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

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ABSTRACT

In both mouse and human, Notch1 activation is the main initial driver to induce T-cell development in hematopoietic progenitor cells. The initiation of this developmental process coincides with Notch1-dependent repression of differentiation towards other hematopoietic lineages. Although well described in mice, the role of the individual Notch1 target genes during these hematopoietic developmental choices is still unclear in human, particularly for *HES4* since no orthologous gene is present in the mouse. Here, we investigated the functional capacity of the Notch1 target genes *HES1* and *HES4* to modulate human Notch1-dependent hematopoietic lineage decisions and their requirement during early T-cell development. We show that both genes are upregulated in a Notch-dependent manner during early T-cell development and that HES1 acts as a repressor of differentiation by maintaining a quiescent stem cell signature in $CD34^+$ hematopoietic progenitor cells. While HES4 can also inhibit natural killer and myeloid cell development like HES1, it acts differently on the T- *versus* B-cell lineage choice. Surprisingly, HES4 is incapable of repressing B-cell development, the most sensitive hematopoietic lineage with respect to Notch-mediated repression. In contrast to HES1, HES4 promotes initiation of early T-cell development, but ectopic expression of *HES4*, or *HES1* and *HES4* combined, is insufficient to induce T-lineage differentiation. Importantly, knockdown of *HES1* or *HES4* significantly reduces human T-cell development. Overall, we show that the Notch1 target genes *HES1* and *HES4* have non-redundant roles during early human T-cell development which may relate to differences in mediating Notch-dependent human hematopoietic lineage decisions.

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Introduction

Multipotent hematopoietic stem cells (HSC) reside in the bone marrow after birth and give rise to all the different blood cell types.[1] This process is orchestrated by integrating cell-intrinsic mechanisms and external stimuli which results in the activation of molecular networks that drive differentiation towards specific hematopoietic lineages.[2,3] Such events are also important during the early stages of intrathymic T-cell development. Following colonization of thymus-seeding progenitors from the bone marrow, Delta-like-ligand-4 (DLL4), which is expressed by thymic epithelial cells, triggers the Notch1 receptor on these multipotent precursors. Activation of Notch1 induces T-cell differentiation through upregulation of T-lineage genes and this also prevents differentiation towards other hematopoietic lineages.[4-6] In mice, the regulatory network that drives early T-cell development has been well-studied. Activation of TCF1 contributes to the induction of T-lineage genes,[7] while *Gata3* upregulation is important to impair B-lineage potential.[8] Subsequently, residual natural killer (NK) cell potential is repressed by BCL11B that completes T-cell commitment.[9] During these steps, Notch activation remains

essential to guide the cells along the T-lineage differentiation pathway and for proliferation and survival. In human, however, the regulatory network that supports these processes is less clear. Due to species-specific gene expression patterns and functions, extrapolation of murine data is often not possible. One important difference between human and mouse is the need for Notch signaling during T-cell development. In both, strong Notch1 activation is necessary to induce T-cell specification at the expense of B- and myeloid lineage potential.[10] In human, however, Notch signaling then needs to be reduced for efficient differentiation towards committed thymocytes,[11] while in mouse high Notch activity is maintained.[12] Thereafter, Notch also differentially modulates the differentiation towards T-cell receptor (TCR)-αβ and TCR-γδ T-cells in mouse *versus* human.[13-15]

Activation of Notch1 results in the expression of many different target genes whose individual roles in controlling hematopoietic lineage decisions are still unclear. These include some members of the *HES* gene family that encode bHLH transcription factors that function as homo- and heterodimers and repress their target genes through recruitment of co-repressors.[16] *HES1* is a well-known target and is essential for the inhibition of myeloid development during early T-lineage differentiation in mouse through repression of C/EBP-α.[17] It also plays a prominent role in Notch-driven T-cell acute lymphoblastic leukemia (T-ALL).[18] Importantly, the human genome also encodes *HES4,* a *HES1* paralogue that is absent in the mouse[19] (*Online Supplementary Figure S1*), but present in *Xenopus* and Zebrafish. It has been documented that, depending on the species, biological setting and tissue, HES4 can have overlapping or antagonistic roles compared to HES1.[20-24] Although *HES4* displays clear Notch-dependent regulation in Notch-driven cancers,[25] it is unclear if HES4 can mediate non-malignant Notch-driven hematopoietic lineage decisions.

Given that the individual roles of the various Notch target genes are still unclear, we studied the function of HES1 and HES4 during the early Notch-dependent hematopoietic lineage decisions in human. We show that they have both different and similar impacts on the differentiation of human hematopoietic progenitor cells (HPC) towards various blood cell types when overexpressed, and that both are essential during early human T-cell development.

Methods

Isolation of CD34$^+$ hematopoietic precursor cells

Umbilical cord blood (CB) was obtained with informed consent and used with approval of the Medical Ethical Commission of Ghent University Hospital (Belgium). Mononuclear cells were isolated through Lymphoprep density-gradient (ELITech) and CD34$^+$ HPC were purified using CD34-magnetic beads (Miltenyi Biotec).

Other methods are available in the *Online Supplementary Material and Methods*.

Results

HES4, like *HES1*, displays Notch-dependent expression and regulation during human T-lineage differentiation

HES4 was shown to be Notch dependent in leukemic T cells,[25] but whether *HES4* displays Notch-dependent expression during non-malignant T-cell development is unclear. Gene expression analysis showed that both *HES1* and *HES4* are expressed during similar Notch-dependent stages[4,14] of human T-cell development (Figure 1A). This Notch-dependent HES4 expression was functionally validated through exposure of CB CD34$^+$Lin$^-$ HPC onto OP9 stromal cells that express different Notch ligands. HES1 and HES4 expression in HPC was only induced in conditions that permit T-lineage differentiation (OP9-DLL1, OP9-DLL4 and OP9-Jagged2 (JAG2)), but not in B-lineage permissive conditions (OP9-GFP and OP9-JAG1; Figure 1B).[26] Similarly, short 48 hour (h) co-culture experiments onto OP9-GFP *versus* OP9-DLL1 stromal cells confirmed the Notch-dependent expression of both *HES* genes in intrathymic CD34$^+$ cells, cells that have already experienced *in vivo* Notch activation (Figure 1C). Publicly available data revealed that, in contrast to *HES1*, *HES4* is not expressed in HSC in the bone marrow (*Online Supplementary Figure S2A*). However, HES4 expression is detected in monocytes while lymphocytes and dendritic cells (DC) express lower levels. Protein staining confirmed the higher HES4 levels in monocytes compared to lymphocytes (*Online Supplementary Figure S2B*). These findings illustrate that both *HES1* and *HES4* are Notch-dependently induced during human T-cell development. Given that no information is available on the role of HES4 in human hematopoiesis, we studied this using viral gene perturbation approaches in human CD34$^+$ HPC that are differentiated towards various hematopoietic lineages in well-established *in vitro* co-culture systems (Figure 1D).

HES4 lacks the potential to repress B-cell development

DLL4-dependent Notch1 activation is essential to induce T-lineage differentiation at the expense of B-cell development.[6,13,27] Since both *HES1* and *HES4* are induced following Notch1 activation, we investigated if these genes have the potential to repress B-cell development through enforced expression in CB HPC. This resulted in 50-fold higher expression levels of each gene compared to *ex vivo* isolated CD34$^+$ thymocytes that reflect physiological Notch activation (*Online Supplementary Figure S3A*), and protein staining suggests that slightly higher levels of HES4 were obtained compared to HES1 (*Online Supplementary Figure S3B*). *HES1*- and *HES4*-transduced HPC were cultured on MS-5 stromal cells in B-lineage differentiation conditions and compared to HPC transduced with the constitutive active form of Notch1 (ICN1) that mimics Notch activation and represses B-cell development.[13,27] Whereas control transduced HPC efficiently differentiate into CD19$^+$HLA-DR$^+$ B-cells, enforced *ICN1* expression inhibited B-cell development (Figure 2A-B). Remarkably, while HES1 also inhibited B-lineage differentiation, continuous *HES4* expression did not impact B-cell development (Figure 2A-B), and combined overexpression of *HES1* and *HES4* showed a dominant repression of HES1 (*Online Supplementary Figure S4A-B*). Consistently, CD7$^+$CD5$^+$ T-cell precursors were generated in cultures where ICN1 was overexpressed, but not within control transduced conditions (Figure 2C-D). In contrast, enforced expression of either *HES1* or *HES4* (Figure 2C-D), or *HES1* and *HES4* combined (*Online Supplementary Figure S4C-D*), was insufficient to induce the development of CD7$^+$CD5$^+$ T-lineage progenitors. These findings show that HES1 and HES4 have distinct capacities to mediate the Notch1-

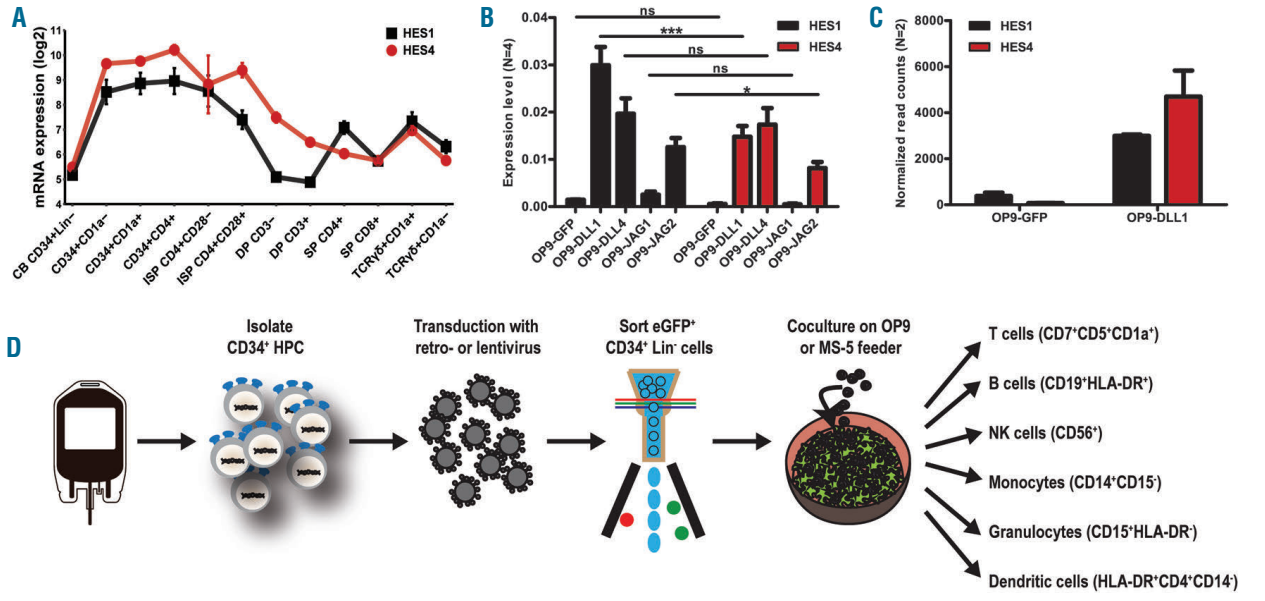


Figure 1. *HES4*, like *HES1*, is expressed Notch dependently during human T-cell differentiation. (A) Line plots show log$_2$ normalized probe intensities for *HES1* (black) and *HES4* (red) gene expression levels during normal T-cell development. Data shows the average expression of two independent experiments from two different donors. Error bars indicate the standard error of the mean (SEM). (B) Quantitative RT-PCR of HES1 (black bars) and HES4 (red bars) in CB CD34$^+$Lin$^-$ precursors cultured for 3 days on OP9-GFP, OP9-DLL1, OP9-DLL4, OP9-JAG1 and OP9-JAG2. Data shows the average expression of four independent experiments, relative to the mean of *GAPDH* and *YHWAZ* mRNA levels. Error bars indicate SEM. ***P<0.001; *P<0.05 (two-way ANOVA); ns: not significant. (C) DESeq2-normalized read counts of *HES1* (black bars) and *HES4* (red bars) in thymic CD34$^+$ cells cultured for 2 days on OP9-GFP and OP9-DLL1. Graphs show the average of two independent experiments and error bars indicate SEM. (D) Schematic overview illustrating the experimental workflow and the differentiation markers used to identify the different hematopoietic lineages.

dependent T- *versus* B-cell lineage decision and that both HES proteins, individually or combined, are insufficient to impose T-cell fate.

HES4, but not HES1, promotes induction of T-lineage differentiation

To further study the impact of HES1 and HES4 on early T-cell development, we performed perturbation experiments in OP9-DLL4 co-cultures that support induction of T-lineage differentiation in human HPC as illustrated through the emergence of CD7$^+$CD5$^+$ T-cell precursors in control transduced cells (Figure 3A). We analyzed these cultures after 2 weeks to focus on the efficiency and kinetics of induction of T-lineage specification and commitment. Ectopic expression of *ICN1* promoted the generation of early CD7$^+$CD5$^+$ and CD5$^+$CD1a$^+$ T-cell precursors from HPC (Figure 3A, C) and yielded higher numbers of these cells (Figure 3B, D). Enforced expression of *HES1*, however, resulted in a significant reduction in the frequency and absolute number of CD7$^+$CD5$^+$ specified and CD5$^+$CD1a$^+$ committed T-cells (Figure 3A-D). In contrast, high levels of HES4 improved the differentiation of HPC towards the T-cell lineage, although less efficient compared to ICN1 overexpression (Figure 3A-D). This positive role of HES4 on early T-lineage differentiation was counteracted by HES1 when both were overexpressed (*Online Supplementary Figure S5A-B*). Because previous work from our lab demonstrated that JAG2, DLL1 and DLL4, but not JAG1, have the potential to initiate T-cell development in human HPC,[26] we investigated if enforced *HES4* expression could alter these Notch ligand requirements to induce T-lineage differentiation. Whereas overexpression of *ICN1* in HPC was sufficient to induce T-cell development in OP9-GFP, OP9-JAG1 and OP9-DLL4 co-cultures, *HES4*-transduced cells, like the control, could only generate CD7$^+$CD5$^+$ T-cell progenitors on OP9-DLL4 stromal cells (*Online Supplementary Figure S6A-D*). Overall, these findings illustrate that, while continuous HES1 expression inhibits T-lineage specification and commitment, HES4 can enhance induction of T-cell development in the presence of Notch signaling when present at higher levels compared to endogenous *HES1* expression. However, HES4 is not sufficient to alter the Notch ligand requirements to induce T-lineage differentiation in HPC.

NK-cell development is severely hampered by HES1 and HES4

In human, Notch1 activation is permissive for NK-lineage differentiation,[28] and our laboratory previously illustrated that this is mediated by the Notch1 target gene *DTX1*.[11] Here, we studied the impact of ectopic *HES1* and *HES4* expression on NK-cell development through co-culture of control, *ICN1*-, *HES1*- and *HES4*-transduced HPC on MS-5 stromal cells with cytokines supporting NK-cell differentiation. As documented previously,[28] enforced *ICN1* expression enhanced NK-cell development and resulted in a significant increase in CD56$^+$ NK-cells that express CD7, a Notch-dependent marker during early T-cell development (Figure 4A, C). In contrast, both HES1 and HES4 reduced NK-cell development, and this was the case for both CD56$^+$CD7$^-$ and CD56$^+$CD7$^+$ subsets (Figure 4A-C). To investigate if *HES4* overexpression can improve the generation of CD56$^+$CD7$^+$ NK-cells in the presence of Notch signaling, analogous to the Notch signaling requirement for HES4 to enhance differentiation towards CD7$^+$ T-lineage precursors, we cultured control and *HES4*-transduced HPC on MS5-DLL4 stromal cells in the presence of NK-lineage supporting cytokines. However, also in these

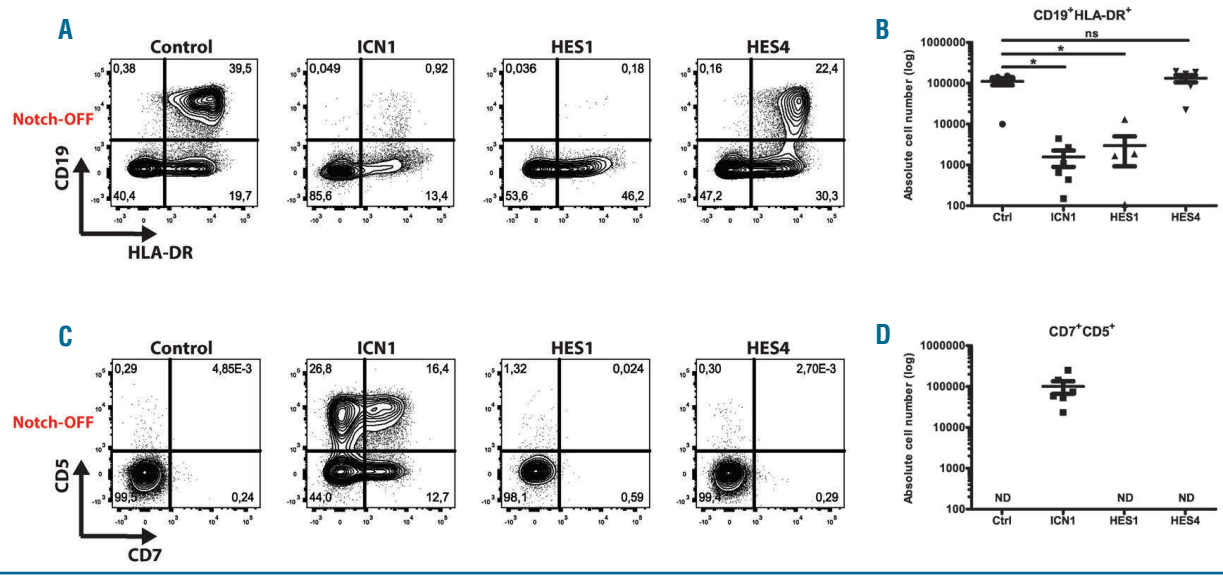


Figure 2. HES4 is not sufficient to inhibit B-cell development. Flow cytometric analysis (A, C) and absolute cell numbers (B, D) of control, *ICN1-*, *HES1-* and *HES4*-transduced CD34$^+$Lin$^-$ CB progenitors cultured on the MS-5 feeder (Notch-OFF) for 3 weeks in the presence of the B-lineage supporting cytokines IL-7 and SCF, showing the development of CD19$^+$HLA-DR$^+$ B-lineage cells (A-B) and CD7$^+$CD5$^+$ T-lineage cells (C-D). Data shows the average of six independent experiments and error bars indicate SEM. *P<0.05 (non-parametric paired Wilcoxon test); ns: not significant; ND: not detectable. Flow cytometry plots shown are representative for six independent experiments.

conditions, continuous *HES4* expression impaired the development of CD56$^+$CD7$^+$ NK-cells (*Online Supplementary Figure S7A-B*). These findings illustrate that both HES1 and HES4 can repress NK-cell development.

HES1 and HES4 inhibit the development of myeloid cells

Notch1-mediated repression of myeloid lineage differentiation is critical following thymic colonization of HPC[4,6] and genetic experiments in mice revealed an important role for HES1 in this process.[17] To investigate if HES1 and HES4 can mediate repression of human myeloid development downstream of Notch1, we cultured control, *ICN1-*, *HES1-* and *HES4*-transduced HPC in conditions that support myeloid differentiation. Consistent with earlier work, enforced expression of *ICN1* reduced myeloid development.[13,27,29] While this was mainly reflected by a decrease in the absolute cell numbers for CD15$^+$HLA-DR$^-$ granulocytes and CD14$^+$CD15$^-$ monocytes, for HLA-DR$^+$CD4$^+$CD14$^-$ DC both the frequency and absolute cell numbers were decreased (Figure 5A-D). In case of continuous *HES1* and *HES4* expression, however, we observed a reduction in both the percentage and absolute cell number of granulocytes (Figure 5A-B) and monocytes (Figure 5A, C). In contrast, while the absolute numbers of DC was decreased by *HES1* overexpression, this was not the case upon ectopic expression of *HES4*. Furthermore, the frequencies of DC were increased in these cultures (Figure 5A, D). Thus, both HES1 and HES4 have the potential to repress monocyte and granulocyte differentiation downstream of Notch1, whereas DC development is less impaired, consistent with the intrathymic development of these antigen-presenting cells.

HES1, but not HES4, maintains a quiescent stem cell signature in CD34$^+$ cells

Enforced expression of *HES1* can maintain a stem cell signature in multiple cell types.[16,30,31] To compare the effect of HES1 and HES4 on HSC maintenance, we tracked the frequency and number of CD34$^+$ HPC, following *ICN1, HES1* or *HES4* transduction, in OP9-DLL4 T-lineage differentiation conditions. In agreement with previous studies,[13,27] ectopic expression of *ICN1* induced T-cell development and resulted in a significant decrease in the percentage and absolute number of CD34$^+$ HPC (Figure 6A-C). Although conditions with elevated *HES1* levels did not yield higher numbers of CD34$^+$ cells (Figure 6C), we observed a significant and more than 2-fold increase in their frequency (Figure 6A-B). In contrast, enforced expression of *HES4* did not affect the fraction of CD34$^+$ HPC (Figure 6A-C). In addition, since earlier work illustrated that overexpression of *HES1* results in the generation of quiescent HSC,[18] we evaluated cellular proliferation. While HPC that express higher levels of *HES1* displayed reduced proliferation, *HES4*-transduced cells proliferated similarly as the control (Figure 6D). These findings suggest that HES1, but not HES4, can induce maintenance of a quiescent stem cell state by repressing differentiation and proliferation of CD34$^+$ HPC.

RNA sequencing reveals HES1- and HES4-induced molecular pathways during early human hematopoiesis

To reveal specific HES1- and HES4-induced molecular changes, we performed RNA sequencing in control, *HES1-* and *HES4*-transduced CD34$^+$Lin$^-$ HPC that were cultured in the absence or presence of Notch signaling on OP9-GFP or OP9-DLL1, respectively. Enforced expression of HES1 in the absence of Notch1 activation (OP9-GFP, Notch-OFF, Figure 7A) resulted in a significant downregulation of genes associated with the differentiation of B-cells (*MYB*,[32] *GM2A*,[33] *DUSP2*[33] and *IGLL1*[34]), NK-cells (*ERI1*,[35] *EMP3*[36] and *SRGN*[37]) and myeloid cells (*EPX*,[38] *CLC*,[39] *SRGN*,[37] *MS4A2*[40] and *MS4A3*[41]) (Figure 7A). *CEBPA* expression was not detected in these experiments. However, given the essential role of HES1 in repression of CEBPA-dependent myeloid differentiation in mouse,[17] we evaluated the

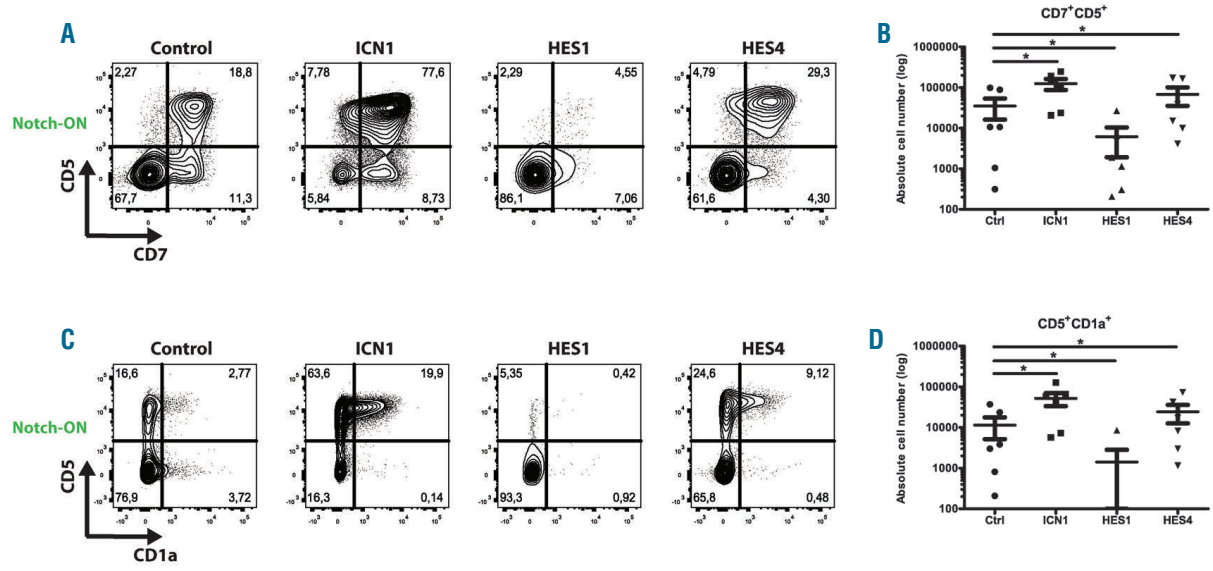


Figure 3. *HES4*, but not *HES1*, promotes induction of T-lineage differentiation. Flow cytometry analysis (A, C) and absolute cell numbers (B, D) of control, *ICN1*-, *HES1*- and *HES4*-transduced CD34$^+$Lin$^-$ CB precursors cultured on the OP9-DLL4 feeder (Notch-ON) for 2 weeks in the presence of the T-lineage specific cytokines IL-7, SCF and FLT3L, showing the development of CD7$^+$CD5$^+$ (A-B) and CD5$^+$CD1a$^+$ (C-D) T-lineage cells. Data shows the average of six independent experiments and error bars indicate the standard error of the mean (SEM). *P<0.05 (non-parametric paired Wilcoxon test). Flow cytometry plots shown are representative for six independent experiments.

impact of HES1 and HES4 on *CEBPA* expression in the HL-60 leukemic cell line and, consistently, HES1 was very potent in repressing *CEBPA* expression, while HES4 was less efficient (*Online Supplementary Figure S8A*). In OP9-DLL1-cultured CD34$^+$ HPC (Notch-ON, Figure 7B), we observed significant repression of Notch signaling and T-lineage associated genes by HES1, including *NOTCH1, RBPJ, MYB*[42] and *DAD1*[43] (Figure 7B). Surprisingly, on OP9-GFP stromal cells where no or little Notch1 triggering occurs, we also observed downregulation of these genes upon *HES1* overexpression (Figure 7C). Also *IRF8* expression, which is induced by Notch signaling for DC deve-lopment,[44] was repressed by HES1 (Figure 7B). On both OP9 stromal co-cultures, expression of *IKZF1*, which is essential for differentiation of lymphoid-primed multipotent progenitors into common lymphoid progenitors (CLP),[45] was downregulated by high levels of HES1 (Figure 7A-B). In contrast, *HES1* overexpression showed significant upregulation of genes associated with stem cell maintenance, such as *CD34*, *CD44*, *HOXB2*[46], *HOXB5*[46], *ESAM*[47] and *ID1*[48], and genes involved in cell cycle arrest, including *IGFBP4, CDKN2D* and *CDKN1C*, which was independent of Notch signaling (Figure 7A-B). These findings were confirmed by gene set enrichment analysis (GSEA) that revealed significant enrichment of HSC markers and genes involved in CD34$^+$ quiescence in the *HES1*-transduced condition (Figure 7D-E). Genes associated with the maintenance of multipotent HSC at the expense of both CLP and granulocyte-monocyte progenitor (GMP) differentiation were also enriched upon *HES1* overexpression (*Online Supplementary Figure S8B-C*). In contrast, *HES4* overexpression did not result in large gene expression changes. Among the significantly downregulated genes, we found *CLC* and *SRGN* (Figure 7A, G), which were previously associated with respectively myeloid and NK-cell development.[37,39] Moreover, upon *HES4* overexpression, we observed a slight, but non-significant upregulation of *NOTCH1, RBPJ* and *IKZF1* in the absence of Notch signaling (Figure 7C), while this was not the case when the cells were cultured on the OP9-DLL1 feeder (Figure 7B). Furthermore, high levels of HES1 or HES4 resulted in a significant downregulation of *HES1* itself (Figure 7F-G). Comparison of the promotor sequences of the putative HES1 and HES4 target genes did not reveal a difference in the relative abundance of E-box, N-box and C-site motifs between HES1 and HES4 targets (*Online Supplementary Figure S9A*). However, using HOMER motif analysis, we observed a difference in the top enriched transcription factor binding sites within HES1 and HES4 target gene promoters (*Online Supplementary Figure S9B-C*). Thus, using RNA sequencing, we identified potential downstream targets of HES1 and HES4 that may modulate Notch-driven lineage decisions during human hematopoiesis and this may involve co-regulation by specific transcription factors.

HES1 and HES4 are both essential for inducing human T-cell development in HPC

To investigate if HES1 and HES4 are required downstream of Notch during early human T-lineage differentiation, we performed *HES1* and *HES4* short hairpin RNA (shRNA)-mediated knockdown experiments in HPC that were cultured on OP9-DLL4 in T-lineage supporting conditions. For both genes, we used two independent shRNA which induced 40-60% knockdown (Figure 8A). Compared to the control, both the frequency and absolute numbers of CD7$^+$CD5$^+$ T-lineage specified and CD5$^+$CD1a$^+$ committed T-cell progenitors were significantly reduced upon knockdown of either *HES1* or *HES4* (Figure 8B-E). However, we did not observe a diversion to the B- or myeloid lineage in these cultures (*data not shown*), probably as a result of insufficient knockdown. Nevertheless, these findings illustrate that both HES1 and

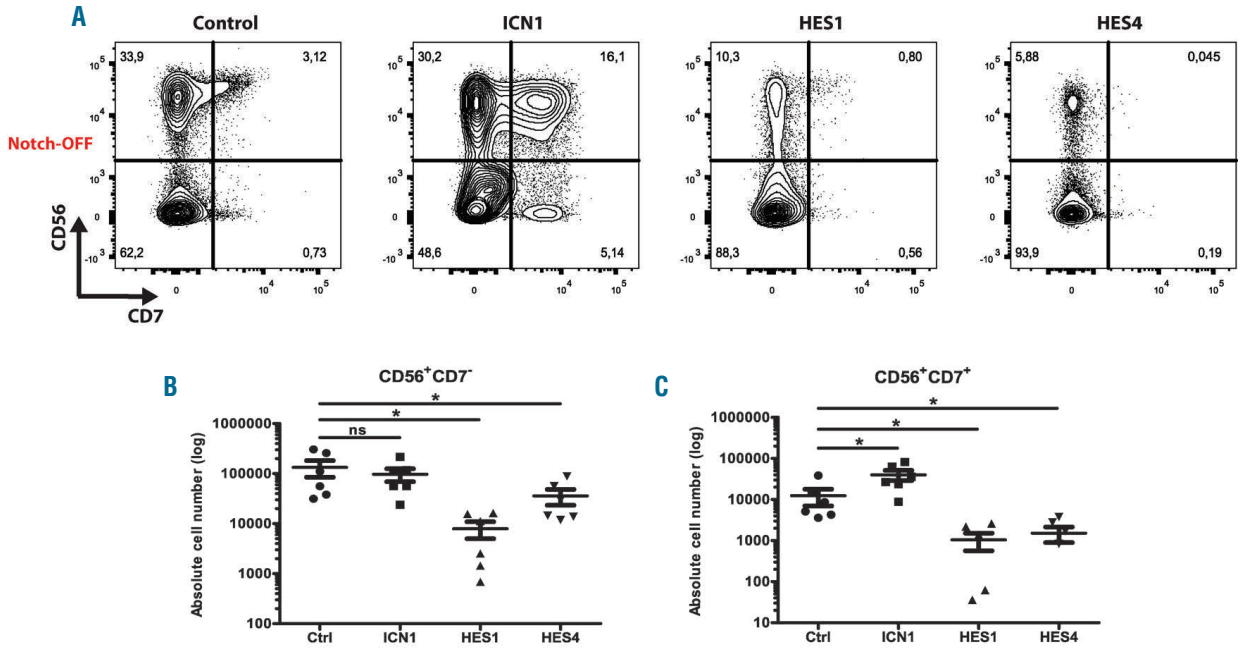


Figure 4. HES1 and HES4 repress NK cell development. (A) Flow cytometric analysis of control, *ICN1-*, *HES1-* and *HES4*-transduced CB CD34$^+$Lin$^-$ precursors cultured on the MS-5 feeder (Notch-OFF) for 3 weeks in the presence of the NK-lineage specific cytokines IL-7, SCF, FLT3L and IL-15, showing the development of CD56+CD7- and CD56$^+$CD7$^+$ NK cells. Plots shown are representative for six independent experiments. (B-C) Absolute numbers of CD56$^+$CD7$^-$ (B) and CD56$^+$CD7$^+$ (C) NK cells generated in corresponding cultures shown in (A). Data shows the average of six independent experiments. Error bars indicate the standard error of the mean (SEM). **P*<0.05 (non-parametric paired Wilcoxon test); ns: not significant.

HES4 have essential and non-redundant roles during early human T-cell development.

Discussion

It is well established that Notch1 activation in thymus-seeding precursors allows progenitor cells to differentiate towards the T-cell lineage, while development towards other hematopoietic cell types is repressed.[4-6,13] However, the individual roles of various Notch1 target genes during these developmental choices are still unknown. Therefore, we investigated the potential of HES1 and HES4 to mediate human Notch1-dependent hematopoietic lineage decisions. We illustrate that both genes are essential downstream of Notch1 to induce human T-cell development and that they have both similar and different potentials to repress the differentiation towards alternative, non T-cell lineages.

In murine hematopoiesis, HES1 is a repressor of intrathymic myeloid differentiation during the Notch-dependent induction of T-cell development.[17] HES1 is also required for sustaining leukemic growth in Notch-driven T-ALL, both in mouse and human.[18,49] Previous overexpression studies in human have shown that HES1 inhibits B- and NK-lineage differentiation, but failed to reveal a block in monocyte development.[27] In agreement with previous work in mice,[17] we show here that HES1 can repress myeloid development in human and that this is mediated through repression of several genes with known roles in monocyte, granulocyte and dendritic cell development, thereby providing novel molecular insights in this process. The discrepancy in monocyte differentiation compared to previous work[27] may relate to differences in expression levels following transduction since we used a codon-optimized construct to facilitate comparison with HES4. This could also explain the more efficient block in B-cell development in our results.

Our novel RNA sequencing data reveal mechanisms through which HES1 specifically represses T-, B- and NK-associated genes, thereby validating the developmental impacts in our co-cultures. In light of the requirement for HES1 downstream of Notch1 during T-cell development, the repression of T-lineage differentiation by HES1 is striking. While this may result from the high expression levels in our perturbation settings, this may also reflect stage-specific requirements for HES1 in this process that are recurrent and may involve HES1 oscillation as observed during embryonic development.[16] Although it is unclear if the latter occurs in developing thymocytes, Notch activation is required at different stages of early T-cell development and recurrent Notch activation events therein seem essential to balance between proliferation and differentiation.[4,50] While HES1 is required to repress myeloid development in early T-cell precursors, it could later be essential to maintain thymocytes in a quiescent state, which is necessary during TCR rearrangements.[4] Although less evident in human, this fits the high expression level of *Hes1* in DN3a thymocytes that are active TCR-rearranging cells.[51] A role for HES1 as a regulator of cell quiescence also concurs with the observation that continuous expression in HPC maintains cells in a CD34$^+$ precursor state, consistent with previous studies.[18,27] Here, we show that this is correlated with the upregulation of genes involved in stem cell maintenance, quiescence and cell cycle arrest. However, while HES1 is dispensable in mice for HSC maintenance,[18] our data suggests that HES1 represses differentiation in human by maintaining a quiescent stem cell signature.

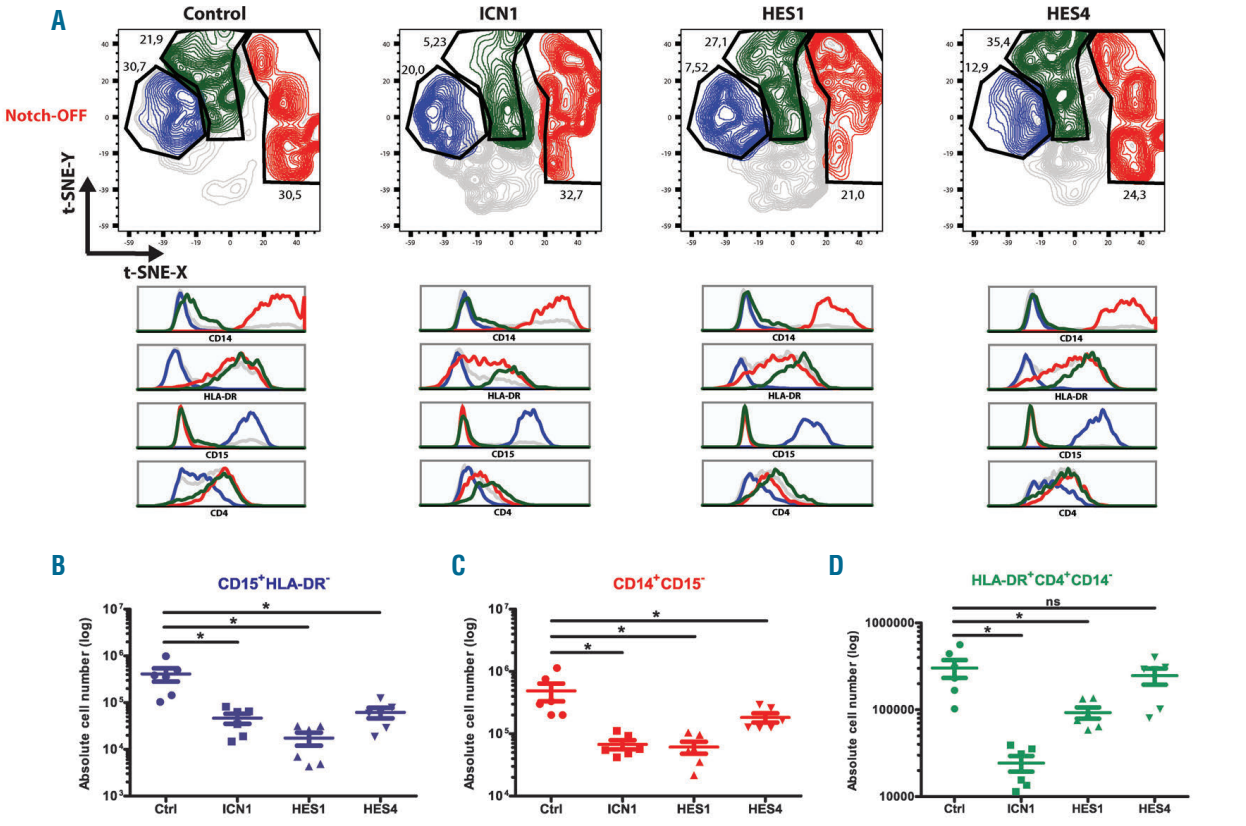


Figure 5. Myeloid differentiation is hampered by HES1 and HES4. (A) Flow cytometry analysis of control, *ICN1*-, *HES1*- and *HES4*-transduced CB CD34$^+$Lin$^-$ progenitors cultured on the MS-5 feeder (Notch-OFF) for 1 week in the presence of the myeloid-lineage specific cytokines SCF, FLT3L, TPO, GM-CSF and G-CSF, showing the development of CD15$^+$HLA-DR$^-$ granulocytes (blue), CD14$^+$CD15$^-$ monocytes (red) and HLA-DR$^+$CD4$^+$CD14$^-$ dendritic cells (green). t-SNE plots shown are representative of six independent experiments. (B-D) Absolute numbers of CD15$^+$HLA-DR- granulocytes (B), CD14$^+$CD15$^-$ monocytes (C) and HLA-DR$^+$CD4$^+$CD14$^-$ dendritic cells (D) generated in corresponding cultures shown in (A). Data shows the average of six independent experiments and error bars indicate the standard error of the mean (SEM). *P<0.05 (non-parametric paired Wilcoxon test); ns: not significant.

For the first time, we study the role of HES4 as a downstream modulator of Notch during non-malignant human hematopoiesis. In agreement with previous studies that compared the function of HES1 and HES4 in brain and bone development,[20,21] our data suggests that both HES proteins have similar, and also different roles during human Notch-dependent hematopoietic lineage decisions. Compared to HES1, which is a strong repressor with a clear impact on HPC differentiation, enforced *HES4* expression has a similar impact on myeloid and NK-cell development, but not on B- and T-cell differentiation. We believe that this lower repressive capacity of HES4 compared to HES1 is not due to lower expression levels following transduction since we obtained a similar mRNA increase for both *HES* genes, and protein staining even suggested higher amounts of HES4 protein compared to HES1. These different repressive capacities may, however, reflect a more stringent role for *HES* genes in the repression of myeloid fate compared to B-lineage differentiation, which is in agreement with a genetic study in mice that showed that intrathymic Notch signaling is more important for the repression of myeloid development than for preventing B-lineage diversion.[52] One exception to the robust repression of myeloid development by *HES* genes concerns their impact on DC development. Although *HES1* overexpression did result in a significant reduction in the absolute number of DC, their frequency was increased compared to control transduced cells. Strikingly, HES4 was not sufficient to robustly repress DC development and this higher tolerance towards *HES* genes is in agreement with the intrathymic and Notch-dependent development of DC.[53] Interestingly, in contrast to HES1, HES4 did promote T-cell development in the presence of Notch1 signaling. This places *HES4* in line with other Notch targets, such as *IL7R* and *TCF7*, that have clear stimulating roles on T-cell development following Notch1 activation.[11,54] However, HES4 by itself, or in combination with HES1, is insufficient to induce T-cell development,[26] indicating that other downstream targets, or combinations thereof, are important for driving this process. Nevertheless, the clear distinct roles of HES1 and HES4 on B- and T-cell development may be important to control the initial Notch-dependent B- *versus* T-cell lineage decision in which HES1 represses the B-cell lineage while HES4 permits further T-cell development. Following this initial bifurcation, both HES1 and HES4 may then be required to repress NK-lineage differentiation while initially still permitting DC development. As such, HES1 and HES4 may have unique roles during these early stages of human T-cell differentiation, which are not-redundant as illustrated through our loss-of-function studies. Unfortunately, these knockdown studies did not result in an increased development of other, non T-cell lineages, but we believe this may result from the incomplete loss of *HES1* and *HES4* expression in these experiments.

In contrast to *HES1*, *HES4* overexpression did not result

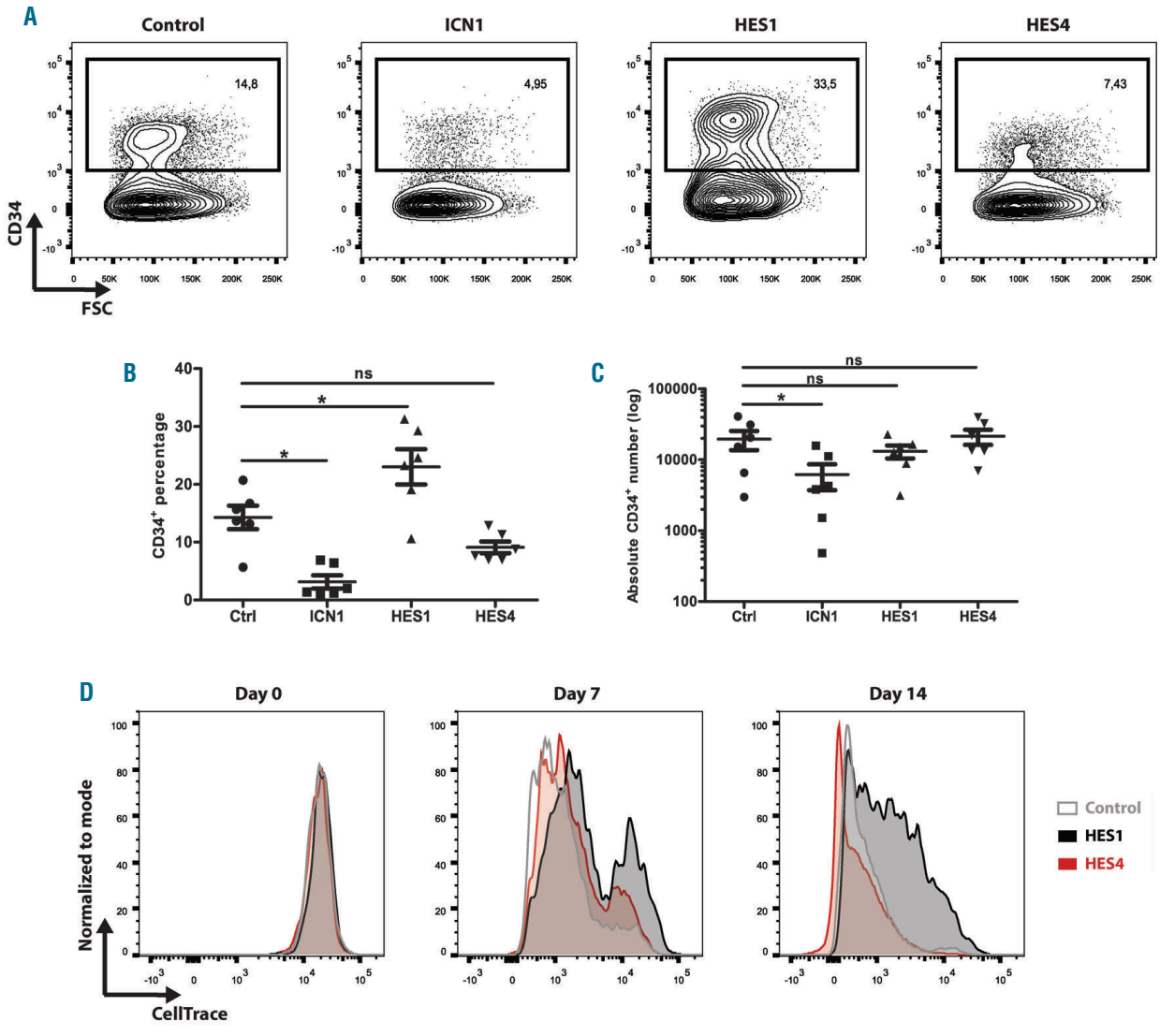
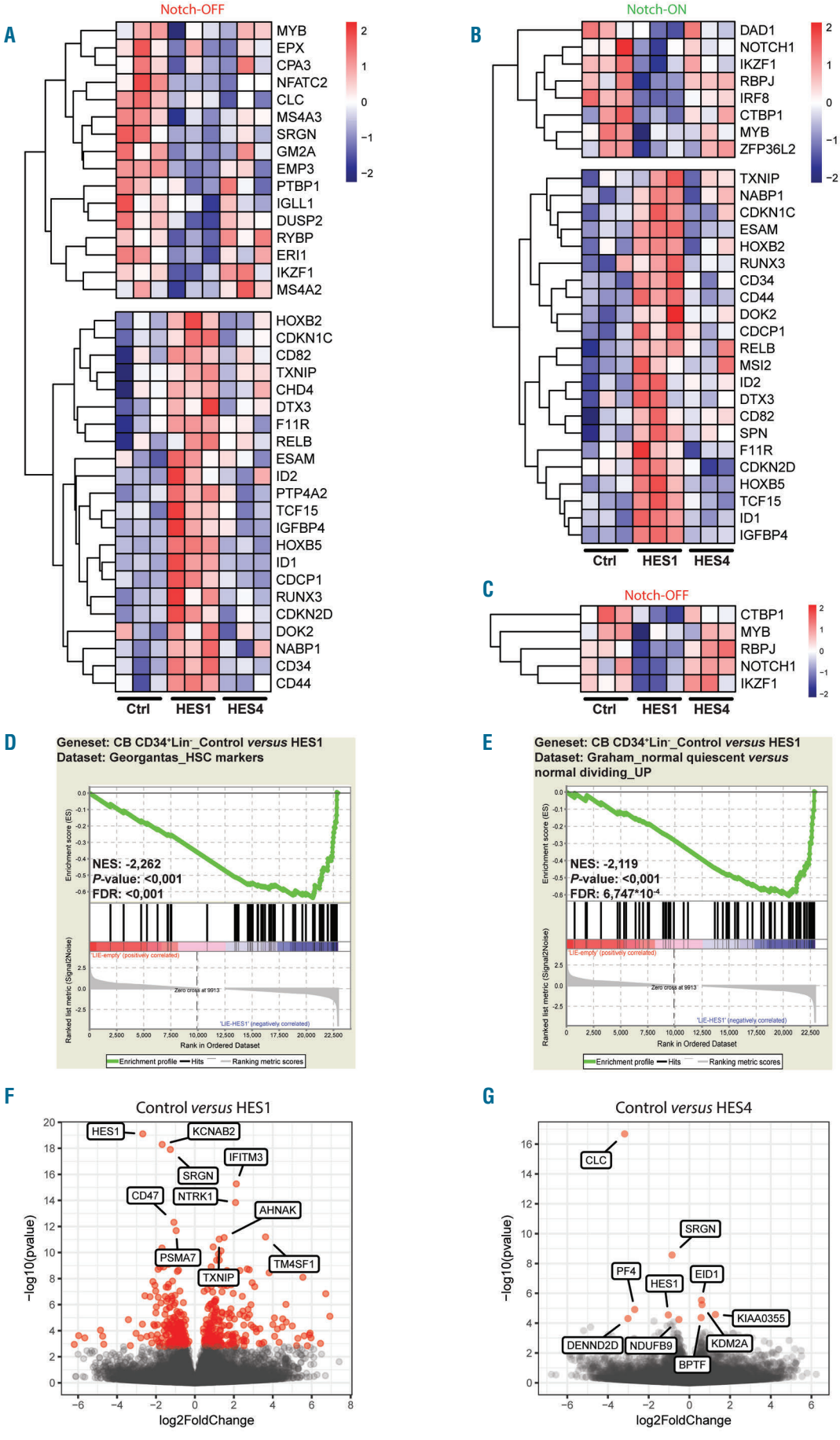


Figure 6. HES1, but not HES4, induces stem cell maintenance and quiescence in CD34⁺ cells. (A) Flow cytometric analysis of control, *ICN1-*, *HES1-* and *HES4*-transduced CB CD34⁺Lin⁻ precursors cultured on the OP9-DLL4 feeder for 2 weeks in the presence of the T-lineage specific cytokines IL-7, SCF and FLT3L, showing the maintenance of CD34⁺ HPC. Plots shown are representative of six independent experiments. (B-C) Frequency (B) and absolute numbers (C) of CD34⁺ cells maintained in corresponding cultures shown in (A). Data shows the average of six independent experiments. Error bars indicate the standard error of the mean (SEM). *P<0.05 (non-parametric paired Wilcoxon test); ns: not significant. (D) Flow cytometry analysis of control (grey), *HES1* (black)- and *HES4* (red)-transduced CB CD34⁺Lin⁻ HPC cultured on the OP9-DLL4 feeder for 2 weeks in the presence of the T-lineage supporting cytokines IL-7, SCF and FLT3L, showing proliferation as measured by the dilution of the CellTrace Violet dye at indicated time points.

in large gene expression changes. This might reflect the immature state of the transduced HPC since gene expression profiling was performed 48 h after transduction in an effort to discover direct regulatory events. However, the lack of *HES4* expression in HSC suggests a minimal role for this protein at this immature stage, and the precise role for HES4 in gene regulation may therefore only be revealed at later differentiation stages, such as following Notch1 activation in immature thymocytes. Other genome-wide approaches, such as ChIP-seq in *HES4*-expressing thymocytes, will be required to identify other HES4 target genes. Remarkably, while we did not observe clear differences in the relative abundance of specific HES binding motifs (E-box, N-box and C-site) in the promotors of the predicted HES1 and HES4 target genes, both HES proteins may differentially regulate downstream target gene expression through interactions with other transcription factors that are distinct for HES1 and HES4. Such mechanism could explain their different impact on hematopoietic lineage decisions. Intriguingly, we identified *HES1* as a downstream target of both HES1 and HES4, thereby confirming the autoregulation of HES1[16] and suggesting a novel feedback loop between HES4 and HES1 that may also contribute to a possible transient repression of HES1, thereby allowing early thymocytes to escape from the repressive effects of HES1 to support their further differentiation. Such a direct repressive role of HES4 on *HES1* expression seems essential given that protein interactions through the formation of HES1-HES4 heterodimers seemingly fail to counteract the strong repressive activity of HES1 homodimers. We anticipate that single cell (sc)RNA sequencing will provide more detailed insights into the actual sequence of *HES1* and *HES4* expression during early human T-cell development.


A
Notch-OFF
MYB
EPX
CPA3
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CLC
MS4A3
SRGN
GM2A
EMP3
PTBP1
IGLL1
DUSP2
RYBP
ERI1
IKZF1
MS4A2
HOXB2
CDKN1C
CD82
TXNIP
CHD4
DTX3
F11R
RELB
ESAM
ID2
PTP4A2
TCF15
IGFBP4
HOXB5
ID1
CDCP1
RUNX3
CDKN2D
DOK2
NABP1
CD34
CD44
Ctrl
HES1
HES4
B
Notch-ON
DAD1
NOTCH1
IKZF1
RBPJ
IRF8
CTBP1
MYB
ZFP36L2
TXNIP
NABP1
CDKN1C
ESAM
HOXB2
RUNX3
CD34
CD44
DOK2
CDCP1
RELB
MSI2
ID2
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CD82
SPN
F11R
CDKN2D
HOXB5
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Geneset: CB CD34+Lin-_Control versus HES1
Dataset: Georgantas_HSC markers
NES: -2,262
P-value: <0,001
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Dataset: Graham_normal quiescent versus normal dividing_UP
NES: -2,119
P-value: <0,001
FDR: 6,747*10-4
Enrichment score (ES)
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Control versus HES1
HES1
KCNAB2
IFITM3
SRGN
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NTRK1
AHNAK
PSMA7
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TXNIP
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Control versus HES4
CLC
SRGN
PF4
HES1
EID1
KIAA0355
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NDUFB9
KDM2A
BPTF
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Figure 7 (previous page). Gene expression analysis of HES1 and HES4 during human hematopoiesis. (A) Heat map representation of differentially expressed genes in control *versus HES1*-, or *HES4*-transduced CB CD34$^+$Lin$^-$ cells cultured for 1 day on OP9-GFP (Notch-OFF), highlighting genes involved in the differentiation towards the B-, natural killer (NK)- and myeloid lineage (top) and HSC maintenance and quiescence (bottom). (B) Heat map representation of differentially expressed genes in control *versus HES1*-, or *HES4*-transduced CB CD34$^+$Lin$^-$ progenitors cultured for 1 day on OP9-DLL1 (Notch-ON), highlighting genes involved in the differentiation towards the T-lineage (top) and HSC maintenance and quiescence (bottom). (C) Heat map representation of differentially expressed genes in control versus *HES1*-, or *HES4*-transduced CB CD34$^+$Lin$^-$ cells cultured for 1 day on OP9-GFP (Notch-OFF), highlighting genes associated with Notch signaling and T-cell development. (D-E) GSEA shows a significant enrichment of HSC markers (D) and genes involved in quiescence (E) in the *HES1*-transduced condition compared to control. (F-G) Volcano plot representation of control *versus HES1* (F)-, or HES4 (G)-transduced CB CD34$^+$Lin$^-$ precursors cultured for 1 day on OP9-GFP, highlighting the expression of the top 10 differentially expressed genes. Red dots indicate significance (padj<0.05; n=3).

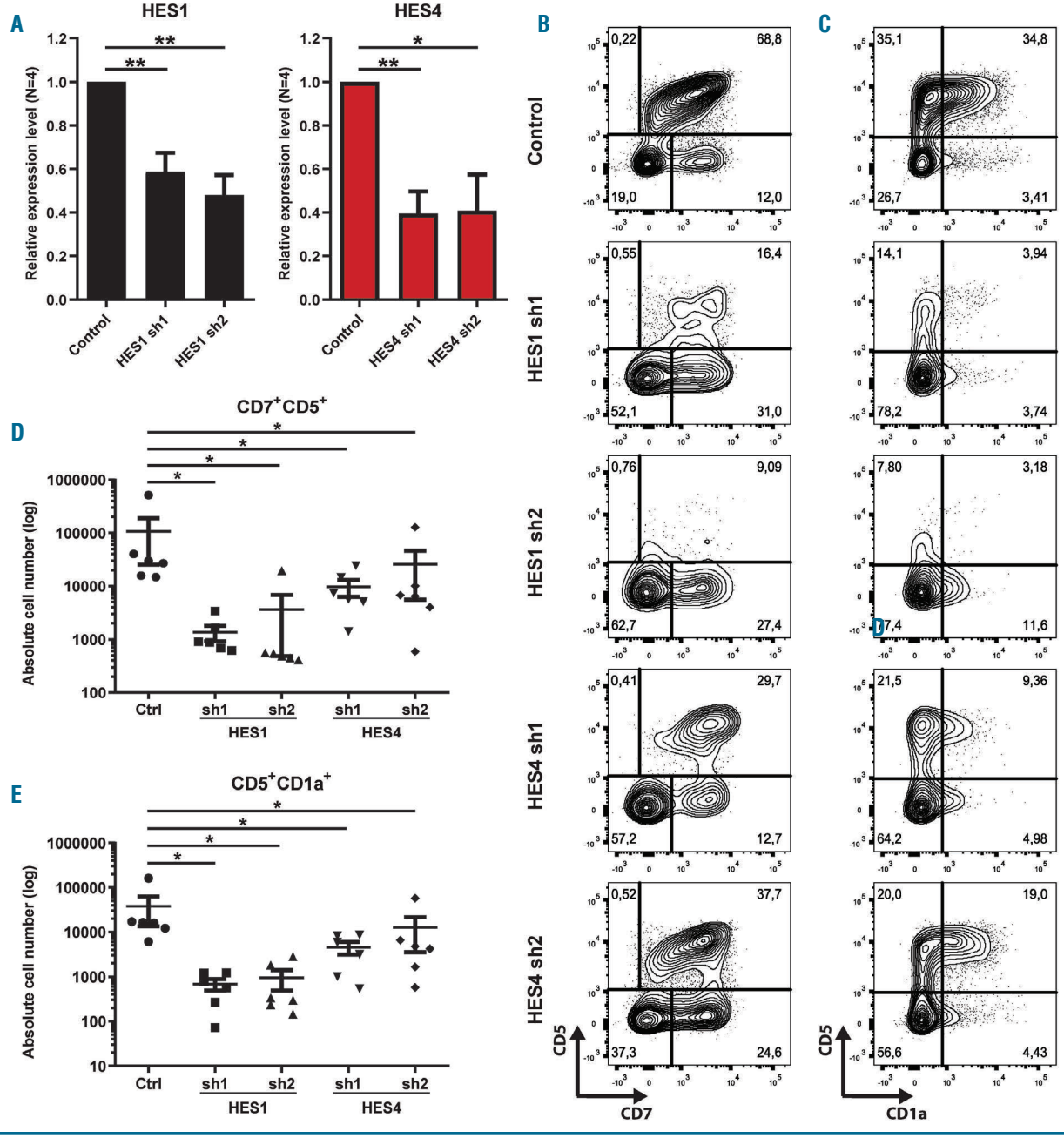

Figure 8. Non-redundant roles for HES1 and HES4 during early human T-cell development. (A) Quantitative RT-PCR of *HES1* (black bars) and *HES4* (red bars) in CB CD34$^+$Lin$^-$ cells transduced with a control or two different *HES1* or two different *HES4* single hairpin RNA (shRNA), respectively, and cultured for 3 days on OP9-DLL4 to induce Notch activation. Data shows the average expression of four independent experiments, relative to the mean of *ACTB* and *GAPDH* mRNA levels, and normalized to expression in the control shRNA condition. Error bars indicate the standard error of the mean (SEM). $^{**}P<0.01$; $^{*}P<0.05$ (paired Student's *t*-test). (B-C) Flow cytometry analysis of control, *HES1* and *HES4* shRNA-transduced CD34$^+$Lin$^-$ hematopoietic progenitor cells (HPC) cultured on the OP9-DLL4 stromal cells for 2 weeks in the presence of the T-lineage supporting cytokines IL-7, SCF and FLT3L, showing the development of CD7$^+$CD5$^+$ (B) and CD5$^+$CD1a$^+$ (C) T-cell precursors. Contour plots shown are representative for six independent experiments. (D-E) Absolute numbers of CD7$^+$CD5$^+$ T-lineage specified (D) and CD5$^+$CD1a$^+$T-lineage committed (E) progenitors generated in corresponding cultures shown in (B) and (C), respectively. Data shows the average of six independent experiments and error bars indicate the standard error of the mean (SEM). $^{*}P<0.05$ (non-parametric paired Wilcoxon test).

Given that HES4 is not present in the mouse genome, this work thus improves our understanding of the mechanisms through which Notch activation controls human blood cell development. This is an important issue since we have previously documented that Notch activation has different impacts on T-cell development in human *versus* mouse.[4,14,55] Our work shows that both HES1 and HES4 are important to support the Notch1-dependent induction of human T-cell development and that this may be regulated through both different and similar repressive mechanism on other hematopoietic lineages. These findings thus underscore the distinct regulatory functions of each member of the complex network of Notch1 downstream target genes.

Disclosures

No conflicts of interest to disclose.

Contributions

MDD performed experiments; MDD, ML, JR and LT analyzed data; BV, GL and PVV provided critical reagents; FVN performed RNA sequencing experiments; MDD and TT designed research and wrote the paper.

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