SUPPLEMENTARY INFORMATION for

Interaction of c-Myb with p300 is required for the induction of acute myeloid leukemia (AML) by human AML oncogenes


SUPPLEMENTARY METHODS

Plasmid construction

MSCV-AML1-ETO-I-GFP was a gift from Dr. Hamish Scott, MSCV-AML1-ETO9a-I-GFP was obtained from Dr. Dong-Er Zhang through Addgene (Addgene plasmid 12433). MSCV-Luc-I-NRAS and MSCV-MLLAF9-I-Venus was a gift from Dr. Scott Lowe. MSCV-MLLAF9-I-GFP was a gift from Dr. Michelle Perugini and MSCV-MLL-ENL-neo was obtained from Dr. Robert Slany through Addgene (Addgene plasmid 20873). The MLL-ENL fragment was cloned into the EcoRI sites of MSCV-I-GFP. MSCV-AML1-ETO9a-I-NRAS-I-GFP was generated by amplifying the IRES-NRAS portion of the MSCV-Luc-I-NRAS plasmid and cloning it into the MSCV-AML1-ETO9a-I-GFP plasmid using MfeI cohesive ends. pMYS-Myb-I-Venus, pMYS-L302AMyb-I-Venus, pMYS-E308GMyb-I-Venus were generated by PCR amplification of the IRES-Venus fragment from MSCV-MLLAF9-I-Venus and cloning into the NotI and SalI ends of the pMYS-I-GFP vectors.

RT-PCR analysis of oncogene expression in transduced fetal liver cells

RNA was extracted from 5 x 10^5 fetal liver cells immediately prior to transplantation in recipient mice and used for cDNA synthesis and reverse-transcription PCR. Primers used were as follows. AML1-ETO9a: 5’-TGGAGAGGGAAAAGCTTCA-3’ (forward), 5’-ATTGCGTCTTCACATCCACA-3’ (reverse); MLL-AF9: 5’-AGGATCAGAGTGACTTTAAG-3’ (forward), 5’-TGCTTGTCACATTGACC-3’
Semi-quantitative allele-specific PCR analysis of bone marrow reconstitution
Mutagenically separated PCR (MS-PCR: ref. 1) was used to estimate the proportions of WT and Boo bone marrow cells. 1ng of genomic DNA (gDNA), extracted from bone marrow cells (Qiagen), was added to fluorescently-labelled, allele-specific reverse primers (WT: 5’-NED-TGCCTGATGTCCCTTCAACT-3’; Booreana (Boo): 5’-VIC-TTACTGTTACTGCCCCGTGCCCTTCCCTCATCC-3’), following which WT and Boo alleles were amplified in a single MS-PCR. Fluorescently-labelled PCR products were separated by capillary electrophoresis using an Applied Biosystems (AB) 3730 DNA Analyser and DNA fragment chromatograms were analysed with AB GeneMapper (v4.0) software. Area under the peak (relative fluorescence units) was measured for WT and Boo allele PCR products, and background signals. In samples where only one allele was amplified, such as the homozygous WT control, a maximum background fluorescence peak was measured and assigned to the minor allele. Homozygous WT and homozygous Boo genotype control samples were used to demonstrate allele-specific amplification of the WT and Boo alleles. The heterozygous genotype control demonstrates that the MS-PCR amplifies both the WT and Boo alleles, when present, in a single sample and allows calculation of the relative efficiencies of amplification of the WT and Boo alleles.

Co-immunoprecipitation
Bone marrow cells obtained from WT C57Bl6 and Booreana mice were enriched for c-Kit expressing cells using MACS CD117 microbeads (Miltenyi). Cells were then lysed using a modified RIPA buffer using previously described procedures 39 and incubated with either an
α-CBP or α-p300 antibody (Santa Cruz) for 2 hours at 4°C on a rocker. The mixture was then incubated with 20ul of Protein A/G PLUS-Agarose beads (Santa Cruz; sc-2003) and incubated overnight at 4°C on a rocker. Immunoprecipitates were collected by centrifugation at 1000g for 5 minutes at 4°C and washed 5 times in modified PBS. Pelleted beads containing protein were analysed by SDS-PAGE and immunoblotting using a Myb 1.1 antibody at 1:500 dilution.

**Flow cytometric analysis and cytocentrifugation**

For all in vitro analyses, cells from WT C57Bl6 and *Booreana* mice were stained with anti-c-Kit PECy7 or c-Kit APC (eBioscience), Gr-1 APC (Biolegend), CD11b-PE or CD11b-Pacific Blue (Biolegend), antibodies and analyzed on a FACS Diva or Cyan ADP cytometer and analysed using FlowJo. Cytocentrifugation was carried out as previously described and cell were stained with May-Grunwald-Geimsa.

For analysis of leukemia induction in mouse transplant experiments, the following antibodies (all From Biolegend) were used – c-Kit PE, CD45.1 PerCP/Cy5.5, CD45.2 PE/Cy7, CD11b APC/Cy7 and Gr-1 APC.

For the rescue experiments, transduced haematopoietic cells were sorted for Venus and GFP double-positive cells on a MoFlo (Beckman Coulter) and subject to replating assays on methylcellulose as described above.

**Isolation of cells for expression profiling**

Bone marrow cells were isolated from 4 8-week old *Booreana* and age matched C57Bl6 mice and enriched for c-Kit expressing cells using MACS CD117 microbeads (Miltenyi). Cells were transduced with retroviral supernatants prepared from MSCV-I-GFP and MSCV-AML1-ETO9a-I-GFP. Cells were transduced by retroviral supernatants by spinoculation using fibronectin (Sigma, F-1141) or Retronectin (Takara) using manufacturer’s guidelines.
Cells were cultured in IMDM with 20% fetal calf serum and a cocktail of IL-3, IL-6 and SCF cytokines produced in-house. 48 hours post-transduction, cells were sorted for GFP-positive, c-Kit positive cells on a MoFlo (Beckman Coulter) using an anti-c-Kit PECy7 antibody (eBioscience).

Expression Profiling

The expression profiling experiment was a part of a larger array profiling screen and samples were FACS sorted in 4 different batches. Expression profiling was performed on MouseRef-8 v2.0 Expression BeadChip. Array (Illumina). Raw signal intensity was calculated with Illumina BeadStudio software. Subsequently data was processed with R²/Bioconductor packages lumi⁴ and limma⁵, and annotated using Illumina’s manifest file (http://www.switchtoI.com/pdf/Annotation%20Files/Mouse/MouseRef-8_V2_0_R3_11278551_A-text.zip/). Probe intensities were variance stabilized and robust spline normalized using lumi package. Differentially expressed probes were identified by fitting linear model to normalized intensities with the limma package. P values were adjusted for multiple testing with Benjamini and Hochberg's method.

The expression profiling experiment was a part of a larger array profiling screen and samples were FACS sorted in 4 different batches. This resulted in a batch effect as can be seen from clustering of array data (Fig. S4). The data were corrected for this batch effect with the ComBat algorithm⁶ which is available as an R function (http://jlab.byu.edu/ComBat/Download_files/ComBat.R). The BooV2 sample was removed as an outlier. These gene expression profiling data have been deposited in the GEO database under the accession number GSE34224.

GSEA analysis ⁷,⁸ was performed on the batch corrected data using five Molecular Signatures Database collections (c1 - c5) from Broad Institute. Probe sets were collapsed to the single
gene vector. Metric parameter was changed to t-test and given the small sample size, we performed 1000 permutations on each gene-set.

Differentially expressed probes were determined as probes with adjusted P value < 0.05 and fold-change $\geq 1.5$. Differentially expressed probes for all contrasts with log2FoldChange and adjusted P values are shown in Supplementary Table S2. Genes from contrast III belonging to at least one Myb related gene set (Myb-ChIP-bound, Myb-direct-target and Zuber-Myb) are shown in Supplementary Table S3. Myb-ChIP-bound and Myb-direct-targets genes were obtained from Supplementary Tables S5 and S7 from ref. 9. Genes differentially expressed upon Myb shRNA knock-down in MLLAF9 leukemia model (“Zuber-Myb”) were obtained from the Supplementary Table 1 file from ref. 10

Rank-rank hypergeometric overlap 11 between contrasts I and II was performed after collapsing all probe sets to the single gene vector. (http://systems.crump.ucla.edu/rankrank/). Differentially-expressed genes were functionally annotated using the DAVID functional annotation chart tool 12,13 (http://david.abcc.ncifcrf.gov/) and visualized using ggplot2 library 14. The visualization used in Fig. S7 was inspired by clusterProfiler package 15. Redundancy of GO terms was reduced by GO trimming 16.

SUPPLEMENTARY TABLES

Table S1: Semi-quantitative PCR analysis of bone marrow reconstitution by transduced Boo donor bone marrow

Table S2: List of all differentially-expressed genes

Table S3: Myb targets amongst genes that are differentially expressed between AML1-ETO9a-expressing WT and Boo progenitors
Supplementary Figure S1: c-Myb interacts with p300 through a key residue that is mutated in Booreana (Boo) mice. (A) Illustration showing the key amino acids involved in the interaction of the c-Myb TAD (violet) with the hydrophobic pocket formed by three beta-helices by the p300 KIX domain (green). The E308 residue (blue) is altered to glycine in the Boo mouse strain and the Y630 residue (orange) is altered to asparagine in the Plt6 strain. (B) In vivo co-immunoprecipitation performed using whole cell lysates from c-Kit enriched bone marrow cells from Boo and wild-type mice showing lack of interaction between c-Myb and CBP/p300 in the former but not the latter.
Supplementary Figure S2: FACS analyses of differentiation marker expression. c-Kit-enriched BM cells from WT and Boo mice transduced with empty vector, AML1-ETO9a or MLL-AF9 were cultured in IL-3, IL-6 and SCF for 14 days, following which expression of Gr-1 and CD11b was measured on cells gated on GFP positivity. AML1-ETO9a expressing Boo homozygous cells express higher levels of CD11b compared to WT or Boo heterozygous cells. MLL-AF9 expressing Boo heterozygous and homozygous cells show higher levels of expression of CD11b when compared to WT cells. The reduced number of Gr-1+ cells in cultures from Boo homozygotes is consistent with our previous report (main text ref. 41).
Supplementary Figure S3. Proportions of GFP+/CD11b+ cells (A) and GFP+/Kit+ cells (B) in peripheral blood of mice transplanted with WT or Boo cells transduced with the indicated vectors. Peripheral blood was sampled from several mice of each genotype/vector combination over the entire course of the leukemia induction study (see also Fig. 4). Each data point represents one sample from one mouse; individual mice shown were sampled 1-3 times over the course of the study. The lack of data points for the MLL-AF9 WT mice beyond 90 days reflects the death of all mice in this group by this time (see Fig. 4A).
Supplementary Figure S4: FACS profiles showing GFP and c-Kit expression of cells in the (A) bone marrow and (B) spleens of transplanted recipient mice. AML1-ETO9a and MLL-AF9-expressing wild-type mice show a large percentage of infiltration of GFP+ leukaemic cells.
Supplementary Figure S5: (A) Real-time PCR confirming expression of the AML1-ETO9a (AE) and MLL-AF9 (MA) oncogenes in transduced fetal liver cells from wild-type and Booreana mice. See Supplementary Methods above for details. (B) FACS plots showing initial transduction frequency of fetal liver hematopoietic cells 48 hours after co-culture with packaging cells expressing vector, AML1-ETO9a or MLL-AF9 retrovirus.
Supplementary Figure S6: Time course of (A) GFP and (B) CD45.2 expression 7, 19 and 33 weeks post-transplantation of vector (V), AML1-ETO9a (AE) and MLL-AF9 (MA) transduced fetal liver cells into irradiated recipient mice showing successful reconstitution of transgene-expressing donor cells. Note log scale in (A).
Supplementary Figure S7: Transplantation of transduced bone marrow cells from wild-type and Booreana donor mice. (A) Survival of irradiated recipient mice receiving AML1-ETO9a-expressing wild-type and Booreana donor cells. Only the former succumb to a myeloid leukemia with (B) increased GFP positive, c-Kit positive, FcγRII/III positive cells in their bone marrow. P < 0.05 (Fisher’s exact test). (C) FACS analyses of peripheral blood (taken at the indicated times post-transplant) from irradiated mice transplanted with WT or Boo cells that had been transduced with control or AML1-ETO9a retroviruses. Note the similar percentages of GFP+ve cells in all groups at 8 weeks, and the enhanced proportion in WT/AML1-ETO9a recipients at 20 weeks.
Supplementary Figure S8: Hierarchical clustering of microarray samples before and after correction for batch effect. Note that correction did not affect aberrant clustering of an outlier (sample BooV2), which was removed from all subsequent analyses.
Supplementary Figure S9: (A) Gene Set Enrichment Analysis of WT\textsuperscript{Ctrl} vs WT\textsuperscript{AMLETO} contrast (Set I). A GSEA enrichment plot (top) for the highest-ranked gene set and list of top-scoring gene sets (below), showing extensive enrichment of AML1-ETO related gene sets labeled with red arrows. (B) Rank-rank hypergeometric overlap between contrasts (I) and (II) showing extensive representation of AML1-ETO induced genes in AML1-ETO transduced Boo cells. Color represents log10-transformed hypergeometric P-value. Red represents higher than expected number of overlapping genes, blue indicates lower than expected number of overlapping genes. See Supplementary Methods for details.
Figure S10

- Clec4e
- Ccl4
- Carhsp1
- Rnase4
- Marcksl1
- Ets2
- P2ry6
- Spp1
- Acta2
- Clec4d
- Il12a
- Gpx3
- Sdc4
- Trl2
- Mmp13
- Cd14
- LOC100048556
- Saa3
Supplementary Figure S10. Expression levels (log2 intensity, derived from microarray data) of selected genes in WT\textsuperscript{Ctrl} (n = 4), WT\textsuperscript{AMLETO9a} (n = 4), Boo\textsuperscript{Ctrl} (n = 3) and Boo\textsuperscript{AMLETO9a} (n = 4) samples. The genes shown are the 58 that overlap between sets I and III but are not present in set II (see Fig. 5A), plus \textit{Gata2} and \textit{Gfi1b}. 
Supplementary Figure S11: Enrichment of GO (gene ontology biological process) terms across contrasts I, II, III and IV. The size of each individual circle represents the number of genes corresponding to each term and color indicates $-\log_{10}$ transformed p value, as indicated in the adjacent keys. Enrichment analysis was performed using the DAVID functional annotation tool.
SUPPLEMENTARY REFERENCES


