Supplementary Information

PU.1 cooperates with IRF4 and IRF8 to suppress pre-B cell leukemia

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Supplementary Figures

Supplementary Figure 1. Analysis of B cell development in the absence of PU.1 and IRF8. (**A**-**B**) BM cells were isolated from mice of the indicated genotypes and analyzed for CD19, B220 and IgM expression by flow cytometry. Representative flow cytometric plots show the gates used to calculate the number of (**A**) total $CD19^{+}B220^{+}$ B cells and (**B**) $CD19^{+}$ BM compartments based on B220 and IgM expression used to define pro/pre-B cells immature, transitional and recirculating B cells. (**C**-**G**) Total cell number of each cell population were calculated from the gating shown in (**A**) and (**B**). A simplified genotype nomenclature is shown below the graphs with symbols representing the existence of two (+) or no (-) functional alleles for the indicated genes. The genotypes are presented in the same order as in (**A**). Data are mean ± SD from 3 to 13 mice per genotype. p values compare the indicated groups using a paired t test (two tailed). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Supplementary Figure 2. Analysis of the pro- and pre-B cell compartments in the absence of PU.1 and IRF8. BM cells isolated from mice of the indicated genotypes were analyzed for the frequency of (A) $CD19^{+}B220^{+}IgM^{-}c\text{-}Kit^{+}$ pro-B and (B) $B220^{+}IgM^{-}CD25^{+}$ pre-B cells. (C-D) Fold change (normalized to the wild-type value set as 1) in the total number of each cell population from each genotype were quantified from the gating shown in (A-B). A simplified genotype nomenclature is shown below the graphs with symbols representing the existence of two (+), one (+/-) or no (-) functional alleles for the indicated genes. The full genotypes are presented in the same order as in (A). The data are mean ± SD from 3 to 13 mice per genotype. p

values compare the indicated groups using a paired t test (two tailed). * $p < 0.05$, ** $p < 0.005$.

Supplementary Figure 3. Analysis of pro and pre-B cell compartments in the BM. (A-B) BM cells isolated from mice of the indicated genotypes were analyzed for pro- and pre-B cells. CD43 antibody was utilized to confirm the analysis of pro- and pre-B cells by c-Kit and either with or without CD25 surface receptors.

Supplementary Figure 4. Histological section of spleens from moribund mice lacking PU.1, IRF4 or IRF8. (A) 10X magnification. (B) 40X magnification.

Supplementary Figure 5. Ectopic expression of Blnk does not reduce the growth of pre-B-ALL in culture. (A) Ectopic expression of Blnk in IL-7 dependent proliferation of pre-B ALL. Leukemic cells from mice of the indicated genotypes were infected with Blnk-expressing or control MIGR1 (GFP) retroviral vectors. Flow cytometry was used to determine the percentage of GFP^+ cells over the indicated time course. Data are the mean ± SD of 2 to 4 independent B-ALL samples per genotype and were normalized for relative frequency of GFP expression at day 1 (set as 1). p values compare the indicated data point to the MIGR1 control using a paired t test (two tailed* $p < 0.05$). (B) RNA samples from infected cells were subjected to RT-qPCR to measure the relative expression of mRNA transcript for *Blnk*. Transcript frequencies were normalized to *Hprt* transcript levels. The data are mean ± SD

Supplementary Table 1.

Cell number	*PU.1*$^{fl/fl}$ *Cd19cre Irf8*$^{-/-}$ B-ALL number	Recipients that developed B-ALL	Cell number	*PU.1*^{Mb1cre} *Irf4** B-ALL number	Recipients that developed B-ALL
1 x 10^6	93	3/3	1 x 10^6	404	1/1
	198	3/3		405	1/1
	225	2/2		506	1/1
	312	1/1	5 x 10^5	313	1/3
	316	6/6	1 x 10^5	404	1/1
	325	3/3		405	1/1
1 x 10^4	225	2/2		506	1/1
	312	1/2	1 x 10^4	404	1/1
	316	2/3		506	1/1
	325	3/3	1 x 10^3	278	3/3
1 x 10^2	225	2/2		506	1/1
	312	2/2			
	325	2/3			
1 x 10^1	225	1/2			
	325	2/3			

* *PU.1*^{Mb1cre} *Irf4* tumor genotypes; 404, 405 *PU.1*$^{fl/+Mb1cre}$ *Irf4*$^{-/-}$, 506 *PU.1*$^{fl/flMb1cre}$ *Irf4*$^{-/-}$, 313 *PU.1*$^{fl/+Mb1cre}$ *Irf4*$^{+/-}$ and 278 *PU.1*$^{fl/flMb1cre}$ *Irf4*$^{+/-}$.

Supplementary Table 2.

Fig / *n*	*WT*	*Irf4*$^{-/-}$	*PU.1*$^{fl/+}$ *Mb1cre* *Irf4*$^{-/-}$	*PU.1*$^{fl/fl}$ *Mb1cre* *Irf4*$^{-/-}$	*PU.1*$^{fl/fl}$ *CD19cre*	*Irf8*$^{-/-}$	*PU.1*$^{fl/fl}$ *CD19cre* *Irf8*$^{-/-}$	*ALL*	
2									
A, B, C, D, E, F, G	12	10	9	5					
3									
A, B, D, E	Same as above								
4									
A	3	3	2	2	3	3	3		
B, C	3	3	2	2					
6									
A, B	3		10	4			10		
7								*PU.1*$^{fl/+ \text{ or } fl/fl}$ *Mb1cre* *Irf4*$^{-/-}$	*PU.1*$^{fl/fl}$ *CD19cre* *Irf8*$^{-/-}$
A	9	6	6	6	3	6	9	18	9
B	9	6	6	6	3	3	9	12	12
C	9	6	6	6	3	3	9	12	12
8									
A								5	6
B								5	6
A (lowest panel)	3							4	
B (lowest panel)	3							4	
Supp 1, 2	12				6	6	10		
Supp 3		2				2			
Supp 5									
A								2	2
B	3							4	

Supplementary Material and Methods

Antibodies and flow cytometry

Single cell suspensions were prepared in FACS buffer (balanced salt solution supplemented with 2 % (v/v) fetal calf serum (FCS)). Cells were stained for 30 min on ice with the following fluorescent or biotin-conjugated antibodies; c-Kit (ACK2), CD19 (ID3), B220 (RA3-6B2), IgM (331.12), CD43 (S7), Igκ (187.1), pre-BCR (SL156), IL-7Rα (B12-1), CD25 (PC61). Antibodies were purchased from BD Biosciences, eBioscience or Biolegend. Anti-rat Ig-phycoerythrin- and PECy7-streptavidin (BD Biosciences) were used as secondary detection reagents. Cells were washed with FACS buffer and resuspended with 1 μg/mL propidium iodide to allow for the exclusion of dead cells. For analysis, samples were run on an LSRII or LSR-Fortessa flow cytometer (BD Biosciences). Results were analysed using FlowJo software (Treestar Inc.).

Retrovirus production and infection

Retroviral supernatants were generated by transient transfection of 293T cells with plasmids that encode viral envelope proteins (pMD1-gag-pol and pCAG-Eco) and a specific expression vector encoding for either MSCV-iresGFP (MIGR1), MSCV-Spi-B-iresGFP (MIGR1-Spi-B), MSCV-Blnk-iresGFP (MIGR1-Blnk) or MSCV-Ikaros-ERT2-iresGFP (MIGR1-Ikaros-ERT2) [40]. The MIGR1-Ikaros-ERT2 construct was activated by 500 nM 4-hydroxy-tamoxifen (Sigma-Aldrich). 293T cells were transfected using the calcium phosphate precipitation method (Clontech). Retroviral supernatants were centrifuged onto RetroNectin (Takara)-coated plates for 60 min at

4000 rpm at 4 ^{o}C. Cells were then cultivated with the virus in the presence of 4 μg/mL polybrene (Sigma-Aldrich) for 20-24 h before changing to fresh media for further cultivation.

Histological and microscopic analysis

Organs were fixed in 10 % (w/v) neutral buffered formalin overnight, and histological sections stained with hematoxylin and eosin. Sections were imaged at 10X, 40X and 100X magnifications.

PCR analysis of VDJ rearrangement of the *IgH* locus

DNA was extracted from leukemic cells, and wild-type pre-B-cells as control. PCR conditions were as follows: 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 30 s, 60^{o}C for 30 s and 72^{o}C for 2 min. The primers used were as previously described [41, 42]. PCR products were resolved on 2 % (w/v) agarose gel and the respective rearrangements were determined.

Quantitative RT-PCR expression analysis

Total RNA was prepared with TRIzol® Reagent (Life Technologies) and cDNA synthesized with iScript^{TM} Reverse Transcription Supermix for RT-qPCR (Bio-Rad). qPCR reactions were performed using pre-designed Taqman gene expression assays (*Hprt*, Mm01545399_ml, *Spib* Mm03048233_m1 and *Ikzf1* Mm01187884_ml, Applied Biosystems) or using SYBR green reaction mix (Promega) using the **following primers; Hprt GGGGGCTATAAGTTCTTTGC and** TCCAACACTTCGAGGTCC, *Blnk* CTGCCGCACCATCCCCACTAC and

GTCACAGGCGCCAGCATACCAG, and *Ikzf1* CAATGTCGCCAAAACGTAAGA and GTTGATGGCATTGTTGATGG. qPCR reactions were performed on CFX384 Touch^{TM} Real-Time PCR Detection System (Bio-Rad).

RNA-seq analysis

Pro-B cells (CD19^{+}B220^{+}c-Kit^{+}) were flow cytometrically sorted from the BM of C57Bl/6 (WT), *Irf4*$^{-/-}$, *Irf8*$^{-/-}$ and *PU.1*$^{fl/fl}$ *Mb1*$^{cre/+}$ mice and cultured for 7 days in IL-7 and OP9 stromal cells. B220^{+} pro-B cells were flow cytometrically sorted and RNA prepared using the Qiagen RNeasy Mini Kit.

Read density heat maps

Read densities were calculated using the JNOMICS program[I. Tamir, unpublished]. Associated heat map visualizations were implemented using R (http://www.R-project.org) and were wrapped with customized bash scripts for command line usage.

Motif discovery

For motif discovery, we used the MEME-ChIP suite (version 4.9.1)[20] to discover the most significant motif in the summit centered top 300 SISSRS peaks of each track or track overlap subset.

Peak overlap analysis

All peak overlap analyses were performed with the Multovl program[23] by using a minimal overlap length of one and allowing for all possible overlaps. Results were parsed and converted to tables with custom-made bash, perl and R scripts.

Chromatin Immunoprecipitation (ChIP) sequencing analysis

Bio-ChIP-seq analysis of PU.1 binding. Pro-B cells isolated from the BM of *PU.1* (*Spi1*)$^{ihCd2/ihCd2}$ *Rosa26*$^{BirA/BirA}$ mice were expanded in vitro for 5 days on OP9 cells in the presence of IL-7. Pro-B cells ($10x10^7$) were used for chromatin precipitation by streptavidin pulldown (Bio-ChIP) as previously described [1].

ChIP-seq analysis of histone modifications and transcription factor binding. Pro-B cells from the BM of *Rag2*$^{-/-}$ mice were expanded 4-5 days on OP9 cells in the presence of IL-7 prior to ChIP with an anti-PU.1 (G148-74; from BD Bioscience), an anti-IRF4 antibody (sc6059x; from Santa Cruz) or an anti-IRF8 antibody (sc6058; from Santa Cruz). Approximately $10x10^7$ *Rag2*$^{-/-}$ pro-B cells were used for ChIP as previously described [2].

Illumina deep sequencing. ChIP-precipitated DNA (~5 ng) were used as starting material for the generation of single-end sequencing libraries as described by Illumina's ChIP Sequencing sample preparation protocol. DNA fragments of 200–500 bp were selected. The libraries were amplified by 15 PCR cycles. Completed libraries were quantified with the Agilent Bioanalyzer dsDNA 1000 assay kit and Agilent QPCR NGS library quantification kit. Cluster generation and sequencing were performed by using the Illumina/Solexa Genome Analzyer II and IIx systems according to the manufacturer's guidelines.

PU.1, IRF4 and IRF8 peaks were called using the MACS program version 1.3.6.1 [3] with default parameters, a read length of 36 for ChIP-seq, a genome size of 2,654,911,517 bp (mm9) and the appropriate input control sample. DNase

hypersensitive (DHS) sites and histone modifications were previously mapped by ChIP-sequencing of *in vitro* cultured *Rag2*$^{-/-}$ pro B cells [4].

Western blotting

Total protein extracts were produced from equivalent numbers of cells and subjected to western blotting using standard techniques. Primary antibodies were anti-mouse IgM-HRP and Igκ-HRP (Southern Biotechnology Associates), anti-Actin (I-19, sc1616), PU.1 (T-21, sc352), and IRF8 (C-19, sc6058) from Santa Cruz Biotechnology Inc. HSP-70 (N6) was a gift of Drs. W. Welch and R. Anderson. The monoclonal antibodies against Pax5 (1H9) [5], and IRF4 (3E4) [6] were produced in house.

Supplementary References

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