SUPPLEMENTARY APPENDIX

# Long-term observation reveals high-frequency engraftment of human acute myeloid leukemia in immunodeficient mice

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# Supplementary Material and Methods

## Sample preparation for xenotransplantation assays

For primary samples with blast counts <95% or for isolation of human AML cells from murine organs for secondary transplantations, enrichment using the MACS technology (Miltenyi, Bergisch-Gladbach, Germany) for CD33 (in 13/14 of samples) or CD38 (1/14) was performed. Post-MACS purities exceeded 95% (not shown).

## Flow cytometric cell sorting of patient samples

For three patients, leukemic blasts were separated by flow cytometric cell sorting into CD34+ and CD34- cells using a FACS Aria III (BD Biosciences, Franklin Lakes, NJ, USA). Post-sort purities exceeded 95% (not shown).

### Histopathology

For histopathological analyses, murine organs were fixed in 4% phosphate buffered formalin and then embedded in paraffin. Immunohistochemical analysis was performed as previously described (1), using H&E staining and antibodies against CD33 (Ventana, Tucson, Arizona, USA, Mouse monoclonal, Clone: QBEnd 10, RTU.), CD34 (Ventana, Mouse monoclonal, Clone: QBEnd 10, RTU) and CD117 (DAKO, Glostrup, Denmark; A4502, Rabbit polyclonal, Dilution 1:50) respectively.(1)

## Limiting dilution and homing assays

Limiting dilution assays were accomplished by transplanting 1 x $10^6$, 0.5 x $10^6$ and 0.1 x $10^6$ cells intravenously, and LSC frequencies then calculated using L-Calc 1.1 software from StemCell Technologies. For homing assays, purified AML blasts were labeled with CFSE (CellTrace CFSE Cell Proliferation Kit, ThermoFisher Scientific, Waltham, MA, USA) and 1x$10^6$ cells per mouse injected via the tail vein. 8 hours after transplantation mice were euthanized and BM analyzed by flow cytometry recognizing CFSE and human antigens (see below).

### Next generation sequencing

NGS analysis was performed both on patient-derived AML cells and corresponding mouse-derived leukemic cells obtained by MACS purification of pooled BM samples from all engrafted animals transplanted with one AML. DNA for NGS was isolated using ZR-Duet DNA/RNA MiniPrep kit (ZymoResearch, Irvine, CA, USA) according to the manufacturer's protocol and NGS analysis performed as previously described (2). Patient NGS libraries were prepared using the AML community panel from Thermo Fisher containing 19 genes frequently mutated in AML (see ampliseq.com). Patient libraries were sequenced using the Ion PGM platform and analyzed using the Ion Reporter AML pipeline (version 5.0). The average coverage per sample was ~1.400-fold. Sensitivity for calling a mutation was set at 3%. If a mutation was observed in a "post-transplantation" sample but not reported to be present in the initial patient sample, the mapped reads were manually analyzed to assess whether the mutation was present but with a lower allelic

burden than 3%. The sensitivity of such retrospective analysis is dependent on the coverage of the specific region (normally around 0.25%).

**Microarray analyses**

Microarray gene expression analyses were performed on RNA extracted in triplicates from CD34+ and CD34- sorted patient blasts (Patients 8 and 16, inv(16) AML). RNA was extracted with the RNeasy Mini Kit (Qiagen). Concentration and purity of RNA samples were determined with a NanoDrop photometer (peqlab), and integrity confirmed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only RNA samples with RIN values ≥ 7.5 were considered. Per condition, 100 ng of RNA were used to prepare cyanine-3-labeled cRNA for hybridizations, which were performed according to standard protocols using Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarrays. After extensive washing, fluorescence intensities were detected with the Scan Control A.8.4.1 software (Agilent) on an Agilent DNA Microarray Scanner and extracted from images using Feature Extraction 10.7.31 software (Agilent). Quantile normalization was applied to the data s et and correlation analysis was performed. After normalization, the microarray probes were filtered according to the flag information provided by Feature Extraction 10.7.3.1 software. Fold changes were calculated and differentially expressed RNAs identified. The log2-normalized data was linearized and used as input for Gene set enrichment analysis (GSEA, Broadinstitute.org). One gene matrix transposed file was generated containing the transcripts from up- and down-regulated genes in the comparison between CD34+ and CD34- blasts from the two inv(16) patients and the 42 transcripts of the LSC

signature of Eppert et al. (3). GSEA computes if the gene set is enriched in the generated gene expression data.

# Supplementary Tables

| pat # | AML type (FAB, WHO°, genetic†), risk group | age (years) | sex | karyotype | marker profile | molecular abberation | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | FLT3 | NPM1 | Other |
| 1 | M4,with mutated NMP1, intermediate-I | 62 | m | normal | 33/13/117/HLA-DR | ITD | MUT | - |
| 2 | therapy-related, adverse | 75 | f | complex, -7 | 34(85%)/38/33/133/13 | WT | WT | EVI1 |
| 3 | M3, acute promyelocytic leukemia° | 58 | f | 46, t(15;17) | 33/13/15$^{lo}$/117 | WT | WT | PML-RARα |
| 4 | M5, NOS, intermediate-I | 41 | f | normal | 34(68%)/133/33/117/13/15/HLA-DR | ITD | WT | - |
| 5 | M5, with mutated NPM1, intermediate-I | 53 | m | normal | 33/14/13/133/117 | ITD | MUT | - |
| 6 | M5, NOS, intermediate-I | 84 | m | 47, XXY | 34(37%)/33/117/CD15$^{lo}$/CD133$^{lo}$/HLA-DR | ITD | WT | - |
| 7 | M2, adverse | 64 | m | 46, complex | 34(50%)/38/33/133/117 | WT | WT | - |
| 8 | M4eo, with inv(16)(p13.1q22), favorable | 37 | m | 46, inv(16)(p13.1q22) | 34(45%)/38/33/13/117/HLA-DR | WT | WT | CBFB-MYHA11A |
| 9 | M4eo, with inv(16)(p13.1q22), favorable | 32 | m | 46, inv(16)(p13.1q22) | 34(15%)/33/13 | WT | WT | CBFB-MYHA11A |
| 10 | M7, NOS, intermediate-I | 51 | f | normal | 34(73%)/38/33/117/13/HLA-DR | ITD | MUT | - |
| 11 | myelodysplasia-related changes#, adverse | 67# | m | 46, complex | 38/33/117/13/HLA-DR | ITD | MUT | - |
| 12 | M3, acute promyelocytic leukemia° | 49 | m | n.d. | 33/38/117/13 | n.d. | n.d. | PML-RARα |
| 13 | M5, with mutated NPM1, intermediate-I | 48 | m | 46, del(9)(q13q22) | 33/38/117/13/15/HLA-DR | WT | MUT | - |
| 14 | M5, NOS, intermediate-I | 69 | f | normal | 33/38/117$^{lo}$/13/15 | ITD | WT | - |
| 15 | M5, with mutated NPM1, intermediate-I | 55 | m | 46, del(9)(q13q22) | 33/38/117/13/15/HLA-DR | WT | MUT | - |
| 16 | M4eo, with inv(16)(p13.1q22), favorable | 56 | f | 46, inv(16)(p13.1q22) | 34(30%)/33/13 | WT | WT | CBFB-MYHA11A |
| 17 | M5#,with t(9;11)(p21.3;q23.3), intermediate-II | 66# | m | 46, t(9;11)(p22;q23) | 33/38/13/15/HLA-DR | WT | WT | - |
| 18 | therapy-related, with t(8;21)(q22;q22.1), favorable | 59 | f | 46, t(8;21)(q22;q22) | 34(46%)/38/33/117/15/HLA-DR | WT | WT | RUNX1/RUNX1T1 |
| 19 | M2, NOS, intermediate-I | 56 | f | 47, XXY | 34(95%)/38/33/133/117/13/15/HLA-DR | WT | WT | - |

standard engrafters (37%)

long latency engrafters (58%)

° according to Arber et al. Blood , 2016

† accoding to Mrozek et al. Journal of Clinical Oncology, 2012

#relapse sample

**Suppl. Table 1**

**Supplementary Table 1: Patient's and AML characteristics.** Long latency (distinguished in grey) and standard engrafters are indicated by lateral arrows.

| pat # | puncture week 8-10 | puncture week 12-14 | puncture week 16 | last negative BM puncture (weeks post-tx) | first positive BM puncture (weeks post-tx) | final assessment of leukemia (weeks post-tx) |
|---|---|---|---|---|---|---|
| 1 | + | n.a. | n.a. | n.a. | 7 | 7 |
| 2 | + | n.a. | n.a. | 4 | 8 | 9 |
| 3 | + | n.a. | n.a. | 4 | 8 | 10 |
| 4 | - | + | n.a. | 8 | 12 | 13 |
| 5 | - | - | n.a. | 12 | 15 | 15 |
| 6 | - | + | n.a. | 12 | 13 | 15 |
| 7 | - | + | n.a. | 11 | 14 | 16 |
| 8 | - | - | n.d. | 12 | 17 | 17 |
| 9 | - | - | - | 16 | 20 | 21 |
| 10 | - | - | - | 16 | 20 | 22 |
| 11 | - | - | - | 20 | 24 | 25 |
| 12 | - | - | - | 24 | 26 | 26 |
| 13 | - | - | - | 24 | 26 | 27 |
| 14 | - | - | - | 20 | 24 | 28 |
| 15 | - | - | - | 24 | 26 | 28 |
| 16 | - | - | - | 25 | 28 | 30 |
| 17 | - | - | - | 32 | 36 | 38 |
| 18 | - | - | - | 36 | 39 | 39 |
| 19 | - | - | - | 50 | n.a. | n.a. |

n.d. = not determined
n.a. = not assessable
+ = human cells detected (≥1% of murine BM)
- = no human cells detected (<1% of murine BM)

**Suppl. Table 2**

**Supplementary Table 2**: Overview of routinely performed BM punctures indicating the weeks in which the puncture was performed, if the puncture was positive (“+”) or negative (“-“) and at which week post-transplantation the last negative and respectively the first positive puncture occurred. Shown are furthermore the weeks of final assessment for each transplanted AML case.

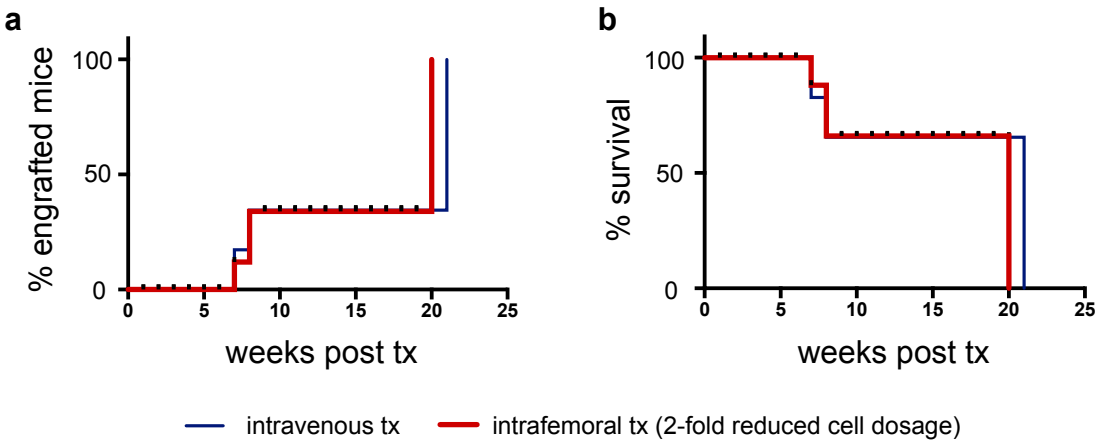
| No. | gene | mutation | allele frequency (%) | |
|---|---|---|---|---|
| | | | pre-tx | post-tx |
| 1 | **WT1** | p.Ser381Ter | 47 | 46 |
| 2 | **KRAS** | p.Gly13Asp | 42 | 47 |
| | **CEBPA** | p.Asp80HIS | 52 | 54 |
| 5 | **DNMT3A** | p.Arg882His | 50 | 42 |
| | **NPM1** | insCTCTG, p.Trp288fs | 57 | 57 |
| 6 | **DNMT3A** | c.2645G>A, Arg882His | 47 | 54 |
| 7 | **KIT** | p.Asp816Val | 20 | 24 |
| | **TET2** | pHis1219Leu | 99 | 100 |
| | **PTPN1** | p.Ala72Thr | 8 | 7 |
| | **CEBPA** | p.Pro34Gln | 44 | 46 |
| | **CEBPA** | p.His24fs | 19 | 16 |
| | **RUNX1** | p.Thr111Pro | 67 | 71 |
| 8 | **NRAS** | p.Gln61Hi | 41 | 53 |
| | **NRAS** | p.Gln61Lys | 6 | 0 |
| | **CEBPA** | p.Ala9Val | 0 | 13 |
| 9 | no AML specific mutations detected in pre- and post-tx samples | | | |
| 10 | **NPM1** | c.859_860insTCTG, Trp288fs | 45 | 48 |
| | **WT1** | 1385G>C, Arg462Pro | 47 | 50 |
| | **CEBPA** | 589_590insACCCGC, Pro196_Pro197insHisPro | 73 | 69 |
| 11 | **DNMT3** | c.2645G>A, Arg882His | 52 | 48 |
| | **NPM1** | c.859_860insTCTG, Trp288fs | 32 | 33 |
| 13 | **DNMT3A** | p.Arg882His | 43 | 18 |
| | **IDH1** | p.Arg132His | 7 | 4 |
| | **NPM1** | p.Trp288fs | 45 | 20 |
| | **Flt3** | p.Asp835Tyr | 25 | 11 |
| | **IDH2** | p.Arg140Gln | 15 | 6 |
| 15 | **DNMT3A** | p.Arg882Cys | 50 | 53 |
| | **NPM1** | p.Trp288fs | 46 | 48 |
| | **ASXL1** | p.Gln588Arg | 0 | 4 |
| 18 | **BRAF** | p.Val600Glu | 32 | 34 |
| | **KRAS** | p.Gly12Val | 8 | 8 |
| | **ASXL1** | p.Glu1102Asp | 48 | 53 |

**Suppl. Table 3**

**Supplementary Table 3: Allele frequencies from patient PB-derived (pre-transplant) vs. mouse BM-derived (post-transplant; xenogeneic) samples**. Paired pre- and post-transplantation samples from 12 patients were available for NGS analysis. Indicated are allele frequencies of specific mutations in each sample. Note that pooled BM of mice engrafted with each

AML case where analyzed; please see Table 1 for numbers of engrafted mice for each case. Long latency (distinguished in grey) and standard engrafters are indicated by lateral arrows. Note that for patient #9 (AML with inv(16)) no AML specific mutations were detected in pre- and post-transplant samples using this panel.

## Supplementary Figures



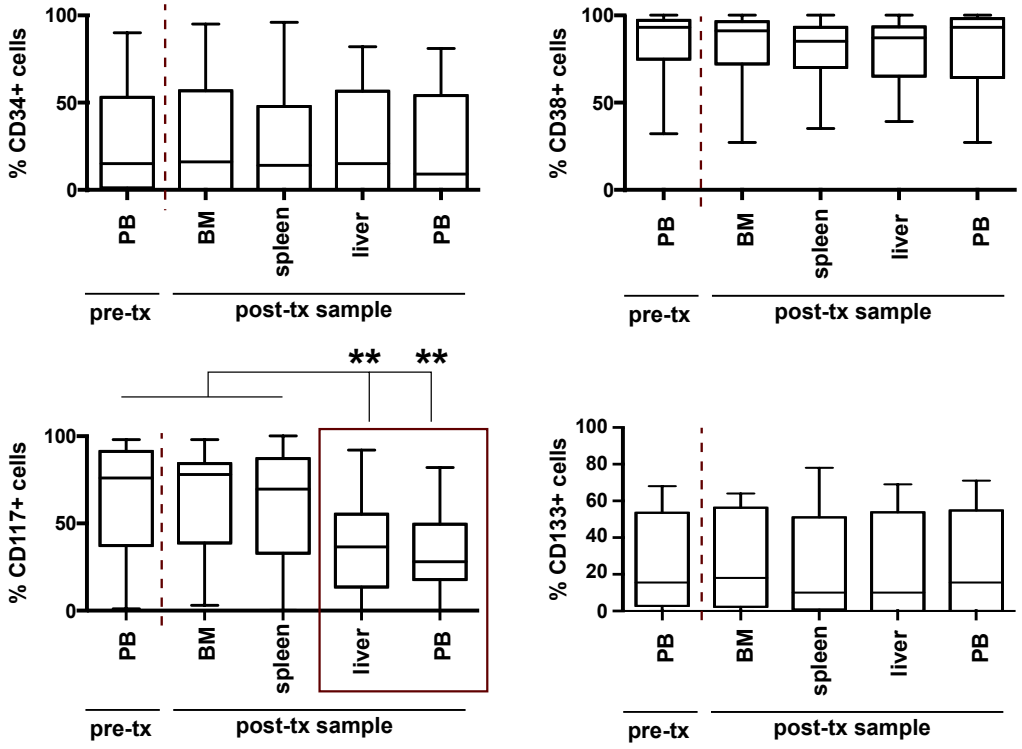

**Suppl. Figure 1**

**Supplementary Figure 1: Intravenous vs. intrafemoral transplantations.** Shown are summarized data from three patient samples (pat #1, #2, #9) transplanted intravenously and intrafemorally side-by-side with a 2-fold reduced cell dosage for intrafemoral injections. Both transplantation procedures result in similar time-to-engraftment and overall survival of mice (statistically not significant).

**a**

| No. | % CD34+ | | % CD38+ | | % CD34+CD38- | | % CD117+ | | % CD133+ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pre-tx | post-tx* | pre-tx | post-tx* | pre-tx | post-tx* | pre-tx | post-tx* | pre-tx | post-tx* |
| 1 | 15 | 11 ± 3 | 77 | 82 ± 4 | 11 | 14 ± 4 | 85 | 79 ± 12 | 13 | 18 ± 7 |
| 2 | 82 | 88 ± 11 | 94 | 82 ± 7 | 3 | 1 ± 1 | 73 | 79 ± 8 | 67 | 62 ± 5 |
| 3 | 2 | 0 ± 0.5 | 33 | 41 ± 9 | 2 | 1 ± 0.5 | 91 | 88 ± 4 | 0 | 0 ± 0 |
| 4 | 68 | 59 ± 9 | 97 | 91 ± 2 | 21 | 16 ± 4 | 66 | 72 ± 11 | 68 | 64 ± 11 |
| 5 | 2 | 0 ± 0 | 92 | 98 ± 3 | 1 | 0 ± 0 | 24 | 17 ± 10 | 14 | 18 ± 7 |
| 6 | 48 | 56 ± 18 | 95 | 91 ± 7 | 4 | 1 ± 1 | 86 | 83 ± 5 | 17 | 12 ± 6 |
| 7 | 90 | 95 ± 3 | 100 | 98 ± 2 | 0 | 0 ± 0 | 38 | 32 ± 3 | 52 | 56 ± 4 |
| 8 | 45 | 43 ± 7 | 94 | 97 ± 3 | 3 | 1 ± 1 | 88 | 79 ± 7 | 27 | 31 ± 8 |
| 9 | 15 | 21 ± 6 | 97 | 96 ± 5 | 1 | 0 ± 0 | 98 | 96 ± 6 | 0 | 0 ± 0 |
| 10 | 73 | 79 ± 13 | 83 | 74 ± 18 | 3 | 4 ± 3 | 97 | 88 ± 14 | 0 | 0 ± 0 |
| 11 | 1 | 0 ± 0 | 98 | 100 ± 3 | 0 | 0 ± 0 | 67 | 58 ± 3 | 4 | 8 ± 6 |
| 12 | 0 | 0 ± 0 | 98 | 95 ± 4 | 0 | 0 ± 0 | 77 | 68 ± 6 | 58 | 64 ± 11 |
| 13 | 1 | 0 ± 0 | 96 | 92 ± 5 | 0 | 0 ± 0 | 96 | 98 ± 3 | 5 | 3 ± 2 |
| 14 | 3 | 1 ± 1 | 32 | 27 ± 9 | 0 | 0 ± 0 | 10 | 17 ± 8 | 63 | 57 ± 9 |
| 15 | 0 | 0 ± 0 | 84 | 91 ± 5 | 0 | 0 ± 0 | 1 | 3 ± 2 | 3 | 4 ± 2 |
| 16 | 34 | 39 ± 2 | 85 | 81 ± 17 | 18 | 12 ± 8 | 35 | 41 ± 6 | 2 | 0 ± 0 |
| 17 | 0 | 0 ± 0 | 62 | 66 ± 6 | 0 | 0 ± 0 | 92 | 78 ± 11 | 17 | 23 ± 7 |
| 18 | 46 | 52 ± 5 | 68 | 60 ± 9 | 13 | 19 ± 9 | 75 | 78 ± 3 | 31 | 19 ± 13 |
| 19 | 95 | - | 99 | - | 0 | - | 94 | - | 75 | - |

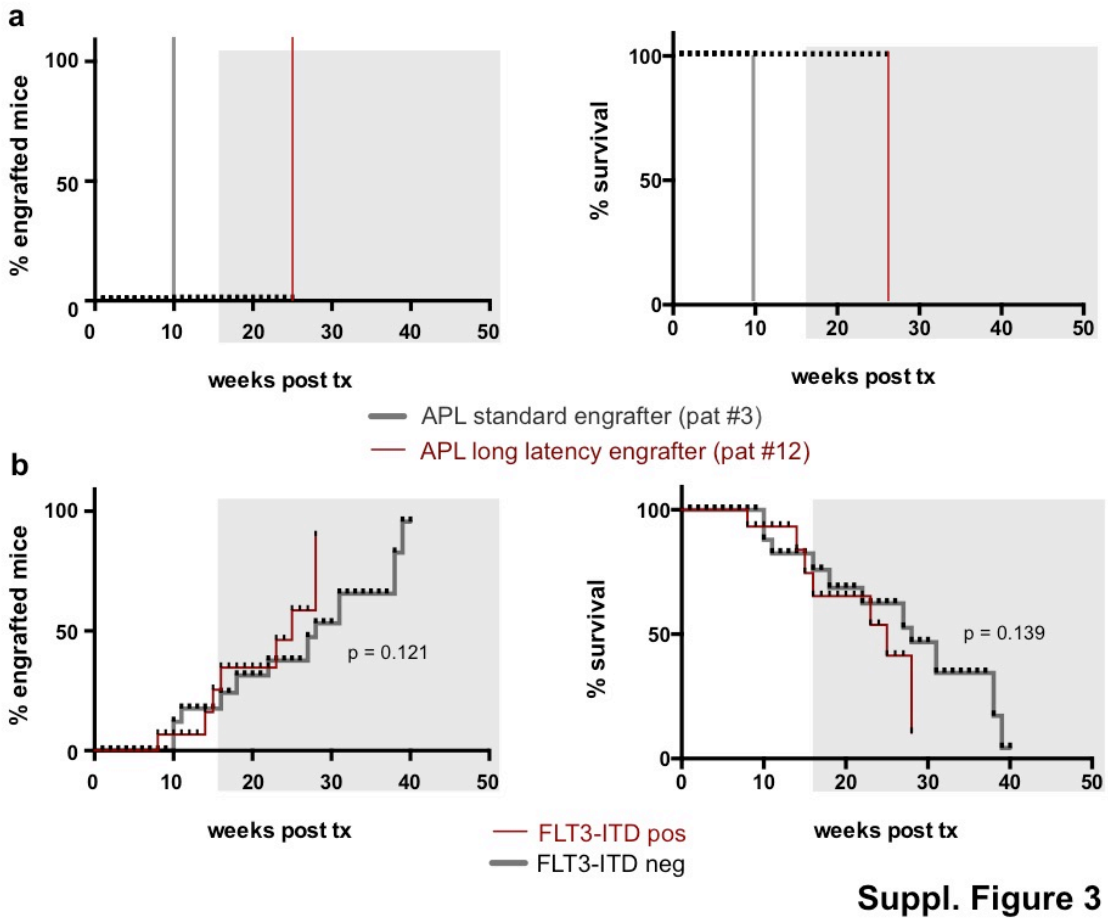
**b**




**Suppl. Figure 2**

**Supplementary Figure 2: Phenotypic quantification of pre- and post-transplantation leukemic blasts retrieved from murine BM, PB and organs. (a)** Quantification of % human CD34+, CD38+, CD34+CD38-, CD117+ and CD133+ cells in patient-derived (pre-transplant) leukemic blasts versus post-transplant leukemic cells retrieved from murine BM. Note that flow

cytometry analysis were performed individually in every animal. Shown are mean values ± standard deviation for each condition (meaning all animals engrafted with one AML case; for numbers see Table 1). Note that largely conserved phenotypic features are observed in all cases. **(b)** Additional flow cytometric quantification of CD34, CD38, CD117 and CD133 surface expression in patient peripheral blood-derived (pre-transplant) and post-transplant samples derived from mouse BM, spleen, liver and PB. Shown are summarized results of all engrafted AML cases. No changes in CD34, CD38 and CD133 were observed whereas a significant decrease in CD117 expression in post-transplant samples of liver and PB was detected.

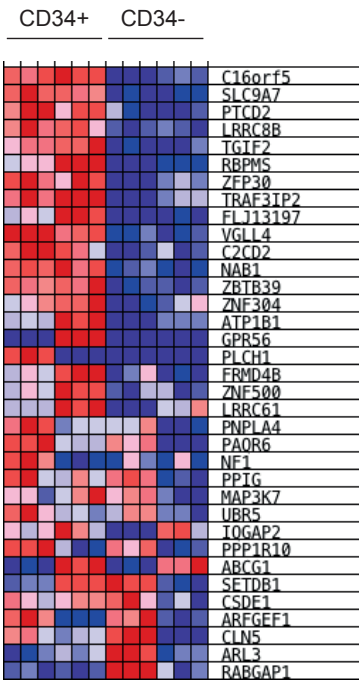



**Supplementary Figure 3: Time to engraftment and survival analysis for acute promyelocytic leukemia (APL) and FLT3-ITD mutated versus non-mutated AML cases. (a)** The two engrafted APL samples show heterogeneous behavior, one belonging to the standard engrafters (grey) and one to the long latency engrafters (red). **(b)** Comparison of mouse engraftment (left) and respectively overall survival (right) with FLT3 mutated versus FLT3 non-mutated AML (red: FLT mutated, n=7; grey: FLT3 non-mutated, n=11). No significant differences were detected.

**a**

| PROBE | RANK IN GENE LIST | RANK METRIC SCORE | RUNNING ES | CORE ENRICHMENT |
|---|---|---|---|---|
| C16orf5 | 154 | 2.465419531 | 0.07043456 | Yes |
| SLC9A7 | 311 | 2.076884508 | 0.12891641 | Yes |
| PTCD2 | 317 | 2.068343401 | 0.1920381 | Yes |
| LRRC8B | 355 | 2.003180027 | 0.25212738 | Yes |
| TGIF2 | 511 | 1.789841652 | 0.3018592 | Yes |
| RBPMS | 581 | 1.707986116 | 0.35187793 | Yes |
| ZFP30 | 609 | 1.676597357 | 0.4022995 | Yes |
| TRAF3IP2 | 764 | 1.574861765 | 0.44548613 | Yes |
| FLJ13197 | 818 | 1.541956902 | 0.4909443 | Yes |
| VGLL4 | 1033 | 1.4199332 | 0.5274432 | Yes |
| C2CD2 | 1071 | 1.399352074 | 0.56905746 | Yes |
| NAB1 | 1485 | 1.235013843 | 0.59343934 | Yes |
| ZBTB39 | 1922 | 1.099680901 | 0.612934 | Yes |
| ZNF304 | 2078 | 1.060677409 | 0.64035594 | Yes |
| ATP1B1 | 2112 | 1.053190708 | 0.6715087 | Yes |
| GPR56 | 2770 | 0.924979746 | 0.6784849 | Yes |
| PLCH1 | 3069 | 0.883759856 | 0.69585234 | Yes |
| FRMD4B | 3438 | 0.836307108 | 0.7094958 | Yes |
| ZNF500 | 3618 | 0.817054152 | 0.7286848 | Yes |
| LRRC61 | 4774 | 0.692577958 | 0.7123862 | Yes |
| PNPLA4 | 4809 | 0.689265311 | 0.7323717 | Yes |
| PAQR6 | 6717 | 0.505503476 | 0.6859408 | No |
| NF1 | 8255 | 0.397508323 | 0.6482151 | No |
| PPIG | 10586 | 0.257991254 | 0.58048147 | No |
| MAP3K7 | 10971 | 0.235171527 | 0.575213 | No |
| UBR5 | 11336 | 0.214815646 | 0.5699709 | No |
| IQGAP2 | 11595 | 0.201218992 | 0.5677533 | No |
| PPP1R10 | 12177 | 0.166215613 | 0.55398077 | No |
| ABCG1 | 13426 | 0.092002168 | 0.5162881 | No |
| SETDB1 | 13659 | 0.078915656 | 0.51117235 | No |
| CSDE1 | 13956 | 0.063768975 | 0.50351584 | No |
| ARFGEF1 | 15404 | -0.002081853 | 0.45661277 | No |
| CLN5 | 16291 | -0.033561509 | 0.4288818 | No |
| ARL3 | 26520 | -0.684934437 | 0.11785747 | No |
| RABGAP1 | 26922 | -0.73296541 | 0.12726794 | No |

**b**




**Suppl. Figure 4**

**Supplementary Figure 4:** Table of genes and heat map of the genes enriched in the LIC signature of AML with inv(16) CD34+ blasts.

## Supplementary references:

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