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## Germline *PAX5* mutation predisposes to familial B acute lymphoblastic leukemia

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### Abstract:

In recent years, through whole genome analyses, convincing evidence for the contribution of genetic predisposition to childhood B-cell precursor - acute lymphoblastic leukemia (BCP-ALL) due to altered *PAX5* has been provided. A recurrent mutation p.Gly183Ser affecting the octapeptide domain has been described in three unrelated families and a p.Arg38His mutation affecting the DNA-binding paired domain reported in another one.

We strengthen here the assumption of the inherited character of familial BCP-ALL by identifying the *PAX5* p.Arg38His mutation in a family in which the three children developed BCP-ALL. One relapsed two years after his initial diagnosis and was allografted with his brother's cells before the latter developed BCP-ALL. The patient allografted relapsed later from donor-related cells.

By syngeneic transplantations in mice, we showed that p.Arg38His expression does not abrogate the engraftment capacity of transduced *Pax5*<sup>-/-</sup> pro-B cells unlike wild type *PAX5*-rescued *Pax5*<sup>-/-</sup> pro-B cells and can predispose to BCP-ALL. Through functional and molecular analyses, we demonstrated that p.Arg38His acts as a hypomorphic variant altering the pattern of expression of *PAX5* target genes. Our data highlight the importance of transcriptional deregulation, particularly of genes involved in B cell differentiation in familial BCP-ALL. We demonstrated that inherited genetic basis of susceptibility to BCP-ALL has been underestimated and should be considered before any familial allograft.

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## **Germline *PAX5* mutation predisposes to familial B acute lymphoblastic leukemia**

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We describe here a family with a high incidence of B-cell precursor-acute lymphoblastic leukemia (BCP-ALL) affecting 3 children (II.1, II.2 and II.3) at 11, 17 and 25 years old, respectively (Figure 1A, C and Supplemental Figure 1). Both parents were asymptomatic. After a first relapse, the proband (II.2) underwent a familial allogeneic hematopoietic stem cell transplantation (HSCT) with his 11-year-old younger brother (II.3) leading to a complete remission (CR) for 20 years but relapsed a second time and died rapidly after intracranial hemorrhage. His sister (II.1) developed BCP-ALL for which she was treated by chemotherapy leading to a CR even 30 years after her initial diagnosis. II.3 developed BCP-ALL 14 years after being HSCT donor for II.2 and died from infectious complications after HSCT from an unrelated donor (Supplemental Figure 1).

As the family history was compatible with a germline transmission of the disease, a whole exome sequencing was performed on II.3, which identified a *PAX5* germline heterozygous c.113G>A mutation resulting in a R38H substitution. The R38H substitution is predicted to alter the protein affinity to DNA<sup>1</sup> and concerns a highly conserved residue located within the N-terminal DNA-binding Paired domain (Supplemental Figure 2A-C and Supplemental Tables 1 and 2). This mutation is associated with leukemic progression in murine models with *Pax5* haploinsufficiency<sup>2</sup> or *PAX5-ELN* fusion<sup>3</sup>. This mutation was subsequently detected in all affected subjects, at the BCP-ALL diagnosis and at remission, and in one asymptomatic parent without history of malignancy (Figure 1B, Supplemental Figure 1 and Supplemental Table 3). *PAX5* somatic alterations are present in one third of sporadic BCP-ALL<sup>4,5</sup> but only recently, *PAX5* germline mutations leading to BCP-ALL have been identified<sup>6-8</sup>. *PAX5* encodes a critical transcription factor for B-cell differentiation<sup>9-11</sup> repressing B-lineage 'inappropriate' gene expression and promoting the transcription of specific B-cell genes<sup>10-14</sup>. As described in other germline *PAX5* pedigrees<sup>6-8</sup>, the existence of asymptomatic carriers and the absence of immunodeficiency before the onset of BCP-ALL suggest the requirement of additional genetic alterations leading to the development of leukemia. Subjects II.2 and II.3 had a normal karyotype at the BCP-ALL stage. Additional cytogenetic abnormalities were found in II.2 at his first (before transplant) and second (after transplant with his brother,

consequently named II.<sup>3/2</sup>) relapses. Somatic *CDKN2A* homozygous loss and *RAS* pathway mutations, recurrent features of BCP-ALL, were detected in all analyzed BCP-ALL samples (II.2, II.3 and II.<sup>3/2</sup>) (Supplemental Figure 3 and Supplemental Tables 3 and 4). A second *PAX5* mutation was detected at diagnosis: Y371fs in II.1, R140L in II.3 and II.<sup>3/2</sup> (Figure 1D-F and Supplemental Table 3). Next-Generation Sequencing of II.3 patient cDNA showed that these two *PAX5* mutations were mainly detected on different alleles (data not shown).

More than 20 years separate HSCT allograft in II.2 from his donor-related (II.<sup>3/2</sup>) leukemia and fourteen years from the allograft to II.3's diagnosis of BCP-ALL (Supplemental Figure 1). The II.2 and II.3 leukemic samples harbored distinct *IGH* clonal rearrangements (Supplemental Figure 4) suggesting that the R140L mutation occurred independently in both patients. Interestingly, the R140L mutation is recurrently associated to the R38H mutation in sporadic BCP-ALL since 10/11 of patients reported in the literature with R140L mutation have also the R38H mutation suggesting rather a cooperation between these two mutations<sup>15</sup> than just a biallelic inactivation of *PAX5*<sup>16</sup>.

The onset of BCP-ALL in patients with *PAX5* G183S germline mutations located in the octapeptide domain is very early with a median age of 2 years old and associated with a loss of the chromosome 9p leading to the simultaneous losses of the second *PAX5* allele and *CDKN2A*<sup>6,7</sup>. In contrast, the germline R38H variant (this work and <sup>8</sup>) is associated with an older age of onset (11 to 25 years old) and a normal karyotype (Supplemental Figure 5 and Supplemental Table 5).

To functionally address the impact of the R38H mutation on the B-cell differentiation, we transduced with *PAX5* WT and/or R38H murine fetal liver *Pax5*<sup>-/-</sup> B-cells (Figure 2A) and the murine plasmacytoma 558L $\mu$ M cell line that does not express *Pax5* and *Cd79a* (Supplemental Figure 6). As expected, *PAX5* WT restored the expression of *Cd19* in *Pax5*<sup>-/-</sup> cells and their capacity to differentiate in contrast to R38H (Figure 2B). R38H was also unable to rescue surface IgM expression in 558L $\mu$ M cell line (Supplemental Figure 6). In

addition, R38H exhibited a weak competitive or additive effect on PAX5 WT when co-expressed in primary cells or in 558L $\mu$ M cell line respectively (Figure 2B and Supplemental Figure 6). PAX5 WT significantly increased the *in vitro* clonal activity of transduced *Pax5*<sup>-/-</sup> B220<sup>+</sup> cells and led to the expression of *Cd19* unlike R38H (Supplemental Figure 7), confirming that R38H is not able to trigger B-cell differentiation. Furthermore although no difference in frequencies of positive wells between PAX5 R38H- and MIG-infected cells was observed, the absolute number of B cells obtained per well was significantly higher (Supplemental Figure 7). Altogether, our data demonstrate that R38H disables B-cell differentiation and partially maintains PAX5-dependent cell growth properties without overt dominant-negative effect on the normal PAX5 function.

The transcriptome of R38H-transduced *Pax5*<sup>-/-</sup> cells (Figure 2C-E) is similar, although not identical, to the negative control (MIG) and clearly distinct from the PAX5 WT condition (Figure 2C). Gene set enrichment analyses confirmed that R38H is unable to regulate PAX5 target genes and to lead to a pro-B-cell gene expression program in contrast to PAX5 WT<sup>17</sup> (Figure 2D and Supplemental Figure 8). Worthnoting, the comparison of the residual transcriptional activity of PAX5 germline mutants showed that R38H has a stronger hypomorphic effect than G183S (Supplemental Figure 9). Although the R38H transcriptome shares similarities with the one of PAX5 WT, supervised comparative analyses identified molecular profile specific to R38H (Figure 2E). In particular, the Leukemia Inhibiting Factor Receptor gene (*Lifr*)<sup>18</sup>, implicated in stemness, is downregulated in PAX5 WT condition and upregulated in R38H condition (Figure 2E). Our results suggest that PAX5 R38H confers some stemness features to the B cells. We thus transplanted transduced CD45.2<sup>+</sup> *Pax5*<sup>-/-</sup> B cells (Figure 2A, F-H). As previously described<sup>19,20</sup>, MIG-transduced cells were able to home back to the bone marrow (BM) and to efficiently long-term engraft with an average of 30% CD45.2<sup>+</sup>/GFP<sup>+</sup> cells in BM 18 weeks after transplantation (Figure 2F-G). In contrast, PAX5 WT-transduced cells abrogated their long-term engraftment capacity in the BM and spleen of recipient mice (Figure 2G). Interestingly, the ectopic expression of R38H led to an intermediate phenotype 3.5 weeks after transplantation and as expected, none of engrafted

GFP<sup>+</sup> cells acquired Cd19 (data not shown) since they do not express PAX5 WT. Interestingly, 2 out of 4 mice maintained their engraftment 18 weeks post-transplantation and one (PAX5 R38H #4) developed a clonal B220<sup>+</sup>/IL7R<sup>+</sup>/Kit<sup>-</sup> leukemia (Figure 2H and Supplemental Figure 10) with acquisition of a *Jak3*<sup>V670G</sup> mutation (Supplemental Table 6).

In conclusion, we report a germline PAX5 p.R38H mutation in a family in which three children developed BCP-ALL. We demonstrate that R38H acts as a hypomorphic variant, does not abrogate the engraftment capacity of transduced *Pax5*<sup>-/-</sup> pro-B cells and can predispose to BCP-ALL. The existence of asymptomatic patients without evidence of immunodeficiency before the onset of BCP-ALL shows that *PAX5* germline cases might be underestimated and should be considered prior to any familial allograft.



## Figure legends

### Figure 1: **Familial BCP-ALL with heterozygous germline *PAX5* R38H mutation.**

(A) The germline R38H variant was shown to be inherited from one parent (I.2) who is so far an asymptomatic carrier with no history of cancer at 68 years old. The proband (II.2, indicated by an arrow) developed BCP-ALL at 17 years old. He relapsed two years after his initial diagnosis and was allografted with his brother (II.3) as a donor 14 years before II.3 also developed BCP-ALL. II.2 relapsed at 40 years old and rapidly passed away after intracranial hemorrhage. His sister (II.1) developed BCP-ALL at 11 years old for which she received chemotherapy without HSCT. She is still in CR more than 30 years after her initial diagnosis. The proband's younger brother (II.3) developed also BCP-ALL at 25 years old and died from infectious complications after HSCT from an unrelated donor. The symbol (+) indicates the presence of the R38H germline mutation (B) Sanger sequencing of *PAX5* mutation in samples of I.1, I.2, II.1, II.2 and II.3 with the location of the mutation indicated by an arrow demonstrating a germline origin. The nucleotide and protein sequences are indicated at the bottom. (C) Representative image of May-Grunwald-Giemsa-stained bone marrow smear at BCP-ALL diagnosis in individual II.1. (D) Somatic *PAX5* mutations in leukemic samples from subjects II.1 (top) and II.3 (bottom). Positions of mutations are indicated with red arrows. (E-F) Leukemic architecture at BCP-ALL diagnosis in subject II.3 (donor, (E)) and at donor-related BCP-ALL in subject II.2 (recipient, (F)). Variant allele frequency (VAF) for each mutation is indicated.

### Figure 2: ***PAX5* R38H behaves as a hypomorphic variant and can predispose to BCP-ALL in mice**

(A) Experimental scheme of retroviral complementation assays. (B) *Pax5*<sup>-/-</sup> cells were co-transduced with either empty MIG (MSCV-IRES-GFP) and MImCherry (labeled as empty vectors), empty MImCherry and MIG *PAX5* WT (labeled as *PAX5* WT), empty MImCherry and MIG-*PAX5* R38H (labeled as *PAX5* R38H) or MImCherry-*PAX5* WT and MIG-*PAX5*

R38H (labeled as WT + R38H) retroviral vectors. The proportions of cell markers were evaluated by FACS for each condition (n=8 per condition). Data are representative of two independent experiments (n=4 independent infections per experiment). Percentages indicate proportions of GFP/mCherry-double-positive that are CD19-positive or surface IgM (sIgM)-positive as indicated. Results are expressed as mean  $\pm$  SD; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001. (C) Principal Component Analysis (PCA) of the 2 best components for the 2000 most differentially expressed genes between the three conditions. Black dots represent empty MIG condition, blue dots MIG-PAX5 WT condition and red dots MIG-PAX5 R38H condition. (D) GSEA of PAX5 target gene and pro-B cell gene sets<sup>17</sup> in expression profiles of *Pax5*<sup>-/-</sup> cells transduced with PAX5 WT versus PAX5 R38H with False Discovery Rate FDR < 0.01. NES: Normalized Enrichment Score. (E) Comparative supervised heatmap using a Z-score and spearman correlation clustering displaying the 44 most differentially expressed coding genes between PAX5 R38H-expressing *Pax5*<sup>-/-</sup> pro-B cells and MIG condition (fold > 1.5; q-value < 0.05), with side comparison (lower panel) of corresponding gene expression in PAX5 WT-expressing *Pax5*<sup>-/-</sup> pro-B cells. (F) Representative FACS plots showing engraftment of the GFP<sup>+</sup> donor cells in bone marrow (BM) samples at 3.5 and 18 weeks and spleen samples at 18 weeks post-transplantation. Mice were numbered with #x. (G) Quantification of engraftment of CD45.2<sup>+</sup>/GFP<sup>+</sup> donor cells over time in BM at 3.5, 11.5 and 18 weeks post-transplantation and in spleen 18 weeks post-transplantation. Each dot represents individual mice (n=4 to 6). Data show medians of engrafted mice (CD45.2<sup>+</sup>/GFP<sup>+</sup> cell proportion >1% of total cells). (H) Representative FACS plots showing B220 (CD45R) and Kit (CD117) expression in CD45.2<sup>+</sup>/GFP<sup>+</sup> cells of BM or spleen of PAX5 R38H- or MIG-transduced cells 18 weeks after transplantation. Mouse #4 shows BCP-ALL phenotype.

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## Author contributions

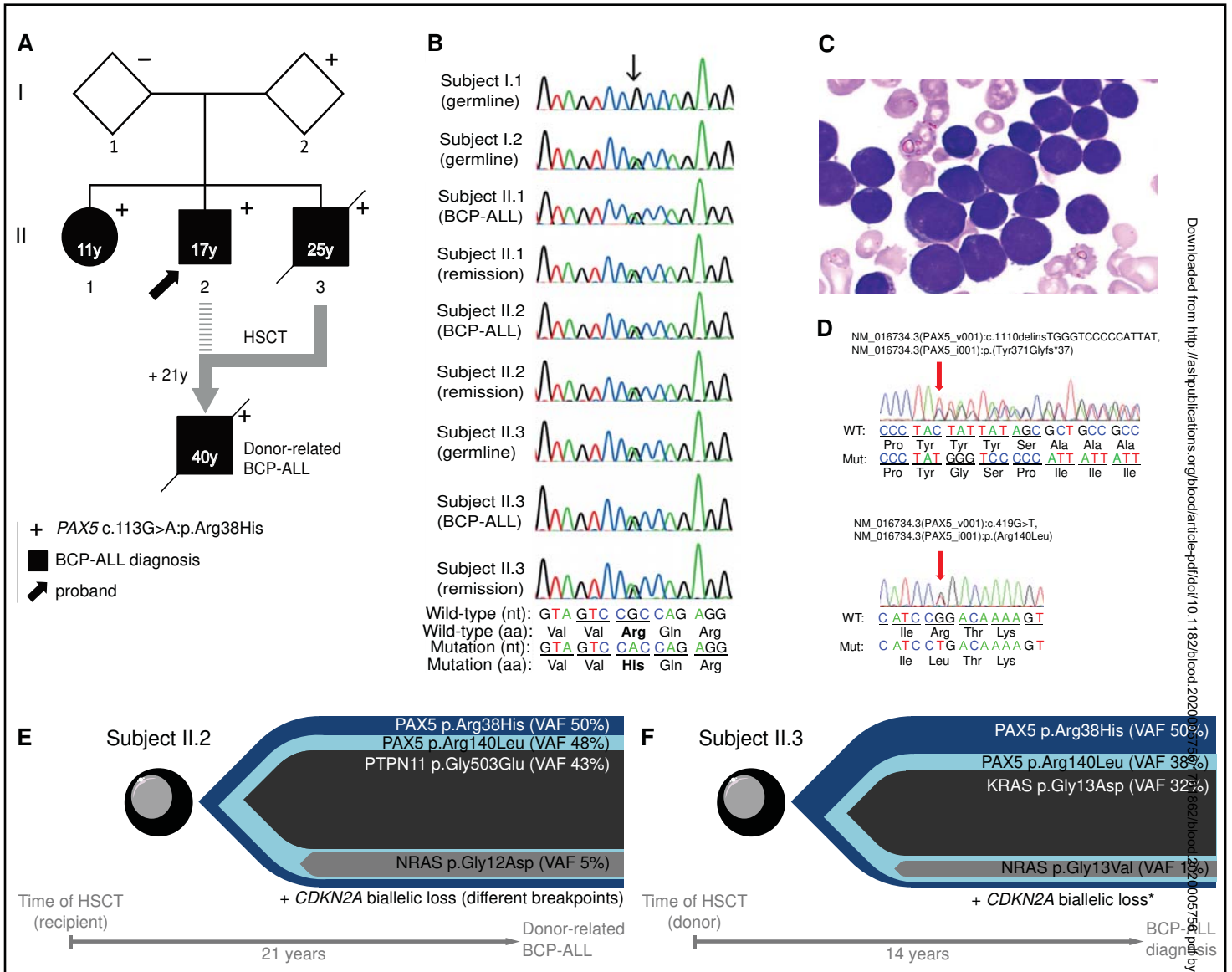
N.D., L.J., B.G., E.D., C.P. and C.Br. conceived and designed the experiments. N.D., L.F., N.H., S.G., A.C., A.M-R., C.R-L., C.V. and M.F. performed molecular and cytogenetic analyses. L.J., C.H., S.H., and B.G. performed and analyzed *in vivo* and *in vitro* studies. N.P. and S.D. performed the microarray experiments. L.L. analyzed the mouse NGS data. V.F. analyzed the transcriptomic data. S.L. provided genetic counseling. S.P., C.Be., B.N. and J.F. provided samples and clinical data. N.D., L.J., B.G., E.D., C.P. and C.Br. wrote the manuscript with feedback from all authors.

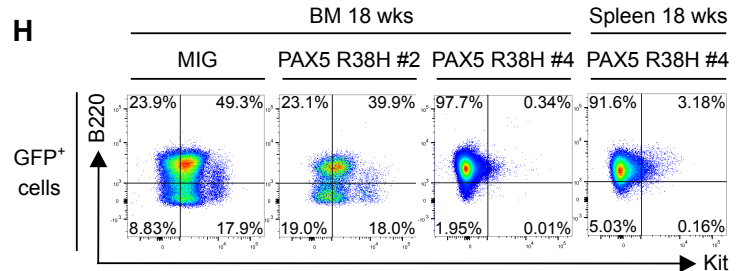
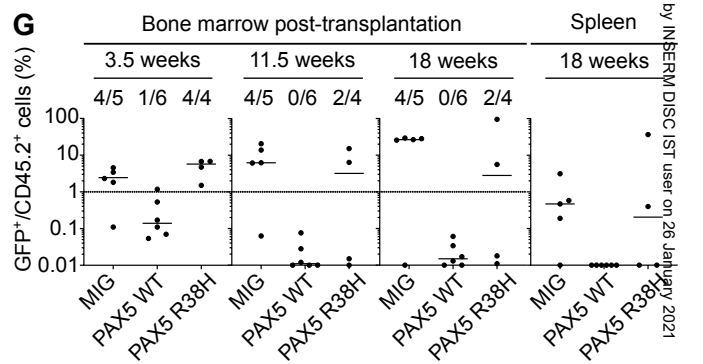
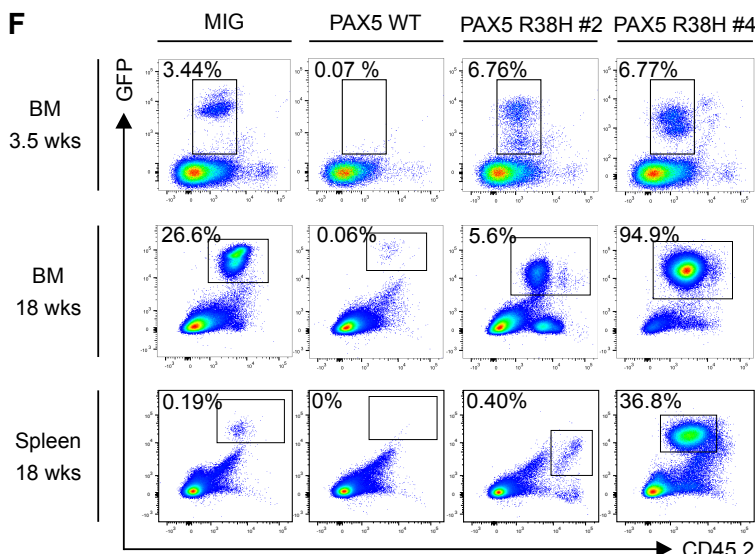
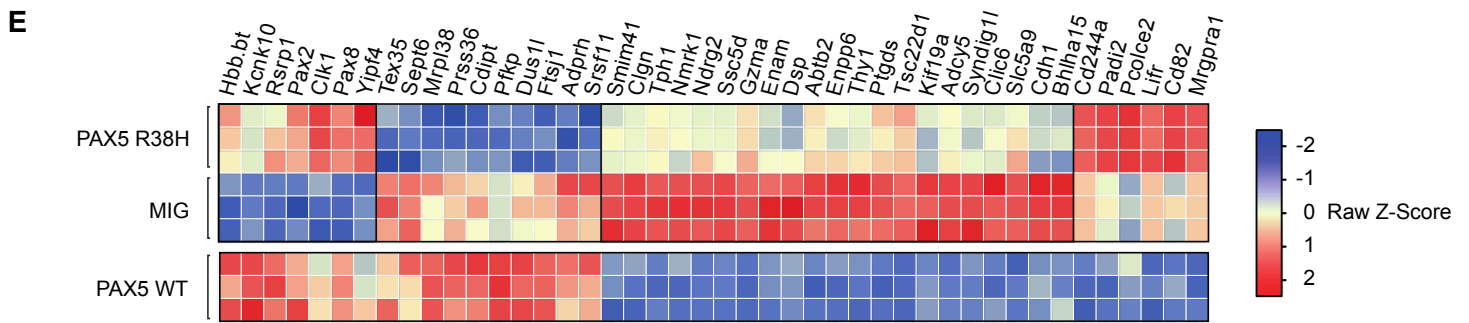
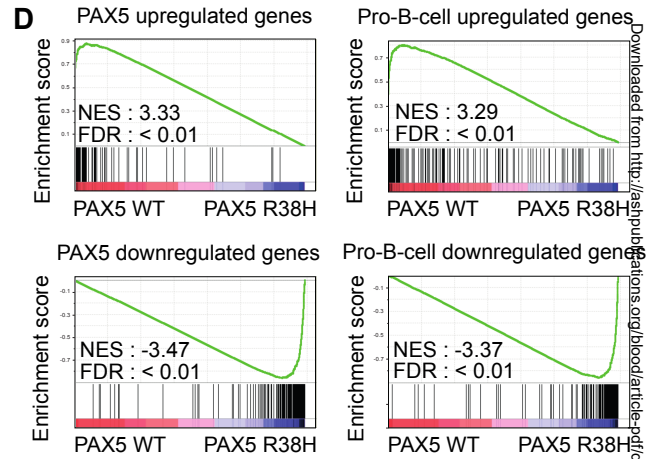
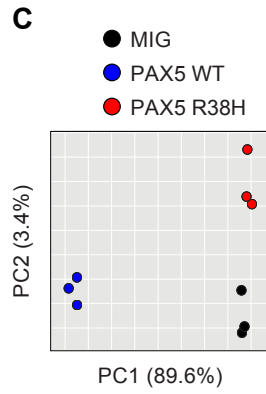
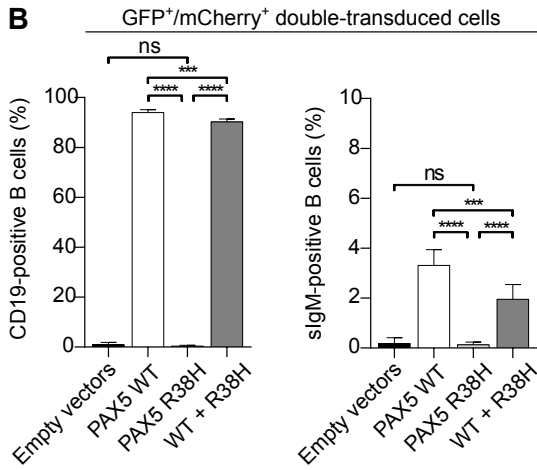
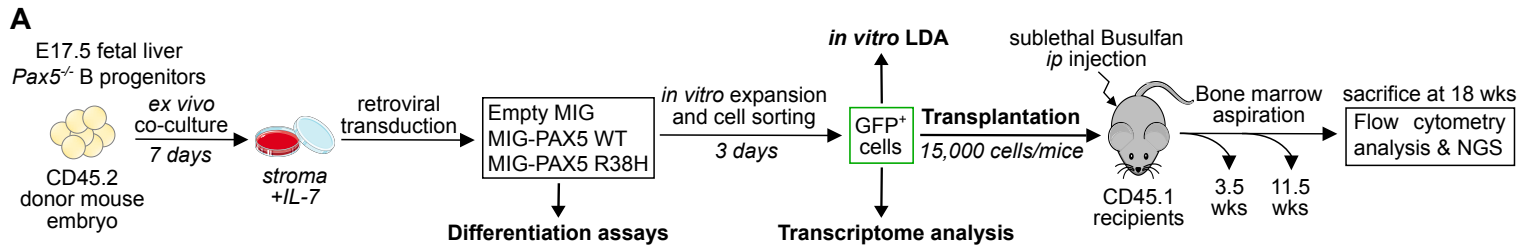
## Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

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