CSL–MAML-dependent Notch1 signaling controls T lineage–specific IL-7Rα gene expression in early human thymopoiesis and leukemia

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Notch1 activation is essential for T-lineage specification of lymphomyeloid progenitors seeding the thymus. Progression along the T cell lineage further requires cooperative signaling provided by the interleukin 7 receptor (IL-7R), but the molecular mechanisms responsible for the dynamic and lineage-specific regulation of IL-7R during thymopoiesis are unknown. We show that active Notch1 binds to a conserved CSL-binding site in the human *IL7R* gene promoter and critically regulates *IL7R* transcription and IL-7R α chain (IL-7Rα) expression via the CSL–MAML complex. Defective Notch1 signaling selectively impaired IL-7Rα expression in T-lineage cells, but not B-lineage cells, and resulted in a compromised expansion of early human developing thymocytes, which was rescued upon ectopic IL-7Rα expression. The pathological implications of these findings are demonstrated by the regulation of IL-7Rα expression downstream of Notch1 in T cell leukemias. Thus, Notch1 controls early T cell development, in part by regulating the stage- and lineage-specific expression of IL-7Rα.

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Abbreviations used: γc, common cytokine receptor γ; CB, cord blood; ChIP; chromatin immunoprecipitation; CompE, compound E; DN, double negative; dnMAML1, dominant-negative MAML1; DP, double positive; ETP, early thymic progenitor; FTOC, fetal thymic organ culture; GABP, GA binding protein; GSI, γ-secretase inhibitor; ICN1, intracellular Notch1; MEF, mouse embryonic fibroblast; MFI, mean fluorescence intensity; mRNA, messenger RNA; T-ALL, T cell acute lymphoblastic leukemia.

The development of T cells is a tightly regulated process guided by inductive signals provided by the thymic microenvironment (1). The interaction of Notch1 with Delta-like ligands expressed by the thymic epithelium is an initial obligatory event for lymphomyeloid progenitors seeding the thymus to undergo T cell specification and diversion away from alternative cell fates (1–3). Thereafter, recurrent Notch–ligand interactions are required for intrathymic early thymic progenitors (ETPs) to maintain T cell specification and to support further development along the T cell lineage (4–6). During this maturation process, ETPs that are CD4^{-}CD8^{-} double negative (DN) and either CD44^{+}CD25^{-}CD117^{+} or CD34^{+}CD1a^{-}CD33^{+} in mice or humans, respectively, differentiate into DN2 (CD44^{+}CD25^{+} or CD34^{+}CD1a^{+}) and DN3 (CD44^{-}CD25^{+} or CD4^{+}CD3^{-} immature single positive) thymocytes (1, 3, 7). Progression beyond the DN3 stage and irreversible T cell commitment is accomplished by signaling through a pre-TCR that promotes survival, proliferation, and further differentiation to the CD4^{+}CD8^{+} double-positive (DP) stage (8). This developmental checkpoint, known as β selection, also depends on cooperative signaling provided by Notch1 (9). Therefore, Notch activation is crucial early in thymopoiesis for the induction and maintenance of T cell specification within the DN intrathymic compartment and, later on, for the functional outcomes of β selection.

Besides the pre-TCR–dependent phase of thymocyte expansion at the β-selection checkpoint, there is an early phase of extensive proliferation of the intrathymic pool of T cell precursors

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in response to IL-7 (10, 11). Binding of IL-7 to its receptor (IL-7R), which is composed of an α-chain (IL-7Rα) associated to the common cytokine receptor γ (γc) chain (12), plays a conserved nonredundant role by promoting the survival and proliferation of DN progenitors (10, 13–16). IL-7, however, is dispensable for differentiation beyond the DN3 stage, although it may be required later on during positive selection of CD8$^+$ cells (11, 17, 18). Thus, besides Notch1 signals, IL-7–IL-7Rα interactions provide additional thymic signals that are critical for the development of thymocytes before the DP stage.

The stage-specific function of IL-7 during intrathymic development is accomplished by a tight regulation of IL-7Rα expression. IL-7Rα is first induced during thymopoiesis in late ETPs in transit to DN2, it declines steadily after the DN2 stage and must be terminated before transition to the DP stage, but it is reexpressed after positive selection in single-positive thymocytes (2, 11, 17–20). Still, the molecular bases of the dynamic regulation of IL-7Rα expression during thymopoiesis remain poorly understood. In early lymphoid precursors and B cell progenitors of mice, IL-7Rα gene (*Il7ra*) transcription is regulated by the Ets family transcription factor PU.1 (21). However, PU.1 down-regulation is specifically required for progression in the T cell lineage (22), and another Ets factor, GA binding protein (GABP), was shown to regulate IL-7Rα expression in T cells (23). Nonetheless, neither expression nor function of GABP is T-lineage specific. Rather, GABP regulates IL-7Rα expression in pre-B and committed B cells as well and has recently been proven to be a critical regulator of B cell development (24, 25). Therefore, the molecular mechanism responsible for the dynamic and T-lineage–specific regulation of IL-7Rα expression remains to be identified. In this paper, we provide evidence that Notch1 accomplishes this function during T cell development. We show that active Notch1 directly regulates human IL-7Rα gene (*IL7R*) transcription and critically controls the IL-7–dependent expansion of the intrathymic pool of early DN T cell progenitors in human thymopoiesis as well as the IL-7–induced proliferation of T cell leukemias.

RESULTS

Notch1 signaling up-regulates IL-7Rα expression in hematopoietic precursors

In both mouse and human thymopoiesis, Notch1-induced T-lineage specification parallels the induction of IL-7Rα expression and IL-7 dependency. We thus wanted to investigate whether IL-7Rα expression in early thymopoiesis is a direct consequence of Notch1 activation rather than a byproduct of progression toward the T cell lineage. To this end, we first analyzed the impact of Notch1 signaling on surface levels of IL-7Rα expressed on human ETPs developing in a hybrid human/mouse fetal thymic organ culture (FTOC). Thus, sorted ETPs were infected either with a bicistronic retroviral vector encoding the intracellular active form of Notch1 (intracellular Notch1 [ICN1]) and GFP as a reporter, or with a GFP-only control vector (26), and IL-7Rα expression was then analyzed by flow cytometry on the ETP progeny arising in a FTOC assay. Supporting a direct role of Notch1 in IL-7Rα expression, we found that ectopic expression of ICN1 consistently resulted in the generation of DP thymocytes with up-regulated IL-7Rα, as compared with the GFP-transduced controls (fold increase of mean fluorescence intensity [MFI] ± SEM: 2.26 ± 0.28 from three independent experiments; Fig. 1 A). More importantly, similar approaches showed that IL-7Rα was induced de novo on the major cell progeny (>95%) arising in multicytokine cultures (27) from ICN1-transduced human CD34$^+$ cord blood (CB) multipotent precursors, which displayed a homogeneous lineage-negative (Lin$^-$) phenotype (i.e., CD1a$^-$, CD2$^-$, CD3$^-$, CD4$^-$, CD5$^-$, CD7$^-$, CD8$^-$, CD13$^-$, CD14$^-$, CD19$^-$, CD33^{lo}, CD34$^-$, CD56$^-$, CD116$^-$, CD122$^-$, and TCR-β$^-$). In contrast, no IL-7Rα was expressed on the equivalent Lin$^-$ population derived from control CB precursors transduced with GFP (Fig. 1 B), which represented a minor proportion (5%) of the GFP$^+$ progeny (95% CD13$^+$ myeloid cells). Loss-of-function experiments were then performed to establish whether Notch-deficient progenitors had defects in IL-7Rα expression. Thus, Notch signaling was inhibited in CD34$^+$ CB cells by ectopic expression of a dominant-negative mutant form of the Notch coactivator MAML1 (dominant-negative MAML1 [dnMAML1]) fused to GFP (28), and dnMAML1$^+$ cells were then analyzed for their capacity to acquire surface IL-7Rα under optimal culture conditions, using the OP9-DL1 coculture system (29). As shown in Fig. 1 C, control CD34$^+$ progenitors transduced with GFP-only vectors gave rise to a major Lin$^-$ progeny (i.e., CD3$^-$, CD13$^-$, CD19$^-$, and CD56$^-$; 90% by day 22), which expressed IL-7Rα (>85% of cells). In contrast, the equivalent Lin$^-$ progeny of dnMAML1$^+$ precursors (70%) were markedly impaired in their capacity to express IL-7Rα (<25%). Overall, these results indicate that Notch1 signaling can up-regulate IL-7Rα expression in primary human hematopoietic progenitors.

Inhibition of Notch1 signaling specifically impairs *IL7R* gene expression in T-lineage cells

To next investigate whether up-regulation of IL-7Rα by Notch1 resulted from direct induction at the transcriptional level, we first used the T cell line Jurkat as a clonal model in which ectopic ICN1 expression resulted in IL-7Rα up-regulation at the cell surface (27). We found that *IL7R* messenger RNA (mRNA) expression was markedly increased in ICN1-transduced cells, as compared with GFP-only–transduced controls (Fig. S1), indicating that Notch1 signaling is able to control *IL7R* gene expression. Because *IL7R* transcription is a hallmark of lymphoid progenitors developing along either the T or the B cell lineages, it was important to investigate whether regulation of *IL7R* mRNA expression by Notch1 is common to T and B cell lymphocyte precursors or restricted to T-lineage cells. Thus, we analyzed two human cell lines that constitutively express surface IL-7Rα, namely SupT1 and REH (Fig. 2 A), as prototypes of pre-T (30, 31) and pre-B cells (32), respectively, and asked whether *IL7R* mRNA expression was affected upon Notch signaling inhibition by dnMAML1. We found that disruption of Notch1 signaling, as assessed by decreased expression of *HES1*, resulted in a marked down-regulation of surface IL-7Rα on SupT1 pre-T cells, which correlated with decreased *IL7R* mRNA levels; however, dnMAML1 did not affect IL-7Rα and mRNA expression in REH pre-B cells (Fig. 2, A and B). Quantitative PCR analyses showed that expression of *IL2RG* gene encoding the IL-7R γc chain remained essentially unchanged in either cell line (Fig. 2 B), supporting a specific effect of Notch1 on *IL7R* expression in SupT1 cells. Consistently, SupT1 cells, but not REH cells, expressed detectable levels of endogenous active Notch1 (Fig. S1). As a whole, these data, together with similar results obtained from additional T-lineage (CUTLL1) and B-lineage (NALM-6) cell lines (Fig. S1), support the notion that regulation of *IL7R* expression by Notch1 is T-lineage specific.

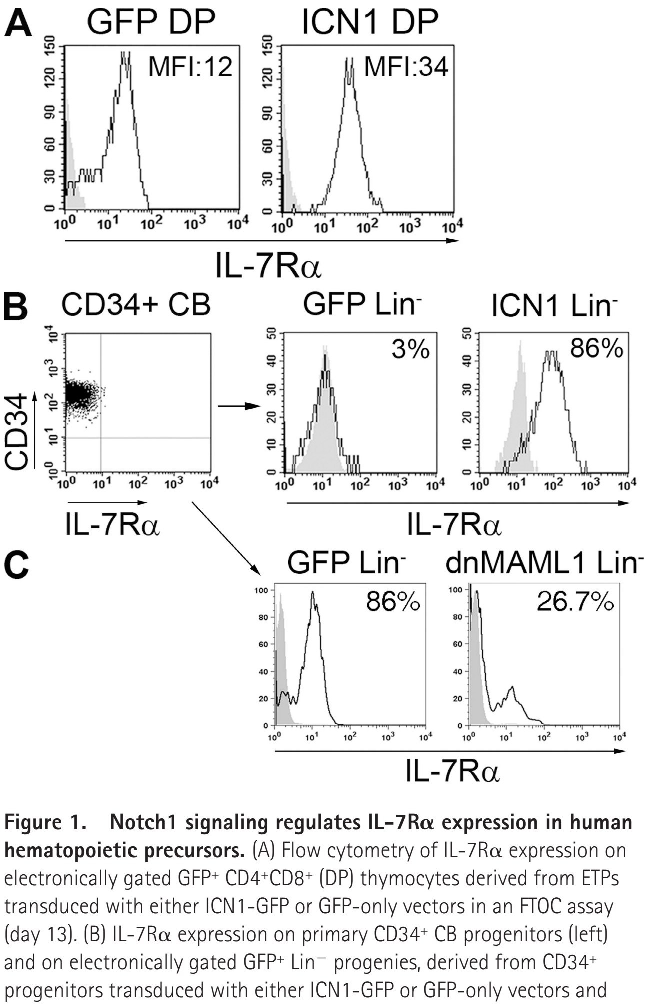

Figure 1. Notch1 signaling regulates IL-7Rα expression in human hematopoietic precursors. (A) Flow cytometry of IL-7Rα expression on electronically gated GFP$^+$ CD4$^+$CD8$^+$ (DP) thymocytes derived from ETPs transduced with either ICN1-GFP or GFP-only vectors in an FTOC assay (day 13). (B) IL-7Rα expression on primary CD34$^+$ CB progenitors (left) and on electronically gated GFP$^+$ Lin$^-$ progenies, derived from CD34$^+$ progenitors transduced with either ICN1-GFP or GFP-only vectors and cultured with multilineage-supportive cytokines for 15 d (right). (C) IL-7Rα expression levels on electronically gated GFP$^+$ Lin$^-$ progenies derived from CD34$^+$ progenitors transduced with either dnMAML1-GFP or GFP-only vectors and cocultured on OP9-DL1 stroma for 22 d. Shaded histograms represent background staining with irrelevant isotype-matched antibodies. Numbers in quadrants represent MFI values (A) and percentages of positive cells (B and C). Results are representative of at least three independent experiments.

Active Notch1 interacts with a CSL-binding site in the *IL7R* promoter and induces *IL7R* transcription

To examine whether Notch1 directly activates *IL7R* gene expression, luciferase reporter assays were performed using a vector in which we cloned a 2-kb fragment encoding the 5′ upstream regulatory region of human *IL7R* (Fig. 3 A). Cotransfection of this reporter, along with a retroviral vector encoding ICN1 in two distinct cell lines, 293T and Jurkat, resulted in a significant increase of luciferase activity compared with GFP-transfected controls (Fig. 3, B and C). Notably, cotransfection of dnMAML1 with ICN1 abrogated *IL7R* promoter activation (Fig. 3 C). Overall, these data support a direct effect of ICN1 on *IL7R* transcription.

Notch receptors can induce gene transcription by two alternative mechanisms either dependent or independent of ICN1 binding to the transcription factor CSL (CBF-1/RBP-Jκ suppressor of Hairless, and Lag-1) and subsequent recruitment of a coactivation protein complex including p300, CBP, and MAML1 (3). Supporting a CSL-dependent mechanism of Notch1-induced *IL7R* gene activation, we identified a putative CSL-binding site (CTTGGGAA) in the *IL7R* promoter that was conserved between human and mouse at positions −936 and −996 bp upstream of the transcription initiation site, respectively (Fig. 3 A). Formal proof that CSL was in fact involved in ICN1-induced *IL7R* promoter activation was obtained from luciferase reporter assays performed in mouse embryonic fibroblasts (MEFs) derived from RBP-J$\kappa^{-/-}$ homozygous mice or RBP-J$\kappa^{+/-}$ heterozygous controls (33). We found that ectopic ICN1 expression markedly induced *IL7R* promoter activity in RBP-J$\kappa^{+/-}$ MEFs but promoter activation was severely impaired in CSL-deficient RBP-J$\kappa^{-/-}$ MEFs (Fig. 3 D). Moreover, site-directed mutagenesis (CTTGGGAA to CTGTACCA) at the CSL-binding site resulted in impaired transcription from the *IL7R* reporter construct in 293T cells (Fig. 3 E). Therefore, ICN1-induced activity of the *IL7R* promoter is dependent on the CSL-binding site.

To directly test whether ICN1 associates to the CSL-binding motif of *IL7R* in vivo, we performed chromatin immunoprecipitation (ChIP) assays using an antibody against human Notch1. DNA fragments spanning the CSL-binding site of the *IL7R* promoter were enriched in ICN1 immunoprecipitates from SupT1 and CUTLL1 T-lineage cell lines, as well as from primary DN2 human thymocytes, but not from REH, NALM-6, and HPB-NULL pre-B cell lines. As a control, we also observed a selective enrichment of the Notch target gene *HES1* in the former cells (Fig. 3 F, top). Therefore, endogenous ICN1 can bind constitutively to the CSL-binding site of *IL7R* and *HES1* promoters in T-lineage cells, although with different efficiencies that may depend on stage-specific differences in chromatin contexts. However, no binding of ICN1 could be detected in any pre-B cell line. Because *IL7R* expression is regulated by the Ets transcription factor PU.1 in developing B cells (21), ChIP assays were performed using an anti-PU.1 antibody as well. In contrast to ICN1, PU.1 bound to the *IL7R* promoter in all analyzed pre-B cell lines but not in pre-T thymocytes or cell lines (Fig. 3 F, bottom). Therefore, ICN1 binds in vivo to the CSL site of the

IL7R promoter in human pre-T cells, whereas PU.1 associates with the *IL7R* promoter in pre-B cells.

Notch1 signaling regulates IL-7Rα expression and controls progenitor expansion in early human T cell development

Our finding that *IL7R* is a direct transcriptional target of Notch1 pointed to a fundamental role of Notch1 in the regulation of IL-7Rα expression during T cell development. In fact, expression and activity of Notch1 measured by *HES1* transcriptional levels paralleled *IL7R* mRNA expression throughout human thymocyte development (Fig. 4). *IL7R* expression also correlated with mRNA levels of its target *BCL2* and with those of the Notch1 target pTα (*PTCRA*). However, expression of the *SPI.1* gene that encodes PU.1 was inversely correlated with *IL7R* mRNA expression. Also, we did not find correlation between *IL7R* and *GABPA*, the gene encoding the Ets factor GABPα, which was uniformly expressed along T cell development (Fig. 4). Therefore, Notch1 activation, but neither PU.1 nor GABPα expression, correlated with *IL7R* expression along human T cell development.

To directly investigate the contribution of Notch1 to the regulation of IL-7Rα expression during human thymopoiesis, ETPs from human thymus were transduced with the pan-Notch inhibitor dnMAML1 fused to GFP, and development of thymocytes incapable of Notch signaling was analyzed in an FTOC assay using GFP as a tracer. We found that proportions of

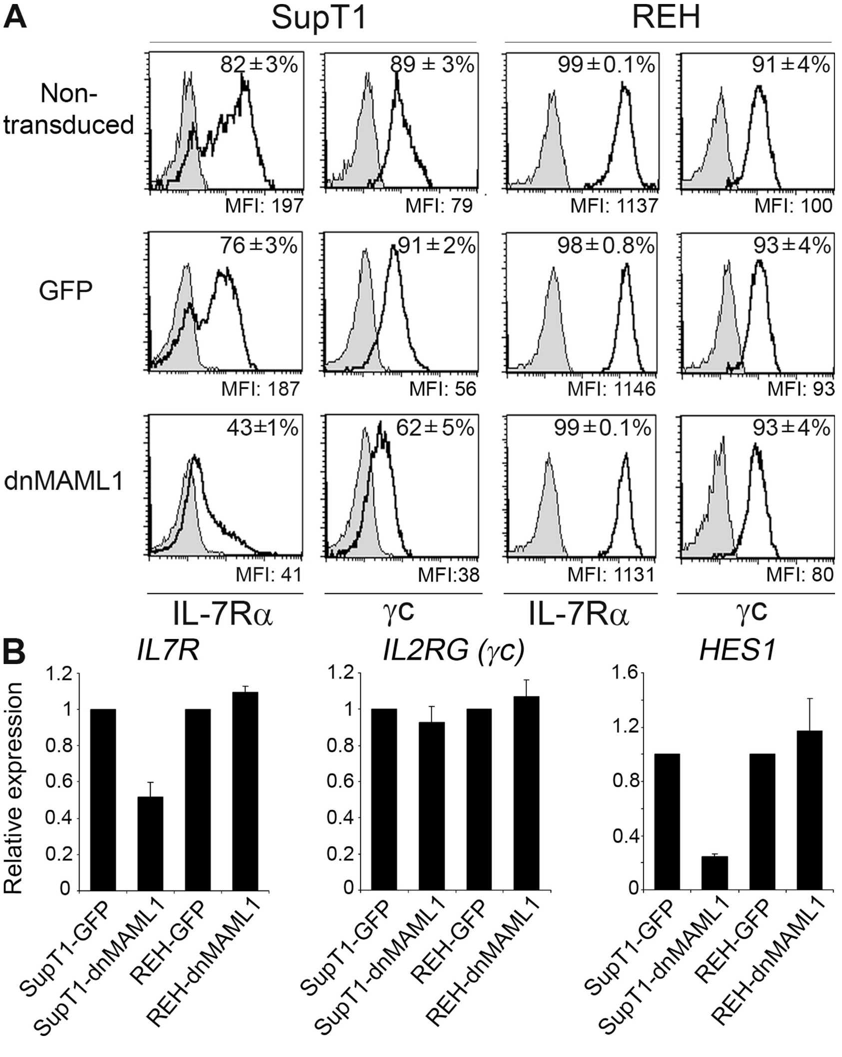

Figure 2. Inactivation of Notch signaling by dnMAML1 results in T-lineage–specific inhibition of IL-7Rα protein and *IL7R* mRNA expression. SupT1 pre-T cells and REH pre-B cells were transduced either with a retroviral vector encoding dnMAML1 fused to GFP or with a GFP-only control vector. (A) Surface expression of IL-7Rα and γc chains was analyzed by flow cytometry on electronically gated GFP$^+$ and dnMAML1$^+$ cells 6 d after transduction or on total nontransduced cells. Shaded histograms represent background staining with an irrelevant isotype-matched antibody. Numbers in quadrants are means ± SEM of percentages of positive cells from three independent experiments. MFI data of this particular experiment are shown at the bottom of each histogram. (B) Real-time quantitative PCR analysis of *IL7R* (IL-7Rα), *IL2RG* (γc), and *HES1* mRNA expression in SupT1 and REH cells transduced with dnMAML1-GFP or GFP only. Results were normalized to *GAPDH* expression values. Bar graphs represent means ± SEM of triplicate samples. Results are representative of three independent experiments.

dnMAML1$^+$ thymocytes decreased markedly with time in FTOC compared with GFP-only–transduced controls (Fig. 5 A). This was likely a result of a growth disadvantage of thymocytes with impaired Notch signaling because absolute numbers of dnMAML1$^+$ cells remained essentially constant during the first 2 wk of FTOC, although they dropped abruptly thereafter and altogether by day 25. In contrast, GFP-transduced controls increased steadily throughout culture (Fig. 5 B). Notably, impaired proliferation of dnMAML1$^+$ thymocytes consistently correlated with undetectable IL-7Rα expression levels on

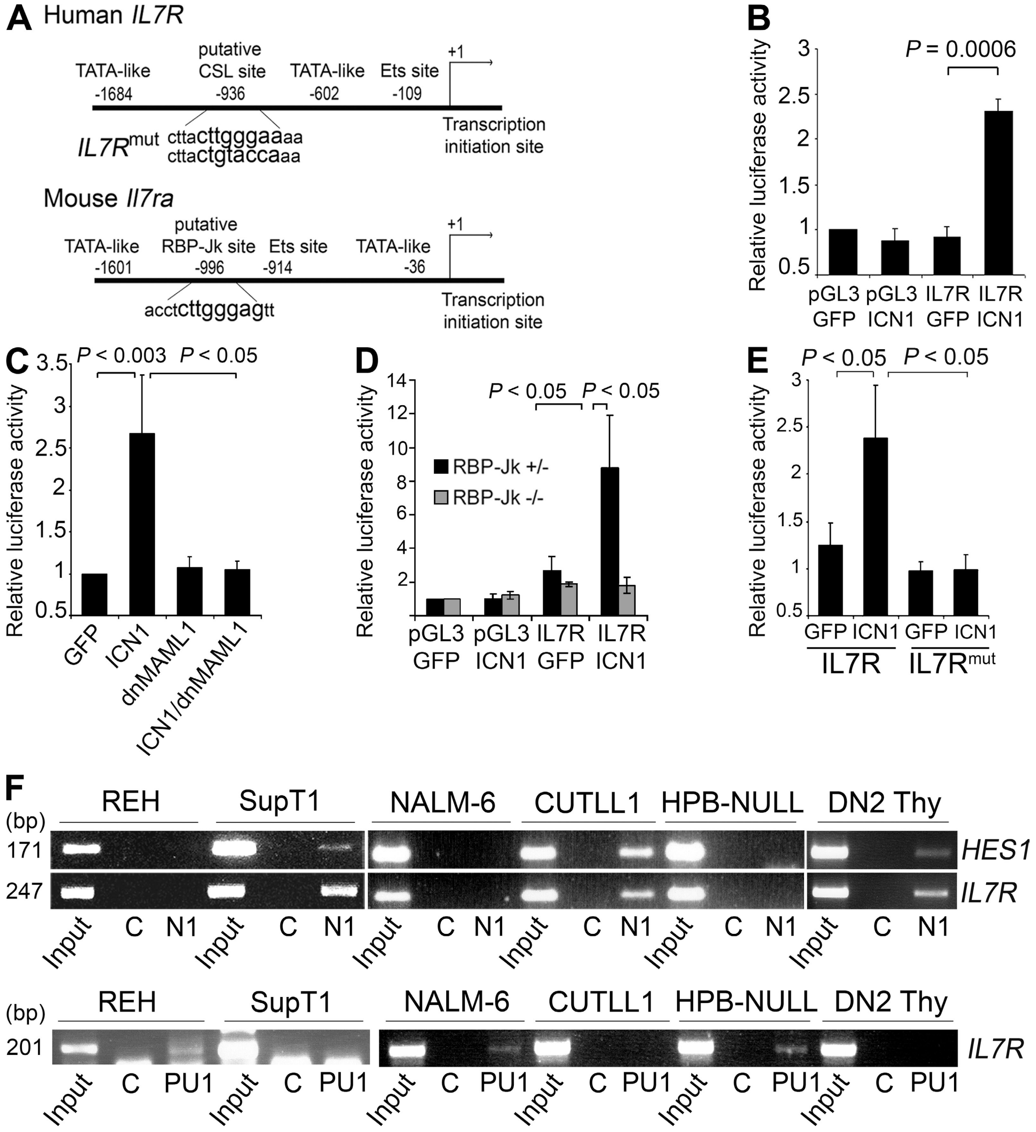

Figure 3. CSL/MAML-mediated transcriptional activation of *IL7R* by active Notch1. (A) Identification of a conserved CSL-binding site in the 5′ regulatory region of human *IL7R* and mouse *Il7ra*. Numbers indicate distances in base pairs from the transcription initiation site. (B) Luciferase reporter assays in 293 T cells cotransfected with a reporter construct containing the 5′regulatory region of *IL7R* shown in A, along with either a retroviral vector encoding ICN1 and GFP (ICN1) or a GFP-only control vector (GFP). Data are represented as fold induction over luciferase activity of control cells cotransfected with an empty reporter vector (pGL3B) and the GFP-only vector. (C) MAML-dependent activation of *IL7R* transcription. Reporter assays were performed in Jurkat cells cotransfected with the *IL7R* reporter and ICN1-GFP, and with or without dnMAML1-GFP. Data are represented as fold induction over luciferase activity of control cells transfected with the GFP-only vector. (D and E) Notch-induced *IL7R* promoter activity requires an intact CSL-binding site. Reporter assays were performed in RBP-J$\kappa^{+/-}$ and RBP-J$\kappa^{-/-}$ MEFs cotransfected with the *IL7R* reporter along with either ICN1-GFP or GFP-only vectors (D) and 293 T cells cotransfected with ICN1-GFP along with a reporter vector containing either the wild-type sequence of the CSL-binding site in the *IL7R* promoter or the mutated (mut) CSL sequence shown in A (E). Bar graphs represent means ± SEM of triplicate samples from at least four independent experiments. (F) ICN1 binds to the *IL7R* promoter in vivo. Formaldehyde cross-linked chromatin from primary DN2 human thymocytes, SupT1 pre-T cells and CUTLL1 T-lineage cells, and REH, NALM-6, and HPB-NULL pre-B cells was subjected to ChIP with specific antibodies against human Notch1 (N1; top), or PU.1 (bottom). Goat or rabbit Igs were used, respectively, as controls. PCR was done on input DNA and on immunoprecipitated DNA with primers pairs spanning the CSL sites of *HES1* and *IL7R* (top) or the Ets site of *IL7R* (bottom). Results are from one representative out of two to three independent experiments.

$\sim$50% of thymocytes before day 12 (54.6 ± 10.1 and 51.7 ± 8.6% by days 4 and 11, respectively; Fig. 5 C) and with reduced numbers of cycling cells (up to 14-fold by day 11; Fig. 5 D). Moreover, those dnMAML1$^+$ cells that still displayed surface IL-7Rα had significantly diminished IL-7Rα surface levels as compared with GFP controls (MFI: 12.7 vs. 22.8 and 12.6 vs. 20.0 at days 5 and 11, respectively; Fig. 5 E).

In terms of differentiation, dnMAML1 overexpression resulted in a complete block in the generation of DP CD3$^+$ thymocytes expressing either the pre-TCR or the TCR-αβ (Fig. 5, F and G) together with a parallel increase in both DP immature thymocytes lacking CD3 and non-T cells (Fig. 5 F and Fig. S2). This pattern resembles that found in FTOC assays in which treatment with a γ-secretase inhibitor (GSI) impaired Notch signaling and hampered cytoplasmic TCR-β (TCR-βic) expression (Fig. S2) (34, 35). Thus, we can conclude that CSL/MAML-dependent Notch1 signaling is absolutely required for progression through the β-selection checkpoint in humans, as reported in mice (9). In contrast, ETPs with impaired Notch signaling were capable of progressing along the initial differentiation stages upstream of β selection with relative efficiencies equivalent to those of controls. Indeed, proportions of DN2 and DN3 thymocytes arising during the initial 2 wk were similar in dnMAML1$^+$ and GFP$^+$ FTOCs (Fig. 5 F), although absolute numbers were markedly decreased in the former (53 ± 13% [$P = 0.0164$] and 74 ± 11% [$P = 0.002$] reduction of control DN3 cells by days 4 and 11, respectively), and essentially no dnMAML1$^+$ cells were recovered by day 25 (Fig. 5 G). Down-regulated IL-7R levels may thus be sufficient for maintaining survival of thymocytes upstream of β selection but unable to sustain cellular expansion in response to mouse IL-7 produced locally in the thymic lobes. Supporting this possibility, neither numbers of apoptotic cells nor expression levels of antiapoptotic Bcl2 molecules changed significantly in Notch-deprived FTOCs before day 12. However, down-regulated IL-7Rα levels expressed on Notch-deprived thymocytes showed a diminished function, as assessed by STAT5 phosphorylation, compared with controls (Fig. S3). Collectively, these data indicated that impaired Notch signaling had two independent stage-specific effects during T cell development: first, a down-regulation of IL-7Rα expression that resulted in an impaired proliferation from DN1 to DN3 stages; and second, a developmental arrest at the β-selection checkpoint. We thus concluded that Notch1 signaling has a critical role in sustaining proliferation between T cell specification and commitment, whereas it is thereafter obligatory for β selection.

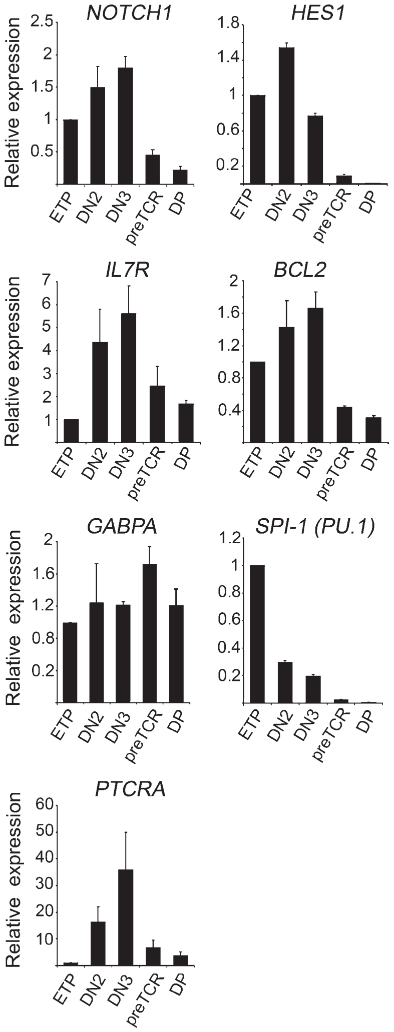

Figure 4. Regulated gene expression in early human T cell development. Expression of the indicated genes was analyzed by real-time quantitative PCR using specific Taqman probes. Total RNA was isolated from sorted human thymocyte cell subsets representative of successive developmental stages: ETP (CD34$^+$CD1a$^-$CD33$^+$), DN2 (CD34$^+$CD1a$^+$), DN3 (CD4$^+$CD3$^-$), pre-TCR (CD4$^+$CD8$^+$ pre-TCR$^+$), and DP (CD4$^+$CD8$^+$CD3$^+$ TCR-αβ$^+$). Samples were normalized to the expression of 18S ribosomal RNA. Bar graphs represent means ± SEM of duplicate samples from at least two independent experiments.

Enforced expression of IL-7Rα rescues impaired proliferation of DN thymocytes incapable of Notch1 signaling

To investigate whether restoration of IL-7Rα expression might be sufficient to rescue defective development of Notch-deprived thymocytes, ETPs were transduced with a retrovirus encoding IL-7Rα and GFP, or with a GFP-only vector, and T cell development was then analyzed in an FTOC treated with the GSI compound E (CompE) (36) or in untreated cultures. Because IL-7Rα overexpression on DP thymocytes has been shown to disrupt thymopoiesis in mice as a result of an impaired supply of local IL-7 for DN cells (19), hIL-7 was exogenously provided to our FTOC assays. IL-7Rα overexpression did not significantly affect IL-7–mediated proliferation of thymocytes with intact Notch signaling, as proportions of GFP- and IL-7Rα–transduced thymocytes remained constant throughout culture in GSI-untreated FTOCs (Fig. 6 A). In contrast, proportions of IL-7Rα–transduced cells increased significantly over non-transduced thymocytes in GSI-treated lobes during the first 2 wk of culture (Fig. 6 A), indicating that enforced IL-7Rα expression provided a competitive growth advantage to early developing thymocytes with inactive Notch. Accordingly, ectopic IL-7Rα expression significantly rescued the reduced cell recovery observed during the initial 2 wk of culture in GSI-treated lobes (Fig. 6 B and Fig. S3). Restored cellularity was associated with increased proportions of IL-7Rα$^+$ thymocytes (Fig. 6 C) and elevated numbers of cycling cells

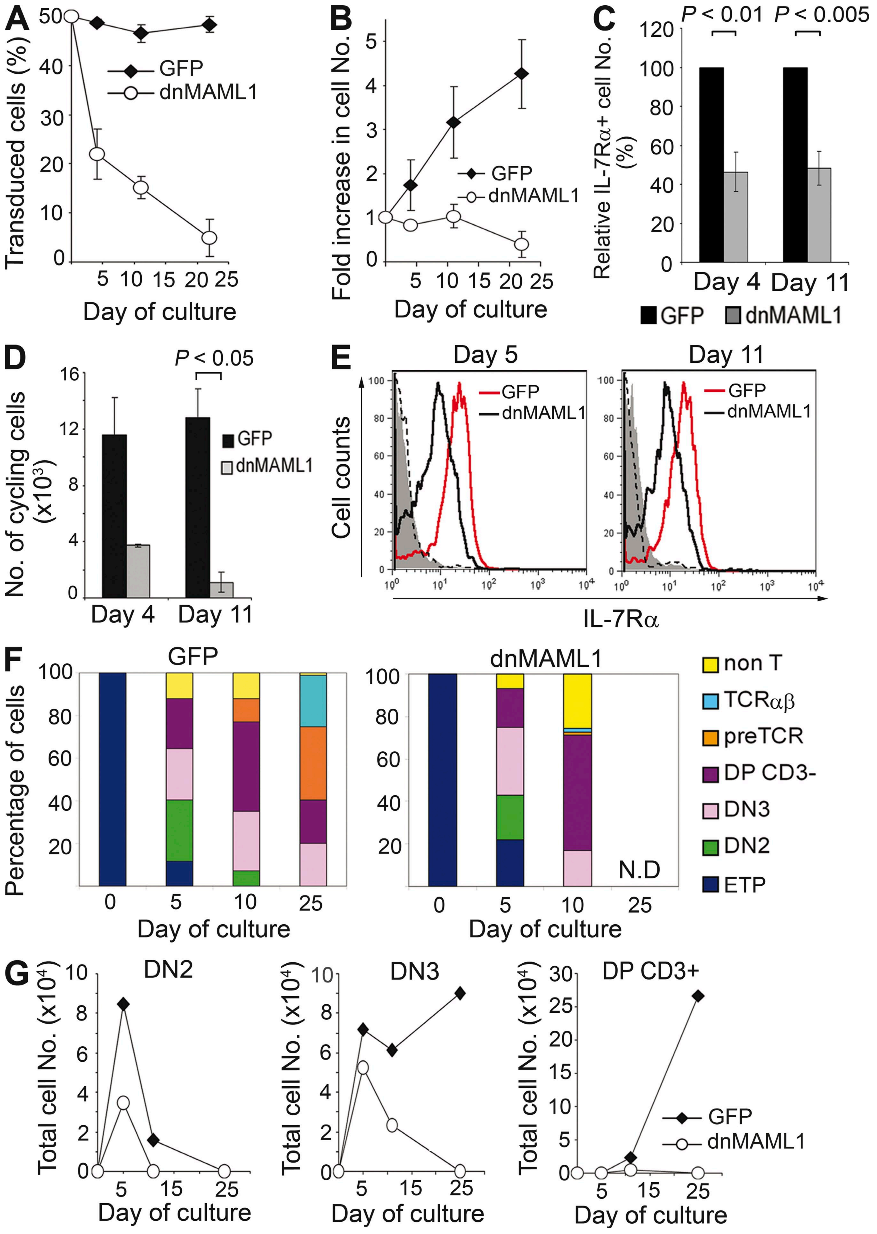

Figure 5. Notch inhibition by dnMAML1 down-regulates IL-7Rα expression and impairs DN progenitor expansion in early human T cell development. Human ETPs were retrovirally transduced with dnMAML1-GFP (dnMAML1) or GFP-only (GFP) vectors and cultured in a FTOC assay. (A) Percentages of electronically gated GFP$^+$- and dnMAML1$^+$-transduced cells recovered at the indicated days were normalized to 50% of transduced cells at day 0. (B) Absolute numbers of GFP$^+$- and dnMAML1$^+$-transduced thymocytes are represented as fold increase normalized to input cell numbers (10^4) of transduced cells. (C) Notch inhibition results in reduced numbers of IL-7Rα$^+$ cells in FTOC. Relative numbers of IL-7Rα–expressing cells generated by days 4 and 11 of FTOC were determined on electronically gated GFP$^+$- and dnMAML1$^+$-transduced cells and normalized to 100% expression in GFP-transduced controls. (D) Absolute numbers of cells in S-G2-M phases of cell cycle were determined by DRAQ5 staining on gated GFP$^+$- and dnMAML1$^+$-transduced cells. Results in A–D are means ± SEM of at least three independent experiments. (E) Surface IL-7Rα expression levels analyzed by flow cytometry on electronically gated GFP$^+$ and dnMAML1$^+$ cell progenies generated by days 5 and 11 of FTOC. Background fluorescence (shaded) was determined with an irrelevant isotype-matched antibody. (F) Percentages of thymocyte cell subsets generated from ETPs were calculated on gated GFP$^+$- and dnMAML1$^+$-transduced cells at the indicated times of FTOC. Non-T refers to CD13$^+$ or CD56$^+$ cells. ND, not determined because of low cell recovery. (G) Total numbers of DN2, DN3, and DP CD3$^+$ thymocytes generated in F. Results in E–G are representative of at least three independent experiments.

(Fig. 6 D) and with a rescued production of DN2 and DN3 thymocytes (Fig. 6 E). Still, levels of apoptosis or expression of Bcl2 remained unchanged regardless of IL-7Rα ectopic expression in GSI-treated lobes (Fig. S3), thus confirming that proliferation, rather than survival, is compromised in the absence of Notch signaling before the β-selection checkpoint. Thereafter, however, enforced IL-7Rα expression was unable to rescue Notch-deprived thymocytes from GSI-induced

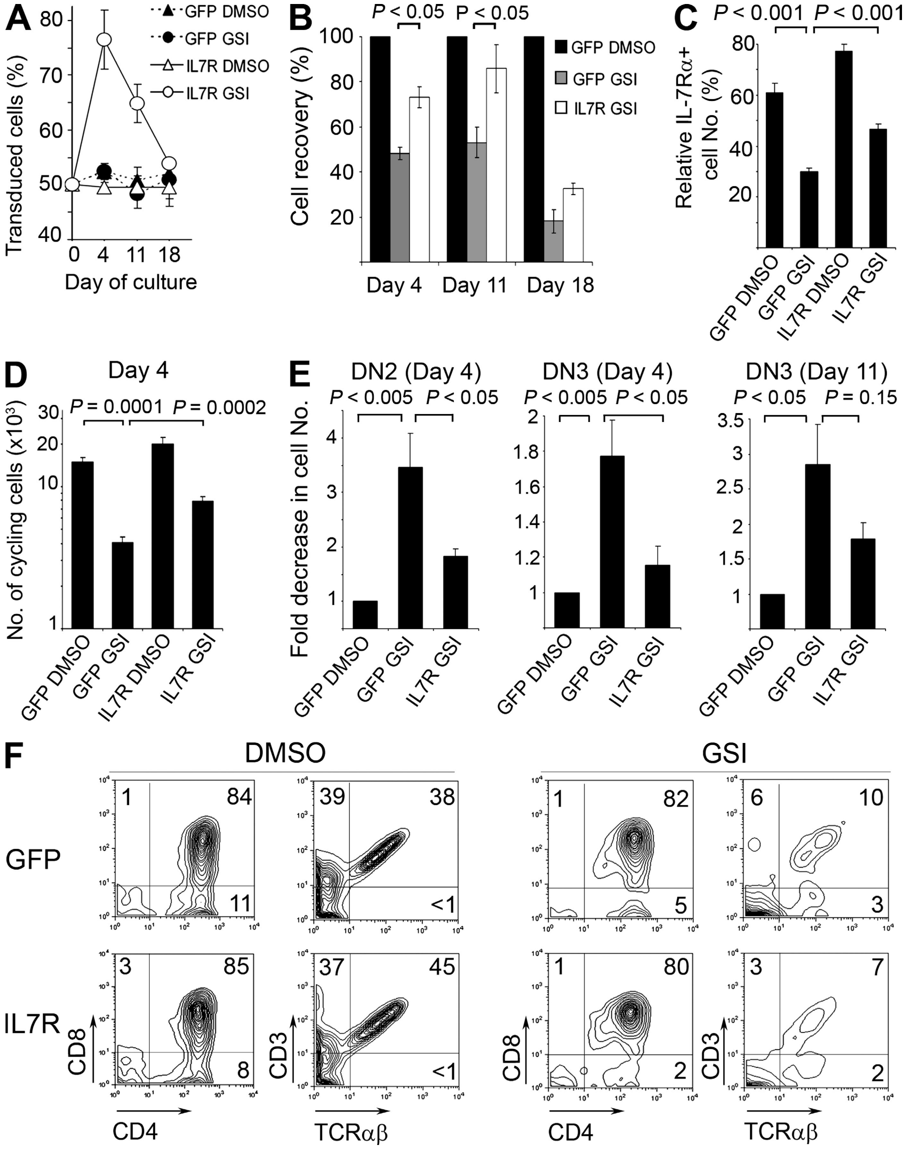

Figure 6. Ectopic IL-7Rα expression rescues defective proliferation of early DN thymocytes incapable of Notch signaling but cannot substitute for Notch at the β-selection checkpoint. Human ETPs transduced either with a retroviral vector encoding IL-7Rα and GFP or with a GFP-only vector were cultured in an FTOC assay supplemented with recombinant human IL-7 and either the GSI CompE or DMSO vehicle. (A) Percentages of electronically gated GFP$^+$- and IL-7Rα^+–transduced cells recovered at the indicated days were normalized to 50% of transduced cells at day 0. (B) Relative cell numbers of electronically gated GFP$^+$- and IL-7Rα^+–transduced cells recovered from GSI-treated FTOCs were normalized to 100% cell recovery of GFP$^+$-transduced control thymocytes in DMSO-treated FTOCs. (C) Percentages of IL-7Rα–expressing cells were determined by flow cytometry on electronically gated GFP$^+$- and IL-7Rα^+–transduced cells by day 11 of FTOC. (D) Numbers of cells in S-G2-M phases of cell cycle were determined on gated GFP$^+$- and IL-7Rα^+–transduced cells by day 4. (E) Relative production of DN2 and DN3 thymocytes was determined by flow cytometry on gated GFP$^+$- and IL-7Rα^+–transduced cells at the indicated days of FTOC. Data are represented as fold reduction of absolute numbers of GFP$^+$ and dnMAML1$^+$-transduced thymocytes in GSI-treated FTOCs normalized to numbers of control GFP$^+$ cells in DMSO-treated FTOCs. Results in A–E represent means ± SEM of three independent experiments. (F) Flow cytometry of CD4 versus CD8 and TCR-αβ versus CD3 expression was performed on gated GFP$^+$- and IL-7Rα^+–transduced thymocytes by day 19 of FTOC. Numbers in quadrants indicate percentage of positive cells. Total cell recoveries from 2×10^4 input cells per lobe were 203,290 and 45,074 GFP$^+$ cells in DMSO- and GSI-treated lobes, respectively, and 177,345 and 55,254 IL-7Rα^+ cells in DMSO- and GSI-treated lobes, respectively. Results from one out of three independent experiments are shown.

apoptosis, and absolute cell numbers dropped abruptly along the third week of FTOC (Fig. S3). This effect concurs with a profound developmental block at the β-selection checkpoint, marked by the impaired production of TCR-$\alpha\beta^+$ DP thymocytes (Fig. 6 F) and the aberrant generation of DP $CD3^-$ thymocytes lacking TCR-βic (Fig. S2). Collectively, these results demonstrate that ectopic expression of IL-7Rα can restore proliferation of Notch-deprived thymocytes placed upstream of β selection but cannot substitute for Notch signaling at the β-selection checkpoint.

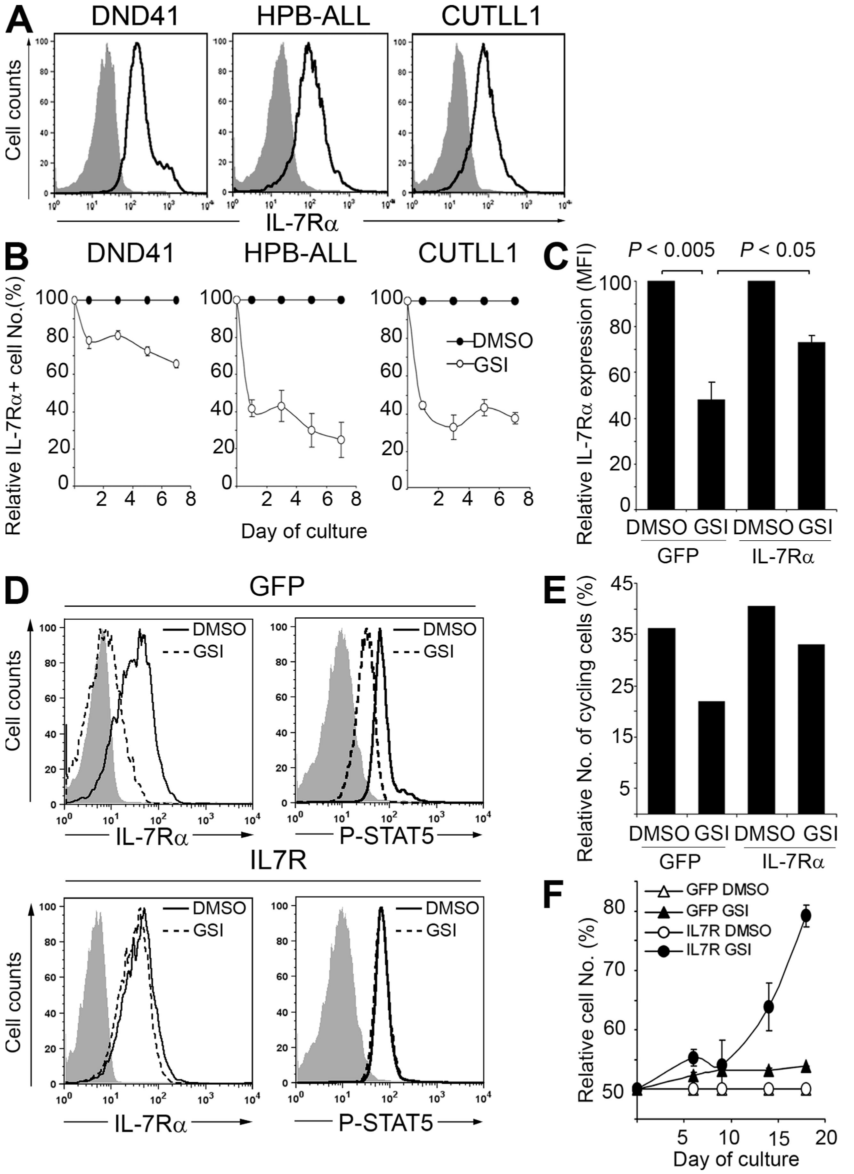

Figure 7. Notch1 regulates IL-7Rα expression and IL-7–dependent proliferation in T-ALL. (A) Surface IL-7Rα expression analyzed by flow cytometry on DND41, HPBALL, and CUTLL1 T-ALL cell lines. Background fluorescence (shaded) was determined with an irrelevant isotype-matched antibody. (B) Percentages of IL-7Rα–expressing T-ALL cells cultured with the GSI CompE were determined by flow cytometry and normalized to 100% IL-7Rα$^+$ cells recovered from DMSO-treated controls at the indicated times. (C) Relative IL-7Rα expression levels on CUTLL1 cells transduced either with a retrovirus encoding IL-7Rα and GFP or with a GFP-only vector were determined by flow cytometry upon culture with either GSI CompE or DMSO for 10 d. MFI values were normalized to IL-7Rα expression values on GFP-transduced CUTLL1 cells treated with DMSO. (D) Relative protein level (left) and function (right) of IL-7Rα receptors expressed on CUTLL1 cells transduced and cultured as in C were determined by flow cytometry after surface staining of IL-7Rα and intracellular staining of phosphorylated STAT5 with specific mAbs. Background fluorescence (shaded) was determined with irrelevant isotype-matched mAbs. (E) Relative numbers of cells in S-G2-M phases of cell cycle from a representative experiment in (C) were determined on gated GFP$^+$- and IL-7Rα$^+$–transduced CUTLL1 cells by day 18. (F) Percentages of GFP$^+$ and IL-7Rα$^+$ CUTLL1 cells transduced and cultured as in C were normalized to 50% of transduced cells at day 0. Data in B, C, and F are means ± SEM of at least three independent experiments. Results in D and E are from one of three independent experiments performed on different days.

Notch1 regulates IL-7Rα expression and IL-7–dependent proliferation in T cell leukemias

Similar to normal immature thymocytes, leukemic blasts from T cell acute lymphoblastic leukemia (T-ALL) patients can express functional IL-7Rs that support proliferation in response to IL-7 (37). Because gain-of-function mutations in Notch1 are common in T-ALL (38), we decided to investigate whether Notch signaling also controls IL-7Rα expression in T-ALLs. To this end, we analyzed the ability of CompE to inhibit IL-7Rα expression in three GSI-sensitive T-ALL cell lines (DND41, HPB-ALL, and CUTLL1) that display constitutive IL-7Rα expression (Fig. 7 A). As previously described (36), CompE treatment resulted in Notch1 inhibition and impaired proliferation of T-ALLs (Fig. S4). Notably, these effects paralleled a gradual down-regulation of IL-7Rα expression, which resulted in an up to 70% reduction of cells expressing IL-7Rα during the first week of treatment (Fig. 7 B). Similar results were obtained using additional T cell lines including SupT1 (Fig. 2), Jurkat (Fig. S1), and Peer (not depicted). Therefore, Notch signaling controls IL-7Rα expression in T-ALLs.

To assess whether IL-7Rα expression is relevant to T-ALL proliferation independently of Notch, we analyzed responsiveness to IL-7 of GSI-treated CUTLL1 cells transduced with *IL7R*. As shown in Fig. 7 C, *IL7R* transduction significantly restored surface IL-7Rα expression to levels sufficient to rescue diminished STAT5 phosphorylation of GSI-treated T-ALLs (Fig. 7 D) and to support IL-7–induced proliferation, as indicated by the increased proportions of cycling cells (Fig. 7 E). Moreover, ectopic IL-7Rα expression provided a competitive growth advantage to T-ALL cells with impaired Notch signaling in response to IL-7, as proportions of IL-7Rα–transduced cells increased significantly over nontransduced cells throughout culture, as compared with GFP-transduced controls (Fig. 7 F). Collectively, these results demonstrate that the regulation of IL-7Rα expression downstream of Notch1 is not restricted to normal developing thymocytes but is also common to human T-ALL cells. Moreover, they indicate that IL-7R signaling is important for proliferation of Notch-dependent T-ALL cells, suggesting that cooperation between Notch1 and the IL-7R pathway may play a fundamental role in the pathophysiology of T-ALLs.

DISCUSSION

Notch1 and IL-7R signaling are both critical in early T cell development (27), but a functional relationship between both pathways has not been established. Supporting such a direct link, in this paper we identified IL-7Rα as a new transcriptional Notch1 target and showed that IL-7Rα expression is regulated by Notch1 in a T-lineage– and developmental stage–specific manner. We also provided evidence that developmental regulation of IL-7Rα expression by Notch1 during human thymopoiesis is critical to controling expansion of the early T cell progenitor compartment in response to IL-7. Moreover, we found that active Notch1 also regulated IL-7Rα expression and IL-7–dependent proliferation of human T-ALLs, suggesting that cross talk of both pathways may be relevant for leukemogenesis.

Active Notch1 was shown to specifically transactivate the *IL7R* promoter in a CSL/MAML-dependent manner. This finding provides the molecular basis for understanding the differential transcriptional regulation of *IL7R* expression in T and B cell lineages and offers new insights into the dynamic regulation of IL-7Rα expression during thymopoiesis. In mouse B cell development, IL-7Rα expression is regulated by two Ets transcription factors, PU.1 and GABP, which appear to function sequentially in a developmental stage–specific manner (21, 24, 25). PU.1 is required as well for survival of early thymic immigrants, but PU.1 down-regulation is obligatory for T cell specification and progression in the T cell lineage (22). We show in this paper that PU.1 down-regulation concurs with up-regulation of Notch activity in human thymopoiesis, as shown in mice (20), and restriction of PU.1 function by Notch1 appears to be a particular aspect of T-lineage specification in mice (39). It is thus possible that IL-7Rα expression is initially supported by PU.1 in early thymic immigrants but needs to be maintained after T-lineage specification by Notch1. Alternatively, *IL7R* transactivation in the thymus may be specifically induced only after T cell specification downstream of Notch1 because the earliest thymus precursors still lack IL-7Rα (5, 27). Supporting a direct role of Notch1 in *IL7R* transcription de novo, we showed that induction of IL-7Rα expression in CB multipotent progenitors was critically regulated by Notch1 signaling. In any of these scenarios, factors other than Notch1 may contribute to sustain IL-7R expression in more mature T cell–committed thymocytes, as Notch1 activity is drastically down-regulated in post–β-selected thymocytes before transition to the DP stage. In this regard, Xue et al. (23) have shown that GABP regulates *IL7R* expression in mouse developing thymocytes and that it is required for a normal DN to DP transition after β selection (24). Because *GABPA* mRNA expression is maintained at high levels throughout human thymopoiesis, and particularly in pre-TCR$^+$ and DP thymocytes with down-regulated Notch (Fig. 4), it is possible that GABP is actually contributing to *IL7R* gene expression in β-selected thymocytes also in humans. Thus, GABP could act in concert with lineage-specific *IL7R* regulators to control stage-specific *IL7R* expression in human thymopoiesis, as occurs during B cell development in mice (24, 25).

Defective proliferation of Notch-deprived thymocytes in our FTOC assays could be rescued by enforced expression of IL-7Rα. This was a stage-specific effect restricted to thymocytes within the early DN progenitor compartment, but IL-7Rα failed to replace Notch1 signals at the β-selection checkpoint, when cell survival requires a proper pre-TCR function (8). Therefore, crucial checkpoints controlling cellular expansion in human thymopoiesis are independently set by the signaling functions of the IL-7R and the pre-TCR, as proposed in mice (40), and both seem highly dependent on Notch1 activity. Indeed, we show in this paper that Notch1 controls IL-7R–dependent proliferation of the DN progenitor pool, and Maillard et al. (41) recently demonstrated an absolute requirement of Notch for cell survival/proliferation during β selection in vivo that was independent of the pre-TCR, as was previously shown in vitro (9). Thus, Notch and pre-TCR should act in parallel pathways that synergize during β selection (41). Still, TCR-β rearrangement and/or expression could be Notch dependent because DN4-like thymocytes lacking TCR-βic accumulated in Notch1-deficient mice (42) as well as in our dnMAML1$^+$ and GSI-treated FTOCs. Collectively, we can propose that, besides the conventional roles reported for Notch1 as a commitment factor very early in thymopoiesis and as a trophic factor during β selection, Notch1 serves a more unconventional role as a regulator of IL-7 responsiveness and T cell progenitor expansion before acquisition of the pre-TCR. Thus, the two main phases of cellular growth characterized in postnatal thymic lymphopoiesis, involving either the IL-7R or the pre-TCR, are independently impacted by Notch1 signals.

Our gene expression analyses support the idea that the exquisite stage-specific dependence of IL-7 during thymopoiesis is the result of the coordinated regulation of Notch1 activity and IL-7Rα expression, and a similar mechanism can be inferred from available data in mice (6, 20). We found that Notch target genes and *IL7R* simultaneously reached maximal expression at the DN2 and DN3 stages, and both became down-regulated before transition to the DP stage. Accordingly, maximal IL-7 responsiveness and massive expansion occurs in vivo at the DN2 to DN3 transition (10, 11, 13), whereas developing thymocytes become insensitive to IL-7 between the β-selection and positive selection checkpoints (18). Besides transcriptional regulation, active suppression of cytokine signal transduction ensures termination of IL-7R signals required for progression to the DP stage in mice, and then IL-7Rα expression and signaling are restored by positive selection (17). Such a strict control may be necessary to avoid IL-7–mediated survival/proliferation signals in preselection DP thymocytes and to escape from overactivity of a cytokine receptor, which can contribute to thymocyte malignancy (43). Our observation that ectopic IL-7Rα could rescue the growth arrest induced by Notch deprivation not only in normal thymocytes but also in T-ALLs is, thus, remarkable. Importantly, IL-7R signaling significantly contributes to T-ALL proliferation by activation of PI3K (37), and constitutive PI3K activation has recently been shown to induce resistance to Notch1 inhibition in T-ALL (36). Therefore, downstream effectors of IL-7R, such as PI3K, represent suitable molecular targets for therapeutic intervention. Overall, these results implicate IL-7Rα as a major regulator for cell cycle progression induced by Notch1 in early human thymopoiesis and support a cooperative role between Notch1 and IL-7Rα in leukemogenesis that deserves further studies.

It is currently believed that dynamic regulation of IL-7Rα expression determines efficient responses to limited amounts of IL-7 locally supplied by the thymic microenvironment (19). In mice made transgenic for Lunatic Fringe, a modulator of Notch activation, DN thymocytes must continuously compete for limiting Notch1 expansion signals in vivo (44). We can thus propose that by regulating lineage- and stage-specific expression of IL-7Rα, Notch could serve a crucial role devoted to enhancing competitiveness for limiting IL-7 production in the thymus and also in the bone marrow niches, which would finally result in selective expansion of ETPs and leukemic blasts under physiological and pathological conditions, respectively.

MATERIALS AND METHODS

Thymus and CB precursor isolation and flow cytometry. Experiments were performed, and thymus and CB samples were obtained, in accordance with procedures approved by the Consejo Superior de Investigaciones Científicas (CSIC) Bioethics Committee. Informed consent was obtained in accordance with the Declaration of Helsinki. ETPs, DN2, and DN3 thymic progenitors were isolated using the Dynal CD34 selection system (Invitrogen) in combination with cell sorting using a FACS Vantage SE (BD). DP subsets (CD3$^+$ TCR-αβ$^+$ and CD3$^+$ pre-TCR$^+$) and CB CD34$^+$ progenitors were selected using Percoll density gradients (Thermo Fisher Scientific) and magnetic cell sorting (AutoMACS; Miltenyi Biotec) as previously described (35).

Antibodies used were the following: CD1a-PE, CD4-PE-Cy5, CD13-PE-Cy5, CD33-PE-Cy5, CD34-PE-Cy5, CD56-PE-Cy5, IL-7Rα –PE, and TCR-αβ–PE-Cy5 (Beckman Coulter); CD3-PE, γc-biotin, CD34-FITC, Bcl2-PE, IL-7Rα, and goat anti–mouse IgGs-APC (BD); and CD8-PE (Invitrogen). TCR-βic expression was assessed using the Cytofix Cytoperm kit (BD) and the βF1 mAb (provided by M. Brenner, Brigham and Women's Hospital, Boston, MA). Intracellular expression of PSTAT5 was assessed after paraformaldehyde/methanol fixation and PSTAT5–Alexa Fluor 647 staining according to the manufacturer's instructions (BD). DRAQ5 (Enzo Biochem, Inc.) was used for cell cycle analysis. Staining with biotin-coupled Annexin V (Roche) plus Streptavidin-PE (Invitrogen) and 7-AAD (BD) was used for apoptosis analysis. Flow cytometry was performed in a FACSCalibur (BD). Irrelevant isotype-matched antibodies (Invitrogen) were used as controls.

Retrovirus constructs and retroviral infections. Retrovirus vectors encoding the ICN1 Notch1 domain and GFP from a bicistronic transcript (MigR1-ICN1), GFP alone (MigR1-GFP) (26), and the dnMAML1 fused to GFP (MigR1-dnMAML1) (32) were provided by J.C. Aster (Brigham and Women's Hospital, Boston, MA). Full-length human IL-7Rα complementary DNA was cloned into the EcoRI site of MigR1-GFP. Viral supernatant production and retroviral infections were performed as previously described (35). Phoenix (Ampho) packaging cells were provided by G. Nolan (Stanford University School of Medicine, Stanford, CA) and H. Spits (University of Amsterdam Academic Medical Center, Amsterdam, Netherlands).

FTOC assays and cell cultures. FTOC assays were performed as previously described (34). In brief, thymic lobes from 14.5-d-old Swiss mouse embryos were treated with deoxyguanosine (d-Guo; Sigma-Aldrich) and cocultured with transduced human ETPs (1–2 × 10^4 cells/lobe). For inhibition of Notch1 signaling, the GSI CompE (Enzo Biochem, Inc.) was added to FTOCs at a final concentration of 100 nM. DMSO vehicle was used as control. When indicated, FTOCs were supplemented with 200 IU/ml of recombinant human IL-7 (National Institute of Biochemical Standards and Controls). Animal procedures were approved by the Institutional Animal Care Committee.

Human ETPs and CB CD34$^+$ progenitors transduced with either ICN1-GFP or GFP-only vectors were cultured with multilineage-supportive cytokines as previously described (27). GFP- and dnMALM1-transduced CD34$^+$ CB cells were cocultured with OP9-DL1 stroma as reported (29). T-lineage cell lines (Jurkat, CUTLL1, HPB-ALL, and SupT1) and pre-B cell lines (REH, NALM6 [both provided by A. de la Hera and E. Sanz, University of Alcalá, Madrid, Spain], and HPB-NULL [provided by W. Schamel, Max Planck Institute for Immunobiology, University of Freiburg, Freiburg, Germany]) were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FCS.

Quantitative PCR. Real-time PCR quantification of complementary DNA synthesized from TRIzol-extracted (Invitrogen) total RNA using oligo (dT) primers (Roche) was performed using TaqMan Gene Expression Assays (Applied Biosystems), according to the manufacturer's instructions, in a ABI PRISM 7900 HT Sequence Detection system (Applied Biosystems).

Luciferase reporter constructs and luciferase assays. A 2-kb fragment encoding the 5′ upstream regulatory region of human *IL7R* (NM_002185) was amplified by PCR using the Pfu Turbo polymerase system (Agilent Technologies) and cloned in the KpnI and XhoI sites of pGL3Basic luciferase reporter vector (Promega). Site-directed mutagenesis in the CSL-binding site was performed using specific primers (Table S1) and conventional PCR techniques.

For luciferase reporter assays, Jurkat cells were cotransfected by electroporation with the *IL7R* luciferase reporter vector and MigR1-GFP, MigR1-ICN1, and/or MigR1-dnMAML1 plus the constitutively active *Renilla reniformis* luciferase-producing vector prL-CMV (Promega). 293T cells and RBP-Jk$^{+/-}$ or RBP-Jk$^{-/-}$ MEFs (33) were cotransfected by calcium phosphate or by lipofection (Lipofectamine Reagent; Invitrogen), respectively, with the *IL7R* luciferase reporter vector and MigR1-GFP or MigR1-ICN1, plus the prL-CMV *Renilla* vector. Firefly and *Renilla reniformis* luciferase activities were determined in triplicates using the Dual Luciferase Reporter Assay system (Promega) in a Berthold Sirius luminometer and expressed as fold induction relative to transfection with control plasmids.

ChIP. Cells were fixed with 1% paraformaldehyde at room temperature for 15 min. The reaction was stopped by adding glycine up to 0.125 M, and cells were washed in PBS and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl, and protease inhibitor cocktail [Roche]). Lysates were sonicated and diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl, 167 mM NaCl, and protease inhibitors). Polyclonal antibodies against either the C-terminal domain of Notch1 or PU.1 (Santa Cruz Biotechnology, Inc.) were used to label Notch–DNA or PU.1–DNA complexes. Goat or rabbit antibodies were used, respectively, as controls. Immune complexes were precipitated with protein A–agarose and eluted with 0.1 M $NaHCO_3$ and 1% SDS. DNA was extracted using phenol/chloroform after treatment with 20 µg/ml of proteinase K. Unbound chromatin (input) and immunoprecipitated DNA samples were analyzed by semiquantitative PCR with primers amplifying the CSL-binding site either of *HES1* or *IL7R* promoters or the PU.1-binding site (Table S1).

Statistics. Statistical significance was determined with the two-tailed Student's *t* test, with the α level set at 0.05.

Online supplemental material. Fig. S1 shows the specific regulation of *IL7R* gene expression by Notch1 in T-lineage, but not B-lineage, cell lines, and the analysis of constitutive expression of active Notch1 in SupT1 pre-T cells. Fig. S2 shows that inhibition of Notch signaling results in the specific blockade of human intrathymic T cell development at the β-selection checkpoint. Fig. S3 shows that ectopic expression of IL-7Rα rescues impaired proliferation of Notch-deprived thymocyte progenitors placed upstream of β selection but it cannot substitute for Notch signaling at the β-selection checkpoint. Fig. S4 shows the inhibition of Notch1 activation and the impaired proliferation of T-ALL cells treated with the GSI CompE. Table S1 shows the oligonucleotide primers used for RT-PCR, CSL-binding site-directed mutagenesis, and ChIP assays. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081922/DC1.

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