LSD1/KDM1A and GFI1B repress endothelial fate and induce hematopoietic fate in induced pluripotent stem cell-derived hemogenic endothelium

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Supplementary Methods

iPSC lines:

The following iPSC lines were utilized in this study: MML-6838-cl2, SANi001-A, SANi002-A, all derived from a healthy donor, and two unique clones - SANi005-A and BEL-5-Cl.2, each containing a dominant negative GFI1B mutation ($GFI1B^{Q287*}$)[1–4]. The control iPSC lines (MML-6838-cl2, SANi001-A, SANi002-A) were subjected to LSD1 inhibition. The iPSC line MML-6838-cl2 was chosen for HE culture, GFI1B overexpression and single-cell RNA sequencing (scRNAseq) experiments.

Generation of *GFI1B* lentiviral construct

The wild type GFI1B coding sequence, complete with a preceding Kozak sequence and an XbaI restriction site, as well as a following BamHI restriction site (gBlocks from IDT), was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (ThermoFisher Scientific). After transformation, plasmids were isolated and digested with XbaI and BamHI. The band corresponding to the size of GFI1B was visualized on an agarose gel, after which it was subsequently purified and inserted into the LentiCRISPRv2GFP vector (Addgene #82416) following the excision of the Cas9 sequence using XbaI and BamHI. For the creation of an empty vector (EV) control, a double-stranded DNA sequence with overhangs of XbaI and BamHI, was inserted into the LentiCRISPRv2GFP vector to replace the Cas9 sequence cut by XbaI and BamHI.

Flow cytometry and sort

For flow cytometry experiments of the differentiating iPSC colonies, supernatant was harvested into a separate tube and attached cells on the plate were washed 2x with PBS before treatment for 5 minutes with TryplE Select. Cells were collected and passed through a 70µm cell strainer (NUNC) into a 50ml tube containing 10ml of IMDM with 20% FCS and washed with PBS. Both cells from the supernatant and the plate were stained with antibodies (Supplemental Table 1) for 30 minutes. They were then washed with FACS buffer (PBS+0.1% BSA+0.5mM EDTA) and analyzed using the LSD-II (BD Bioscience). For flow cytometry of HE cultures and transduction, cells were stained with CD43 and CD73 antibodies (Supplemental Table 1) and analyzed on FACSymphony™ A5 (BD Bioscience). For scRNAseq, sorting was performed with the BD FACSAria III (BD Bioscience) at room temperature after which cells were washed 3x with chilled DPBS and fixated with 80% chilled methanol. Cells were incubated for 20 minutes at -20°C after which they were submitted for scRNAseq. For outgrowth experiments (Supplemental Fig. 1A), $CD144^{+}CD309^{+}$ cells with either CD73 or CD43 positivity were sorted on day 8 of differentiation and cultured in day 6 iPSC differentiation media for an addition 5 days on cell culture treated plates. FlowJo was used for flow cytometry data analysis.

Reference:

1. Hansen M, Varga E, Wüst T, et al. Generation and characterization of human iPSC line MML-6838-Cl2 from mobilized peripheral blood derived megakaryoblasts. Stem Cell Res. 2017;18:26–28.
2. Hansen M, Varga E, Wüst T, et al. Generation and characterization of human iPSC lines SANi001-A and SANi002-A from mobilized peripheral blood derived megakaryoblasts. Stem Cell Res. 2017;25:42–45.
3. Hansen M, Varga E, Wüst T, et al. Generation and characterization of a human iPSC line SANi005-A containing the gray platelet associated heterozygous mutation p.Q287* in GFI1B. Stem Cell Res. 2017;25:34–37.
4. Van Oorschot R, Hansen M, Koornneef JM, et al. Molecular mechanisms of bleeding disorderassociated GFI1BQ287* mutation and its affected pathways in megakaryocytes and platelets. Haematologica. 2019;104(7):1460.

Supplemental Table 1. antibodies used in this study.

Product	Conjugate	Manufacturer	Dilution	Clone	Cat#
CD34	APC	Biolegend	1:500	581	343510
CD34	BV510	Biolegend	1:300	581	343528
CD31	APC-eF780	eBioscience	1:800	WM-59	47-031942
CD144	FITC	BD	1:100	55-7H1	560411
CD309	PE	Miltenyi	1:800	ES8-20E6	130-093-598
CD41	PE-Cy7	Biolegend	1:1000	HIP8	303718
CD41	Pacific Blue	Biolegend	1:100	HIP8	303714
CD43	PE-Cy7	Biolegend	1:200	CD43-10G7	343208
CD235	PE	Acris	1:2500	JC159	DM066R
CD42	FITC	Diaclone	1:50	HIP1	853.231.010
CD73	APC	Biolegend	1:100	AD2	344006

Supplemental Table 2. Analysis of day 8 CD144$^+$/CD31$^+$ scRNAseq data. (A) Modules of genes differentially expressed between clusters in Fig. 4B. **(B)** Cell types predicted from gene modules in A. **(C)** Differential gene expression between cluster III and cluster II in Fig. 4B. **(D)** GSEA result of C8 cell type gene sets for differentially expressed genes between cluster III and II in Fig. 4B. **(E)** GSEA result of C2 curated gene sets for differentially expressed genes between cluster III and II in Fig. 4B. **(F)** Differential gene expression between cluster II and cluster I in Fig. 4B. **(G)** GSEA result of C8 cell type gene sets for differentially expressed genes between cluster II and I in Fig. 4B. (see excel file)

https://1drv.ms/x/c/96eafeebb0308bd4/EaQ-PDG_RR9EihOyVoma9CEBOyNz5QhcPvWDsrcS1eoL1A

Supplemental Table 3. Bulk RNAseq analysis of EV/GFI1B ectopically expressed CD34$^+$ iPSC-HE. (A) Differential gene expression results between the EV and GFI1B groups on day 4 following transduction. **(B)** Gene Ontology results for differentially expressed genes between the EV and GFI1B groups. **(C)** GSEA result of C8 cell type gene sets for differentially expressed genes between the EV and GFI1B groups. (see excel file)

https://1drv.ms/x/c/96eafeebb0308bd4/EVUgEfWpOR1OspPrLmCjNR0Bp70zyZfjjyQv2MFguSh5kQ

Supplemental Fig.1. GFI1B and LSD1 affect hematopoietic and endothelial specification from hematopoietic/endothelial precursors. (A) CD144$^+$CD309$^+$ cells (left flow plot) were sorted based on the expression of CD73 and CD43 (middle flow plot). These cells were reseeded and cultured for five days. Images on the right, photographed using the EVOS-XL core, 4x objective, are a representative of day 8 sorted cells after five days of culturing. Note the presence of endothelial like adherent cells in the CD144$^+$CD309$^+$CD73$^+$ cultured population and nonadherent hematopoietic blasts in the CD144$^+$CD309$^+$CD41$^+$ cultured population. **(B)** The percentage of CD73$^+$ cells in CD31$^+$ population within the differentiated iPSC colonies (n=8-9 for control, 5-7 for GFI1B^{Q287*}, and 3 for GSK-LSD1). Data represent mean +/- SD, analyzed using two-way ANOVA, Tukey's multiple comparisons test. Significance levels: * < 0.05, ** <0.005, *** <0.0005, **** <0.00005. Representations: Black circles = control iPSC; Red triangles = iPSC treated with GSK-LSD1 inhibitor; Purple squares = GFI1B^{Q287*} iPSC. **(C)** Absolute number of suspension cells in paired control (filled circle) and GFI1B^{Q287*} (purple square) cultures. **(D)** Absolute number of suspension cells in paired control (filled circle) and GSK-LSD1 (red triangle) treated cultures. **(E)** Percentage of CD43$^+$/CD73$^+$ cells within the CD144$^+$/CD31$^+$ population from day 5 to day 9 of iPSC differentiation (mean +/- SD; n=5). Representative flow plot for day 8 shown on the left.

Supplemental Fig.2. Genes and gene modules used to identify CD31$^+$/CD144$^+$ populations. (A) Heatmap displaying scaled expression of gene modules, grouped based on differentially expressed genes identified between clusters from CD31$^+$/CD144$^+$ single cell data (Fig. 4B). **(B)** The expression of gene modules 3, 5, 8, 9, 10, and 11 on UMAP. **(C)** Gene set enrichment plot depicting highly enriched expression of genes involved in hematopoietic stem cell function in cluster II compared to cluster III. **(D)** Enrichment of hematopoietic cell types in cluster II (top plot) and endothelial cell types in cluster I (bottom plot) by gene set enrichment analysis (GSEA).

Supplemental Fig.3. Marker genes expression in CD31$^+$/CD144$^+$ clusters. Dot plot showing the scaled expression of top 15 differentially expressed genes from every cluster depicted in Fig. 4B.

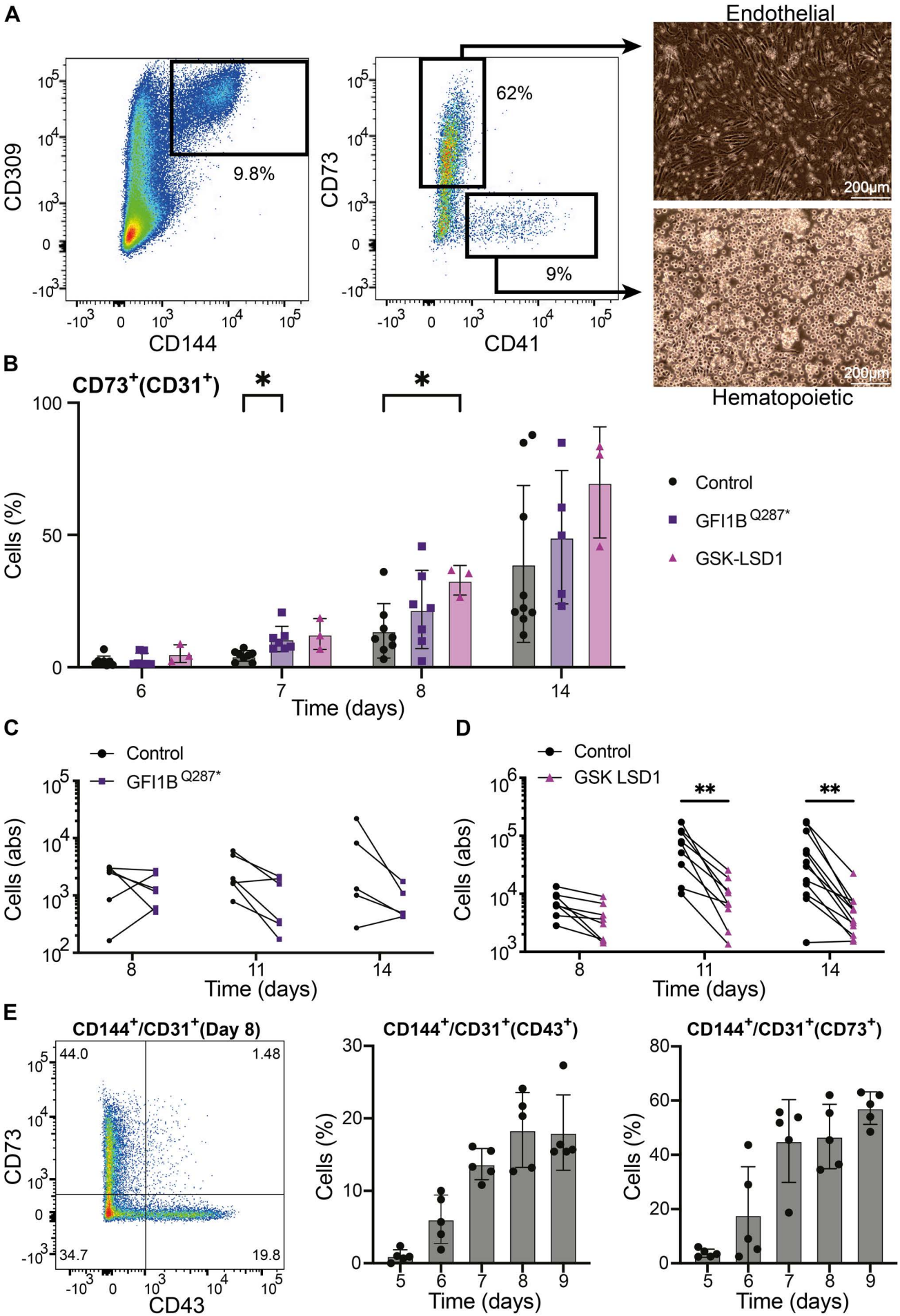
Supplemental Fig.4. Genes expression on UMAP of CD31$^+$/CD144$^+$ populations. The expression of hematopoietic (first row), hemogenic endothelial (second row), endothelial (third row), lymphoid (CD3E, CD3G, CD7), and neuronal (PTPRZ1, SOX2) cluster specific genes on UMAP.

Supplemental Fig.5. The presence of known EHT regulators and GFI1B cofactors in iPSC-HE. (A) Expression of *LCOR*, *ERG*, *SPI1*, *HOXA5*, and *HOXA9* over pseudotime. **(B)** Expression of *LCOR*, *ERG*, *SPI1*, *HOXA5*, and *HOXA9* on UMAP. **(C)** Expression of known GFI1B cofactors on UMAP. Note their presence in the hemogenic endothelial population (Fig. 4B).

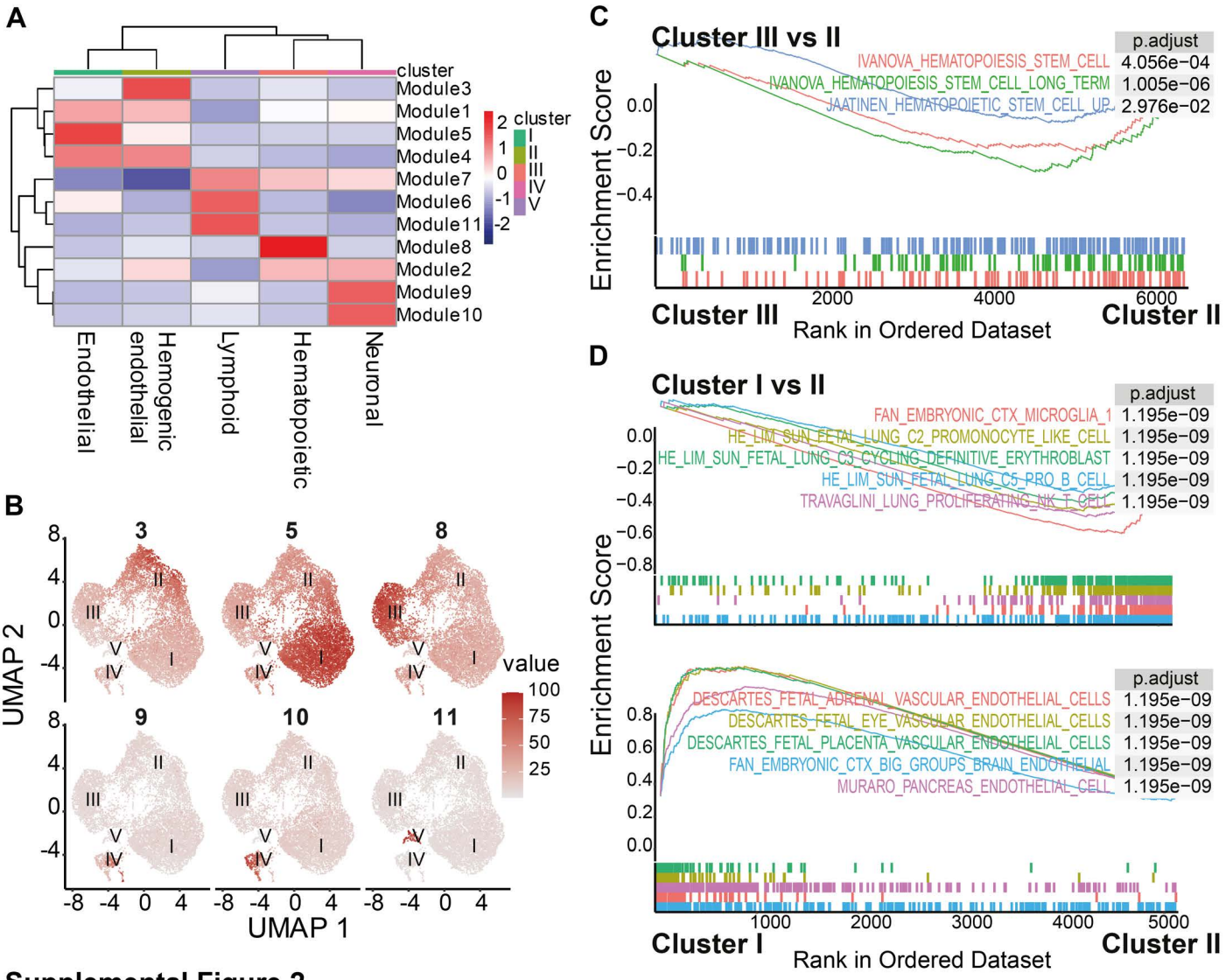
Supplemental Fig.6. LSD1 inhibition results in the absence of hematopoietic population in CD31$^+$/CD144$^+$ single cell data. Total number of cells in the hematopoietic, hemogenic endothelial (HE), and endothelial clusters from Fig. 4B for both control (in black) and GSK-LSD1 treated conditions (in red).

Supplemental Fig.7. Overexpression of GFI1B in CD34$^+$ iPSC-HE increases cell proliferation. (A) Integration of day 5 and day 8 sorted CD144$^+$/CD31$^+$ single cells on UMAP. **(B)** Expression of *GFI1B* and *LSD1* on UMAP of day 5 and day 8 integrated CD144$^+$/CD31$^+$ cells. **(C)** Percentage of GFP positive cells at day 5 after lentiviral transduction on day 5 sorted CD34$^+$ iPSC-HE cells (d5+5). Total number of **(D)** all cells, **(E)** CD43$^+$ and CD73$^+$ cells, in the empty vector (EV) and the GFI1B overexpressed CD34$^+$ iPSC-HE, counted after 4 days of transduction. **(D-E)** Data represent three independent experiments with mean +/- SD. Mann-Whitney tests were performed, with p = 0.1 for all. EV condition is shown red, and GFI1B condition is shown in blue.

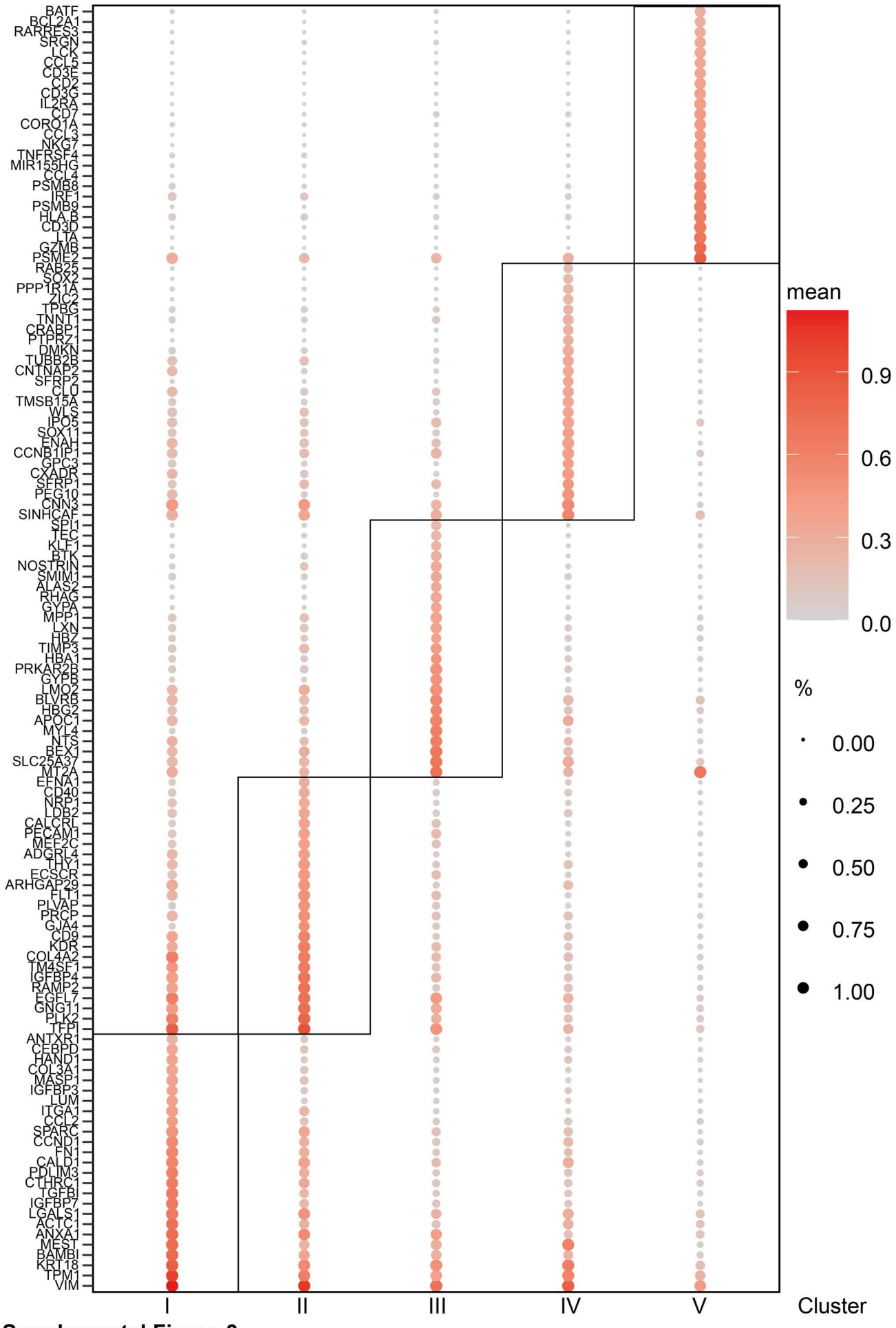
Supplemental Fig.8. Ectopic expression of GFI1B in iPSC-HE upregulates hematopoietic genes and downregulates endothelial genes. (A) Principal component (PC) analysis of RNA sequencing data from day 4 transduced hemogenic endothelium. PC1 and PC2 account for 76% and 11% of the variance separately. **(B)** Volcano plot displaying differentially expressed genes (DEGs) in the GFI1B overexpressed HE comparing to the EV group. Threshold for log2FC and p value are set at 1 and 0.01 respectively. Upregulated genes are colored in red and downregulated in blue. Names of the top 10 DEGs are highlighted. **(C)** Normalized CPM (count per million) of *GFI1*, *GFI1B*, *HOXA9*, *HOXA10*, *CD43*, *GATA2*, *RUNX1*, *CD73*, and *SOX17* for both the EV and GFI1B overexpressed CD34$^+$ iPSC-HE.



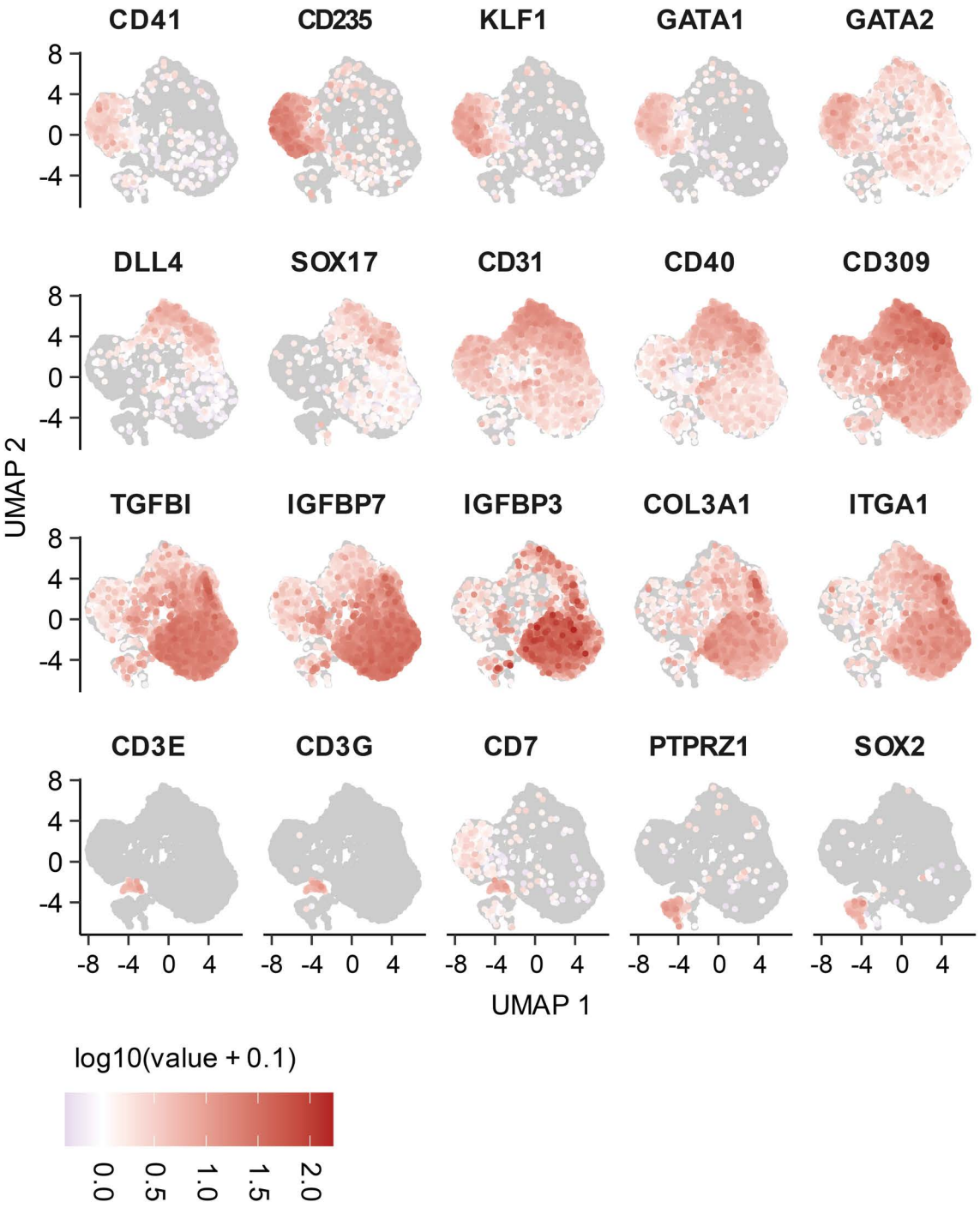
Supplemental Figure 1



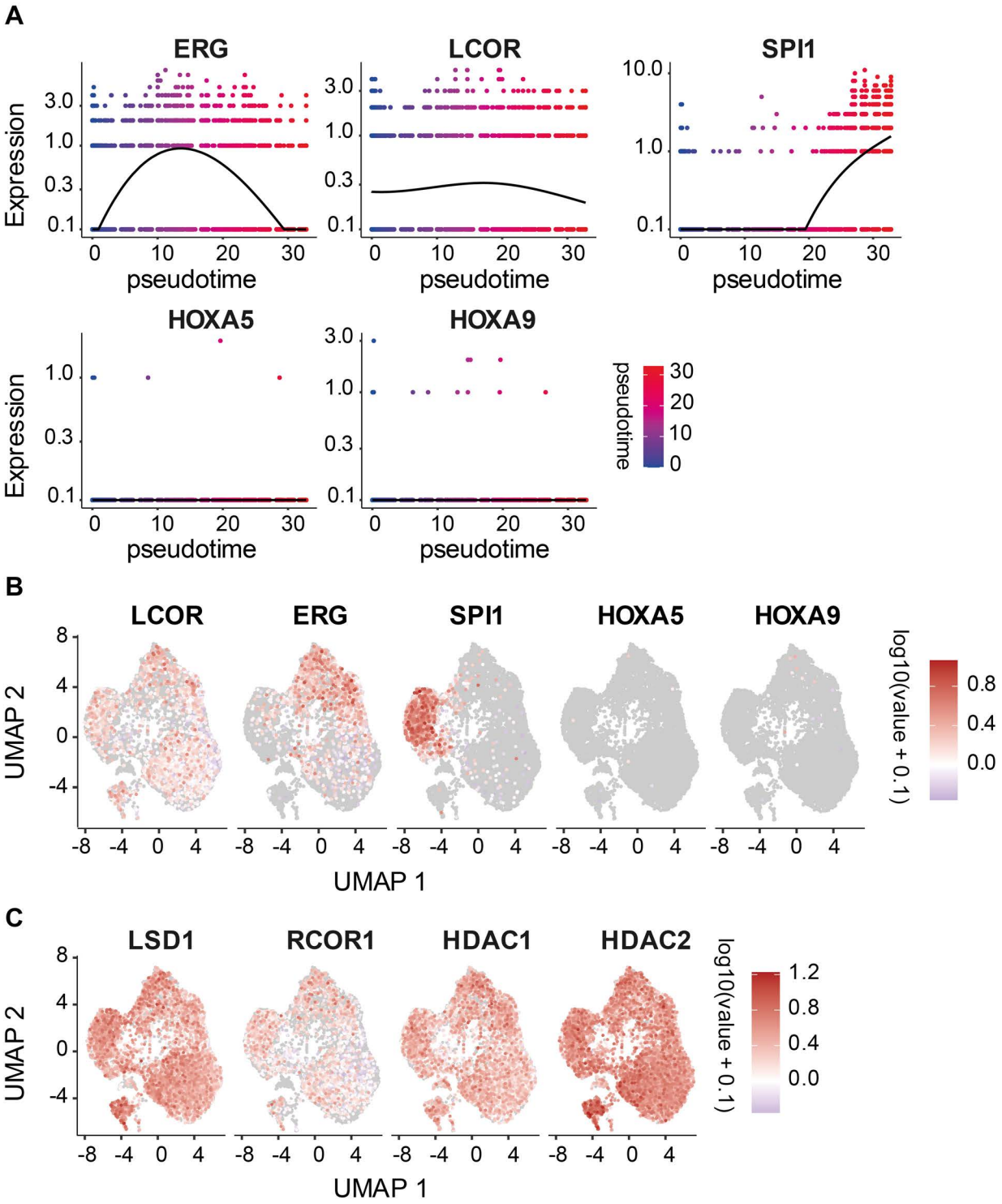
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



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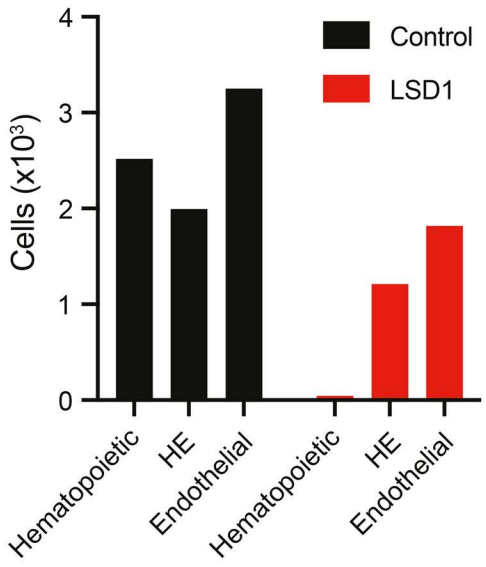

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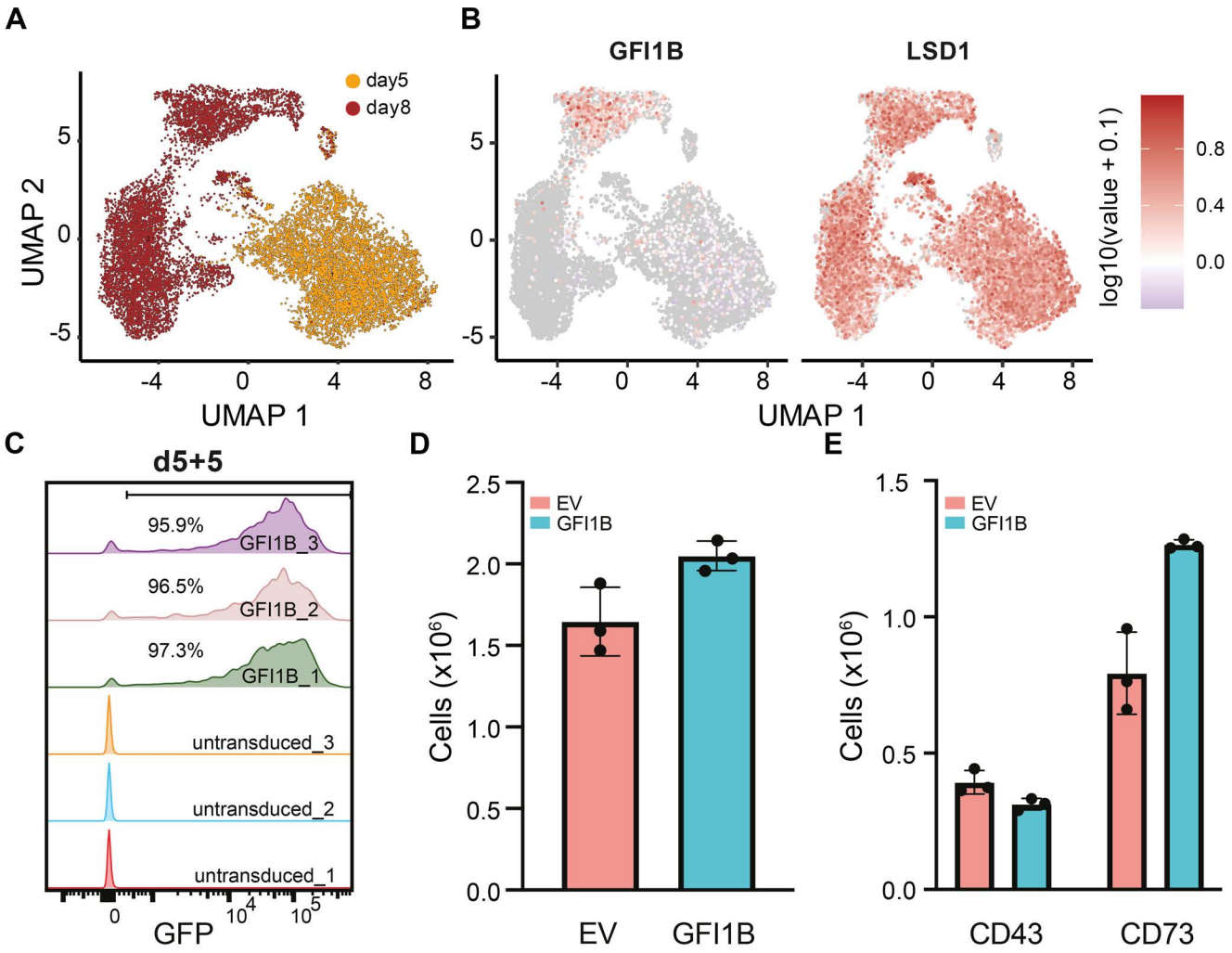
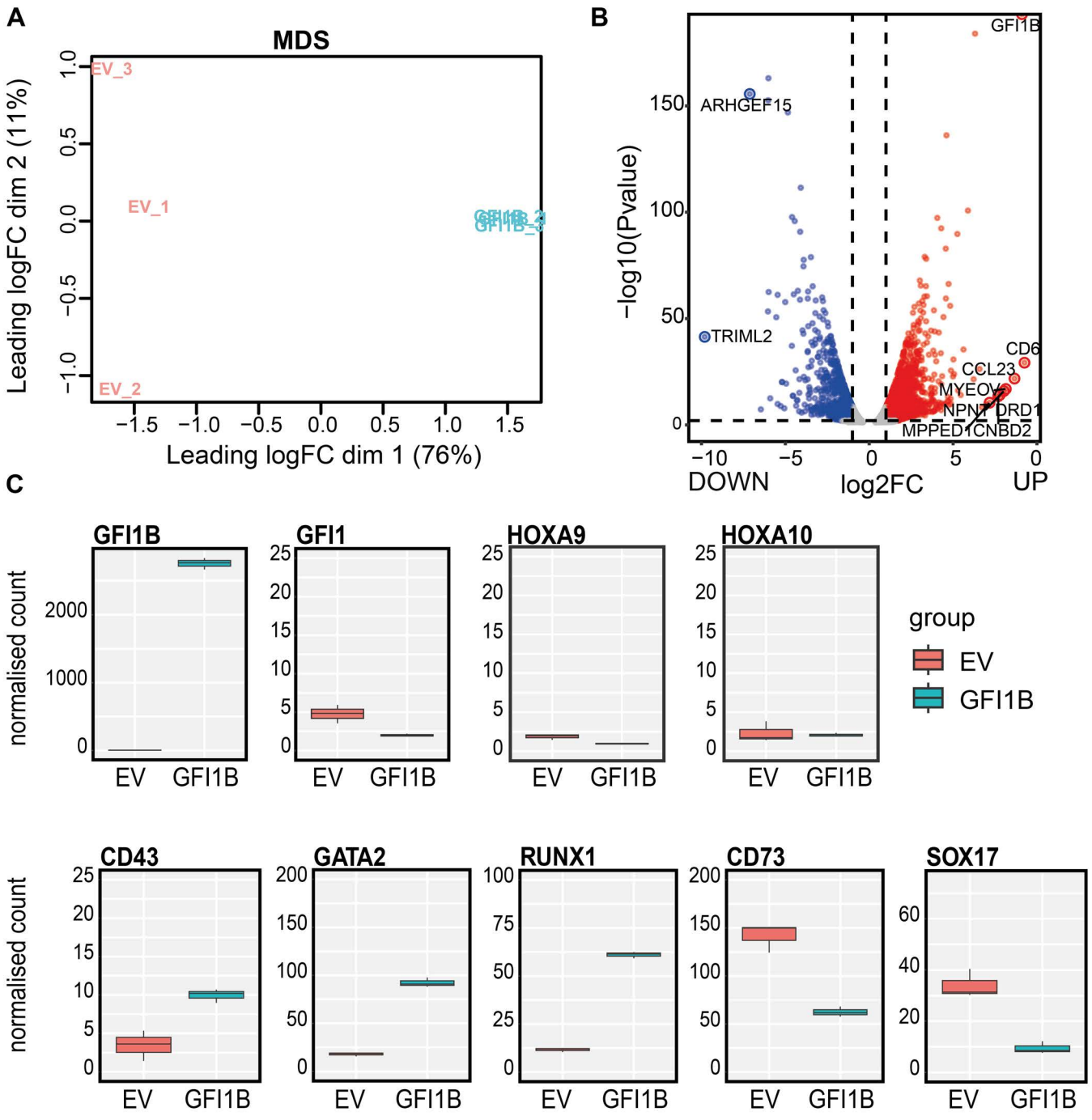


Supplemental Figure 7



Supplemental Figure 8