript levels compared to shNEG (Figure 1B), which translated into a greater knockdown at the protein level (Figure 1A). Protein amounts were similar to those seen in primagraft ALL samples with mono-allelic *CREBBP* deletions (Figure 1C and D). To elucidate the functional effect of CREBBP knockdown, acetylation of CREBBP target residue, histone 3 lysine 18

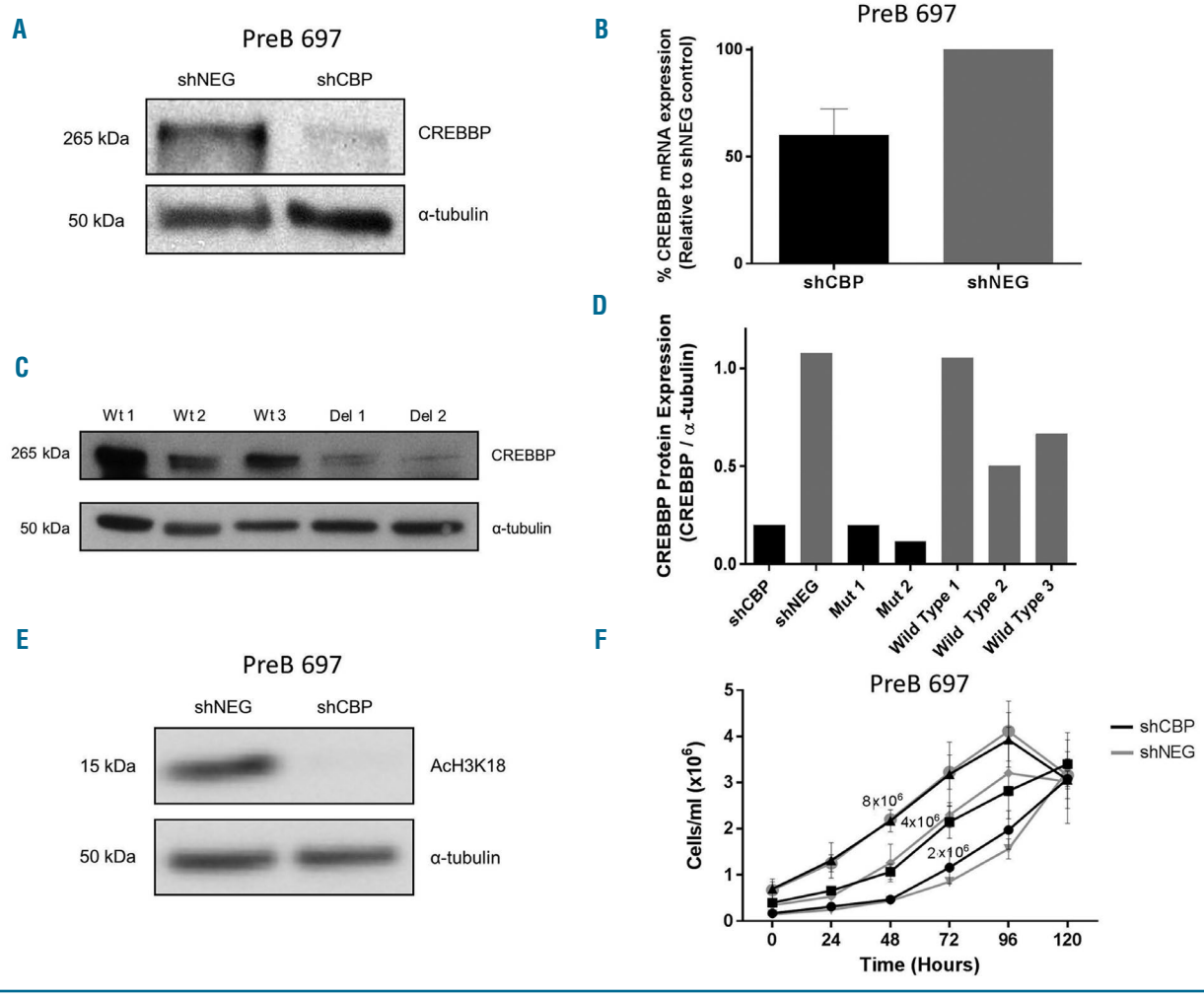

Figure 1. Generation and functional characterization of a stable CREBBP knockdown PreB-acute lymphoblastic leukemia (ALL) cell line. (A) Western analysis of CREBBP in PreB 697 shCBP and shNEG cells with α-tubulin serving as a loading control. (B) Histogram shows mean±SD (n=3) of *CREBBP* mRNA expression relative to *TBP* by RQ-PCR in PreB 697 shCBP and shNEG cells. (C) CREBBP protein expression relative to α-tubulin analyzed by western blotting in a panel of ALL primagrafts carrying wild-type (Wt 1, 2 and 3) or mono-allelic deletion in CREBBP (Del 1 and 2). (D) Histogram of the relative expression of CREBBP in PreB 697 cell lines (left two bars) and primagraft samples normalized to α-tubulin after densitometric analyses. (E) Western analyses of AcH3K18 relative to α-tubulin in PreB 697 shCBP and shNEG cells. (F) Growth curves of PreB 697 shCBP and shNEG cells at various seeding densities. Each point represents the mean cell number±SD (n=3).

(H3K18), was assessed by western blotting and was significantly reduced compared to shNEG cells (Figure 1E). There was no effect of CREBBP knockdown on cell growth or viability (mean±SD; shCBP 32.4 hours±5.4; shNEG 30.7 hours±4.8; *P*=0.7 by Student *t*-test) (Figure 1F).

Given the important role of CREBBP in cAMP-dependent signaling, CREBBP knock-down cells were next studied for expression of cAMP-dependent target genes in response to increased intracellular cAMP. Cells were treated with 100 μM IBMX and 10 μM forskolin to produce a maximal intracellular cAMP response, and analyzed by RQ-PCR for expression of a selection of cAMP-dependent genes. These genes included those previously identified in mouse embryonic fibroblasts (MEF)[15] and also those determined by GEP of PreB 697 cells treated with IBMX and forskolin (*Online Supplementary Table S3*). While induction of gene expression was shown for all selected genes, there was no significant difference in the level of induced gene expression between shCBP and shNEG cells (*P*>0.3) (Figure 2). To investigate the effect of CREBBP knockdown on global gene expression, GEP was performed and the expression of 28 genes was found to be significantly altered in shCBP cells compared to shNEG (*Online Supplementary Table S4*). Ingenuity pathway analysis of the data set showed that transcription of NR3C1, the gene encoding the glucocorticoid receptor (GR) may be affected in CREBBP knockdown cells, based on differential expression of the upstream genes *SCG2, OAT* and *DNAJC15* (*P*=0.04), indicative of a link between CREBBP and GC response.

Stable *CREBBP* knockdown impairs expression of GR target genes but does not alter response to GC

To assess the effect of CREBBP knockdown on GC response, shCBP cells were analyzed for induced expression of the classical GR transcriptional targets, *GILZ* (*TSC22D3), FKBP5, NR3C1 (GR)* and *ITGA9*, following 24-hour exposure to either 17 nM (GI_{50} for PreB 697 cells) dexamethasone or CV. ShCBP cells showed a significant impairment in the GC-induced expression of *GILZ* (*P*=0.009), *FKBP5* (*P*=0.03) and *NR3C1* (*P*=0.0003), with expression levels approximately half of that seen in shNEG cells, but no such effect was seen for *ITGA9* (*P*=0.9) (Figure 3A). However, there was no significant effect on sensitivity to dexamethasone, assessed by viability assay following 96-hour exposure (GI50 values, mean±SD; shCBP 16.6nM±5.5 and shNEG 16.7nM±3.1; *P*=0.9) (Figure 3B). There was also no difference in drug response to the class I/II HDAC inhibitor, vorinostat (GI50 values, mean±SD; shCBP 485 nM±27.8 and shNEG

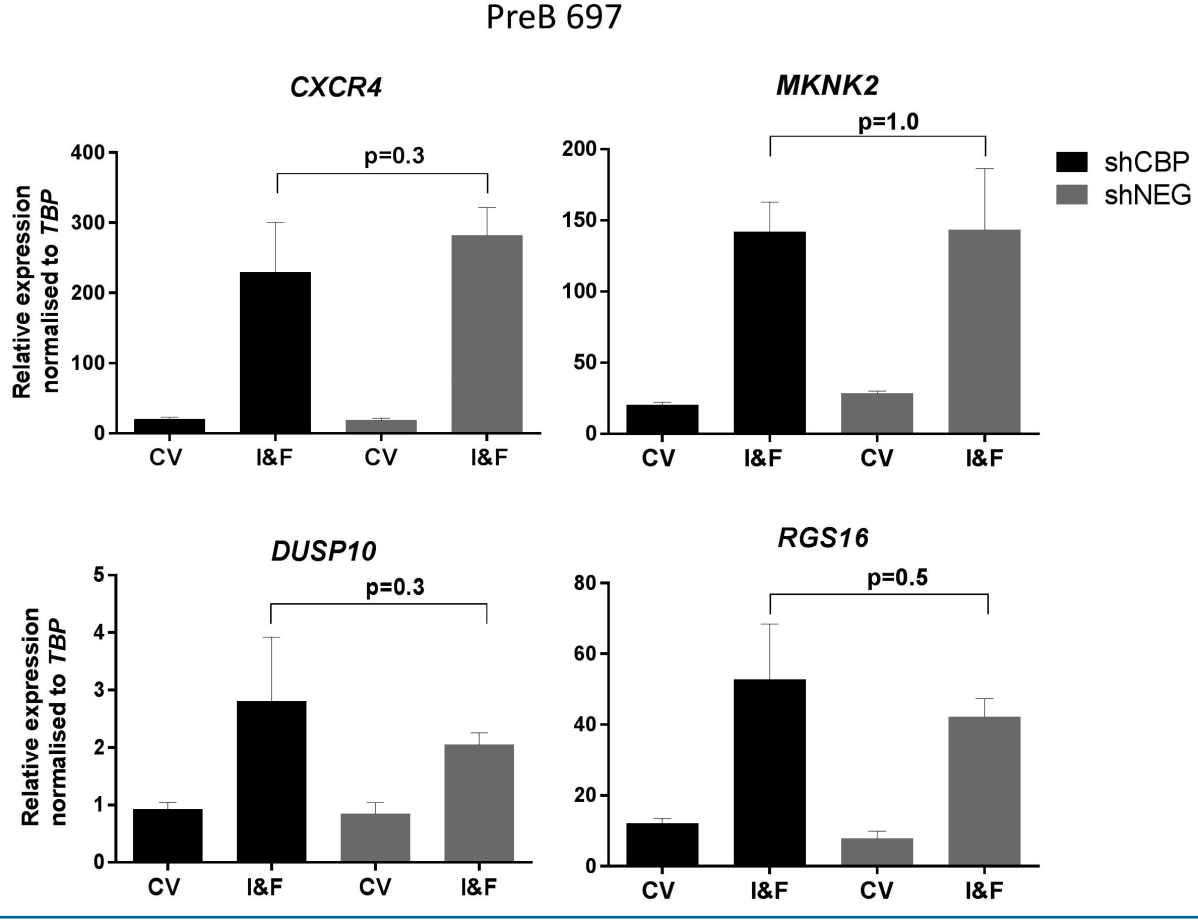

Figure 2. Stable CREBBP knockdown in PreB 697 cells and induced expression of cAMP-dependent genes. Histogram of RQ-PCR data showing mean and SD of cAMP-dependent gene expression relative to the reference gene TBP in PreB 697 shCBP and shNEG cells following 90-minute dosing with control vehicle (CV) or IBMX/forskolin (I&F) treatment (n=3).

471 nM±48.75; *P*=0.9) or to a combination of dexamethasone and vorinostat (*P*=0.8) (Figure 3B). Further to this, there was no significant differential sensitivity to the common ALL therapeutics, daunorubicin, vincristine, methotrexate and 6-thioguanine (*P*>0.07) (*Online Supplementary Figure S1*).

Transient knockdown of *CREBBP* in PreB 697 and MHH-CALL-2 cells has no impact on *GR* target gene induction or GC response

To confirm these data, we used a pool of small interfering RNA (siRNA) targeting CREBBP transfected into PreB 697, as well as MHH-CALL-2 cells. Results were similar for both cell lines. For PreB 697 cells, CREBBP knockdown was associated with reduced acetyl H3K18 (AcH3K18) levels (Figure 4B) but there was no significant impairment in cAMP-dependent target expression (Figure 4C). Unlike in the stably transduced PreB 697 cells, siRNA did not show a significant alteration of GC-induced expression of GR targets; *GILZ, FKBP5* and *ITGA9* (PreB 697 *GILZ*; *P*=0.6, *FKBP5*; *P*=0.6, *ITGA9*; *P*=0.2). There was, however, a statistically significant increase in the expression of NR3C1 (*P*=0.01) (Figure 4D) and an accompanying significant sensitization to dexamethasone in PreB 697 siCBP cells (GI_{50} values, mean±SD; siCBP 17 nM±1.6, control 34.7 nM±2.7; *P*=0.0006) (Figure 4E). In MHH-CALL-2 cells, transient CREBBP knockdown was associated with reduced AcH3K18 expression, but neither impairment in induced cAMP-dependent nor GR target gene expression (*CXCR4, MKNK2, DUSP10* and *RGS16*; *P*>0.2 and *GILZ, FKBP5, NR3C1* and *ITGA9*; *P*>0.7, respectively). In line with this, there was no difference in sensitivity to dexam-

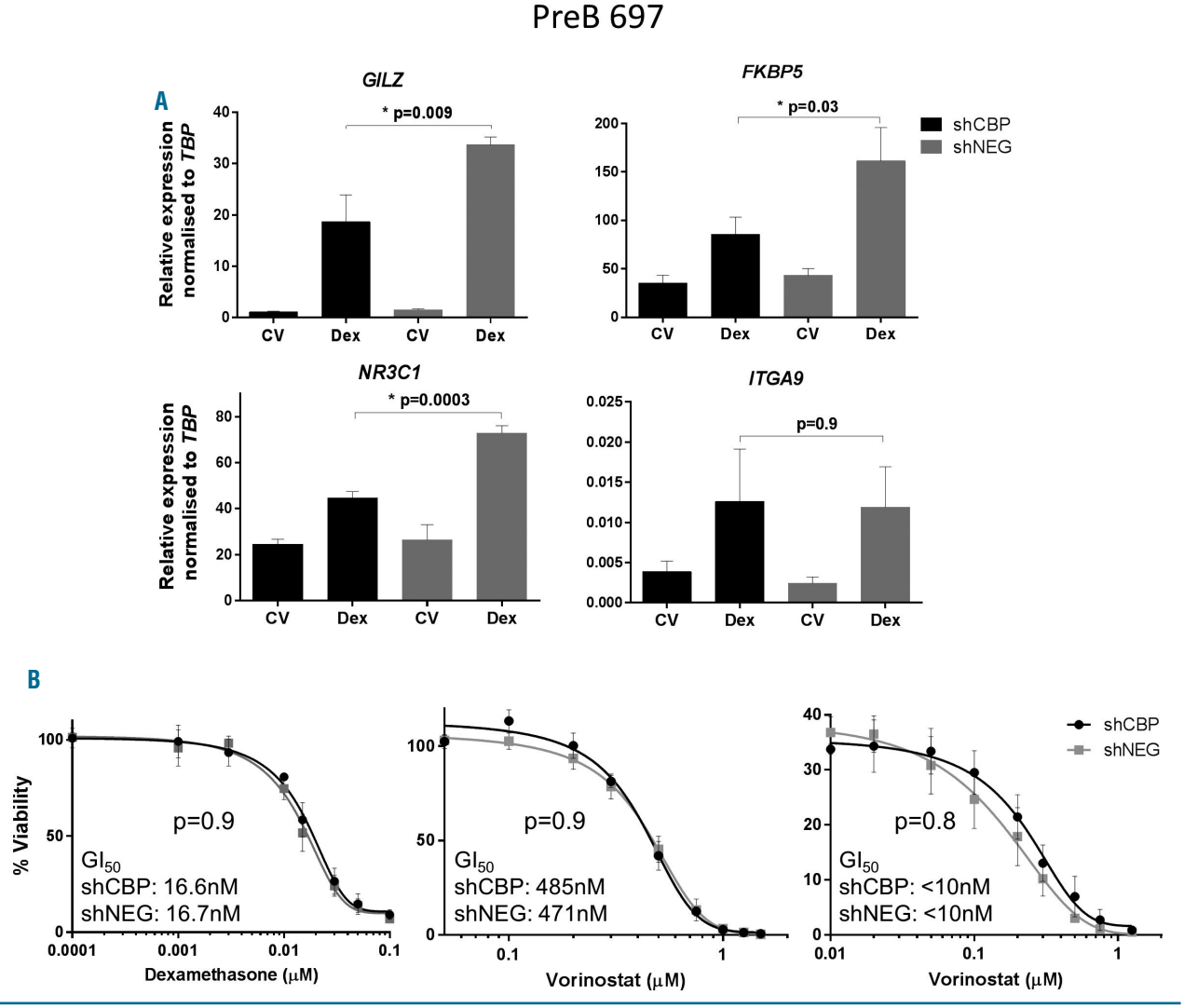

Figure 3. Stable CREBBP knockdown in PreB 697 cells leads to impaired expression of glucocorticoid receptor (GR) target genes, but does not influence glucocorticoid (GC) sensitivity. (A) Histogram of RQ-PCR data showing mean and SD of mRNA expression of GR target genes relative to *TBP* in PreB 697 shCBP and shNEG cells following 24-hour exposure to 17 nM dexamethasone (n=3). (B) Viability curves of PreB 697 shCBP and shNEG cells after dosing with dexamethasone, vorinostat or a combination of both, in which the concentration of vorinostat was varied and that of dexamethasone kept constant at 17 nM. Viability curves show mean±SD (n=3).

ethasone (GI_{50} values, mean±SD; siCBP 2.8 nM±0.59; control 3.8 nM±0.7; *P*=0.1) (Figure 5A-E).

CREBBP knockdown in primary HHD ALL cells does not alter response to GC

To add further biological relevance to the study, CREBBP knockdown using siRNA pool transfection was carried out in HHD ALL primagrafts. Knockdown was confirmed by western analyses (Figure 6A) and in 2 of 3 primagraft samples was associated with a reduction in AcH3K18 levels (a representative example is shown in Figure 6B). Overall, CREBBP knockdown had a minimal effect on the induced expression of cAMP-dependent targets or of GR targets (Figure 6C and D). Importantly, as shown in all other models, CREBBP knockdown in all 3 HHD primagraft samples showed no significant effect on sensitivity to dexamethasone compared to vehicle-treated control cells (GI_{50} values, mean±SD; L779 siCBP 65.2 nM±38.3 *vs.* control 90.8 nM±18.2; *P*=0.4, L829R >10 μM for both; L914 siCBP 6.1 nM±2.2 *vs.* control 5.6 nM±0.41; *P*=0.7) (Figure 6E).

KRAS is a substrate of CREBBP and knockdown of CREBBP is associated with increased Ras signaling

Recent studies have shown that *CREBBP* mutations frequently co-occur with RAS mutations in childhood HHD ALL.[18,24] To test for acetylation of KRAS by CREBBP, we performed *in vitro* acetylation assays using recombinant proteins. Direct acetylation of KRAS was shown in the presence of CREBBP, identifying KRAS as a substrate for CREBBP (Figure 7A). The functional impact of CREBBP attenuation on Ras pathway signaling was investigated by RNAi-mediated knockdown of CREBBP in PreB 697 cells which harbor NRAS (G12D) mutation and in 3 Ras pathway mutant HHD ALL primagrafts (L779, L829R and L914) with *NRAS* (Q61R), *KRAS* (G13D) and *CBL/FLT3* (Δ836) mutations (*Online Supplementary Table S1*). Western blotting showed an increase in p-ERK levels in PreB 697 shCBP cells compared to shNEG cells and in L829R and L914 primagraft ALL samples after siCBP knockdown, suggesting that CREBBP reduction enhances Ras pathway activation (Figure 7B and C). To assess the influence on sensitivity to MEK inhibition (MEKi), PreB shCBP and shNEG cells were dosed with the benchmark MEKi, U0126. CREBBP knockdown had no effect on sensitivity to U0126 (GI_{50} values, mean±SD; shCBP 9.8 μM±3.5 *vs.* shNEG 10.4 μM±SD 1.7; *P*=0.8) (Figure 7D). CREBBP knockdown in MHH-CALL-2 cells, which lack RAS pathway mutations, did not alter p-ERK levels or MEKi sensitivity (*Online Supplementary Figure S2*).

Discussion

While *CREBBP* mutations were initially identified in lymphoid malignancies, including relapsed childhood

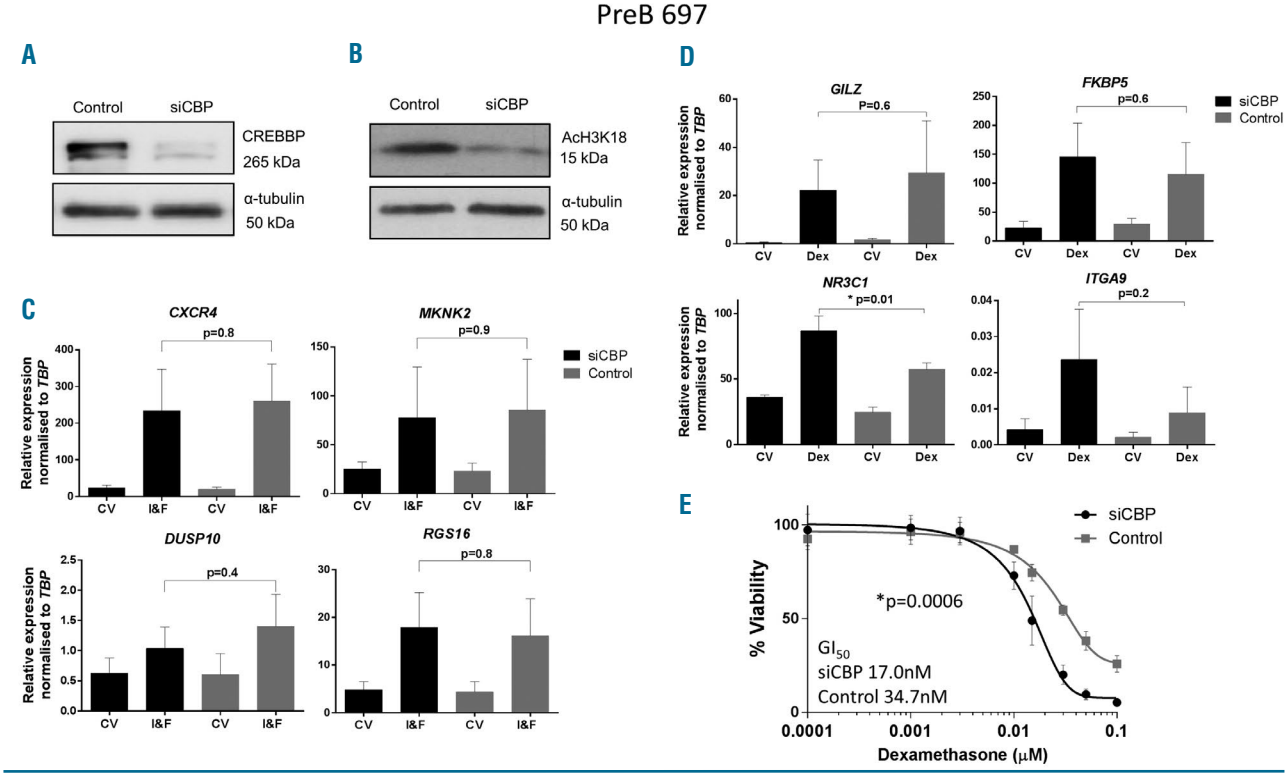

Figure 4. Transient CREBBP knockdown in PreB 697 cells does not affect induced expression of cAMP-dependent or glucocorticoid receptor (GR) target genes and has marginal effect on glucocorticoid (GC) sensitivity. Representative western blot of CREBBP (A) and AcH3K18 (B) in siCBP and control treated PreB 697 cells 24 hours post transfection, with α-tubulin used as loading control. (C) Histograms of RQ-PCR data showing mean and SD of cAMP-dependent gene expression relative to the reference gene TBP in siCBP and control treated PreB 697 cells, following 90-minute dosing with control vehicle (CV) or IBMX/forskolin (I&F) treatment (n=3). (D) Histograms of RQ-PCR data showing mean and SD of mRNA expression of GR target genes relative to TBP in siCBP and control treated PreB 697 cells (n=3). (E) Viability curves of siCBP and control treated PreB 697 cells after dosing with dexamethasone. Values plotted represent the mean % viability±SD (n=3).

ALL, diffuse large B-cell lymphoma and follicular lymphoma,[15,25-27] it is becoming increasingly apparent that *CREBBP* is inactivated in a range of solid tumor types, including bladder, medulloblastoma, lung, adenoid carcinoma, esophageal cancer and thalamic glioma.[28-36] To date, experimental data on the functional consequences of *CREBBP* inactivation in oncogenesis and disease progression are sparse, with this study being one of the first in any tumor type. CREBBP knockdown was performed in ALL cell lines both stably and transiently. Knockdown was also achieved in primagrafts from ALL patients within the HHD cytogenetic subtype, known to be commonly associated with *CREBBP* mutations. In the majority of cell models, induction of cAMP-dependent gene expression was unaffected by CREBBP knockdown, suggesting that remnant CREBBP protein levels were sufficient to maintain cAMP-dependent signaling. While GEP of PreB 697 shCBP cells showed differential expression of genes upstream of the GR (i.e. *SCG2, OAT, DNAJC15*) and significant impairment of GR target gene expression in response to GC treatment, no impact was seen on GC-induced apoptosis. In fact, CREBBP knockdown in both ALL cell lines and all ALL primagrafts tested did not impact on GC sensitivity. However, expanding the range of cell lines and primary samples to include wild-type and other RAS pathway activating mutations, may clarify some of the variability we observed on the transcription of cAMP and GR targets. Nevertheless, our data are in line with a recent clinical study of HHD ALL cases, which showed that patients with *CREBBP* mutation did not have a poor response to prednisone in BFM protocols.[18]

Prompted by the observation that *CREBBP* mutations frequently co-occur with Ras pathway activating mutations, including *KRAS, NRAS, PTPN11* and *FLT3*,[18] we investigated the influence of CREBBP knockdown on Ras signaling. Both cell lines and primagraft ALL cells, with different Ras pathway mutations, showed increased levels of p-ERK after CREBBP knockdown, suggesting that CREBBP attenuation enhances Ras signaling. We show that KRAS is directly acetylated by CREBBP, a secondary modification shown to have a negative regulatory effect on RAS activity by altering conformational stability of the Switch II domain and thus interaction with guanine nucleotide exchange factors.[37] The link we have identified between mutations in epigenetic modifiers such as *CREBBP* and enhanced oncogenic signaling is supported by a recent study in early thymocyte precursor ALL which found that inactivation of the methyltransferase, EZH2, co-operates with mutant RAS to induce hyperactive cytokine signaling, principally through STAT3, and was associated with a reduced sensitivity to JAK inhibitors.[38] Further to this, inactivation of another methyltransferase, *SUZ12*, has also been shown to co-operate with Ras signaling in a number of solid tumors, acting here to enhance downstream oncogenic Ras transcriptional signatures.[39]

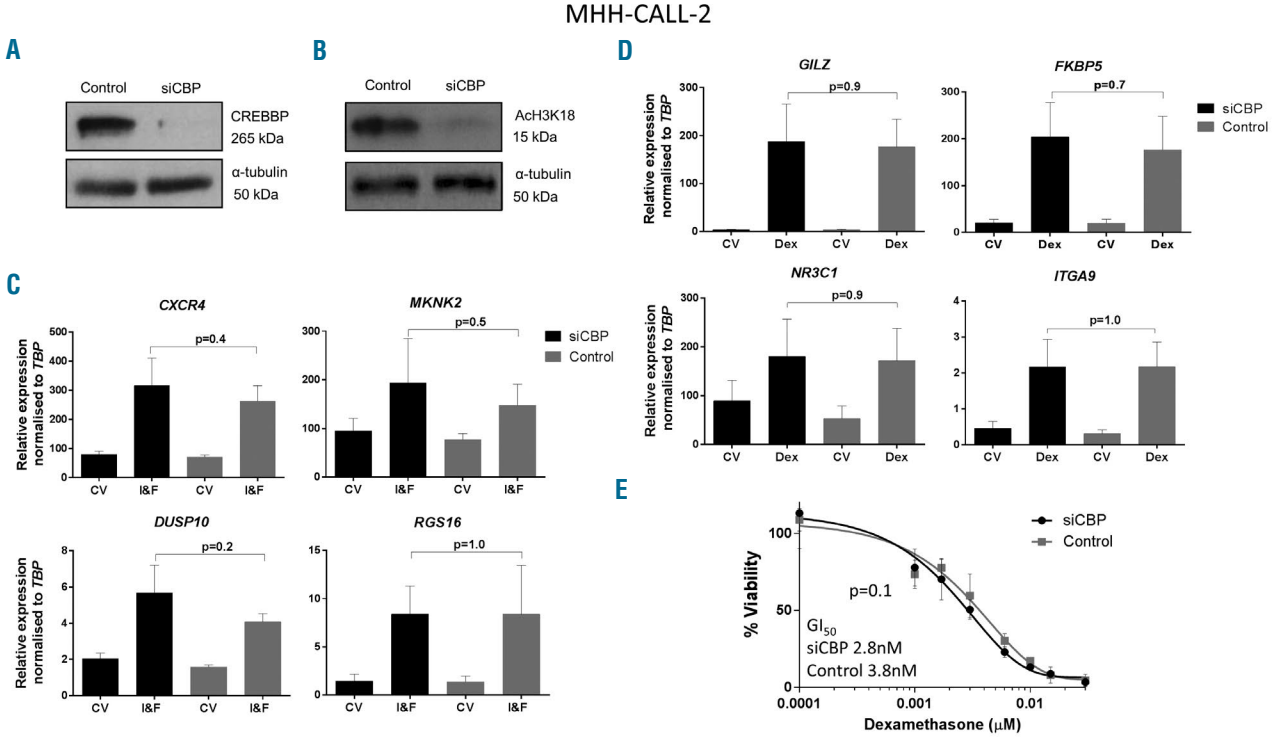

Figure 5. Transient CREBBP knockdown in MHH-CALL-2 cells does not affect induced expression of cAMP-dependent or glucocorticoid receptor (GR) target genes and has no effect on glucocorticoid (GC) sensitivity. Representative western blot of CREBBP (A) and AcH3K18 (B) in siCBP and control treated MHH-CALL-2 cells 24 hours post transfection, with α-tubulin used as loading control. (C) Histograms of RQ-PCR data showing mean and SD of cAMP-dependent gene expression relative to the reference gene *TBP* in siCBP and control treated MHH-CALL-2 cells, following 90-minute dosing with control vehicle (CV) or IBMX/forskolin (I&F) treatment (n=3). (D) Histograms of RQ-PCR data showing mean and SD of mRNA expression of GR target genes relative to *TBP* in siCBP and control treated MHH-CALL-2 cells following 24-hour exposure to 17 nM dexamethasone (n=3). (E) Viability curves of siCBP and control treated MHH-CALL-2 cells after dosing with dexamethasone. Values plotted represent the mean % viability±SD (n=3).

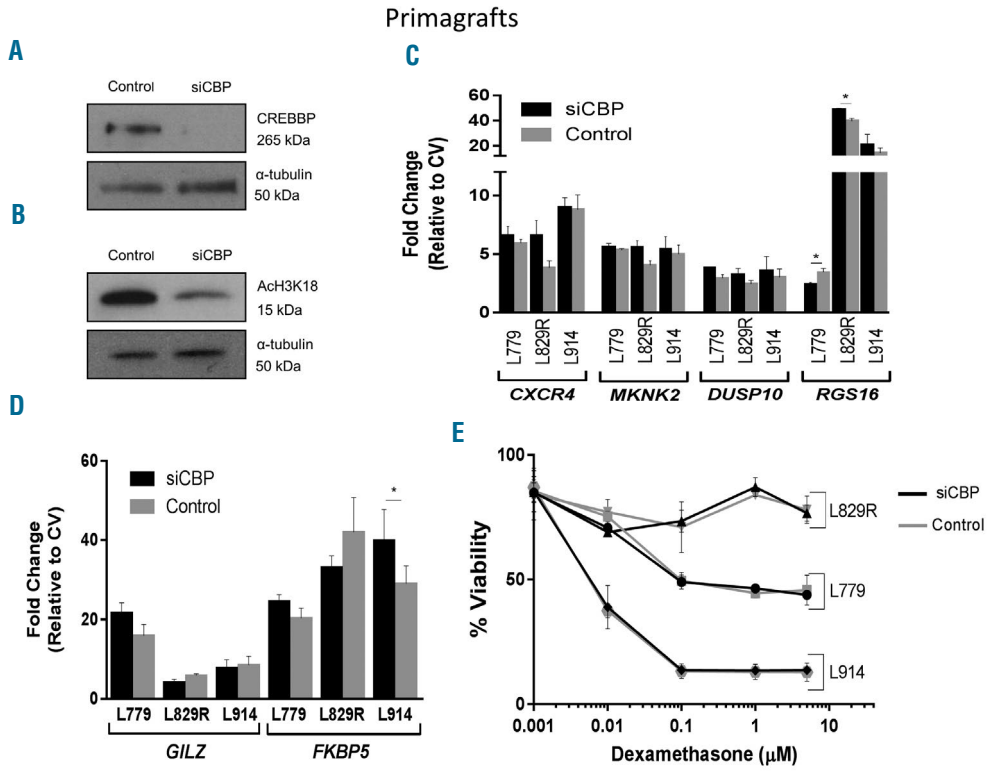

Figure 6. CREBBP knockdown in HHD primagraft ALL cells does not significantly impair the induced expression of cAMP-dependent or glucocorticoid receptor (GR) target genes and does not impact on glucocorticoid (GC) sensitivity. Representative western blot of CREBBP (A) and AcH3K18 (B) in siCBP and control treated HHD primagraft ALL cells 24 hours post transplantation, with α-tubulin used as loading control. (C) Histograms of RQ-PCR data showing mean and SD of the fold change relative to CV in cAMP responsive genes in siCBP and control treated HHD primagraft ALL cells, following 90-minute dosing with CV or IBMX/forskolin (I&F) treatment. TBP was used as reference gene. Histograms show means of triplicate wells±SD for L779 and L829R and triplicate primagraft samples for L914 (n=3). (D) Histograms of RQ-PCR data showing mean and SD of the fold upregulation of GR target genes relative to CV in siCBP and control treated HHD primagraft ALL cells following 24 hours exposure to 1 μM dexamethasone. Histograms show intra-assay for mean±SD L779 and L829R and inter-assay for L914 (n=3). (E) Viability curves of siCBP and control treated HHD primagraft ALL cells after dosing with dexamethasone. Values plotted represent the mean % viability±SD of triplicate wells for L779 and L829R and triplicate experiments for L914.

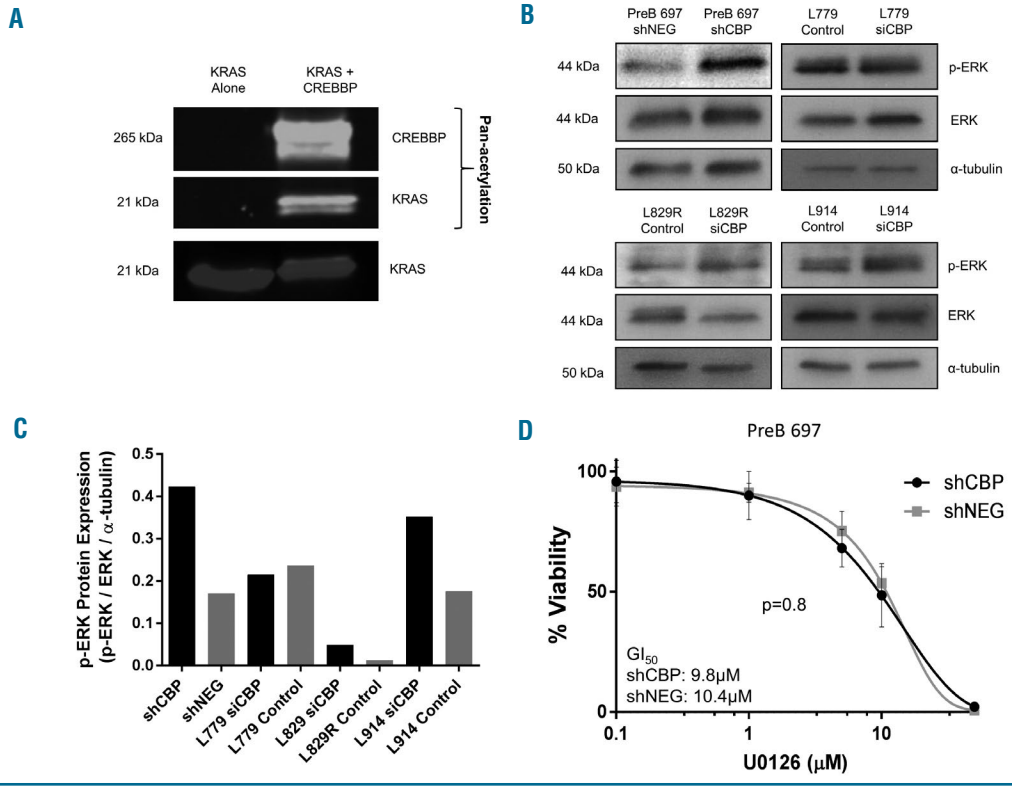

Figure 7. CREBBP acetylates KRAS and attenuation of CREBBP increased Ras pathway signaling without altering sensitivity to MEK inhibitors. (A) Western blot showing the acetylation of the recombinant KRAS by CREBBP as well as the autoacetylation of CREBBP, using acetylated lysine antibody (top) and KRAS antibody (bottom) to confirm equal loading. (B) Western blot for p-ERK, ERK and α-tubulin in CREBBP knockdown PreB 697 (shCBP and shNEG), L779, L829R and L914 cells. (C) Histogram of the relative expression of p-ERK in PreB 697 (shCBP and shNEG) and CREBBP knockdown primagraft samples normalized to ERK and α-tubulin after densitometric analyses. (D) Viability curves of PreB 697 shCBP and shNEG cells after 96-hour dosing with U0126. Values plotted represent the mean±SD of 3 independent experiments.

Here, we show that, despite an enhanced Ras pathway activation in CREBBP attenuated ALL cells, sensitivity to MEKi was retained, which may have important clinical implications. Our group has recently reported on the preclinical evaluation of the MEKi, selumetinib, and demonstrated significant differential sensitivity in Ras pathway-mutated ALL compared to ALL without *RAS* mutations, both *in vitro* and in an orthotopic xenograft model engrafted with primary ALL cells.[23,40] Clinical trials of selumetinib for relapsed Ras pathway mutated ALL cases are underway, and the data presented here suggest that patients with co-occurring *CREBBP* and *RAS* mutations might still be good candidates for MEKi therapy.

CREBBP plays a complex role in a wide array of cellular functions through its acetylation of numerous histone and non-histone proteins, which now includes KRAS. Additional substrates include the tumor suppressor p53 and the oncogene BCL6, with acetylation leading to activation and inactivation, respectively.[7,8] In follicular lymphoma, aberrant acetylation of both p53 and BCL6 was shown in CREBBP mutated samples, suggesting an effect on tumor suppressor/oncogene balance.[25] Another study implicated CREBBP mutation as an early event in follicular lymphoma, contributing to immune evasion due to decreased antigen presentation.[41] Thus, CREBBP mutations may have wide-ranging but varying effects across tumor types. Understanding the oncogenic mechanism of CREBBP is paramount as it may serve as a therapeutic target. Rebalancing of the physiological acetylation levels resulting from loss of HAT activity with HDAC inhibitors is an obvious therapeutic strategy, particularly since vorinostat and romidepsin are approved for the treatment of recurrent/refractory cutaneous T-cell lymphoma.[42,43] A recent report in lung cancer showed that CREBBP was synthetically lethal with its paralog EP300, and depletion of either gene led to apoptosis.[44] However, no differential sensitivity to vorinostat was evident in our CREBBP knockdown BCP-ALL models. Thus, these data suggest that MEK inhibitors might still be exploited in RAS pathway mutant ALL cases, irrespective of the presence of CREBBP alterations.

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