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Supplemental Information

Hif-1α Deletion May Lead to Adverse Treatment Effect in a Mouse Model of MLL-AF9-Driven AML

Talia Velasco-Hernandez, Shamit Soneji, Isabel Hidalgo, Eva Erlandsson, Jörg Cammenga, and David Bryder

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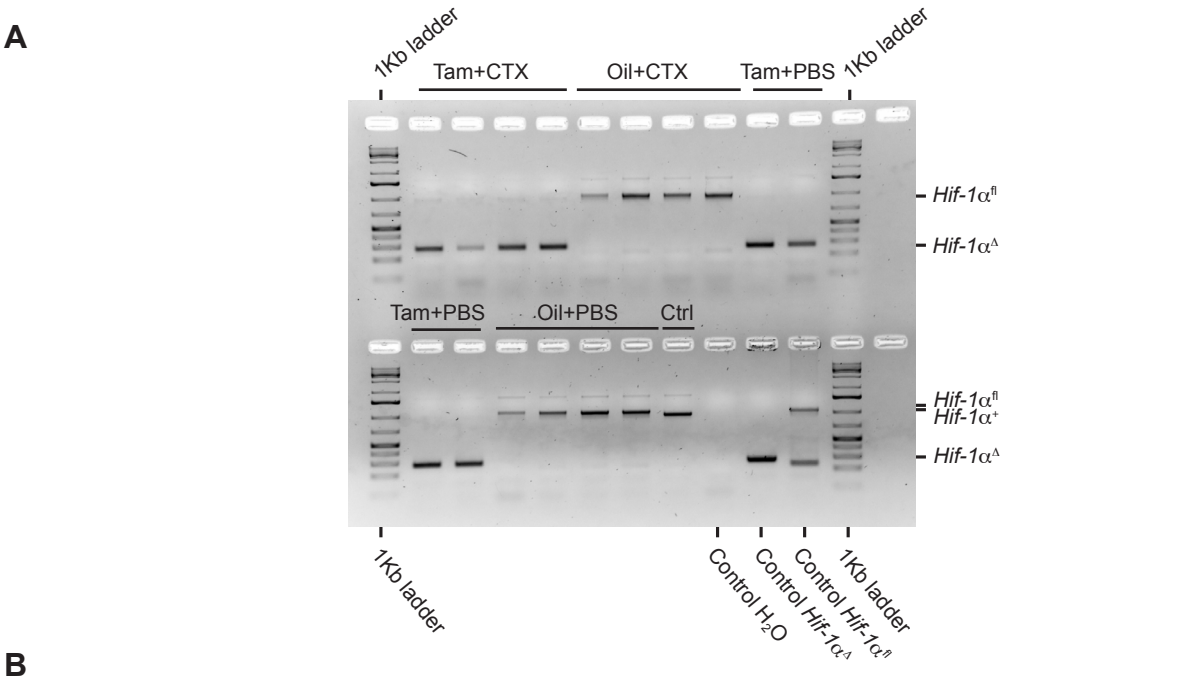
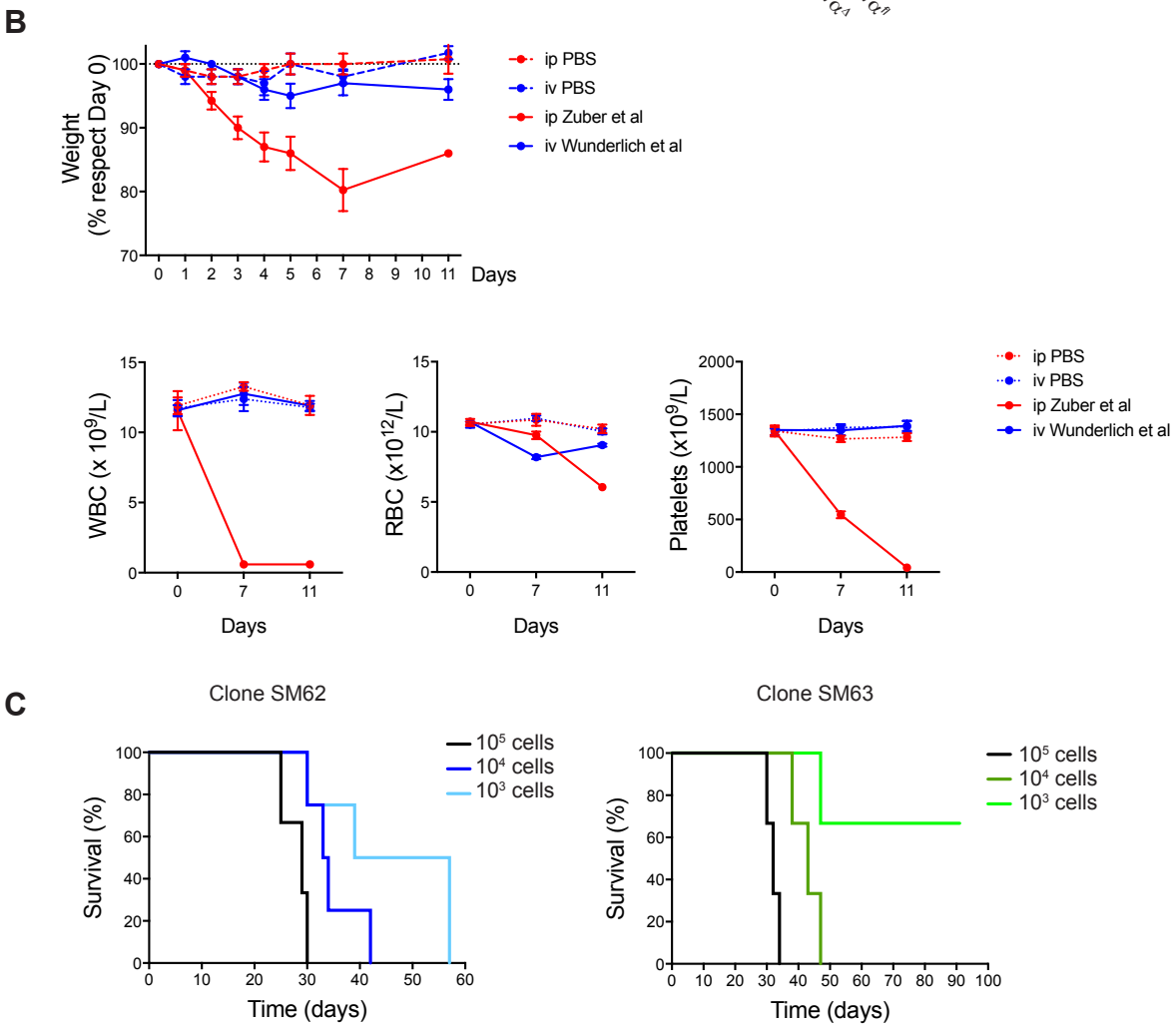
Authors:

Talia Velasco-Hernandez[1*], Shamit Soneji[1], Isabel Hidalgo[1], Eva Erlandsson[1], Jörg Cammenga[2,3] and David Bryder[1*]

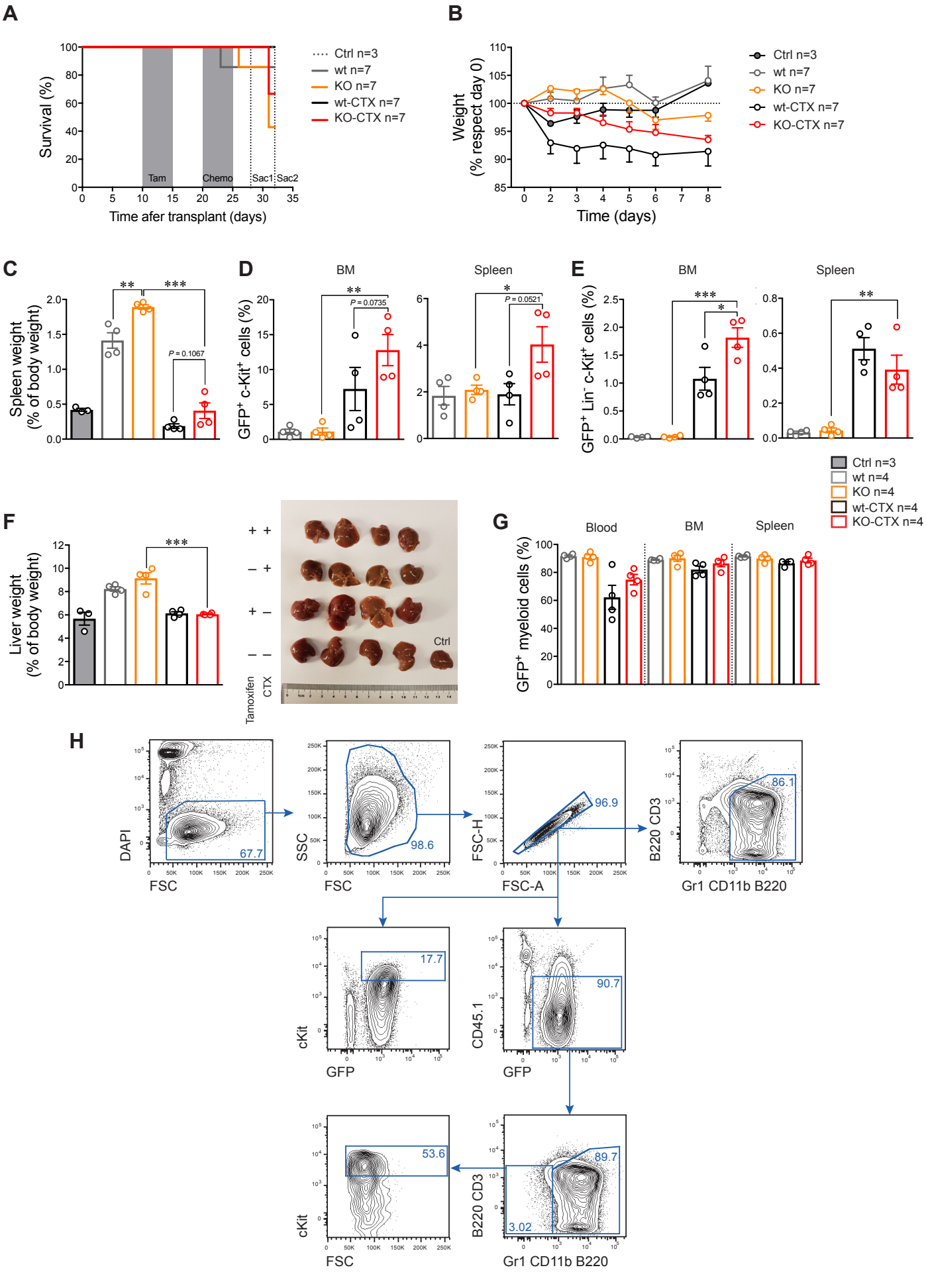
Supplemental Figure S1. Velasco-Hernandez et al





Supplemental Figure 1, related to Figure 1.

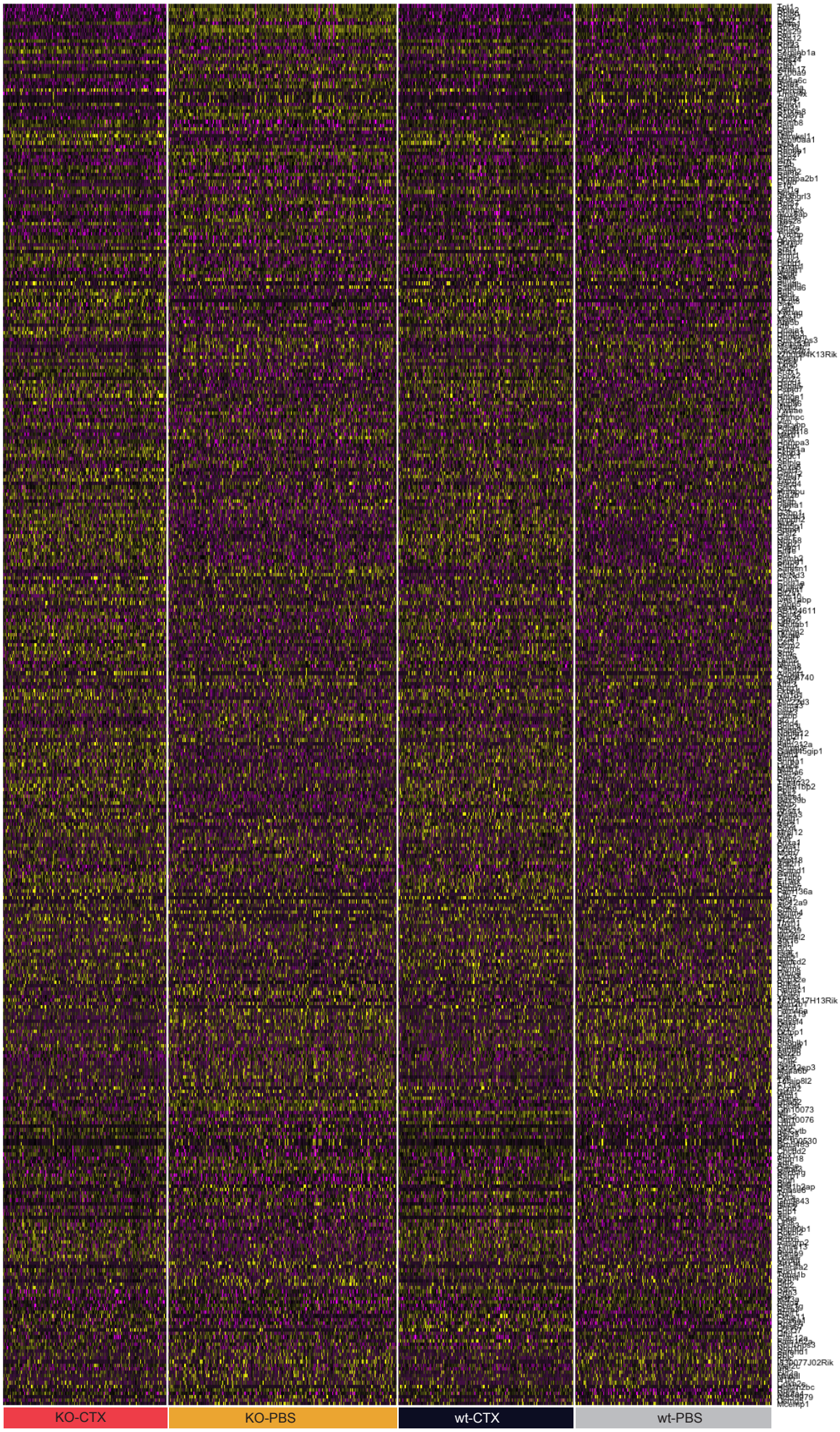
A. PCR analysis to check deletion of *Hif-1α* gene after treatment with tamoxifen. Genomic DNA was extracted from BM cells at day 28 after transplantation and PCR was performed to check deletion of *Hif-1α*. Only in samples from mice treated with tamoxifen we can observe the deletion of the gene and not in the oil-treated mice or control mice samples. Ctrl, control; CTX, chemotherapy; Tam, tamoxifen. **B.** Chemotherapy protocol comparison. We compared the protocols using cytarabine and doxorubicin described by Zuber *et al* (Zuber et al., 2009) and Wunderlich *et al* (Wunderlich et al., 2013). The Wunderlich *et al* protocol was developed for human AML xenografts in chemotherapy-sensitive NSG mice. It showed little effect on our murine AML model, so we decided to use the one described by Zuber *et al,* with a significant effect on blood parameters. Data shown from 1 experiment (n=4 mice/group). **C.** Cell-dose titration. Latency of the disease was analysed with two different clones (SM62 and SM63) and 3 different cell-doses (10^3, 10^4 and 10^5 cells) to adjust the timing of the protocol to the latency of the disease. Data shown from 1 experiment (n=3-4 mice/group). We finally chose to inject 10^4 cells/mouse due to the more homogeneous results: all mice developed the disease and the latency was long enough to administer the two drug regimens. ip, intraperitoneal injection, iv: intravenous injection; WBC, white blood cells; RBC, red blood cells.


A
Survival (%)
Time afer transplant (days)
Tam
Chemo
Sac1
Sac2
Ctrl n=3
wt n=7
KO n=7
wt-CTX n=7
KO-CTX n=7
B
Weight (% respect day 0)
Time (days)
Ctrl n=3
wt n=7
KO n=7
wt-CTX n=7
KO-CTX n=7
C
Spleen weight (% of body weight)
P = 0.1067
D
BM
Spleen
GFP+ c-Kit+ cells (%)
P = 0.0735
P = 0.0521
E
BM
Spleen
GFP+ Lin- c-Kit+ cells (%)
Ctrl n=3
wt n=4
KO n=4
wt-CTX n=4
KO-CTX n=4
F
Liver weight (% of body weight)
Tamoxifen
CTX
Ctrl
G
Blood
BM
Spleen
GFP+ myeloid cells (%)
H
DAPI
FSC
67.7
SSC
98.6
FSC-H
FSC-A
96.9
B220 CD3
Gr1 CD11b B220
86.1
cKit
GFP
17.7
CD45.1
90.7
53.6
89.7
3.02

Supplemental Figure 2, related to Figure 1.

A. Survival curve of the analysed mice along the timeline of the experiment. **B.** Weight of the mice after chemotherapy treatment (first injection at day 1) showing the expected effect of loss of weight in the chemotherapy-treated animals. **C.** Spleen weight of the analysed animals at day 28 after transplantation showing a significant increment in liver size in the PBS-treated animals. **D-E.** Percentage of the GFP^+ $c\text{-}Kit^+$ (**D**) and GFP^+ Lin^- $c\text{-}Kit^+$ (**E**) populations in BM and spleen of the analysed mice at day 28 after transplantation. **F.** Liver weight of the analysed animals at day 28 after transplantation showing a significant increment in liver size in the PBS-treated animals. **G.** GFP^+ myeloid cells in different organs in the analysed mice at day 28 after transplantation indicating a overtaking expansion of the injected cells in the myeloid compartment. Plots represent mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$. CTX, chemotherapy; Ctrl, control; sac, sacrifice; BM, bone marrow. **H.** Gating strategy used to analyse the different studied populations by flow cytometry. For the identification of the desired populations, dead cells were discarded by DAPI staining, intact cells were identified by FSC/SSC, duplets removed by FSC-A/FSC-H, myeloid cells were identified by Gr1 and CD11b staining, donor cells were identified by being negative for CD45.1 and positive for GFP and lineage negative cells were considered as those negative for CD3, B220, Gr1 and CD11b. FSC, forward scatter; SSC, side scatter; FSC-A, forward scatter-area; FSC-H, forward scatter-height.

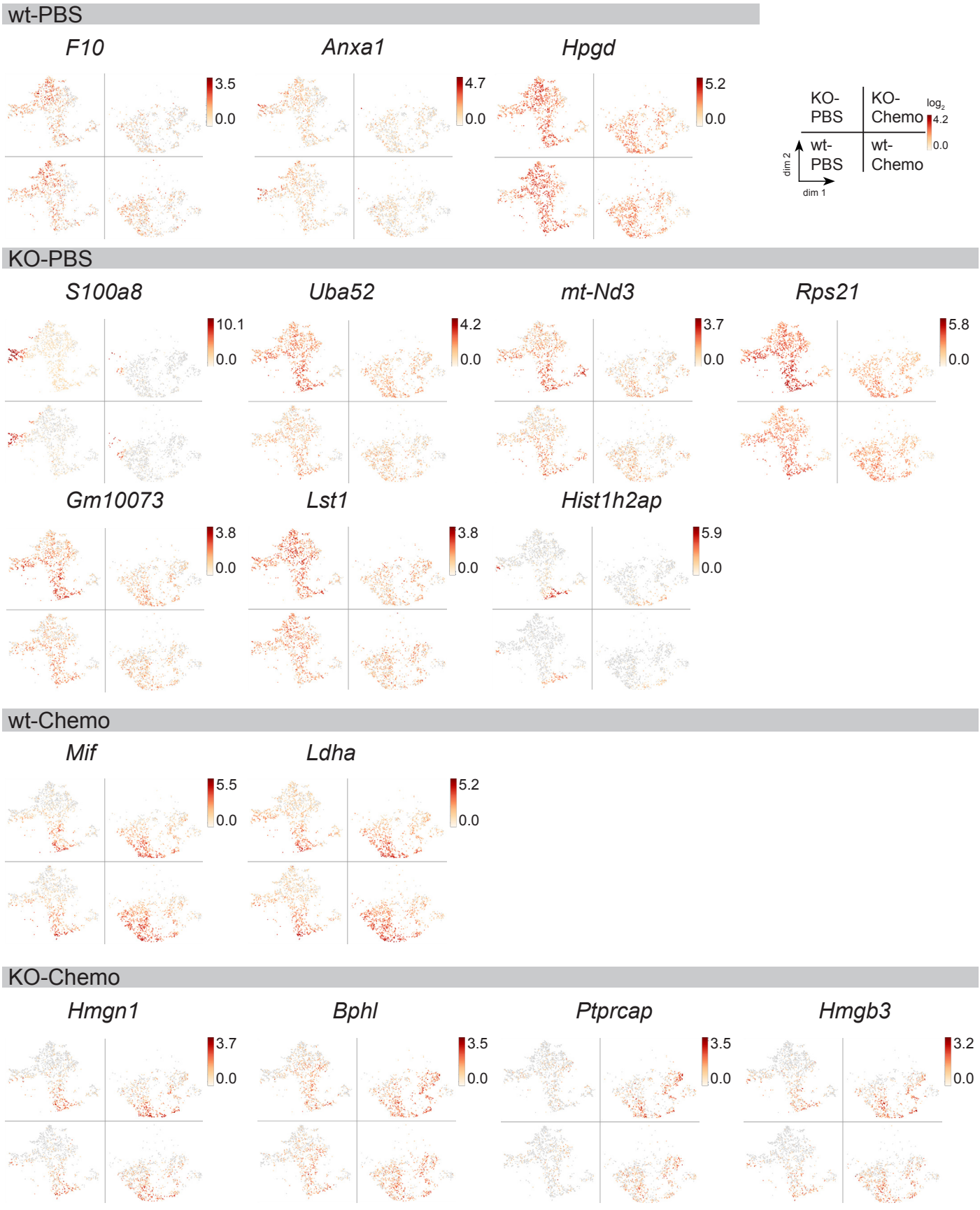
Supplemental Figure S3. Velasco-Hernandez et al



Supplemental Figure 3, related to Figure 3.

Heatmap depicting the 331 genes identified as differentially expressed among groups.

Supplemental Figure S4. Velasco-Hernandez et al



Supplemental Figure 4, related to Figure 3.

t-SNE plots showing the expression of uniquely differentialy expressed genes from each group in each individual cell. The scale at the right of each plot indicates the fold change expression (log_2).

Supplemental Experimental Procedures:

Mice

Hif-1α^{lox} mice (Ryan et al., 2000) (JAX 007561) were crossed with the tamoxifen-inducible Rosa26Cre-ER^{T2} mice (Ventura et al., 2007) (JAX 008463) to generate a combined conditional knock out (KO) model. Mice were maintained at the animal facility of the Biomedical Center at Lund University (Sweden) and all experiments were performed with consent from a local ethics committee.

Retroviral transduction

A MLL-AF9 (MigR1-MLL-AF9-GFP) retroviral vector was used for retrovirus production. Retroviral supernatants were obtained by transient transfection of amphotropic Phoenix cells and supernatants were harvested after 48 h (Velasco-Hernandez et al., 2014).

BM from 8-12-week-old mice was harvested from pooled femora, tibiae and ilia by crushing in phosphate buffer saline (PBS) (Gibco) + 2% fetal calf serum (FCS) (Gibco) using a mortar and pestle. c-Kit+ cells were isolated using a magnetic separation system (MACS) and anti-c-kit magnetic beads (Miltenyi Biotec). Cells were cultured in StemSpan™ serum-free expansion media (Stem Cell Technologies) supplemented with 100 U/mL Penicillin/Streptomycin (Gibco), 20 ng/mL mIL3, 50 ng/ml hIL6, 50 ng/ml hTPO and 50 ng/mL mSCF (all from Peprotech) for 24 h. Transductions were performed by centrifugation of the retrovirus (2 h, 1000 g, 32°C) over retronectin- (Takara) coated plates (according to manufacturer's instructions) and co-culture of the c-Kit^{+} cells over

the virus-coated wells for 24 h at 37°C. We used an MOI of 0.2, resulting in a transduction efficiency of 2% after 48 h. Cells were transplanted 24 h after transduction.

Transplantations, drug administration and monitoring of mice

Eight- to 12-week-old B6SJL x C57BL/6J (CD45.1-CD45.2) recipient mice were lethally irradiated with 900 cGy 4 to 15 h prior to transplantation. 5 x 10^5 cells were intravenously (i.v.) injected into the tail vein of recipient mice together with 1 x 10^5 freshly isolated total BM support cells from B6SJL (CD45.1) mice.

For secondary transplants, 10^4 GFP^+ cells were i.v. injected into sublethally irradiated (500 cGy) recipient mice without support cells. Mice were kept on antibiotics (Ciproxin, Accord) in drinking water for 14 days after transplantation. Clonal leukemic cells were harvested 16 weeks after transplantation when mice started displaying signs of disease.

In secondary recipients, *Hif-1α* deletion was induced at day 10 after transplantation by intraperitoneal injection (i.p.) of 100 µL tamoxifen (10 mg/mL) (Sigma) on 4 consecutive days. As control, the same volume of the vehicle (peanut oil, Sigma) was injected into separate mice. *Hif-1α* deletion was verified by polymerase chain reaction (PCR) analysis of BM cells using the following primers: HIF-24: 5' –GCAGTTAAGAGCACTAGTTG-3', HIF-26: 5' –TTGGGGATGAAAACATCTGC-3'.

Chemotherapy treatment was performed as described by Zuber *et al* (Zuber et al., 2009): i.p. injection of 100 mg/kg cytarabine (Hospira) on 5 consecutive days and 3 mg/kg doxorubicin (Actavis) for 3 days. We started the treatment at day 20 after transplantation.

Three and 5 days after completion of the chemotherapy treatment, samples were collected from PB, BM, spleen and liver. Total white blood cell (WBC) counts were determined by an automated cell counter (KX-21N, Sysmex).

Fluorescence-activated cell sorting analysis

Expansion of leukemic cells was analysed by flow cytometry analysis of PB, BM and spleen cells. PB was lysed with ammonium chloride (Stem Cell Technologies) before staining. 4,6 diamino-2-phenylindole (DAPI) (Sigma) was used to exclude dead cells. The following antibodies were used to determine expansion of the leukemic cells: Gr-1-PE (RB6-8C5), CD11b-PE (M1/70), B220-PE/APC (RA3-6B2), CD3-APC (145-2C11), CD45.1-PECy7 (A20) (BioLegend) and c-Kit-APC-eFluor780 (2B8) (eBioscience). Samples were analysed using a LSRII cell analyser (BD Bioscience) and data analysed with FlowJo software (TreeStar).

Limiting-Dilution Analysis

In vitro

4, 8 or 12 GFP^+ BM cells were sorted into 96 wells of two U-bottom 96-well plates *per* sample with 100 μL of OptiMEM (Sigma) with 10% FCS (Gibco), 100 μM β-mercaptoetanol (Gibco), 50 μg/mL Gentamicin (Gibco), 10 ng/mL mSCF, 5 ng/mL mIL3 and 5 ng/mL G-CSF (all from PeproTech). Live ($DAPI^-$) and GFP^+ cells were sorted using a FACSAriaII cell sorter (BD Bioscience). One plate from each sample was incubated under normoxic conditions (20% O_2, 5% CO_2, 37°C) and other under hypoxic conditions (1% O_2, 5% CO_2, 37°C) for 10 days. Positive wells were scored when a visible colony was present.

In vivo

Eight- to 12-week-old CD45.1 or CD45.1-CD45.2 recipient mice were sublethally irradiated with 500 cGy 4 to 15 h prior to transplantation. 10, 10^2, 10^3 or 10^4 GFP^+ BM cells were injected into the tail vein of recipient mice (n=4-6/group).

Single-cell RNA-sequencing

GFP^+ BM cells were sorted, washed and resuspended into PBS (Gibco) containing 0.04% ultrapure bovine serum albumin (BSA) (Ambion). Four biological replicates per sample were pooled after sorting. Cellular suspensions (target recovery of 1500 cells), Gel Beads and Partitioning Oil were loaded onto the Chromium A Chip (Chromium™ Single Cell A Chip Kit, 10x Genomics), and further into the Chromium Single Cell Controller (10x Genomics) for generation of single-cell Gel Bead-in-EMulsions (GEMs). Reverse transcription (RT) of the mRNA contained in the GEMs and subsequently single cell libraries for 3' RNA-seq, were prepared using Chromium™ Single Cell 3' Library & Gel Bead kit v2 (10x Genomics) according to manufacturer's instructions. In short GEM-RT was performed in a C1000 Touch™ Thermal cycler with 96-Deep Well Reaction Module (Bio-Rad): 53°C for 45 min, 85°C for 5 min; hold at 4°C. After RT, GEMs were broken, and cDNA was cleaned up using DynaBeads® MyOne™ Silane Beads (Thermo Fisher Scientific) and SPRIselect Reagent Kit (Beckman Coulter). cDNA was amplified using the C1000 Touch™ Thermal cycler with 96-Deep Well Reaction Module: 98°C for 3 min; cycled 13x: 98°C for 15sec, 67°C for 20sec, and 72°C for 1 min; 72°C for 1 min; hold at 4°C. After SPRIselect purification, the cDNA was fragmented enzymatically and indexed sequencing libraries were constructed using the reagents in the Single Cell Library Kit and Chromium™ i7

Multiplex Kit (10x Genomics) following these steps: 1) Fragmentation, end repair and A-tailing; 2) Post-fragmentation double sided SPRI selection; 3) Adaptor ligation; 4) Post-ligation cleanup with SPRIselect; 5) Sample index PCR and double sided SPRIselect cleanup. Sample index PCR were performed in a C1000 Touch™ Thermal cycler with 96-Deep Well Reaction Module: 98°C for 45 sec; cycled 14x: 98°C for 20 sec, 54°C for 30 sec, and 72°C for 20 sec; 72°C for 1 min; hold at 4°C. Quality and size of cDNA before fragmentation and post sample index PCR was determined using a High Sensitivity (HS) DNA kit (Agilent Technologies) loaded on a 2100 Bioanalyzer (Agilent Technologies). A final quantification of the barcoded libraries was achieved using quantitative PCR (qPCR) (KAPA Library Quantification Kit for Illumina platforms, KAPA Biosystems), before sequencing using a Nextseq500 (Illumina) was performed. Sequencing ready libraries were denaturated and diluted to 1.8pM according to Illumina instructions before loaded on Illumina NextSeq500 using a 150 cycles High output v2 kit (Illumina). Paired-end sequencing was performed with the following read lengths: Read1; 26 cycles, Read2; 98 cycles and 8 cycles of I7 Index. Two consecutive sequencing runs were performed to achieve enough sequencing depth and the data was combined. Reads were processed and UMIs counted using Cell Ranger v1.3.0 against the mm10 annotation. Downstream analyses were performed using Seurat v2.1.0 for R where the cells were filtered to remove potential doublets and scaled regressing out the effects of mitochondrial content and the number of UMIs attained from each cell. Differentially expressed genes were identified, and the cells were projected on two dimensions using TSNE.

Differential expression analysis and pathway enrichment

t-SNE plots and K-clustering were generated with Loupe software (10X Genomics).

Venn diagrams were generated with BioVenn (www.biovenn.nl) (Hulsen et al., 2008). Gene Functional Classification was performed using DAVID (david.ncifcrf.gov/home.jsp) (Huang da et al., 2009). Functional protein associations, GO and KEGG enrichment analysis were investigated using STRING (string-db.org) (Szklarczyk et al., 2015) and for the visualization of the networks we used Cytoscape (Shannon et al., 2003). A minimum interaction score of 0.5 was used to detect interactions.

Statistical analysis

All data are expressed as the mean ± SEM. Differences between groups were assessed using unpaired Student's *t* tests. All analyses were performed with Prism software, version 7.0 (GraphPad software). Frequency estimations were generated using Extreme Limiting Dilution Analysis (ELDA) software, which takes into account whether the assumptions for LDA are met (http://bioinf.wehi.edu.au/software/elda/) (Hu and Smyth, 2009).

Supplemental References

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