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Repression of Ccr9 Transcription in Mouse T Lymphocyte Progenitors by the Notch Signaling Pathway

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The chemokine receptor CCR9 controls the immigration of multipotent hematopoietic progenitor cells into the thymus to sustain T cell development. Postimmigration, thymocytes downregulate CCR9 and migrate toward the subcapsular zone where they recombine their TCR β-chain and γ-chain gene loci. CCR9 is subsequently upregulated and participates in the localization of thymocytes during their selection for self-tolerant receptor specificities. Although the dynamic regulation of CCR9 is essential for early T cell development, the mechanisms controlling CCR9 expression have not been determined. In this article, we show that key regulators of T cell development, Notch1 and the E protein transcription factors E2A and HEB, coordinately control the expression of Ccr9. E2A and HEB bind at two putative enhancers upstream of Ccr9 and positively regulate CCR9 expression at multiple stages of T cell development. In contrast, the canonical Notch signaling pathway prevents the recruitment of p300 to the putative Ccr9 enhancers, resulting in decreased acetylation of histone H3 and a failure to recruit RNA polymerase II to the Ccr9 promoter. Although Notch signaling modestly modulates the binding of E proteins to one of the two Ccr9 enhancers, we found that Notch signaling represses Ccr9 in T cell lymphoma lines in which CCR9 transcription is independent of E protein function. Our data support the hypothesis that activation of Notch1 has a dominant-negative effect on Ccr9 transcription and that Notch1 and E proteins control the dynamic expression of Ccr9 during T cell development. The Journal of Immunology, 2015, 194: 000–000.
α-chain (19), and *Hes1*, which encodes a transcriptional repressor that limits myeloid potential in ETPs and promotes the survival of DN3 cells (20, 21). The ICN/CSL/MAML complex also activates the transcription of *Tcf7*, which encodes the T cell–specification transcription factor TCF1 (22, 23). Genomic mutations that affect the Notch signaling pathway play a major role in the development of both human and mouse T cell leukemia (24–30). These mutations can be divided into at least two classes: those that lead to the ligand-independent activation of Notch1 and those that stabilize the active form of Notch1. The second category includes mutations in the PEST domain of Notch1 and mutations in the E3 ligase Fbw7, both of which inhibit the rapid degradation of ICN1 by the ubiquitin-proteasome pathway (31). Although many targets of the Notch signaling pathway have been identified, a complete understanding of how this pathway mediates its many biological functions in the thymus has not been achieved.

Notch signaling was proposed to antagonize either the expression or DNA-binding activity of the E protein transcription factors (32–34), which play a critical role in B lymphocyte specification (35). However, the interactions between Notch1 and E proteins are not explained by a simple model in which Notch signaling cannot be explained by a simple model in which Notch signaling subsides. In DP thymocytes, E2A and HEB form dimers that control survival, induce *Hes1* expression in T cell progenitors (39). Therefore, the interaction of E proteins and Notch 1 in immature DN thymocytes cannot be explained by a simple model in which Notch signaling inhibits either the expression or DNA binding of E proteins. The E proteins encoded by the *E2A*/*Tcf3* genes, E12 and E47, promote the development of the lympho-myeloid and common lymphoid progenitors that seed the thymus and are required for the upregulation of *Ccr9* in these cells (38, 40, 41). Another E protein, HEB, is upregulated as ETPs commit to the T cell lineage, and HEB expression peaks at the double-positive (DP) stage (42), after Notch signaling subsides. In DP thymocytes, E2A and HEB form dimers that control survival, induce *Tcrα* rearrangement, and enforce positive selection (43).

In this study, we investigated a role for the Notch1 signaling pathway in the regulation of *Ccr9* in primary T cell progenitors and in Notch1-dependent T cell lymphomas. We show that Notch signaling represses *Ccr9* transcription through the canonical pathway involving the MAML coactivator protein. Notch signaling prevents the recruitment of the histone acetyltransferase p300 and the acetylation of histones at two putative enhancers upstream of *Ccr9* and prevents the recruitment of RNA polymerase (Pol)II to the *Ccr9* promoter. We show that the E protein transcription factors promote CCR9 expression at multiple stages of T cell development and that E2A and HEB bind to the putative enhancers upstream of *Ccr9* in primary cells and lymphoma cell lines. The opposing effects of Notch1 and the E protein transcription factors at the *Ccr9* gene are not mediated by a global effect of Notch1 on either E protein expression or DNA binding, because E proteins bind to one of the two Notch-regulated enhancers, even in the presence of Notch signals. Our data reveal a negative regulatory function for the Notch1 signaling pathway at the *Ccr9* gene and indicate that the integration of Notch signaling and E protein functions cooperatively to guide T cell progenitor migration and T cell development.

Materials and Methods

**Mice**

Mice were housed at The University of Chicago, and all procedures were approved by The University of Chicago Institutional Animal Care and Use Committee. Female liver (FL) MPPs (Ter119+Gr1+CD117+CD272+ cells) were isolated from embryonic day (E)13.5–14.5 embryos derived from timed breedings of C57BL/6 mice. *E47/−* mice were described previously and were on an FvB/N background (44). *Cd4Cre;Hebf/f* mice were on a C57BL/6 background and were described previously (45).

**Isolation and treatment of primary cells and cell lines**

The 531026 T cell lymphoma cell line was described previously (46). The E13.5–E14.5 FL MPPs used to initiate the in vitro cultures were isolated by magnetic bead depletion (MACS, Miltenyi Biotec, San Diego CA) of Gr1+ and Ter119+ cells, followed by flow-assisted cell sorting for CD117+ CD272+ cells. The primary cells were cultured for 7 d on OP9–DL1 stromal cells (plated at 1.5 × 105 cells/well of a six-well plate, 24 h before use) in OPTI-MEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 29.2 mg/ml glutamine, and 80 mM 2-ME; Flt3L (5 ng/ml; PeproTech); IL-7 (1:100 dilution of culture supernatant from the J558-IL7 cell line); and kit ligand (5 ng/ml; PeproTech) prior to analysis. The cells were cultured at 37°C in a humidified incubator with 5% CO2 DMSO (vehicle control) or γ-secretase inhibitor (10 μM) (DAPT, Sigma-Aldrich) were included for the indicated time. The cell lines were cultured in OPTI-MEM supplemented as above, but lacking IL-7, Flt3L, and kit ligand.

**Retroviral transduction**

MigR1, MigR1-ICN, MigR1-Δnop-ICN (DNMAML), and MigR1-HES1 retroviral vectors were described previously (33, 47). Retroviral supernatants were produced in Plat-E cells using the Fugene 6 transfection reagent per the manufacturer’s instructions (Promega). The cells were transduced by spin inoculation, as previously described (26).

**Flow cytometry**

Flow cytometry was performed on a Fortessa flow cytometer (BD Biosciences) using FACSDiva software and analyzed using FlowJo (TreeStar). The cells were sorted on a FACSAria using FACSDiva software.

The bone marrow cells, thymocytes, FL MPPs, and 531026 cells were isolated and stained with Abs from BD Biosciences or eBioscience. Nonpermeabilized Ab staining was blocked by incubating the cells with a CD16/CD32 Ab for 10 min. The cells were then stained with biotin-conjugated Abs and fluorescently labeled Abs and stained with PerCP-Cy5.5-conjugated streptavidin for 15 min. The cells were washed with FACS buffer and resuspended in 500 μl FACS buffer containing propidium iodide to allow dead cells to be excluded from the analysis. The Abs used included CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), TCRβ (H5-597), TCRγδ (UC7-13D5), NK1.1 (PK136), CD11c (HL3), Ter-119 (Ter-119), CD11b (M1/70), Gr1 (RB6-8C5), B220 (RA3-6B2), CD19 (1D3), CD25 (PC6.2), CD121 (CD5, 5B4), Sca-1 (D7), Flt3 (A2F1D), IL-7Rα (A7R34), and CCR9 (CW1.2). The lineage mixture included CD8, CD3ε, TCRβ, TCRγδ, NK1.1, CD11c, Ter-119, CD11b, Gr1, B220, and CD19.

**Quantitative real-time PCR**

The RNA from 5,000–20,000 FACS-sorted primary cells was extracted using the RNeasy Micro Kit (QIAGEN), per the manufacturer’s protocol. The RNA from 500,000–5 × 10^6 T lymphoma cells was extracted using TRIzol reagent, per the manufacturer’s protocol (Invitrogen). Quantitative real-time PCR (QPCR) reactions contained 1 μl cDNA or chromatin immunoprecipitation (ChIP) DNA, gene-specific primers, and SYBR Green Master Mix (Bio-Rad) in a total volume of 25 μl. The amplification was performed in a MyiQ iCycler (Bio-Rad). The data were analyzed in Microsoft Excel using the ΔΔCT method with *Hprt* as a reference gene for normalization. The primers used were *Ccr9* forward: 5′-CAATA TCG TGG ATG AGC TCA TAA AAC AAC-3′; *Ccr9* reverse: 5′-ACC AAA AAC AAC CAA CTG CCG CG-3′; *Hprt* forward: 5′-ACC AAA AAC AAC CAA ACT TGT CCT GA-3′; *Hprt* reverse: 5′-ACC CCA AAC CCT TTT AGC-3′. The data for the ChIP samples are displayed for normalization. The primers used were *Ccr9* forward: 5′-TCG ATGG TGG TTT GCA GGA TGA-3′; *Hprt* forward: 5′-TCC TGA CCG CAA ACT TTC-3′; *Hprt* reverse: 5′-AGG GGA TGA CAC TGG TTC AGG-3′; and *Del1* exon forward: 5′-ATCG ATG TGG TTC GGA GTG-3′, *Del1* exon reverse: 5′-CCT CAT AGC CAG ATG TCT TGA ACC-3′; the data for the ChIP samples are displayed relative to the signal for input DNA. The primers used were −88.5 kb forward: 5′-AGT CCA TAC TAC GGA CAC GAG G-3′; −88.5 kb reverse: 5′-ATCG GCC CAA GAA ACT GAA CAC CAA GAC G-3′; −13 kb forward: 5′-AGA TGG CCT TGG AGC ACC AG-3′; −13 kb reverse: 5′-GGC CCT CAG CAT TAC GCT AC-3′; PI forward: 5′-ACC CTG GTG GTC ATT AGC TT-3′; PI reverse: 5′-TGG GTT TTC TCA GCA GAC AGC AG-3′; P2 forward: 5′-TAC TCC GCC AGT
Ten million cells were fixed with 1% formaldehyde in 1× PBS at room temperature for 15 min. The reaction was quenched by the addition of 2 M glycine to reach a final concentration of 0.125 M, and the samples were incubated for an additional 5 min at room temperature. The cells were washed three times with 1× PBS containing 1 mM PMSF and 0.1% protease inhibitor mixture and then sonicated to shear the DNA into 400–800-bp fragments. For the histone modification ChIPs, the sonication was performed in 0.8 M RIPA buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.8 M NaCl, 0.1% SDS). The protein–DNA complexes were detected using m-Chromatin immunoprecipitation (ChIP). The complexes were precipitated by an overnight incubation on a rotator with 20 μl Protein A (Millipore)-conjugated magnetic beads. The protein–DNA complexes also were precipitated with Abs against acetyl histone H3 (Millipore: 06-599), trimethyl histone H3 lysine 4 (Millipore: 07-473), and trimethyl histone 3 lysine 36 (Millipore: ABE305) in 1 ml ChIP dilution buffer containing Protein A magnetic beads and were incubated overnight at 4°C with rotation. The protein–DNA complexes were washed multiple times and reverse cross-linked, and the DNA was purified and eluted using a PCR purification kit (QIAGEN), per the manufacturer’s protocol. The specific DNA enrichment was quantified by real-time PCR. ChIP sequencing for HEB was performed with thymocytes from 4-wk-old wild-type (WT) mice, and the enrichment peaks were generated using the MACS algorithm (model-based analysis of ChIP sequencing [ChIP-seq] data) with the appropriate input controls, as described previously (48).

**Results**

The chemokine receptor CCR9 is repressed upon thymic immigration coincident with Notch signaling

We examined CCR9 expression on the surface of MPPs and their thymic progeny using flow cytometry to gain insight into the regulation of CCR9. We found that CCR9 was expressed on a very small fraction of lymphoid-primed MPPs (LMPPs) and on a larger fraction of common lymphoid progenitors (CLPs) (Fig. 1A–D).

However, the ETPs and DN2 cells, which had recently immigrated to the thymus, expressed almost no CCR9 on their surface (Fig. 1E, 1F). Approximately 25% of the thymocytes re-expressed CCR9 at the DN3 stage, a number that coincides with the frequency of cells that have passed through β-selection. The frequency of CCR9-expressing cells increased after the DN4 stage,
and essentially all of the DP cells were CCR9+ (Fig. 1G, 1H). These data demonstrate that CCR9 expression is transiently decreased when Notch signaling is most active in T cell progenitors and led us to hypothesize that Notch signaling controls the expression of CCR9.

**Notch signaling represses Ccr9/CCR9 expression in primary MPPs**

We cultured WT FL-derived MPPs on the stromal cell line OP9-DL1, which expresses the DL1 Notch1 ligand, to determine whether Notch signaling could inhibit the expression of CCR9 on T cell progenitors (49). After 7 d in culture, the CD25+ T lymphocyte lineage-specified and CD25+ MPPs were isolated by flow cytometry and examined for their expression of CCR9 (Fig. 2A). Regardless of their expression of CD25, <11% of the cells expressed CCR9 on their surface (Fig. 2B). In contrast, CCR9 expression was substantially increased when these cells were cultured with a γ-secretase inhibitor (GSI) for the final 48 h of culture (Fig. 2B). In the presence of GSI, 42.7% of CD25+ cells and 47.8% of CD25+ cells expressed CCR9. Ccr9 mRNA levels also increased in the CD25+ and CD25+ populations after treatment with GSI compared with treatment with DMSO (Fig. 2C).

GSIs inhibit the cleavage event that liberates ICN1 (16). However, γ-secretase also can cleave other targets that might impact Ccr9/CCR9 expression. We transduced primary MPPs with a retrovirus encoding a constitutively activated form of ICN1, which is impervious to GSI, to demonstrate that the GSI-induced increases in Ccr9 mRNA and CCR9 protein were a consequence of reduced Notch signaling. Surface CCR9 expression was strongly induced by GSI treatment in the CD25+ and CD25+ cells that were transduced with a control virus (MigR1) (Fig. 2D). In contrast, CCR9 was not induced by GSI treatment in the CD25+ and CD25+ cells that were transduced with a control virus (MigR1) (Fig. 2D). At day 7 of culture, Ccr9 transcription was barely detectable in either the CD25+ or CD25+ cells that were constitutively expressing ICN1, regardless of the presence of GSI (Fig. 2E). The repression of basal Ccr9 transcription in DMSO-treated cells by ectopic ICN1 suggests that the background level of CCR9 detected on these T cell progenitors may be a consequence of insufficient Notch signaling.

Although our data demonstrate that Notch signals can repress Ccr9, they do not demonstrate that Notch signaling is essential for the repression of Ccr9. We transduced the day-5 progeny of WT FL MPPs with a retrovirus encoding DNAMAML, which inhibits ICN/CSL coactivator MAML, or a control virus to determine whether Notch signaling was essential to repress Ccr9 (50–52). In comparison with the cells that were transduced with the control retrovirus, both the CD25+ and CD25+ cells that were transduced with DNAMAML upregulated Ccr9 mRNA and CCR9 protein, even in the absence of GSI (Fig. 2F). Both the frequency and the intensity of CCR9 expression increased in the CD25+ and CD25+ cells that were transduced with DNAMAML (Fig. 2F). Ccr9 transcripts also were increased in CD25+ and CD25+ cells that had been transduced with the DNAMAML retrovirus (Fig. 2G). These data demonstrate that Notch signaling represses Ccr9 mRNA and CCR9 protein expression in the progeny of FL MPPs. Our findings are consistent with the hypothesis that the decreased expression of CCR9 on ETPs following thymic immigration is a consequence of Notch signals.

**CCR9 expression on primary MPPs and thymocytes requires E protein transcription factor activity**

We demonstrated previously that CCR9 expression is E protein dependent in LMPPs (38). We transduced FL MPPs with a retrovirus encoding ID2, an inhibitor of E protein DNA binding, to determine whether the induction of CCR9 on the progeny of primary FL MPPs after the inhibition of Notch signaling also requires E protein activity (35). The transduction of MPPs cultured on OP9-DL1 for 5 d with ID2-producing virus did not influence the frequency of CD25− cells expressing CCR9 after 48 h

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**FIGURE 2.** Notch signaling represses Ccr9 mRNA and CCR9 protein expression in primary T cell progenitors. (A) Gating strategy for identification of Lin−CD45+CD25− and Lin−CD45+CD25+ progeny of FL MPPs after 7 d of culture to which DMSO or GSI (10 μM) was added for the final 48 h. The frequency of cells in each of the indicated gates is shown. (B) Expression of CCR9 on Lin−CD45+CD25− (CD25−) and Lin−CD45+CD25+ (CD25+) cells cultured on OP9-DL1 for 7 d, including a 48-h treatment with DMSO (shaded graphs) or GSI (DAPT, 10 μM; open graphs). The frequency of CCR9+ cells in GSI-treated (black text) and DMSO-treated (gray text) samples is indicated. (C) QPCR analysis for CCR9 in Lin−CD45+CD25− and Lin−CD45+CD25+ FL MPPs cultured in vitro for 7 d, including a 48-h treatment with DMSO or GSI, as in (B). Hprt mRNA was used for normalization. Error bars show SD. (D) FL MPPs were transduced with MigR1 or MigR1-ICN on day 4 of culture and treated with DMSO (shaded graphs) or GSI (open graphs) for 48 h starting on day 5 of culture. CCR9 on Lin−CD45+GFP−CD25− (CD25−) and Lin−CD45+GFP−CD25+ (CD25+) cells was determined in the progeny of FL MPPs treated with DMSO or GSI, as described in (D). Postinfection with MigR1 or MigR1-ICN-CD45+GFP+CD25+ (CD25+) cells cultured on OP9-DL1 for 7 d, including a 48-h treatment with DMSO or GSI, as in (B). Hprt mRNA was used for normalization. Error bars show SD. (D) FL MPPs were transduced with MigR1 or MigR1-ICN on day 4 of culture and treated with DMSO (shaded graphs) or GSI (open graphs) for 48 h starting on day 5 of culture. CCR9 on Lin−CD45+GFP−CD25− (CD25−) and Lin−CD45+GFP−CD25+ (CD25+) cells was determined. (E) QPCR analysis for CCR9 mRNA in the Lin−CD45+GFP−CD25− and Lin−CD45+GFP−CD25+ progeny of FL MPPs treated with DMSO or GSI, as described in (D). Postinfection with MigR1 or MigR1-ICN. (F) CCR9 expression on the Lin−CD45+GFP−CD25− and Lin−CD45+GFP−CD25+ progeny of FL MPPs that were transduced with MigR1 (shaded graphs) or MigR1-DNMAML (open graphs) on day 4 of culture (D) and analyzed on day 7. (G) QPCR analysis for CCR9 mRNA in the Lin−CD45+GFP−CD25− and Lin−CD45+GFP−CD25+ progeny of FL MPPs, as described in (F), postinfection with MigR1 or MigR1-DNMAML. All experiments are representative of more than three. *p < 0.05, **p < 0.01.
There was a slight increase in the frequency of ID2-producing virus–transduced CD25+ cells that expressed CCR9 (Fig. 3A, 3B). Control virus–transduced cells and untransduced cells both strongly upregulated CCR9 expression upon treatment with GSI. In contrast, the induction of CCR9 expression upon GSI treatment was blunted in ID2-expressing cells (Fig. 3A, 3B). The frequency of CCR9-expressing cells was increased ∼5-fold in control virus–transduced CD25− and CD25+ cells by 48 h after GSI treatment. In contrast, the frequency of CCR9-expressing ID2 virus–transduced CD25− cells was increased ∼2-fold. There was almost no increase in the frequency of CCR9-expressing CD25+ ID2 virus–

In comparison with control virus–transduced cells (Fig. 3A, 3B), there was a slight increase in the frequency of ID2-producing virus–transduced CD25+ cells that expressed CCR9 (Fig. 3A, 3B). Control virus–transduced cells and untransduced cells both strongly upregulated CCR9 expression upon treatment with GSI. In contrast, the induction of CCR9 expression upon GSI treatment was blunted in ID2-expressing cells (Fig. 3A, 3B). The frequency of CCR9-expressing cells was increased ∼5-fold in control virus–transduced CD25− and CD25+ cells by 48 h after GSI treatment. In contrast, the frequency of CCR9-expressing ID2 virus–transduced CD25− cells was increased ∼2-fold. There was almost no increase in the frequency of CCR9-expressing CD25+ ID2 virus–

The E protein transcription factors contribute to CCR9 expression on primary T cell progenitors. (A) FACS analysis for CCR9 on CD25− and CD25+ Lin− CD45− GFP FL MPPs cultured in vitro for 3 d prior to infection with MigR1 or MigR1-ID2 retrovirus and treated with DMSO (shaded graphs) or GSI (open graphs) on day 5 for 48 h. The frequency of CCR9+ cells on GSI-treated (black text) or DMSO-treated (gray text) cells is indicated. One of four representative experiments is shown. (B) Average (± SD) percentage of CD25− and CD25+ Lin− CD45− GFP+ cells expressing CCR9 after treatment as in (A) (n = 4). (C) FACS analysis for CCR9 protein on Lin− CD44+ CD117+ CD25− DN4 thymocytes and CD4+ CD8+ DP thymocytes isolated from an E2A+/+ or E2A−/− mouse. One of three experiments is shown. (D) FACS analysis for CCR9 protein on DP thymocytes from a Cd4Cre− or Cd4Cre+ Hebf+/− mouse. In (C) and (D), the shaded histograms represent the isotype control. **p < 0.01, ***p < 0.001.

Identification of E protein binding sites near the Ccr9 gene. (A) Occupancy by the HEB transcription factor, as well as H3K4me1 and H3K4me2, at the Ccr9 locus, as determined by ChIP-seq and visualized by the University of California, Santa Cruz browser. A schematic diagram of the Ccr9 gene is shown below the ChIP-seq graphs. The University of California, Santa Cruz track of sequence conservation in mammals is also shown, and the −13-kb and −10-kb conserved regions are indicated. The chromosome locations are indicated above the tracks. (B) Enlarged view of the −13-kb conserved region showing the overlap among HEB binding, H3K4me1, and H3K4me2. (C) QPCR analysis of chromatin immunoprecipitated by Abs directed against the E proteins HEB and E2A from day-7 Lin− CD45+ progeny of FL MPPs treated for 48 h with DMSO or GSI. DNA was amplified using primers within the −13-kb and −10-kb conserved regions or using primers to β-globin or Ebf1, which served as negative controls. Data are expressed as enrichment normalized to input and are averaged from three independent experiments. *p < 0.05.
transduced cells (Fig. 3A, 3B). These data indicate that E protein transcription factors contribute to the expression of CCR9 on FL MPPs following the withdrawal of Notch signaling.

We next tested whether E proteins were required for CCR9 expression on thymocytes. We found that CCR9 was substantially reduced on DN4 thymocytes in the absence of E2A (Fig. 3C). There was a small decrease in the relative mean fluorescence intensity of CCR9 expression on E2A−/− DP thymocytes compared with WT controls (Fig. 3C). Because the E protein HEB is highly expressed in DP thymocytes, we tested whether HEB was required for CCR9 expression in DP thymocytes. We found a mild decrease in CCR9 expression on DP thymocytes in Cdk4creHeg1−/− mice, similar to the decrease observed in E2A−/− DP thymocytes (Fig. 3D). These data indicate that the E2A proteins play an essential role in regulating CCR9 expression on DN thymocytes and that E2A and HEB have either nonessential or possibly redundant functions in regulating CCR9 expression on DP thymocytes.

E proteins bind putative enhancers near Ccr9

To determine whether E proteins could bind to the Ccr9 gene, we examined HEB ChIP-seq data from DP thymocytes. We identified two peaks of HEB binding ~13 kb and 10 kb upstream of the Ccr9 transcription start site in regions that are highly conserved among multiple species (Fig. 4A, 4B). In addition, these conserved regions overlapped with regions containing the histone modifications H3K4me1 and H3K4me2, both of which are enriched at enhancers (Fig. 4A, 4B). Additional experiments revealed that p300 binding, a definitive marker of enhancer activity, also was enriched in these regions. Based on these findings, we identified these two HEB-binding regions as putative enhancers for Ccr9 in DP cells.

To determine whether these putative enhancers were bound by E proteins in T cell progenitors, we performed ChIP on the progeny of FL MPPs that were expanded on OP9-DL1 for 5 d and then treated with either DMSO or GSI for 48 h. E protein binding was detected above background levels (i.e., binding at the β-globin or the Ebf1 loci) at both the −13-kb and the −10-kb regions when the cells were treated with DMSO (Fig. 4C). The relevant E protein in these cells is likely E47, because that is the major E protein expressed in MPPs. The addition of GSI to block Notch signaling resulted in a 2.2-fold increase in E protein binding at the −13-kb region and a small, but not significant, increase at the −10-kb region (Fig. 4C). The overall higher enrichment of E proteins at the −10-kb region compared with the −13-kb region may reflect the number of E-box sites at these putative enhancers. These data indicate that E proteins can bind to both the −13-kb and the −10-kb regions upstream of Ccr9 and that E protein binding at the −13-kb region is influenced only modestly by Notch signals.

Lymphoma lines also show Notch-dependent repression of Ccr9

We examined Notch-dependent T cell lymphoma cell lines to gain further insight into how Notch signaling regulates Ccr9 transcription. We tested three lymphoma cell lines with differing genotypes (E2A−/−, E2A−/−Rag1−/−, and p53−/−). All of these cell lines expressed very low levels of Ccr9 mRNA and CCR9 protein, but CCR9 and Ccr9 mRNA expression were increased by treatment with GSI (Fig. 5A, 5B, data not shown). We focused our subsequent analysis on the E2A−/−Rag1−/− lymphoma line 531026, because it underwent growth arrest when treated with GSI but remained viable for >96 h (data not shown). In comparison with treatment with DMSO, treatment with GSI led to a rapid increase in Ccr9 mRNA that could be detected as early as 6 h after treatment and continued out to 72 h (Fig. 5A). More than 80% of the cells were positive for CCR9 on their cell surface by 48 h following GSI treatment (Fig. 5B). Importantly, Ccr9 mRNA was not induced by GSI treatment when the lymphoma cells were transduced with a retrovirus encoding ICN1 (Fig. 5C, 5D). Transduction of the lymphoma cells with DNMAML resulted in expression of CCR9 and Ccr9 mRNA without the need for GSI (Fig. 5E, 5F). These data demonstrate that Ccr9 mRNA is repressed by the Notch signaling pathway in both primary MPPs and T cell lymphomas.

Notch1 can repress gene expression by inducing the transcription factor HES1, and Hes1 mRNA levels decline in lymphoma lines after treatment with GSI (Fig. 6A) (21, 53). Therefore, we ectopically expressed HES1 in 531026 cells and examined CCR9 expression after treatment with either DMSO or GSI to determine whether HES1 was sufficient to repress Ccr9. If HES1 was sufficient to repress Ccr9, GSI treatment would not induce CCR9 upregulation in HES1-expressing lymphomas. However, CCR9 protein and Ccr9 mRNA were increased in the HES1-expressing lymphoma line after treatment with GSI (Fig. 6B, 6C). HES1 also

![FIGURE 5. Notch signaling represses Ccr9 mRNA and CCR9 protein expression in immature T cell lymphomas. (A)](http://www.jimmunol.org/)

The 531026 T cell lymphoma line was treated with DMSO or GSI for the indicated times and analyzed for Ccr9 mRNA by QPCR. Hprt mRNA was used for normalization. (B) FACS analysis for CCR9 protein on 531026 cells 48 h after treatment with DMSO (shaded graph) or GSI (open graph). The frequency of CCR9+ cells is indicated for GSI-treated (black text) and DMSO-treated (gray text) cells. (C) Relative expression of Ccr9 mRNA in GFP+ 531026 cells transduced with MigR1 or MigR1-ICN and treated with DMSO or GSI for 48 h. Hprt mRNA was used for normalization. (D) FACS analysis for CCR9 on GFP+ cells isolated 48 h after treatment of MigR1- or MigR1-ICN–infected cells with DMSO (shaded graphs) or GSI (open graphs). The frequency of CCR9+ cells in GSI-treated (black text) and DMSO-treated (gray text) cells is indicated by the gated region. (E) Relative expression of Ccr9 mRNA in GFP+ 531026 cells 40 h after transduction with MigR1 or MigR1-DNMAML. Hprt mRNA was used for normalization. (F) FACS analysis for CCR9 on GFP+ 531026 cells 40 h after being transduced with MigR1 (shaded graph) or DNMAML (open graph). The frequency of CCR9+ cells on MigR1-infected cells (gray text) or MigR1-DNMAML–infected cells (black text) is indicated in the gated region. All experiments are representative of at least three. **p < 0.01, ***p < 0.003.)
failed to repress Ccr9 when ectopically expressed in primary MPPs cultured on OP9-DL1 in the presence of GSI (Fig. 6D, 6E). Importantly, the retrovirally encoded HES1 was functional because it repressed Cebpα mRNA expression in primary MPPs (Fig. 6F) (21). Therefore, although HES1 is a transcriptional repressor induced by activated Notch1, its expression is not sufficient to repress Ccr9.

Notch signaling impacts the transcriptional status of the Ccr9 gene

Our data indicate that Notch signaling influences the expression of Ccr9 mRNA and protein in primary MPPs and T cell lymphomas. We examined the recruitment of RNA PolIII to the Ccr9 promoter and histone modifications associated with active transcription across the Ccr9 gene to determine whether Notch signals impact Ccr9 transcription. In the presence of Notch signaling, RNA PolIII was not highly enriched at the Ccr9 promoter (P2) or at exon 1 (EX1) compared with the negative-control Ebf1 locus (Fig. 7A). However, in the presence of GSI, RNA PolIII binding near the Ccr9 promoter and EX1 increased substantially (Fig. 7A). Our EX1 primers lie immediately downstream of the Ccr9 transcription start site and, therefore, likely detect events occurring at the promoter. We also examined the effect of Notch signaling on H3K4 trimethylation (H3K4me3) and H3 acetylation (AcH3), which are both positively correlated with active promoters (54). The inhibition of Notch signaling was associated with an increase in H3K4me3 and AcH3 at the promoter (P1 and P2) and at EX1. These histone modifications were not as prevalent at exons 2 and 3 of Ccr9, consistent with the enrichment of these modifications at promoters but not at more distal exons (Fig. 7B, C). H3K36 trimethylation (H3K36me3), a marker of histones in actively transcribed exons, was increased at exon 2 and exon 3 after the inhibition of Notch signaling (Fig. 7D). The H3K36me3 modification is generally enriched toward the 3′ end of the gene rather than at the promoter (54, 55). Taken together, these data indicate that Notch signaling prevents the recruitment of RNA PolII to the Ccr9 promoter and inhibits the transcription of Ccr9.

Notch signaling regulates recruitment of the histone acetyltransferase p300 to the −13-kb and −10-kb regions of the Ccr9 gene

Our lymphoma lines are E2A deficient; thus, Ccr9 transcription in these cells is either independent of E proteins or dependent on HEB, unlike in primary MPPs. By ChIP, we found a substantial enrichment of HEB binding at the −10-kb region in this lymphoma, even in the presence of Notch signaling (Fig. 8A). HEB also bound the −13-kb region in the presence of Notch signals although, as in primary MPPs (Fig. 4C), the binding was significantly increased by GSI treatment (Fig. 8A). However, we conclude that Ccr9 transcription is E protein independent in these lymphomas because ectopic retrovirus-driven expression of ID2 did not affect lymphoma cell surface expression of CCR9 after treatment with GSI (data not shown). These findings are consistent with our previous conclusion that Notch represses Ccr9 expression independent of E protein function.

To gain further insight into how Notch signaling might impact Ccr9 transcription, we determined whether p300, a coactivator that modulates chromatin accessibility by histone acetylation at enhancers, was recruited to the putative Ccr9 enhancers. p300-mediated histone acetylation can increase transcription factor occupancy at enhancers, such as we observed with the E proteins (56, 57). In the 531026 lymphoma, we found that Ccr9 transcription after Notch inhibition was associated with strong binding of p300 to the −13-kb and −10-kb regions upstream of Ccr9 (Fig. 8B). Compared with the negative-control loci, p300 also bound these regions in the presence of Notch signals, but to a significantly lesser extent (Fig. 8B). Importantly, AcH3 was only substantially enriched at the −13-kb and −10-kb regions over the
β-globin and Ebf1 control genes when Notch signaling was inhibited (Fig. 8C). Our data indicate that Notch signaling limits p300 recruitment and AcH3 at two regions upstream of the Ccr9 gene. Given that p300 recruitment is a major function of transcriptional enhancers, we propose that the −13-kb and −10-kb regions are enhancers of Ccr9 that are compromised, either directly or indirectly, by Notch signaling.

**Discussion**

The dynamic up- and downregulation of CCR9 controls the migration of lymphocytes into and through the thymus (58, 59). We showed that the E protein transcription factors promote Ccr9/CCR9 expression in MPPs and at the DN stages of T cell development. In contrast, Notch signaling is antagonistic to Ccr9/CCR9 expression in T cell progenitors and is sufficient to account for the immediate downregulation of CCR9 after MPPs enter the Notch ligand–rich thymic environment. We showed that Notch signaling induces multiple proteins that could repress Ccr9, including GATA3 and TCF1 (22, 23). However, neither of these transcription factors remain dependent on Notch signaling after their initial induction; therefore, they are unlikely to be the target of Notch1 that transiently regulates Ccr9. In genome-wide binding studies in mouse leukemia cells, ICN was shown to potentially associate with the transcriptional repressor ZNF143 at a subset of genes that lack evidence of activating histone modifications (60, 61), indicating that ICN does not activate, but could repress, these genes. Nonetheless, we favor a model in which the ICN1/CSL/MAML complex either activates or interacts with a factor that binds to the Ccr9 enhancers and either directly or indirectly prevents the recruitment of p300. Interestingly, inhibition of Notch signaling with GSI resulted in a loss of Ccr9 expression in human T lineage acute lymphoblastic leukemia cell lines (62). Genome-wide binding studies revealed that ICN can have divergent functions in human and mouse leukemic cells and that the supraphysiologic levels of ICN found in leukemic cells can result in divergent functions for Notch signaling in leukemic cells compared with normal cells (63). Therefore, it is important to note that our studies show repression of Ccr9 by Notch signaling in both primary and transformed mouse cell lines. Nonetheless, further studies are needed to determine how ICN impacts the re-

**FIGURE 7.** Notch signaling represses Ccr9 transcription. ChIP was performed using Abs against RNA Pol II (A), AcH3 (B), H3K4me3 (C), and H3K36me3 (D) on extracts from 531026 cells isolated 48 h after treatment with DMSO (gray) or GSI (black). The precipitated DNA was amplified by QPCR using primers located near the Ccr9 promoter (primers P1 and P2) or within the Ccr9 EX1, exon 2 (EX2), and exon 3 (EX3). Primers in a region −88.5 kb upstream of Ccr9 and at the Ebf1 or Deltex1 genes served as controls. Primers to the Deltex1 promoter were used for H3K4me3, and AcH3 and primers to Deltex1 exon 7 were used for H3K36me3. The data are expressed as enrichment normalized to input and are averaged from three independent experiments. *p < 0.05, **p < 0.01.

**FIGURE 8.** Notch signaling prevents recruitment of p300 and E proteins to a distal enhancer of Ccr9. ChIP using HEB (A), p300 (B), and AcH3 (C) Abs was performed on 531026 cells 48 h after treatment with DMSO or GSI. The purified DNA was amplified by QPCR using primers within the −13-kb and −10-kb regions of the Ccr9 locus. Primers to β-globin and Ebf1 served as negative controls. Data are expressed as enrichment normalized to input and are averaged from three independent experiments. *p < 0.05, **p < 0.01.
cributment of p300 to the putative enhancers of Ccr9 and whether ICN functions to regulate Ccr9 in both mouse and human T cell development.

It was proposed that Notch signaling inhibits the function of the E protein transcription factors either directly, by limiting their expression, or indirectly, by inducing the expression of ID proteins that inhibit the ability of E proteins to bind DNA (33, 34, 64). Our data indicate that E proteins bind to the −10-kb region of Ccr9, even in the presence of Notch signals. Therefore, Notch signaling does not globally prevent E protein binding in these cells. However, there was a reduction in E protein binding at the −13-kb region when Notch signaling was active, and this decrease could contribute to the reduced expression of Ccr9 in primary T cell progenitors after the activation of Notch1. Indeed, the E protein E47 is necessary for optimal CCR9 expression on FL MPPs and on DN4 thymocytes. Although the E protein HEB is bound to the putative Ccr9 enhancers in DP thymocytes, neither E47 nor HEB is absolutely required for CCR9 expression, although the absence of either protein caused a mild decrease in CCR9. Therefore, E47 and HEB may play redundant roles in Ccr9 regulation in DP thymocytes, although this possibility remains to be tested. E proteins can bind p300 through their activation domains (65, 66); therefore, they could contribute to the stability of p300 binding to the Ccr9 locus in MPPs and T cell progenitors when Notch signaling is inhibited. However, E protein binding may not be sufficient to maintain p300. Alternatively, the decrease in E protein binding to the −13-kb region may be sufficient to destabilize p300 binding. If an interaction between the two Ccr9 enhancers stabilizes p300, such as via the formation of an “enhancer bridge,” then the loss of E protein binding to one of the putative enhancers may be sufficient to reduce enhancer function. However, further studies are needed to determine whether the decrease in E protein binding at the −13-kb region contributes to the loss of Ccr9 transcription in the presence of Notch signaling. Moreover, how Notch signals impact E protein binding at this region remains to be determined. Notch signaling could induce a factor that binds to this enhancer and occludes E protein binding to some of the E-boxes in this region. Alternatively, factors that cooperate with E proteins to allow their recruitment to this region when it is in a “closed” chromatin configuration (i.e., lacking ACh3) may be lacking when Notch signaling is active.

We found that neither E2A nor HEB was essential for Ccr9 expression in multiple T cell lymphomas after the inhibition of Notch signaling. This observation leads us to suggest that the ability of Notch signals to inhibit the recruitment of p300 to the putative Ccr9 enhancers is not a consequence of a direct effect on E protein function. Rather, Notch signaling likely regulates Ccr9 transcription, either directly or through distinct factors that inhibit Ccr9 enhancer function. The liberation of Ccr9 transcription from E protein function in T cell lymphomas is of interest and suggests that, although E proteins can regulate Ccr9, other factors are able to take over this function. The identity of these factors and their roles in normal and malignant T cell migration could be of interest. Our data reveal opposing roles for E protein and Notch signaling in the dynamic expression of Ccr9 on thymocytes and their progenitors and indicate that both proteins function to control intrathymic T cell progenitor migration.

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Disclosures
The authors have no financial conflicts of interest.

References

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Corrections


The fourth author’s name was published incorrectly. The correct name is Akinola Olumide Emmanuel.

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