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ORIGINAL ARTICLE

Notch1 expression is regulated at the post-transcriptional level by the 3′ untranslated region in hematopoietic stem cell development

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Abstract In hematopoiesis, the expression of critical genes is regulated in a stage-specific manner to maintain normal hematopoiesis. *Notch1* is an essential gene involved in the commitment and development of the T-cell lineage. However, the regulation of *Notch1* in hematopoiesis is controversial, particularly at the level of hematopoietic stem cell (HSC). Here, we found that the expression of *Notch1* is controlled at the post-transcriptional level in HSCs. HSCs express a considerable level of *Notch1* mRNA, but its protein level is very low, suggesting a post-transcriptional suppression for *Notch1*. Using a retroviral sensor vector expressing a fusion mRNA of GFP and 3′ untranslated region (3′UTR) of a target gene, we demonstrated that the *Notch1*-3′UTR had a post-translational suppressive effect only at the HSC but not in the downstream progenitor stages. The sequence motif AUnA was required for this post-transcriptional regulation by the *Notch1*-3′UTR. Interestingly, this *Notch1*-3′UTR-mediated suppressive effect was relieved when HSCs were placed in the thymus, but not in the bone marrow. Thus, the expression of *Notch1* in HSCs is regulated by microenvironment at the post-transcriptional level, which may control T lymphoid lineage commitment from HSCs.

Keywords Notch1 · 3′UTR · Post-transcriptional regulation · Hematopoietic stem cell

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Introduction

Hematopoiesis is a tightly regulated process, in which hematopoietic stem cells (HSCs) undergo cell division, self-renewal, and lineage commitment to various types of blood cells. The hierarchical developmental pathways involved in this process have been resolved by identification of myelo-erythroid and lymphoid progenitors as well as HSCs [1, 2]. To maintain normal hematopoiesis, expression of a set of genes for lineage commitment is tightly controlled. For example, the reciprocal expression of PU.1 and GATA-1 was shown to play a critical role in myelo-erythroid versus lymphoid fate decisions [3, 4].

The Notch pathway has emerged as a crucial player in stem cell regulation. There are four Notch receptors (Notch1-4) and five Notch ligands (Jagged1, 2 and Delta1, 3, 4) in vertebrate [5, 6]. In the lymphoid system, *Notch1* is critical in the T versus B lymphoid lineage decision [7], while *Notch2* is critical for MBZ development [8]. However, the data on the role of Notch signaling in the maintenance and self-renewal of HSCs are controversial. Retroviral transduction with the *Notch1* intracellular domain (Notch-IC) or *Hes1* (a canonical Notch target and transcriptional repressor) into HSCs induced the blockade of differentiation and the enhancement of self-renewal activity [9, 10]. In addition, using a Notch reporter mouse in which the GFP is under the control of a Rbpj response element, Notch signaling was shown to be actually activated in immature

cells in the stem cell niche, and retroviral transduction of HSCs with dnXSu(H) (a dominant-negative Rbpj protein) reduced the long-term reconstitution activity of transplanted bone marrow [11]. In osteoblast-specific activated PTH/PTHrP receptor transgenic mice, the expression of Jagged1 on osteoblasts was enhanced and the number of HSCs was increased with Notch1 activation [12]. In addition, human cord blood CD34+ cells were expanded ex vivo in the presence of Notch1 ligand (Delta1$^{ext-IgG}$), and the expanded progenitors showed enhanced hematopoietic reconstitution activity following myeloablative transplantation [13]. These data suggest that Notch signaling plays an important role in the self-renewal of HSCs.

In contrast to the studies of activated Notch signaling in HSCs, loss of Notch pathway by conditional deletion of *Notch1* or *Jagged1* did not appear to affect HSCs. Using interferon-inducible (Mx-Cre) *Notch1* conditional knockout (cKO) mice, *Notch1*-deleted HSCs displayed an early blockage of T-cell development without impaired reconstitution activity of HSCs [7]. The HSCs also showed a normal repopulating capacity in Mx-Cre *Jagged1* cKO mice [14]. In addition, *Notch2* had no effect on the maintenance or expansion of HSCs in Mx-Cre *Notch2* cKO mice [8]. Furthermore, analysis of transgenic mice carrying a dominant-negative MAML1 gene (dn-MAML1), which specifically blocks all canonical Notch signaling, or mice deficient for *Rbpj* has shown that Notch activity was dispensable for the maintenance of HSCs under physiologic conditions [15]. Similar results were also obtained in human. Notch signals were shown not to be required for in vivo maintenance of HSCs in immunodeficient mice following transplantation of dn-MML1 transduced HSCs from cord blood [16]. Thus, the role of the Notch pathway in the maintenance and self-renewal of HSCs still remains controversial.

In this study, we found that the expression of *Notch1* is regulated at the post-transcriptional level in HSCs via the 3′ untranslated region (3′UTR), and the sequence motif AUnA is required for this post-transcriptional regulation by *Notch1*-3′UTR, suggesting that RNA-binding proteins are likely responsible for inhibiting *Notch1* expression in HSCs. In addition, this suppressive effect mediated by the *Notch1*-3′UTR disappeared once HSCs were placed in the thymic environment. Our study revealed a novel mechanism of the *Notch1* regulation in HSCs and in early cell fate decisions.

Materials and methods

Mice

C57BL/6J (Ly5.2) and congenic B6.SJL-Ptprca Pepcb/BoyJ (Ly5.1) mice were purchased from Jackson Laboratory (Bar Haber, ME, USA), and maintained in the Research Animal Facility at the Dana-Farber Cancer Institute in accordance with the institutional guidelines.

Antibodies, cell staining, and sorting

Sorting of HSCs and common lymphoid progenitors (CLPs) was accomplished by staining bone marrow cells with biotinylated anti-IL-7R chain antibodies, FITC-conjugated anti-Sca-1, APC-conjugated anti-c-Kit, and PE-Cy5-conjugated rat antibodies specific for the following lineage markers: CD3, CD4, CD8, B220, Gr-1, and CD19, followed by avidin-PE. HSCs and CLPs were sorted as IL-7Rα$^-$Lin$^-$Sca-1^{hi}c-Kit^{hi} and IL-7Rα$^+$Lin$^-$Sca-1^{lo}c-Kit^{lo} populations, respectively [2]. For myeloid progenitor sorting, bone marrow cells were stained with PE-conjugated anti-FcγRII/III, FITC-conjugated anti-CD34, APC-conjugated anti-c-Kit, and biotinylated anti-Sca-1, followed by avidin-APC/Cy7. Myeloid progenitors were sorted as IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III^{lo} (CMPs: common myeloid progenitors), IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III^{hi} (GMPs: granulocyte/monocyte progenitors), and IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^-$FcγRII/III^{lo} (MEPs: megakaryocyte/erythrocyte progenitor) as described previously [1]. The sorting of double negative (DN) thymocytes was achieved by staining with anti-CD44, anti-CD25 and anti-c-Kit with PE-Cy5-conjugated rat antibodies specific for the following lineage markers: CD3, CD4, and CD8. All of these cells were double-sorted using BD FACS Aria cell-sorting system (BD Biosciences, San Jose, CA, USA).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA isolated from 2000 cells of each population was reverse transcribed to cDNA, and the mRNA levels were quantified by real-time PCR (Applied Biosystems, Foster City, CA, USA) as described previously [17]. β2-microglobulin (B2M) was used as an internal control. The primers, and 5′-FAM- and 3′-TMRA-labeled probes, for *B2M*, *Notch1* and *Hes1* are were as follows: *B2M*, forward 5′-CATACGCCTGCAGAGTTAAGCA-3′, reverse 5′-TCACATGTCTCGATCCCAGTAGA-3′, and probe 5′-CCAGTATGGCCGAGCCCAAGACC-3′; *Notch1*, forward 5′-TGACTGCATGGATGTCAATGTTC-3′, reverse 5′-CCACTGCAGGAGGCAATCA-3′, and probe 5′-AGGACCAGATGGCTTCACACCCCTC-3′; *Hes1*, forward 5′-TTTTGGATGCACTTAAGAAAGATAGC-3′, reverse 5′-GCTTCACAGTCATTTCCAGAATGT-3′, and probe 5′-CCCGGCATTCCAAGCTAGAGAAGGC-3′.

Western blot analysis

Cells were lysed in 100 µl RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 25 mM sodium-β-glycerophosphate, 1 mM EDTA, 1% SDS, protease inhibitors). Lysates were then denatured in an equal volume of 2× SDS sample buffer, resolved on a 10% SDS-PAGE gel and electro-transferred onto nitrocellulose membranes in non-SDS-containing transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol, pH 8.5). Western blotting was performed with anti-Notch1 (C-20, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-βtubulin (9F3, Cell Signaling Technology, Danvers, MA, USA), followed by a 1:10,000 dilution of anti-rabbit HRP-conjugated IgG. The blots were developed with an ECL Plus kit (GE Healthcare, Little Chalfont, UK).

Retrovirus vectors and retroviral infection

Schemas of the retroviral vectors are shown in Figs. 2 and 3. The 3′UTR regions of *Notch1*, *GATA1*, and *PU.1* were amplified from genomic DNA by PCR, and cloned into retroviral vectors. The primer sets and product sizes for cloning of the 3′UTR region are were as follows (underlined sequences indicate the attached restriction enzyme sites): *GATA1*-3′UTR, forward 5′-AATTCTCGAGAGGTACAC AGAATAGCCTTGACCTTG-3′, reverse 5′-ATTAGATATC CCACTTGACACTGACATTTATTTAACCAAATACC-3′ (491-bp); *PU.1*-3′UTR, forward 5′- GCCCTCGAGAAG ACAGGCGAGGTGAAGAAAG-3′, reverse 5′- ATCTTG AATGAGACACTTCTCTGG-3′ (488-bp); *Notch1*-3′UTR, forward 5′- GCCCTCGAGACCCACATTCCAGAGGC ATTTA-3′, reverse 5′-ATCATTTTCATTACCTACAGT TTTGCAT-3′ (1580-bp). The viral supernatant was obtained from cultures of 293T cells co-transfected with the retroviral vector, and gag-pol and VSV-G expression plasmids using the $CaPO_4$ co-precipitation method. FACS-purified cells were plated onto a recombinant fibronectin-coated culture dish (RetroNectin dish, Takara, Chiba, Japan) with the virus supernatant containing the respective cytokine cocktail (for HSCs: SCF, LIF, IL6 and sIL6R; for DN thymocytes: SCF and IL7) as described previously [3]. Thymocytes were placed onto OP9 stromal layers after infection and cultured for 24–48 h.

Intra-thymic injection

A retroviral sensor vector containing the *Notch1*-3′UTR was introduced into HSCs 2 days prior to injection. Using a Hamilton microsyringe with a 26-gauge needle, 20,000 HSCs from Ly5.2 mice were directly injected into a thymic lobe or bone marrow of congenic mice (Ly5.1) without irradiation as described previously [18]. The thymus and bone marrow were harvested after 12 h and subjected to FACS sorting and analysis.

Results

Discrepancy between mRNA and protein expression of *Notch1* in HSCs

The level of *Notch1* mRNA in each stage of hematopoiesis was evaluated by quantitative PCR assay. The expression of *Notch1* mRNA was substantially expressed at the stage of HSC, and gradually decreased with differentiation into downstream progenitors such as CMPs, GMPs and MEPs. In the lymphoid lineage, the *Notch1* mRNA was expressed in CLPs and in DN thymocytes at a higher level as compared to HSCs (Fig. 1a). However, Western blot analysis showed only a faint band of the Notch1-transmembrane form in the HSCs lane, whereas clear bands for Notch1 protein (full-length and transmembrane form) were detected in CLPs and DN thymocytes (Fig. 1c). This discrepancy between the mRNA and protein expression of *Notch1* in HSC suggests that *Notch1* is post-transcriptionally suppressed at the HSCs stage, presumably via the UTRs that can regulate the translation of mRNA. In parallel with this finding, mRNA of *Hes1*, a canonical Notch target gene, was expressed only at a low level in HSCs, as compared to that in thymocytes (Fig. 1b), suggesting that Notch1 signaling is not activated in the majority of HSCs.

Notch1-3′UTR reveals a post-transcriptional suppressive effect in HSCs

To evaluate an activity of post-transcriptional control by the 3′UTR, we prepared a simple retroviral sensor vector. The conceptual schema of this method is shown in Fig. 2a. In this vector, the 3′UTR region of a gene of interest is cloned into a downstream site of the GFP gene. Since the fusion mRNA of GFP and 3′UTR will be translated into GFP protein under the influence of the 3′UTR, post-transcriptional activity can be monitored by GFP fluorescence intensity using FACS.

Based on this principle, we cloned the 3′UTR region of the *Notch1* gene into this sensor vector and introduced its retrovirus into HSCs. As shown in Fig. 2b, the GFP intensity was markedly suppressed by the presence of the *Notch1*-3′UTR in HSCs, whereas GFP signals were highly expressed in the retroviral vectors with *GATA1*-3′UTR, *PU.1*-3′UTR, or control (no insertion of 3′UTRs). The expression of GFP gradually increased even in the presence of the *Notch1*-3′UTR in progenitors of CMPs and GMPs, and the inhibitory effect of the *Notch1*-3′UTR was decreased in DN thymocytes (Fig. 2c). These data suggest that the post-transcriptional suppressive effect of the *Notch1*-3′UTR

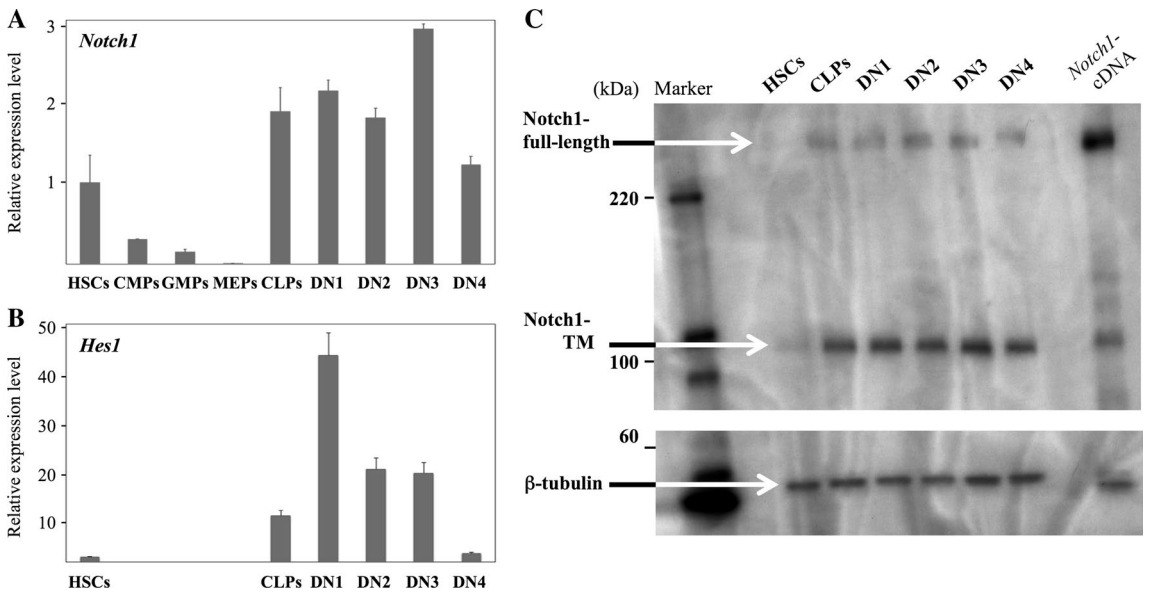

Fig. 1 Expression levels of *Notch1* mRNA and protein in hematopoietic development. Quantitative PCR analysis of *Notch1* (**a**), *Hes1* (**b**) expression. β2-microglobulin was used as an internal control and relative expression levels are represented as the mean ± SD of triplicate experiments. **c** Western blot analysis of Notch1. An arrow in the upper position indicates a full-length Notch1 protein and an arrow in the middle position is a trans-membrane form. Lane of *Notch1*-cDNA indicates a positive control using lysate from a cell line (293T) transfected with *Notch1* cDNA. *HSCs* hematopoietic stem cells, *CMPs* common myeloid progenitors, *GMPs* granulocyte/monocyte progenitors, *MEPs* megakaryocyte/erythrocyte progenitors, *CLPs* common lymphoid progenitors, *DN* double negative thymocytes, which were subdivided into DN1, DN2, DN3 and DN4 based on CD25 and CD44 expression

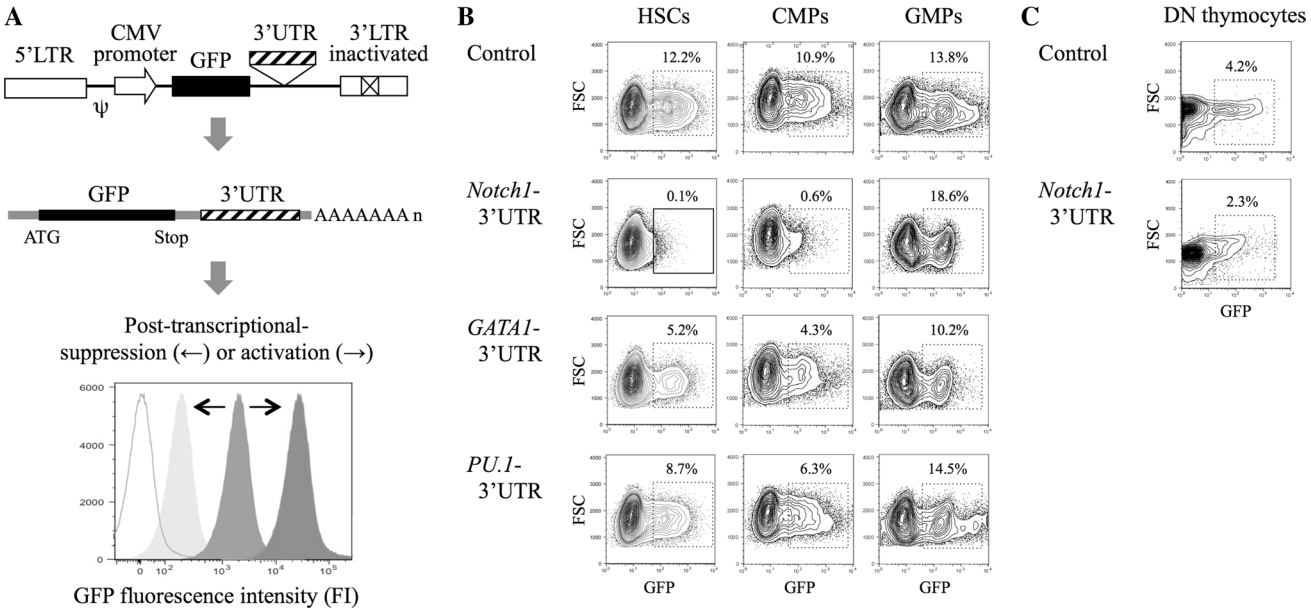

Fig. 2 Functional analysis of 3′UTR-mediated post-transcriptional control by retroviral sensor vector. **a** Schema of the analysis of translational control by retroviral sensor vector. In the retroviral sensor vector, 3′UTR was cloned into a downstream of GFP, and transcribed mRNA of GFP fused with 3′UTR was translated under the influence of 3′UTR. The activity of post-transcriptional control was monitored by the fluorescence intensity of translated GFP. *ψ* packaging signal, *LTR* long terminal repeat, *CMV* promoter, cytomegalovirus promoter, *GFP* green fluorescent protein, *3′UTR* 3′-untranslated region. FACS analysis of fluorescence intensity of GFP in HSCs and progenitors of CMPs and GMPs (**b**), and of DN thymocytes (**c**). Cells were infected with retrovirus from sensor vectors containing either *Notch1*-3′UTR, *GATA1*-3′UTR, *PU.1*-3′UTR, or control (no insertion of 3′UTR) downstream of GFP. GFP expression level was monitored 48 h after retroviral infection. Representative data of four to five independent experiments are shown

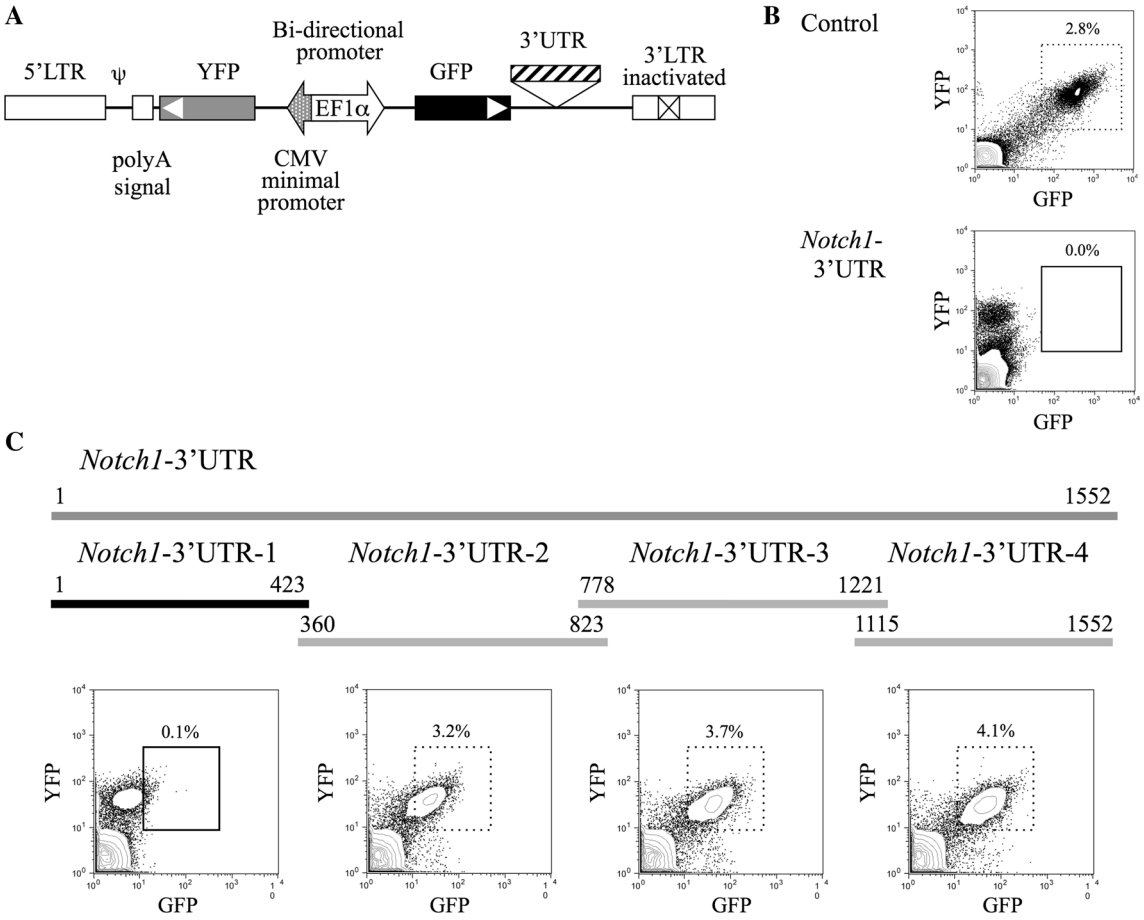

Fig. 3 The second retroviral sensor vector with a bi-directional promoter for functional analysis of post-transcriptional regulation. **a** Structure of second retroviral sensor vector. *ψ* packaging signal, *LTR* long terminal repeat, *GFP* green fluorescent protein, *YFP* yellow fluorescent protein, *3′UTR* 3′-untranslated region. Bi-directional promoter was composed of an *EF1* promoter and a minimal-CMV promoter that was placed in a reverse manner upstream of the *EF1* promoter. **b** This second retroviral sensor vector expressed both GFP and YFP almost equally by the bi-directional promoter, and was used to monitor both the retrovirus transduction efficacy and 3′UTR activity. **c** To narrow down the sequence of the *Notch1*-3′UTR responsible for post-transcriptional inhibition, the *Notch1*-3′UTR of 1552-bp was divided into 4 regions, and each 3′UTR portion was analyzed with the second retroviral sensor vector. The numerals on each bar of 4 regions indicate the position in *Notch1*-3′UTR and the position 1 refers to the first base of the sequence of *Notch1*-3′UTR. Data shown are representative of two to three independent experiments

is evident particularly in the HSC stage. However, this result should be interpreted with caution, since the retrovirus from the retroviral sensor vector containing *Notch1*-3′UTR may result in the loss of transduction activity in HSCs. Therefore, to confirm both of the transduction efficacy of retrovirus and the translational activity of the 3′UTR, we prepared a second sensor retroviral vector with a bi-directional promoter (Fig. 3a), in which a minimal-CMV prompter was placed in a reverse manner upstream of the *EF1* promoter. This sensor vector could express both GFP and yellow fluorescent protein (YFP) in a nearly equal manner. As shown in Fig. 3b, when the *Notch1*-3′UTR was inserted downstream of GFP, its expression was markedly suppressed in HSCs, while YFP was highly expressed as a marker of sufficient transduction of the retrovirus. These data confirmed that the *Notch1*-3′UTR exerts post-transcriptional suppression in HSCs. Using this second system, we further evaluated the sequences of the *Notch1*-3′UTR responsible for the post-transcriptional suppressive effect in HSCs.

The sequence responsible for the post-transcriptional suppressive effect of the *Notch1*-3′UTR

To identify the sequence responsible for the suppressive effect of the *Notch1*-3′UTR in HSCs, we first subdivided the 3′UTR into four parts and tested their suppressive effect of translation, and the suppressive effect was found in the first part of the subdivided *Notch1*-3′UTR as shown in Fig. 3c. We further narrowed down the responsible sequence to a 190-bp fragment, beginning at positions 64–253, located

within the first part of the subdivided *Notch1*-3′UTR (Fig. 4a).

To identify the sequence indispensable for translational suppression, we prepared mutant fragments as shown in Table 1, and cloned these fragments into the second retroviral sensor vector downstream of GFP. The results are plotted as the ratio of the mean fluorescence intensity of GFP to that of control GFP expression in Fig. 4b. Interestingly, one mutant fragment of m-5 revealed a marked relief of post-transcriptional suppression, and two mutants of m-4 and m-8 showed a slight-to-moderate recovery of GFP expression. The relief of suppression was enhanced in mutant clones with replaced sequences of m-4 and/or m-8 as well as m-5. Thus, the sequences corresponding to AUnA (Fig. 4c, Table 1) were shown to be responsible for the *Notch1*-3′UTR-mediated post-transcriptional regulation in HSCs. This sequence motif AUnA is a typical target of RNA-binding proteins. TNFα is a well-studied molecule related to post-transcriptional regulation by RNA-binding proteins such as TPP and HuR [19, 20]. As shown in Fig. 4b, a part of the 3′UTR of *TNF*α that contains several repeats of AUnA sequences also exhibited a potent suppressive effect of mRNA translation in HSCs. These data suggest that translation of *Notch1* mRNA in HSCs is regulated by its 3′UTR, presumably by interacting with as-yet-unidentified RNA-binding proteins.

Relief of *Notch1*-3′UTR-mediated suppression in the thymic environment

To investigate the physiological role of the 3′UTR-mediated suppression of *Notch1* in HSCs, we searched for conditions in which this post-transcriptional suppression could be relieved. We directly injected HSCs (Ly5.2) transduced with the sensor vector into the bone marrow or thymus of Ly5.1 mice, and harvested the HSCs at 12 h after injection. Interestingly, GFP expression was observed when the HSCs were placed in the thymic environment (Fig. 5), whereas suppressive effects were not alleviated in HSCs injected in the bone marrow or cultured in vitro. These data indicated that the suppressive effect of the *Notch1*-3′UTR can be relieved only in the thymus, suggesting that some extrinsic signals from the thymic environment can relieve the post-transcriptional suppression of Notch1 protein in HSCs.

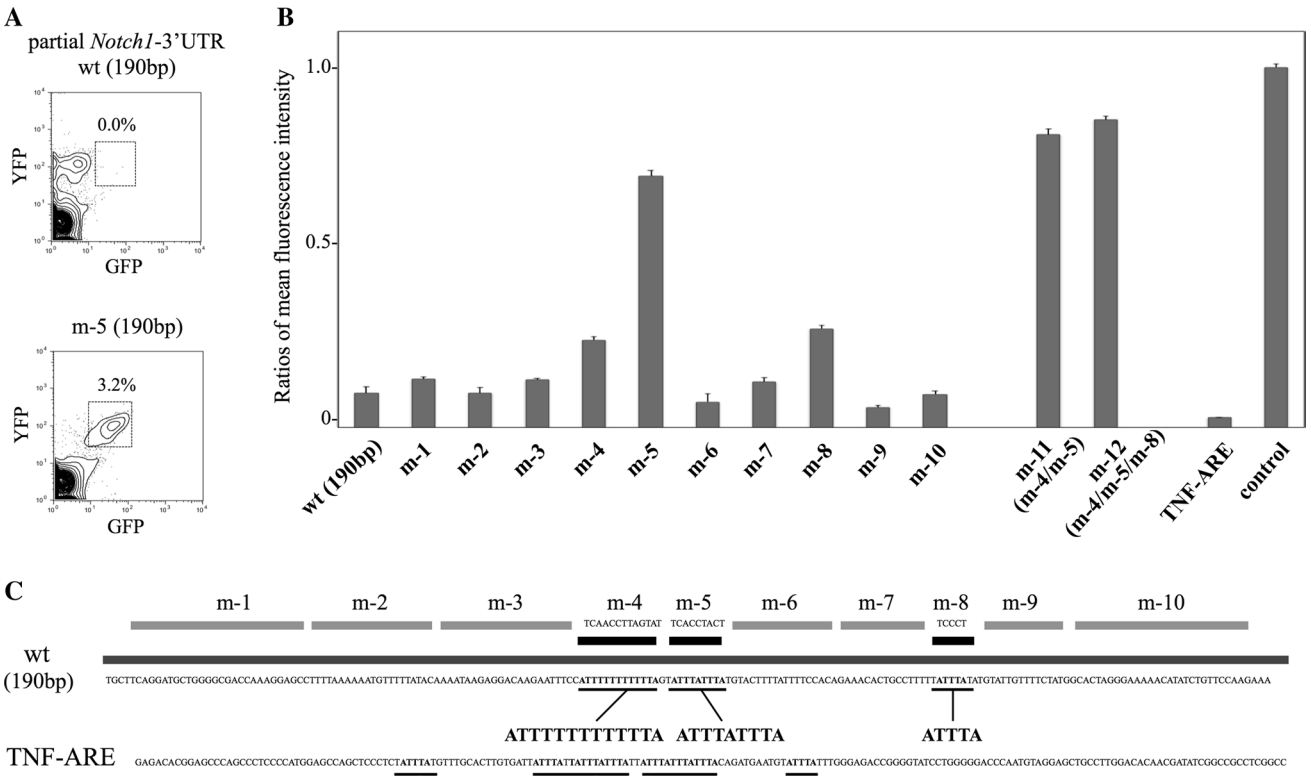

Fig. 4 The sequence responsible for the suppressive effect of the *Notch1*-3′UTR. **a** The sequence responsible for the post-transcriptional suppressive effect of the *Notch1*-3′UTR was narrowed down to 190-bp fragment located within the first part of the subdivided *Notch1*-3′UTR. **b** Modified sequences of this 190-bp region were cloned into the second retroviral sensor vector downstream of GFP and the ratio of the mean fluorescence intensity of GFP to that of control GFP expression was plotted. **c** Schema of positions of modified sequences in the 190-bp region. The sequence marked with a gray bar was replaced with an unrelated sequence as shown in Table 1. TNF-ARE is the sequence of the adenine/uridine-rich element in the *TNF*-3′UTR. Bars indicate mean ± SD of triplicate experiments

Table 1 List of clones of modified sequence

Clones of modified sequence	Sequence of WT	Position in the clone of WT 64–253[a] (190 bp)	Replaced sequence
m-1	CAGGATGCTGGGGCGACCAAA GGAG	69–93	GTCCACAATCTGTTACAGCACAGTT
m-2	TTTTAAAAAATGTTTTTATA	96–115	ATATATACTTCAAAGATAAA
m-3	CAAAATAAGAGGACAAGAA	116–134	TACCCGCCATAAACCCTCC
m-4	ATTTTTTTTTTTA	140–152	TCAACCTTAGTAT
m-5	ATTTATTTA	155–163	TCACCTACT
m-6	GTACTTTTATTTTCCAC	165–181	AATGTAACTCTAATATG
m-7	AGAAACACTGCC	182–193	TCCCTATAACAG
m-8	ATTTA	199–203	TCCCT
m-9	TGTATTGTTTTCT	206–218	AACAAATCAATTC
m-10	GCACTAGGGAAAAACATATCTGTT CCA	222–248	CTCGAACAATCAACAAGTACAACA ACT
m-11 (m-4/m-5)	ATTTTTTTTTTTA/ATTTATTTA	140–152/155–163	TCAACCTTAGTAT/TCACCTACT
m-12 (m-4/m-5/m-8)	ATTTTTTTTTTTA/ATTTATTTA/ ATTTA	140–152/155–163/199–203	TCAACCTTAGTAT/TCACCTACT/ TCCCT

WT wild type

[a] Position 1 refers to the first base of the sequence of *Notch1*-3′UTR

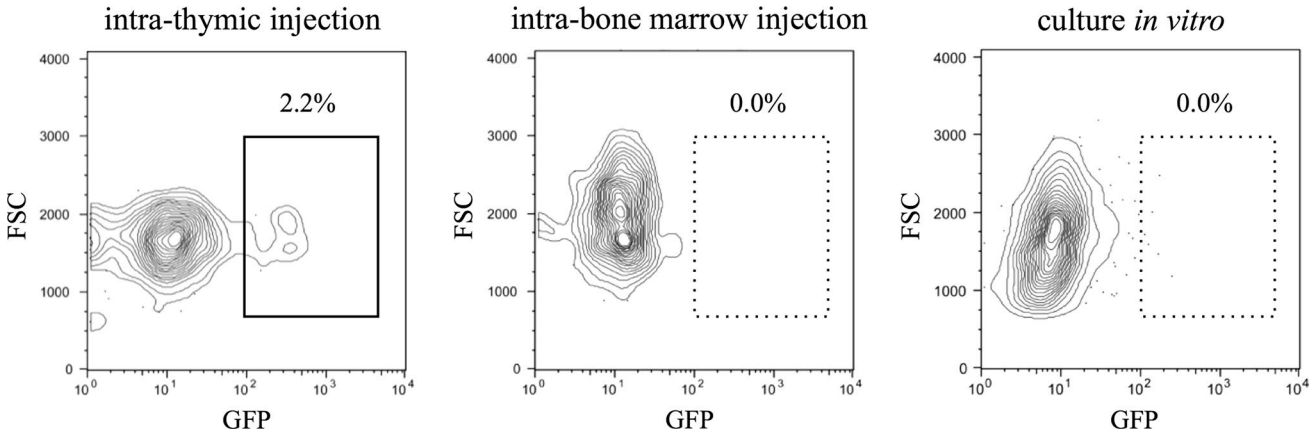

Fig. 5 Relief of the *Notch1*-3′UTR-mediated suppressive effect in thymic environment. HSCs from Ly5.2 mice were infected with a retroviral sensor vector containing the *Notch1*-3′UTR, and were injected into the bone marrow or the thymus of Ly5.1 mice. Cells positive for CD45.2 (Ly5.2) were harvested at 12 h after injection, and the fluorescence intensity of GFP was measured. Representative data of two independent experiments are shown

Discussion

In this study, we found that the expression of Notch1 protein is suppressed at the HSC stage, although its mRNA was substantially expressed. We revealed that the *Notch1*-3′UTR could inhibit the expression of GFP protein in HSCs, although its suppressive effect was not observed in myeloid progenitors or DN thymocytes. We here show that the expression of Notch1 protein is suppressed at the HSC stage post-transcriptionally via the effect of *Notch1*-3′UTR.

The role of the Notch pathway in the maintenance and self-renewal of HSCs is still unclear and controversial. Although the overexpression of Notch signaling has been shown to enhance self-renewal activity [9–13], several studies of cKO mice or dominant-negative inhibition of the Notch pathway revealed that Notch is dispensable in the maintenance or self-renewal of HSCs [7, 8, 14–16]. In our study, the translation of *Notch1* was suppressed to very low levels in HSCs. This regulation could account for previous data, where the deletion of *Notch1* or Notch pathway did not affect the maintenance and self-renewal of HSCs [7, 14]. Concurrently, the translational suppression of *Notch1* is also considered to be significant to avoid aberrant expression of Notch1 protein in HSCs, since the expression of *Notch1* is

initiated with commitment into the T lymphoid lineage. Recent reports proposed that the active Notch pathway supported the maintenance of HSCs at the site of bone marrow niche [21–23]. It is possible that the small amount of Notch1 protein that was shown to be present in HSCs in our experiment could be sufficient for the maintenance of some fractions of HSCs in the niche environment.

In contrast to the findings in HSCs, the post-transcriptional suppression of *Notch1* was not observed in the lymphoid lineage of CLPs and DN thymocytes. The release of the translational suppression of *Notch1* was clearly demonstrated in DN thymocytes by retroviral sensor vector. We failed to apply this sensor retrovirus to CLPs due to a technical limitation. However, both of the mRNA and protein of *Notch1* were expressed in CLPs to the level comparable to that in DN thymocytes, suggesting that the post-transcriptional regulation of *Notch1* is released already at the stage of CLP. CLP is the earliest lymphoid progenitor and can give rise to T, B, and NK cells [2]. The appearance of Notch1 protein at the CLP stage allows this progenitor to undergo the T-cell developmental program. Since the Notch1 signaling has been shown to be critical in the development of T cells [7, 24] with the inhibition of B-cell differentiation [25], the CLP population could be subdivided by the expression of Notch1 protein concordant with T versus B lymphoid lineage decision. In addition, there may be multipotent progenitors with lymphoid lineage potential, upstream of the CLP population, in which post-transcriptional suppression of *Notch1* is relieved to initiate T lymphoid lineage commitment. For example, lymphoid-primed multipotent progenitor (LMPP) [26], granulocyte–monocyte–lymphoid progenitor (GMLP) [27], or HSCs with long-term (LT) or short-term (ST) reconstitution activity are candidate populations in which the regulation of *Notch1* plays a role in their cell fate decision. Further investigations at the single cell level should be required to more clearly delineate the post-transcriptional regulation of *Notch1* in hematopoiesis.

Post-transcriptional regulation of gene expression is mediated by the bindings of microRNAs or RNA-binding proteins to the 3′UTR of genes [19, 20, 28]. Using retroviral sensor vector, we found out that an AUnA sequence in the 3′UTR of *Notch1* is responsible for the translational control in HSCs, and the AUnA sequence is a well-known target motif of RNA-binding proteins [19, 20]. RNA-binding proteins control a variety of genes in different aspects such as tumorigenesis, cell cycle control, inflammation, and cell stress response by affecting stability and mRNA translation of mRNAs. In regard to hematopoiesis, for example, the deletion of HuR was shown to increase the number of HSCs and DN thymocytes [29]. The double KO of ZFP36L1 and ZFP36L2 induced T-cell malignancy, partially by the dis-regulation of *Notch1* in thymocytes [30], although the expression and the effect of these proteins in HSCs remain unclear. It remains unclear as to the RNA-binding protein that controls *Notch1* translation in HSCs. The identification of target RNA-binding proteins is critical to understand the role and the regulation of *Notch1* in hematopoiesis.

Interestingly, the suppressive effect of the *Notch1*-3′UTR was relieved immediately after the intra-thymic injection of HSCs, whereas no such relief of suppression was observed in HSCs injected in the bone marrow or cultured in vitro. Although GFP translation was completely inhibited by the *Notch1*-3′UTR, mRNA of GFP with *Notch1*-3′UTR existed abundantly in HSCs as the CMV promoter drives transcription. Therefore, the rapid appearance of GFP fluorescence after intra-thymic injection should be attributed to the relief of translational inhibition by the 3′UTR, presumably via an environmental signal in the thymus. Notch signaling plays an important role in the cell fate decisions in T lymphoid lineage [7, 24] with the blockage of B-cell development [25]. In addition, the activation of *Notch1* or *Notch2* was shown to inhibit myeloid differentiation in cell lines [31, 32]. Notch1 signaling was shown to counteract the *C/EBP* or *PU.1*-induced myeloid differentiation programs in immature T cells [33, 34]. Thus, the activation of the Notch pathway might promote T-cell development, inhibiting B lymphoid and myeloid lineage differentiation. In our study, we observed the relief of the suppressive effect of *Notch1*-3′UTR in the thymic environment. This phenomenon suggests the possibility that *Notch1* mRNA could be translated soon after the HSCs or multipotent progenitors home into the thymus, and the rapid induction of Notch1 protein may ignite the T cell developmental program.

In conclusion, we demonstrate that the expression of *Notch1* is regulated at the post-transcriptional level in HSCs by its 3′UTR, presumably via RNA-binding protein(s). Our study reveals a novel mechanism of the regulation of *Notch1* in HSCs and in the cell fate decision of the T lymphoid lineage, which may provide insight into a role of post-transcriptional control of critical transcription factors in hematopoiesis.

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Compliance with ethical standards

Conflict of interest The authors declared that no conflict of interest exists.

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