HES1 and HES4 have non-redundant roles downstream of Notch during early human T-cell development

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ONLINE METHODS

Viral constructs and transduction. *HES1*- and *HES4*-encoding gBlocks were codon optimized and synthesized (IDT; Supplemental Table 1). The gBlocks were cloned into the pCR-Blunt vector using the Zero Blunt™ PCR Cloning Kit (ThermoFisher Scientific) and then into the LZRS-IRES-eGFP vector using EcoRI and XhoI restriction sites. In the pLKO vectors encoding *HES1* and *HES4* shRNAs (Sigma-Aldrich, Supplemental Table 2), the puromycin gene was replaced by eGFP using BamHI and KpnI restriction sites. Constructs were validated by sequencing and retro- and lentivirus were produced by transfecting PhoenixA cells using the Calcium Phosphate Transfection Kit (ThermoFisher Scientific) and HEK293FT cells using jetPEI (Polyplus), respectively.

Isolated $CD34^+$ CB cells were cultured in complete Iscove's Modified Dulbecco's Medium (IMDM; ThermoFisher Scientific) containing 10% heat-inactivated fetal calf serum (FCS; Bovogen), 100 U/ml penicillin and 100 µg/ml streptomycin (ThermoFisher Scientific), and 2 mM L-glutamine (ThermoFisher Scientific), supplemented with TPO (20 ng/ml; Tebu-bio), SCF (100 ng/ml; Tebu-bio) and FLT3L (100 ng/ml; Miltenyi Biotec). Two days later, cells were transduced using RetroNectin (Takara)-coated plates. 48 hours after transduction, $eGFP^+CD34^+Lin^-$ HPCs were sorted (FACSAriaII, BD Biosciences) using CD34-APC (BioLegend), CD3-PE (BioLegend), CD14-PE (BioLegend), CD19-PE (BioLegend) and CD56-PE (BioLegend) with a purity >98%.

Coculture experiments. Sorted $eGFP^+CD34^+Lin^-$ HPCs were seeded onto 24-well plates with confluent layers of OP9-GFP, OP9-JAG1, OP9-DLL4, OP9-DLL1, MS-5 or MS-5-DLL4 cells. OP9 cocultures were performed in Minimum Essential Medium α (MEMα; ThermoFisher Scientific) with 20% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. For T-cell development, cells were cultured with 5 ng/ml IL-7 (R&D Systems), 5 ng/ml SCF and 5ng/ml FLT3L. MS-5 cocultures were performed in IMDM with 10% human serum (Innovative Research) and 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Lineage-specific differentiation was induced using these cytokines: 20 ng/ml IL-7 and 20 ng/ml SCF (B-cell development); 5 ng/ml IL-7, 5 ng/ml SCF, 5

ng/ml FLT3L and 10 ng/ml IL-15 (Miltenyi Biotec) (NK cell development); 20 ng/ml FLT3L, 20 ng/ml SCF, 20 ng/ml TPO, 10 ng/ml GM-CSF (Gentaur) and 10 ng/ml G-CSF (myeloid development).

Cocultures were harvested by forceful pipetting and analyzed by flow cytometry using the LSRII following labelling with these antibodies: CD1a-efluor-450 (ThermoFisher Scientific), CD4-PECy7 (BioLegend), CD5-PECy7 (BioLegend), CD7-Alexa Fluor 700 (BD Biosciences), CD14-APC (BioLegend), CD15-PE (BioLegend), CD19-PE, CD34-APC, CD45-PerCPCy5.5 (BioLegend), CD56-PE, HLA-DR-APC-efluor-780 (ThermoFisher Scientific).

Combined overexpression of *HES1* and *HES4*. To obtain combined overexpression of *HES1* and *HES4*, the eGFP marker gene in the LZRS-HES1-IRES-eGFP vector was first replaced by BFP. Subsequently, CB $CD34^+$ cells were cultured for two days in medium and cytokines as described in the Methods section of the main text, prior to transduction with the control eGFP- or *HES4*-eGFP-encoding retrovirus. Two days later, $eGFP^+CD34^+Lin^-$ cells, expressing eGFP or eGFP-*HES4*, were sorted with a purity of >98% and both were subsequently retrovirally transduced with the *HES1*-BFP-encoding virus, using medium with the same composition as during the first round of transduction. 48 hours later, cells expressing only eGFP (control) or eGFP together with *HES1*-BFP (HES1) or only *HES4*-eGFP (HES4) or *HES4*-eGFP together with *HES1*-BFP (HES1-HES4) were sorted based on eGFP and BFP marker gene expression.

Intracellular staining. Cells of interest were first stained with the eBioscience™ Fixable Viability Dye eFluor™ 506 (ThermoFisher Scientific) before fixation and permeabilization using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific). Subsequently, cells were first labeled with the unconjugated mouse α-HES1 (SantaCruz Biotechnology) or mouse α-HES4 (SantaCruz Biotechnology) antibody and subsequently with a PE-conjugated rat α-mouse IgG antibody.

Cell proliferation assay. Cell proliferation was determined using the CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific) and analyzed by flow cytometry at the indicated time points.

RNA extraction, qRT-PCR and gene expression profiling. RNA from sorted cells, either directly after transduction or derived from one-day OP9 cocultures in which CD45 staining was used to prevent OP9 contamination, was extracted using the miRNeasy Micro Kit (Qiagen) and converted into cDNA using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler 480 real-time PCR system (Roche). Gene expression levels were normalized using the *ACTB*, *GAPDH* and *YHWAZ* housekeeping genes (Supplemental Table 3). For RNAseq, the concentration and quality of the total extracted RNA were checked using the Quant-it ribogreen RNA assay (ThermoFisher Scientific) and the RNA 6000 Nano chip (Agilent Technologies), respectively. The RNA sequencing libraries of the three independent samples (from three independent donors) were prepared using the QuantSeq 3' mRNA-Seq Library Prep FWD kit (Lexogen) using 25 ng of input RNA. 17 PCR cycles were used and libraries were quantified by qPCR, according to Illumina's protocol 'Sequencing Library qPCR Quantification protocol guide', version February 2011. A High Sensitivity DNA chip (Agilent Technologies) was used to control the library's size distribution and quality. Sequencing was performed on a high throughput Illumina NextSeq 500 flow cell generating 75 single reads. Normalization and differential expression analysis were performed using the DESeq2 package in R. The data discussed in this publication are accessible through GEO Series accession number GSE130614. *HES1* and *HES4* expression levels during normal human T cell development were retrieved from vsn normalized microarray data, as described previously[1,2]. The public dataset GSE62006[3] was used to assess *HES1* and *HES4* expression through RNAseq in OP9-GFP and OP9-DLL1 cocultured $CD34^+$ thymocytes.

Statistical analysis. Statistical analysis was performed using the non-parametric paired Wilcoxon test, the unpaired or paired Student's *t*-test, or the two-way ANOVA with a significance level of 0.05.

SUPPLEMENTAL TABLES

Supplemental Table 1. *HES1* and *HES4* gBlock sequences.

Name	Sequence
HES1 gBlock (with EcoRI & XhoI restriction sites)	GAATTCATGCCAGCCGATATAATGGAAAAGAACTCTTCTAGCCCCGTCGCAGCAACTCCTGCG TCCGTGAATACTACACCCGATAAGCCTAAGACAGCGAGTGAGCATCGGAAATCCTCAAAACC CATTATGGAAAAACGCCGGCGAGCTCGCATAAACGAGTCTTTGTCTCAACTTAAAACTCTGAT CCTCGACGCCCTTAAAAAAGACAGTAGTAGGCATTCCAAATTGGAGAAAGCGGATATACTTG AAATGACAGTAAAACACCTCAGGAATTTGCAGCGAGCGCAAATGACTGCAGCCCTTTCTACG GATCCGTCTGTTCTTGGTAAGTACCGAGCCGGCTTTTCCGAGTGTATGAACGAGGTCACTCGC TTCCTGAGTACGTGTGAAGGGGTCAATACTGAAGTGAGAACACGGCTTCTCGGTCATCTCGCC AATTGTATGACCCAGATTAACGCAATGACGTACCCGGGCCAGCCACATCCAGCTCTGCAGGC GCCCCCTCCTCCGCCTCCGGGACCCGGTGGACCGCAGCATGCTCCATTTGCGCCCCCCCCTCC ACTCGTACCTATCCCCGGTGGTGCTGCGCCACCTCCGGGCGGTGCCCCCTGCAAGTTGGGGTC ACAAGCAGGAGAAGCTGCCAAGGTGTTTGGCGGTTTTCAGGTTGTTCCGGCACCTGACGGGC AATTCGCATTCCTCATTCCAAACGGAGCATTCGCACATAGCGGTCCCGTAATTCCAGTTTATAC AAGCAATTCAGGCACGAGCGTTGGCCCAAATGCAGTGAGTCCAAGTAGTGGACCGTCATTGA CTGCCGATTCCATGTGGCGCCCTTGGAGGAATTGACTCGAG
HES4 gBlock (with EcoRI & XhoI restriction sites)	GAATTCATGGCAGCTGACACGCCTGGGAAACCTAGCGCATCGCCTATGGCAGGAGCACCTGC AAGCGCAAGCAGAACCCCAGACAAGCCCCGGAGCGCTGCTGAGCACCGCAAATCCTCCAAGC CAGTCATGGAGAAGCGGCGCCGAGCACGTATTAACGAGAGCCTCGCTCAGCTCAAAACCCTC ATCCTGGACGCACTCAGAAAAGAGAGCTCCCGCCACTCGAAGCTGGAGAAGGCAGACATCCT GGAGATGACCGTGAGACACCTGAGAAGCCTGCGTCGCGTGCAGGTGACGGCAGCTCTCAGC GCAGACCCAGCAGTTCTGGGCAAGTACCGCGCAGGCTTCCACGAGTGTCTGGCAGAGGTGA ACCGCTTCCTGGCAGGCTGCGAGGGCGTCCCAGCAGACGTGCGCTCCCGCCTGCTGGGCCAC CTGGCAGCTTGCCTGCGCCAGCTGGGACCATCCCGCCGCCCAGCATCGCTGTCCCCTGCTGCA CCTGCAGAGGCACCAGCACCAGAGGTCTACGCGGGCCGCCCACTGCTGCCATCGCTCGGCGG CCCATTCCCTCTGCTCGCACCACCACTGCTGCCAGGTCTGACCAGAGCACTGCCAGCAGCACC AAGGGCAGGGCCACAGGGCCCAGGTGGACCATGGAGGCCATGGCTGAGACTCGAG

Supplemental Table 2. *HES1* and *HES4* shRNA sequences.

Name	Sequence
HES1 shRNA1	CCGGCCGGACTCTAAACAGGAACTTCTCGAGAAGTTCCTGTTTAGAGTCCGGTTTTT
HES1 shRNA2	CCGGGAAAGTCATCAAAGCCTATTACTCGAGTAATAGGCTTTGATGACTTTCTTTTT
HES4 shRNA1	CCGGCAAGTCCTCCAAGCCGGTCATCTCGAGATGACCGGCTTGGAGGACTTGTTTTT
HES4 shRNA2	CCGGCCACGAGTGTCTGGCGGAGGTCTCGAGACCTCCGCCAGACACTCGTGGTTTTT

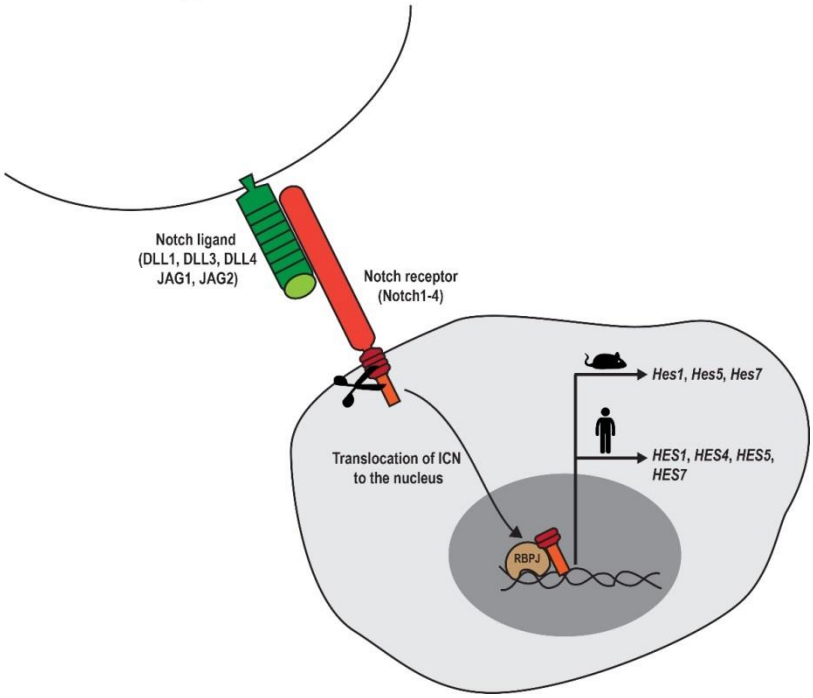
Supplemental Table 3. qRT-PCR primers.

Name	Sequence
ACTB Forward	ATGACCCAGATCATGTTTGAGA
ACTB Reverse	AGAGGCGTACAGGGATAGCA
GAPDH Forward	TCCTCTGACTTCAACAGCGACA
GAPDH Reverse	GTGGTCGTTGAGGGCAATG

YHWAZ Forward	ACTTTTGGTACATTGTGGCTTCAA
YHWAZ Reverse	CCGCCAGGACAAACCAGTAT
HES1 Forward	TGTCAACACGACACCGGATAAA
HES1 Reverse	CCATAATAGGCTTTGATGACTTTCTG
HES4 Forward	CTGGAGAAGGCGGACATCC
HES4 Reverse	GCGGTACTTGCCCAGAAC
CEBPA Forward	GGATAACCTTGTGCCTTG
CEBPA Reverse	CTCCCCTCCTTCTCTCAT

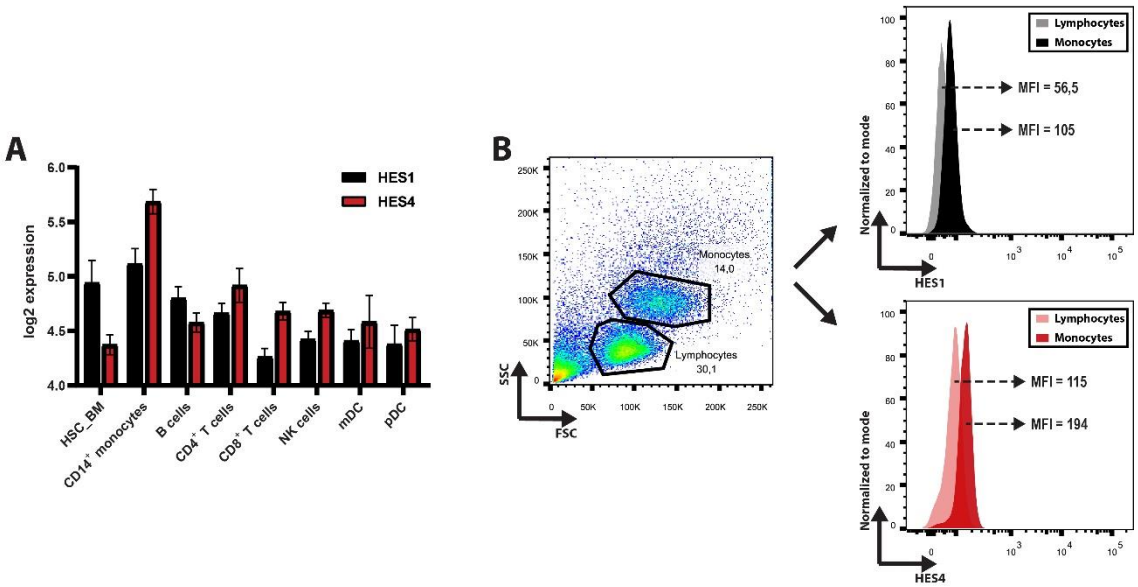
SUPPLEMENTAL FIGURES AND LEGENDS

Supplemental Figure 1



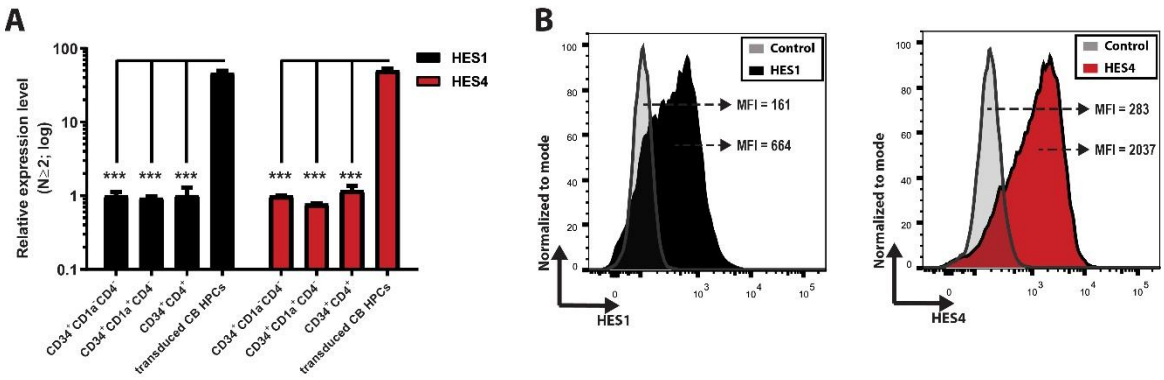
Supplemental Figure 1. Schematic overview illustrating the Notch-dependent *HES* genes in mouse and human. Upon interaction between a Notch ligand (DLL1, DLL3, DLL4, JAG1 and JAG2) and a Notch receptor (Notch1-4), the intracellular domain of Notch is cleaved and subsequently translocates to the nucleus where it interacts with an RBPJ-containing transcriptional complex resulting in the expression of various Notch target genes. Among these targets, it has been shown that the expression of *Hes1*, *Hes5* and *Hes7*, in mice, and additionally of *HES4*, in human, can be induced in a Notch-dependent manner[4].

Supplemental Figure 2



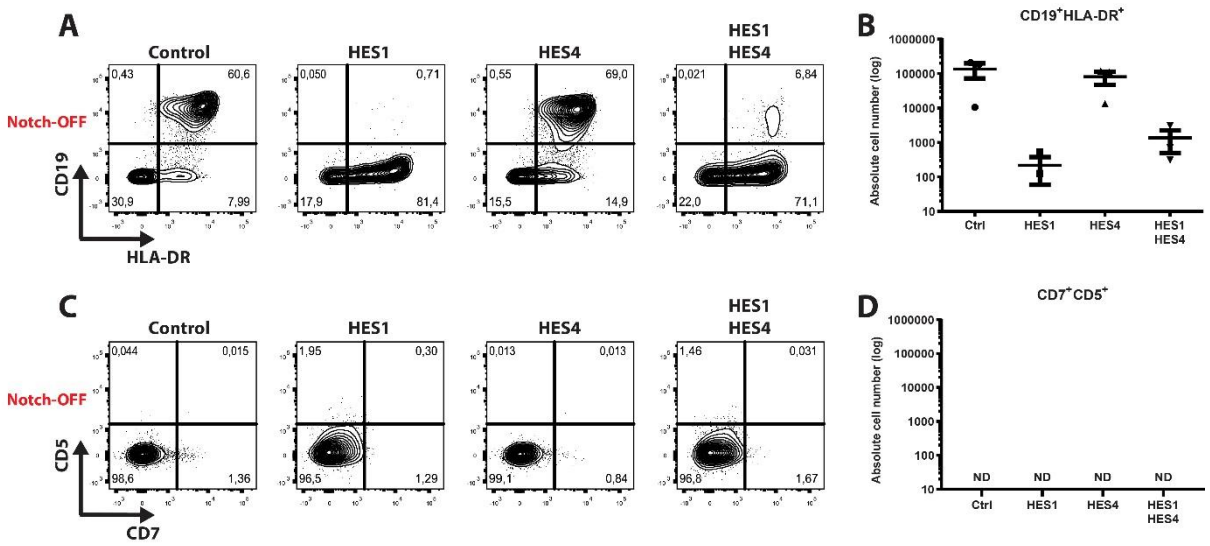
Supplemental Figure 2. *HES1* and *HES4* expression in different blood cell types. (**A**) Publically available data from the BloodSpot website (*www.bloodspot.eu*) showing the log2 expression of *HES1* (black bars) and *HES4* (red bars) in various blood cell types. (**B**) Histograms on the right show flow cytometric analysis of intracellular HES1 and HES4 staining in peripheral blood cells, gated on lymphocytes or monocytes as shown in the density plot on the left; MFI, mean fluorescence intensity. Flow cytometry plots shown are representive for three independent experiments.

Supplemental Figure 3



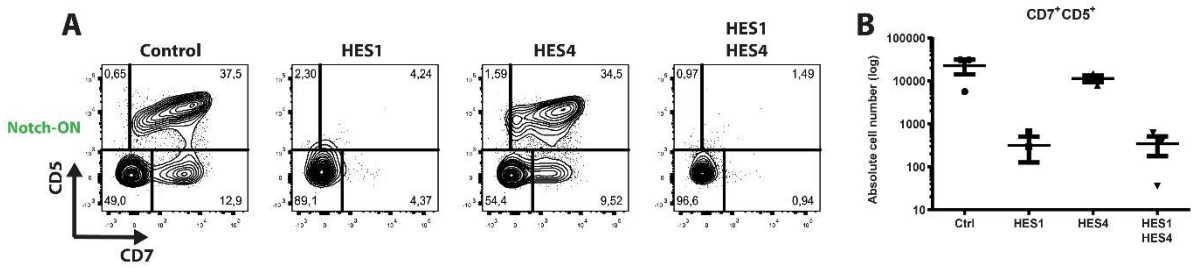
Supplemental Figure 3. Enforced expression of *HES1* and *HES4* in CB HPCs. (**A**) Quantitative RT-PCR of *HES1* (black bars) and *HES4* (red bars) in *HES1*- and *HES4*-transduced CB $CD34^+Lin^-$ HPCs, relative to the mean of *ACTB* and *GAPDH* mRNA levels, and normalized to endogenous expression in thymic $CD34^+CD1a^-CD4^-$ cells. Data shows the average of at least two independent experiments and error bars indicate SEM. ***p<0.001 (unpaired Student's *t*-test). (**B**) Histograms show intracellular flow cytometric analysis for HES1 (left) and HES4 (right) in control (grey), *HES1* (black)- or *HES4* (red)-transduced CB $CD34^+Lin^-$ cells. Data are representative for three independent experiments; MFI, mean fluorescence intensity.

Supplemental Figure 4



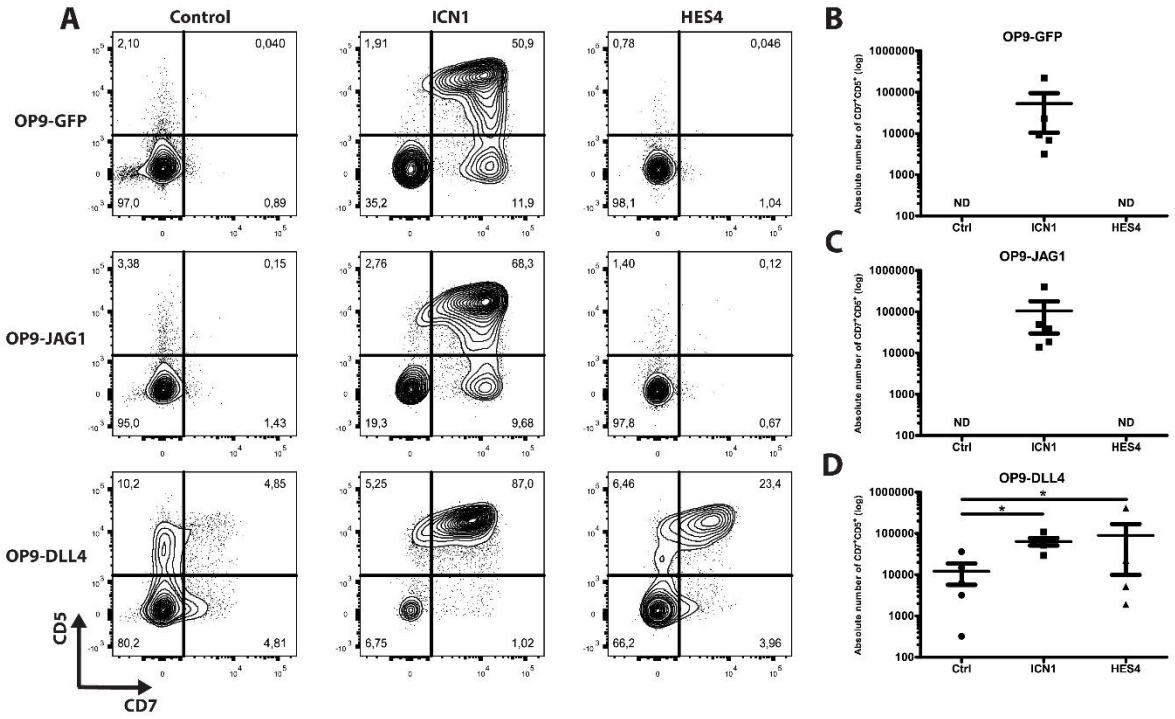
Supplemental Figure 4. Combined overexpression of *HES1* and *HES4* is not sufficient to impose T cell fate in HPCs. Flow cytometry analysis (**A, C**) and absolute cell numbers (**B, D**) of CB $CD34^{+}Lin^{-}$ precursors transduced with control or *HES1* or *HES4* or both *HES1* and *HES4*, and cultured on the MS-5 feeder (Notch-OFF) for three weeks in the presence of the B-lineage supporting cytokines IL-7 and SCF, showing the development of $CD19^{+}HLA\text{-}DR^{+}$ B-lineage cells (**A-B**) and $CD7^{+}CD5^{+}$ T-lineage cells (**C-D**). Flow cytometry plots shown are representative for three independent experiments. Data shows the average of three independent experiments and error bars indicate SEM; ND, not detectable.

Supplemental Figure 5



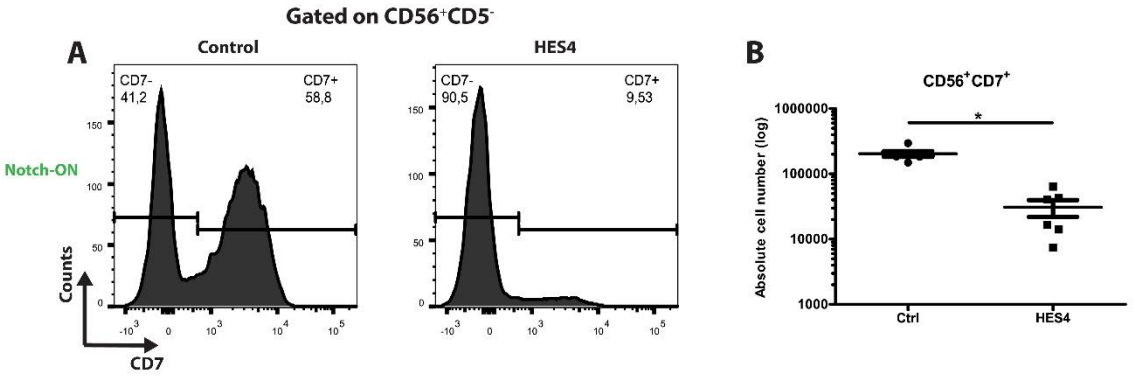
Supplemental Figure 5. Repressive role of HES1 is dominant over HES4. Flow cytometric analysis (**A**) and absolute cell numbers (**B**) of CB HPCs transduced with control or *HES1* or *HES4* or both *HES1* and *HES4*, and cultured on the OP9-DLL4 feeder (Notch-ON) for two weeks in the presence of the T-lineage supporting cytokines IL-7, SCF and FLT3L, showing the development of $CD7^{+}CD5^{+}$ T-lineage precursors. Plots shown are representative for three independent experiments. Data shows the average of three independent experiments and error bars indicate SEM.

Supplemental Figure 6



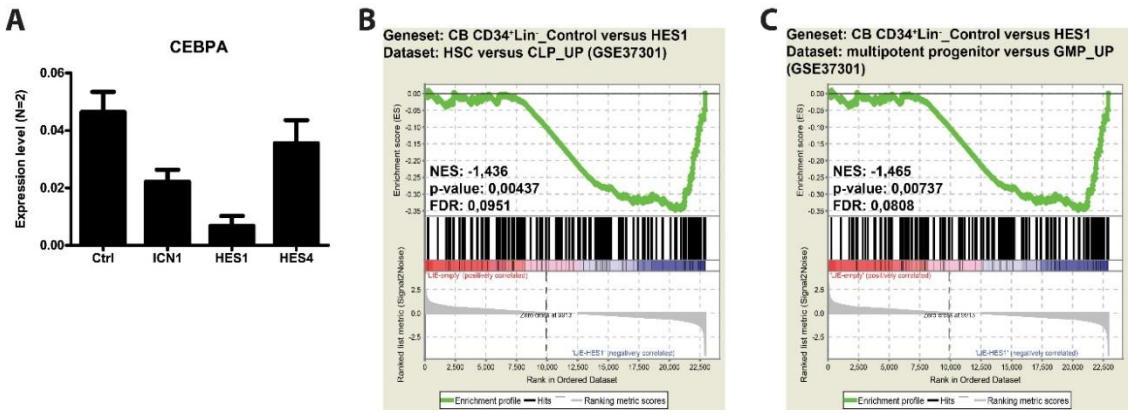
Supplemental Figure 6. HES4 does not alter the Notch ligand requirements to induce T cell differentiation. (**A**) Flow cytometry analysis of control, *ICN1*- and *HES4*-transduced $CD34^{+}Lin^{-}$ CB HPCs cultured on the OP9-GFP, OP9-JAG1 and OP9-DLL4 feeder for two weeks in the presence of the T-lineage specific cytokines IL-7, SCF and FLT3L, showing the development of $CD7^{+}CD5^{+}$ T-lineage cells. Plots shown are representative for five independent experiments. (**B-D**) Absolute numbers of $CD7^{+}CD5^{+}$ T cells generated in corresponding cultures shown in **A**. Data shows the average of five independent experiments. Error bars indicate SEM. *p<0.05 (non-parametric paired Wilcoxon test); ND, not detectable.

Supplemental Figure 7



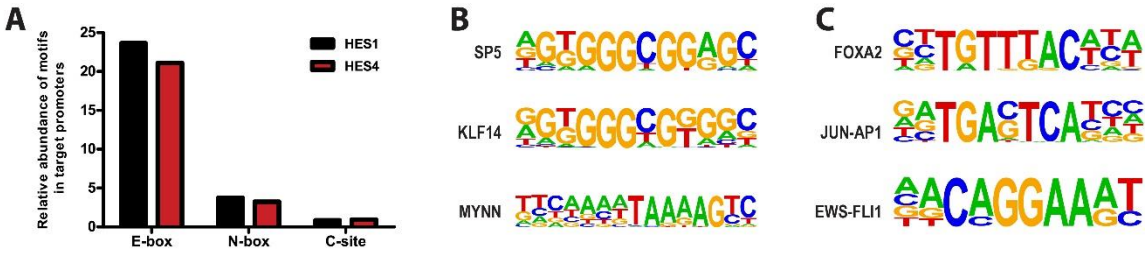
Supplemental Figure 7. HES4-induced *CD7* expression is T cell-specific. (**A**) Flow cytometric analysis of control and *HES4*-transduced CB $CD34^{+}Lin^{-}$ precursors cultured on the MS-5-DLL4 feeder (Notch-ON) for three weeks in the presence of the NK-lineage specific cytokines IL-7, SCF, FLT3L and IL-15, showing the expression of CD7. Histograms were first gated on $CD56^{+}CD5^{-}$ NK cells. Plots shown are representative for six independent experiments. (**B**) Absolute numbers of $CD56^{+}CD7^{+}$ NK cells generated in corresponding cultures shown in **A**. Data shows the average of six independent experiments and error bars indicate SEM. *p<0.05 (non-parametric paired Wilcoxon test).

Supplemental Figure 8



Supplemental Figure 8. HES1 maintains stem cells by repressing CLP and GMP differentiation. (**A**) Quantitative RT-PCR of *CEBPA* in the HL-60 leukemic cell line after transduction with *ICN1*, *HES1* or *HES4*. Data shows the average expression of two independent experiments, relative to the mean of *ACTB* and *GAPDH* mRNA levels. Error bars indicate SEM. (**B-C**) GSEA shows a significant enrichment of genes involved in the maintenance of HSCs and multipotent progenitors versus the differentiation of CLPs (**B**) and GMPs (**C**) in *HES1*-transduced cells compared to control.

Supplemental Figure 9



Supplemental Figure 9. Motif analysis of HES1 and HES4 target gene promoters. (**A**) Relative abundance of E-box, N-box and C-site motifs in the HES1 (black bars) and HES4 (red bars) target gene promoters as identified in Figure 7. (**B-C**) HOMER-derived top 3 enriched transcription factor binding sites in HES1 (**B**) and HES4 (**C**) target gene promoters.

SUPPLEMENTAL REFERENCES

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