SUPPLEMENTARY APPENDIX

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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Supplementary Methods

Formation of Primagrafts

Following intrafemoral injection, mice were monitored for engraftment by tail vein bleed. Blood was red cell lysed (ammonium chloride) and analysed by flow cytometry on a BD FACSCanto II using anti human CD10, CD34 and CD19 and anti-mouse CD45 antibodies (BD Biosciences, Oxford, UK), Once peripheral blood ALL levels reached >30%, mice were euthanised and spleens were removed and resuspended in RPMI 1640 plus 10% foetal bovine serum (FBS). All spleens were >80% ALL cells as assessed by flow cytometry.

Gene Expression Microarray

RNA extracted from PreB 697 shCBP and shNEG cells on three separate occasions was ran on the Affymetrix U133 Plus 2.0 platform by Source Bioscience. Raw data was processed using R software and the GC Robust Multi-Array Average (gcrma) package to obtain expression values, hgu133plus2.db package to annotate gene probes and limma package to carry out differential gene expression analysis. Probe quality was determined by assessment of the normalised expression data and principal component analysis (PCA) and probes with low variation or low signal across arrays were removed. Genes were considered to be significantly differentially expressed where the p value was <0.05 and the fold change between groups was >2. Ingenuity pathway analysis (IPA) was carried out using QIAGEN's IPA software (Qiagen, Manchester, UK).

Table S1. Clinical details of patient samples used to generate primagrafts

Patient ID	Age at Diagnosis	CREBBP Status	Cytogenetics	Other Mutations
L779	5.5 yrs	Wild type	High Hyperdiploid	*NRAS* (Q61R)
L829 Relapse	3.1 yrs	Wild type (Wt 2)	High Hyperdiploid	*KRAS* (G13D)
L914	7.3 yrs	Wild type	High Hyperdiploid	*CBL/FLT3* Large del/Δ836
L885	2.2 yrs	Wild type (Wt 1)	High Hyperdiploid	-
G7578 NN	12 yrs	Wild type (Wt 3)	iAMP21	-
G7578 LN	12 yrs	Mono-allelic deletion (Del 1)	iAMP21	-
G1062 RN	17 yrs	Mono-allelic deletion (Del 2)	Hypodiploid	-

Table S2. List of TaqMan® assay probes (Applied Biosystems)

Probe	Product Number
CREBBP	Hs00231733_m1
GILZ (*TSC22D3*)	Hs00608272_m1
FKBP5	Hs00188025_m1
NR3C1 (*GR*)	Hs00230813_m1
ITGA9	Hs00174408_m1
CXCR4	Hs00607978_s1
MKNK2	Hs00179671_m1
DUSP10	Hs00200527_m1
RGS16	Hs00892674_m1
TBP (Endogenous Control)	4310891E

Table S3. cAMP-dependent genes in PreB 697 cells

Feature	Gene	log2FC	Average Expression	P.Value
217028_at	CXCR4	3.96	10.78	7.9E-08
218205_s_at	MKNK2	2.54	10.69	2.1E-09
215111_s_at	TSC22D1	2.15	9.00	1.1E-08
209201_x_at	CXCR4	4.62	8.85	1.9E-09
202284_s_at	CDKN1A	1.26	8.41	4.6E-08
211919_s_at	CXCR4	4.70	8.16	6.3E-09
201751_at	JOSD1	1.93	8.15	4.1E-08
208622_s_at	EZR	2.34	8.12	6.8E-08
201368_at	ZFP36L2	3.15	7.89	5.7E-09
212430_at	RBM38	2.51	7.44	1.1E-07
223199_at	MKNK2	2.74	7.43	4.4E-08
227846_at	GPR176	1.73	7.42	3.3E-08
209324_s_at	**RGS16**	**4.13**	**7.12**	**2.2E-08**
201473_at	JUNB	3.49	7.08	2.0E-10
201531_at	ZFP36	2.97	6.72	3.5E-08
202499_s_at	SLC2A3	4.61	6.18	2.1E-08
205289_at	BMP2	4.64	5.99	3.5E-09
227410_at	FAM43A	4.17	5.70	5.1E-09
202815_s_at	HEXIM1	2.34	5.56	1.0E-07
41577_at	PPP1R16B	2.96	5.44	1.5E-08
203910_at	ARHGAP29	5.48	5.42	3.9E-08
207630_s_at	CREM	4.68	5.33	3.6E-09
201236_s_at	BTG2	4.68	4.62	2.7E-08
208763_s_at	TSC22D3	5.22	4.60	2.0E-10
202340_x_at	NR4A1	3.75	4.43	4.6E-08
216236_s_at	NA	3.17	4.37	6.0E-10
204491_at	PDE4D	4.68	4.24	4.0E-09
214508_x_at	CREM	5.50	4.23	3.0E-10
217875_s_at	PMEPA1	3.74	4.15	5.1E-08
209967_s_at	CREM	4.78	4.09	7.1E-09
205463_s_at	PDGFA	4.37	4.08	1.0E-11
221563_at	**DUSP10**	**1.48**	**4.04**	**3.3E-08**
216268_s_at	JAG1	2.66	4.01	2.0E-08
214873_at	LRP5L	1.48	3.84	5.4E-08
222088_s_at	NA	4.72	3.76	2.7E-08
209189_at	FOS	5.39	3.76	3.2E-08
202497_x_at	SLC2A3	4.62	3.71	1.0E-10
209305_s_at	GADD45B	3.38	3.51	7.3E-08
209099_x_at	JAG1	3.57	3.36	1.9E-09
230233_at	NA	3.18	3.29	1.0E-11
229072_at	RAB30	3.37	3.23	5.2E-08
223217_s_at	NFKBIZ	2.24	2.96	7.0E-10
223887_at	GPR132	2.06	2.90	2.6E-08
201883_s_at	B4GALT1	2.68	7.10	1.2E-07
224797_at	ARRDC3	2.52	6.44	1.2E-07
202498_s_at	SLC2A3	3.37	3.93	1.2E-07

200605_s_at	PRKAR1A	1.40	9.72	1.3E-07
223218_s_at	NFKBIZ	3.47	3.30	1.4E-07
227558_at	CBX4	3.11	9.11	1.5E-07

PreB 697 cells were treated with 100µM IBMX and 10µM forskolin or CV for 90 minutes. RNA was harvested from these cells and analysed for differential gene expression using the Affymetrix U133 Plus 2.0 microarray platform. Five hundred and twenty two genes were found to be differentially expressed upon cAMP treatment. This list was sorted by largest change in expression (Average Expression column) and the top 50 genes were then sorted by lowest p values (i.e most significant). *CXCR4* and *MKNK2* (highlighted in grey) came out at the top of this list, and are found multiple times on the list. *DUSP10* and *RGS16* (bold text), which have been shown to be cAMP-dependent in mouse embryonic fibroblast cells,[1] were also found in this list. Log2FC: log2 of the fold change.

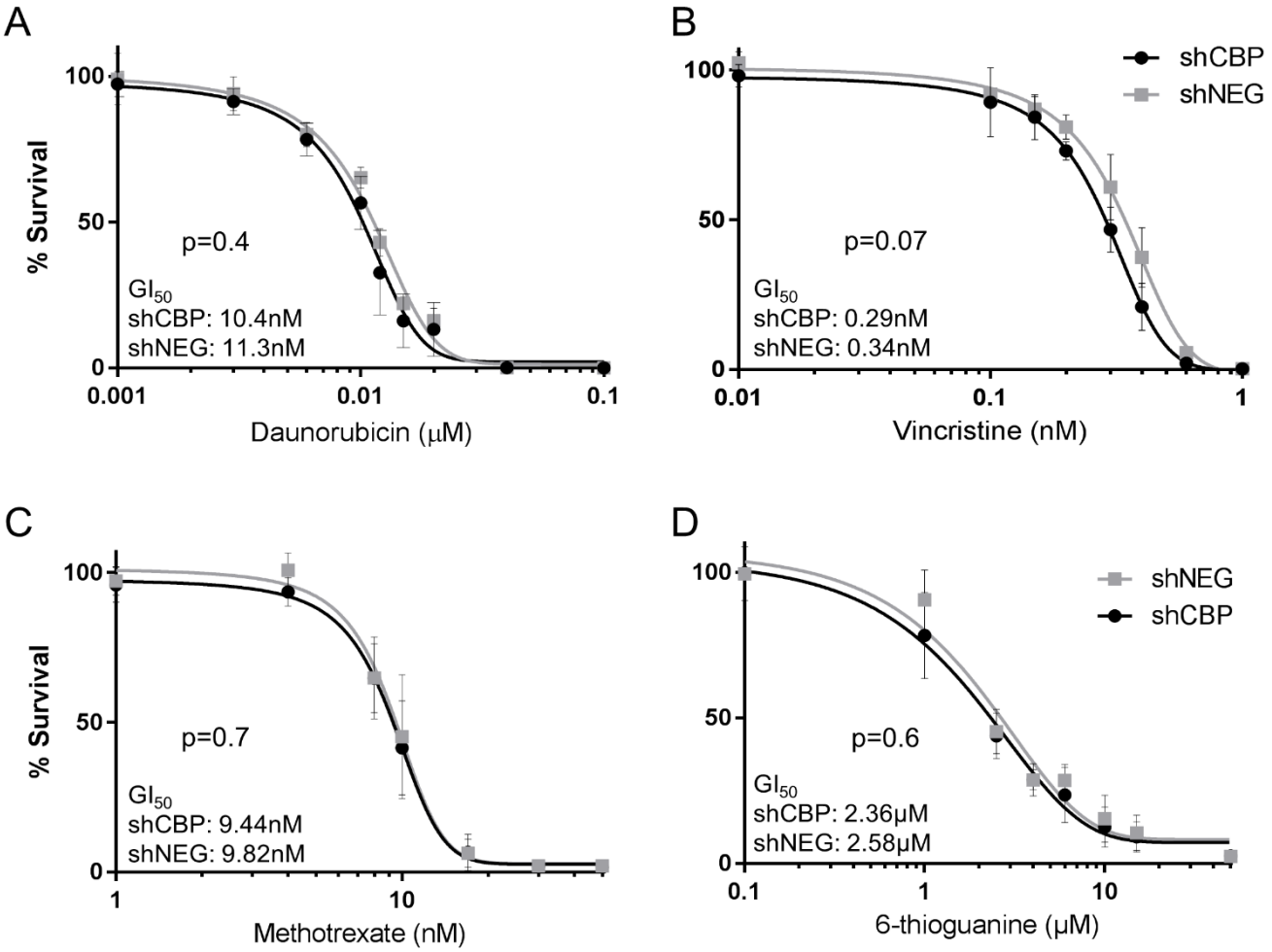
Table S4. CREBBP knockdown by shRNA transfection in PreB 697 cells leads to altered expression of genes upstream of the GR.

Feature	Gene	log2FC	P.Value
204035_at	**SCG2**	**2.81**	**3.3E-05**
232327_at	THSD7B	2.64	1.6E-04
201599_at	**OAT**	**2.59**	**1.7E-04**
234987_at	SAMHD1	1.37	1.6E-04
235048_at	FAM169A	1.23	8.5E-05
210933_s_at	FSCN1	1.17	1.1E-05
204048_s_at	PHACTR2	1.16	2.6E-06
229103_at	WNT3	1.11	3.0E-04
227819_at	LGR6	1.05	8.2E-05
213316_at	KIAA1462	-1.01	6.3E-07
209422_at	PHF20	-1.13	1.2E-04
217485_x_at	PMS2P1	-1.13	6.4E-05
204140_at	TPST1	-1.15	2.4E-04
209970_x_at	CASP1	-1.16	2.3E-05
222288_at	NA	-1.17	1.8E-05
200636_s_at	PTPRF	-1.17	1.6E-04
209321_s_at	ADCY3	-1.19	7.0E-05
208050_s_at	CASP2	-1.28	6.0E-05
223197_s_at	SMARCAD1	-1.33	1.8E-05
212589_at	RRAS2	-1.38	2.7E-04
203857_s_at	PDIA5	-1.50	1.0E-04
228843_at	ARL10	-1.56	2.2E-05
238029_s_at	SLC16A14	-1.61	2.2E-04
211368_s_at	CASP1	-1.61	5.8E-05
232232_s_at	SLC22A16	-1.70	3.3E-05
223204_at	FAM198B	-2.26	3.1E-06
204897_at	PTGER4	-2.48	1.3E-05
218435_at	**DNAJC15**	**-2.50**	**2.6E-06**

RNA samples isolated from PreB 697 shCBP and shNEG on three separate occasions were analysed using the Affymetrix U133 Plus 2.0 platform. Data was normalised and genes whose expression was significantly altered in shCBP compared to shNEG control were identified. Twenty eight genes in total were found to be significantly differentially expressed in CREBBP knockdown cells compared to control (Grey boxes greater expression, white boxes lower expression than control). Ingenuity pathway analysis (IPA) predicted that, based on the

differential expression of *SCG2*, *OAT*, and *DNAJC15* (written in bold) (p=0.045) that upstream expression of *NR3C1,* the gene encoding the glucocorticoid receptor, would be altered.

Figure S1. CREBBP knockdown in PreB 697 cells has no effect on sensitivity to common ALL chemotherapies.

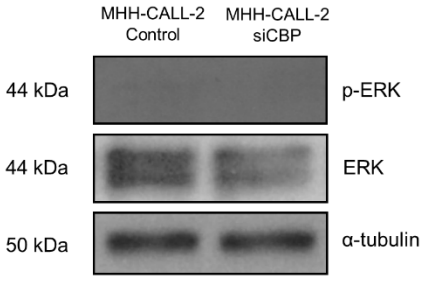


PreB 697 cells with stable CREBBP knockdown were assessed for sensitivity to drugs commonly used in ALL therapy; daunorubicin, vincristine, methotrexate and 6'-thioguanine, compared to isogenic control, by alamar blue drug sensitivity assay over 96 hours. CREBBP knockdown cells showed no differential sensitivity to any of the drugs tested. GI_{50} values, mean ±SD; daunorubicin shCBP 10.4nM ±1.5 versus shNEG 11.3nM ±0.52; p=0.4, vincristine shCBP 0.3nM ±0.02 versus shNEG 0.3nM ±0.04; p=0.07, methotrexate shCBP 9.4nM ±1.6

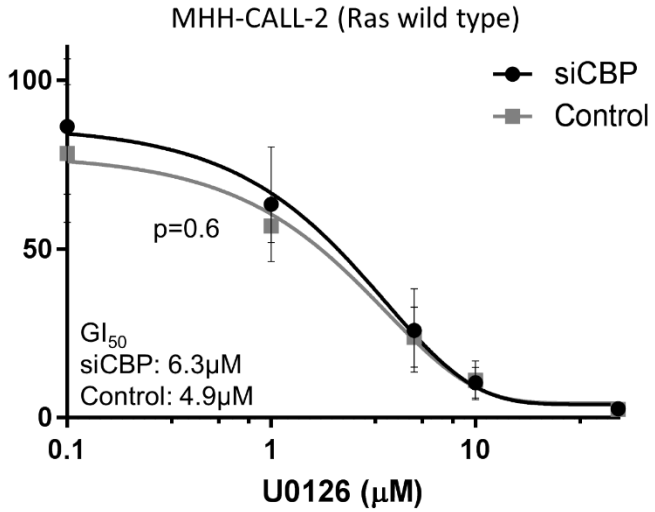
versus shNEG 9.8nM ±1.8; p=0.7 and 6-thioguanine shCBP 2.4µM ±0.58 versus shNEG 2.6µM ±0.32; p=0.6. Histograms show mean ±SD (N=3).

Figure S2. CREBBP knockdown in Ras wild type MHH-CALL-2 cells has no effect on p-ERK expression or sensitivity to the benchmark MEKi U0126

A



B



Ras wild type MHH-CALL-2 cells were assessed for expression of p-ERK and basal ERK by western blotting (**A**). Both control and siCBP transfected cells showed no expression of p-ERK. MHH-CALL-2 cells with transient CREBBP knockdown were assessed for sensitivity to the benchmark MEKi U0126 and showed no differential sensitivity compared to control cells (**B**). GI_{50} values, mean ±SD; siCBP 6.3µM ±1.2 versus control 4.9µM ±0.54; p=0.6. Histogram shows mean ±SD (N=3).

Supplementary References

1. Mullighan CG, Zhang J, Kasper LH, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature. 2011;471(7337):235-239.