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Notch signaling regulates the FOXP3 promoter through RBP-J- and Hes1-dependent mechanisms

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Abstract Evidence has shown that Notch signaling modulates $CD4^+CD25^+$ regulatory T-cells (Tregs). As transcription factor Foxp3 acts as a master molecule governing the development and function of Tregs, we investigated whether Notch signaling might directly regulate Foxp3 expression. Here, we provide evidence that Notch signaling can modulate the FOXP3 promoter through RBP-J- and Hes1-dependent mechanisms. A conserved RBP-J-binding site and N-box sites were identified within the FOXP3 promoter. We show that the Notch intracellular domain (NIC), the active form of Notch receptors, activates a reporter driven by the FOXP3 promoter. Dissection of the FOXP3 promoter revealed bipartite effects of the RBP-J-binding site and the N-boxes: the RBP-J-binding site positively, while the N-boxes negatively regulated the FOXP3 promoter activity. Moreover, in freshly isolated Tregs, NIC-RBP-J complex is bound to the FOXP3 promoter in Tregs. Our results suggest that Notch signaling might be involved in the development and function of Tregs through regulating Foxp3 expression.

Keywords Regulatory T-cells · Notch · Foxp3 · Transcription

Abbreviations

RBP-J	Recombination signal binding protein-Jk
NIC	Intracellular domain of Notch
Treg	Regulatory T-cells
Hes	Hairy and enhancer of split

Introduction

Regulatory T-cells (Tregs) are $CD4^+$ T-cells constitutively expressing CD25 and play pivotal roles in the maintenance of self-tolerance [1]. The forkhead transcription factor Foxp3 is specifically expressed in Tregs [2]. Mutation or deletion of the gene encoding Foxp3 causes severe autoimmune diseases in both human and mice, due to a malfunction of $CD4^+CD25^+$ Tregs [3, 4]. On the other hand, ectopic expression of Foxp3 in conventional T-cells confers immuno-suppressive activities [4, 5]. These findings provided compelling evidences that Foxp3 acts as a master molecule controlling the development and function of Tregs. However, the molecular mechanisms leading to Treg differentiation remain largely unknown. Recently, an initial characterization of the human FOXP3 promoter revealed a basal, T-cell-specific promoter containing several NF-AT and AP-1 binding sites, which could positively regulate FoxP3 expression after triggering of the T-cell receptor (TCR) [6]. More recently, an epigenetic analysis indicated that an evolutionarily conserved region within the

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non-coding part of FOXP3 gene, which was completely and specifically demethylated in Tregs, was associated with the stable expression of Foxp3 [7].

Notch signaling represents a highly conserved pathway regulating cell proliferation and differentiation. Interaction between Notch ligands and receptors triggers a γ-secretase-mediated proteolysis of the receptors and liberation of the Notch intracellular domain (NIC) into cytoplasm. NIC then translocates into nucleus, where it binds to and trans-activates transcription factor RBP-J (also called CBF1). RBP-J recognizes a consensus DNA sequence C(T)GTGGGAA, which exists in multiple downstream genes including Hes family members such as Hes1. Hes proteins are suppressive bHLH molecules that repress many bHLH family transcription factors. Previous studies have shown that Notch signaling regulates lineage commitment at various stages of T-cell maturation [8]. For example, Notch1 is required for T lineage commitment from multipotent hematopoietic progenitors [9, 10]; Subsequently, Notch is required for efficient transition through the β-selection checkpoint [11–13] and may also regulate the development of $\gamma\delta$T-cells [12, 14]; Furthermore, some data propose multiple functions of Notch in peripheral T-cells that include activation [15, 16], tolerance induction [17], and the differentiation of helper T-cells [12]. But potential roles of Notch signaling in the development and functional maintenance of Tregs are still unclear.

Several studies have implicated the participation of Notch signaling in Treg differentiation. Antigen presented by murine APC overexpressing human Serrate1 induced naive peripheral $CD4^+$ T-cells to become regulatory cells, which can inhibit primary and secondary immune responses [18]. Epstein–Barr virus-positive lymphoblastoid cell lines (EBV-LCL) overexpressing the Notch ligand Jagged-1 can induce Tregs and the latter can specifically inhibit the proliferative and cytotoxic memory responses to EBV proteins [19]. Streptozotocin-induced autoimmune diabetes fails to develop in transgenic mice carrying the constitutively active intracellular domain of Notch3 in thymocytes and T-cells, which is associated with an increase of Tregs [20]. But in these above-mentioned studies, it is not clear how Foxp3 expression was regulated.

In the present study, we show that Notch signaling directly targets the FOXP3 promoter. The NIC-RBP-J complex and Hes1 can bind to the highly conserved RBP-J-binding site and N-boxes (Hes-binding site) located in the 5′ region of the FOXP3 gene, respectively. NIC-RBP-J complex is a trans-activator, while Hes1 is a trans-repressor of the FOXP3 promoter in vitro. Furthermore, the NIC-RBP-J complex binds to the FOXP3 promoter in both Tregs and conventional $CD4^+CD25^-$ T-cells. Taken together, our results suggest that Foxp3 expression may be directly regulated by Notch signaling.

Materials and methods

Cloning and construction

The mouse FOXP3 promoter (from −1864 to +316) was amplified by PCR using primers 5′-AGTGCTAGCTGAGGGAAAGAGCAAAGGAGTGTG and 5′-GGCAAGCTTCTGGAGACCAGCAGTTGATAGACA. The amplified promoter fragment was cloned into the pGL3-basic vector (Promega Life Science, Madison, WI) to generate the pGL3-FOXP3. The deletion mutant (−1577 to +316) of RBP-J-binding site was generated by PCR with primers 5′-AGTGCTAGCGATCTTGAATACAAACCTTAAAAC and the downstream primer for full length promoter. Site-directed mutagenesis of the RBP-J-binding site and N-boxes in the FOXP3 promoter was performed using the QuickChange kit (Stratagene), according to the manufacturer's instructions. Reporter constructs derived from these mutant versions of the FOXP3 promoter were named as pGL3-FOXP3-*RBP*, pGL3-FOXP3-RBP⊿, pGL3-FOXP3-*Nbox1*, pGL3-FOXP3-*Nbox2*, and pGL3-FOXP3-*Nbox3* (Figs. 2a, 3a).

Reporter assay

Hela cells or Jurkat cells (2×10^4) were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (Invitrogen). Jurkat cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 4 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, and antibiotics. Cells were transfected with 0.1 mg reporter construct, 0.1 mg pEF-Bos-NIC-neo [21], and 5 ng renilla luciferase vector (phRL-TK; Promega, Madison, WI) using Lipofectamine 2000™ (Invitrogen) or Fugene6™ (Roche). Total amount of transfected DNA was balanced with pEF-Bos-neo. About 48 h after transfection, luciferase activity was assessed using a Luminoskan Ascent (Labsystems, Helsinki, Finland) and a Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol. All luciferase activity was normalized with the renilla luciferase activity. Data were analyzed by *t*-tests. Statistical significance was set at $^{**}P < 0.01$ and $^{*}P < 0.05$.

Isolation and culture of $CD4^+CD25^+$ T-cells

Isolation of mouse $CD4^+$, $CD4^+CD25^-$, and $CD4^+CD25^+$ T-cells was performed by using a mouse Treg isolation kit (Miltenyi Biotec, Bergish Gladcach, Germany) according to the manufacturer's instructions. Briefly, $CD4^+$ T-cells were first enriched from healthy male C57BL/6 mice through negative selection by magnetically removing other types of cells. The $CD4^+$ T-cells were incubated with

magnetic beads conjugated with an anti-CD25 antibody to separate $CD4^+CD25^-$ and $CD4^+CD25^+$ T-cell sub-populations. The purity of the resulting T-cell sub-populations was confirmed to be higher than 95% by flow cytometry.

Western blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were saturated for 1 h at room temperature in TBST supplemented with 5% skimmed milk and immunoblotted overnight at 4°C with anti-Notch1 (NIC, M-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Hes1 (H-140; Santa Cruz Biotechnology), or anti-β-actin antibody (I-19; Santa Cruz Biotechnology). Membranes were then washed, probed with horseradish peroxidase (HRP) conjugated goat anti-rat, rabbit anti-goat, or goat anti-rabbit polyclonal antibodies (Zhong-Shan Biotech., Bei-Jing, China) according to the first antibody, and revealed with enhanced chemiluminescence (ECL; Amersham Biosciences, Uppsala, Sweden).

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a ChIP assay kit following the recommendations of the supplier (Upstate Biotechnology). Anti-NIC and anti-Hes1 antibodies were used for immunoprecipitation of chromatin along with controls. PCR primers for ChIP assays included: 5′-TGAGGGAAAGA GCAAAGGAG and 5′-ACCACCACCTCTTTGCA AGA, for RBP-J.

Results

Ectopic overexpression of NIC can regulate FOXP3 promoter activity

To determine whether Notch signaling directly regulated the FOXP3 promoter, we analyzed the genomic sequence of the mouse (AF277994) and human (AF235097) FOXP3 promoters. An alignment of the mouse FOXP3 promoter (−1864 to +316) with the human counterpart is depicted in Fig. 1a, showing the consensus transcriptional start site (TSS) and TATA box of the FOXP3 promoters. In the 5′ region of both mouse and human FOXP3 promoters, we found a conserved consensus RBP-J-binding motif. Moreover, several Hes consensus binding motifs, defined as N-box (CACNAG), could also be found (Fig. 1b). Notably, one of N-boxes overlapped with TSS in both mouse and human FOXP3 promoters.

To observe whether these putative transcription factor-binding sites modulated transcriptional activity, we amplified the mouse FOXP3 promoter (−1864/+316) and constructed a reporter plasmid pGL3-FOXP3. As T-cells are difficult to transfect, we employed Hela cells. Transfection of Hela cells with pGL3-FOXP3 resulted in little luciferase activity in the cell lysates, consistent with that of

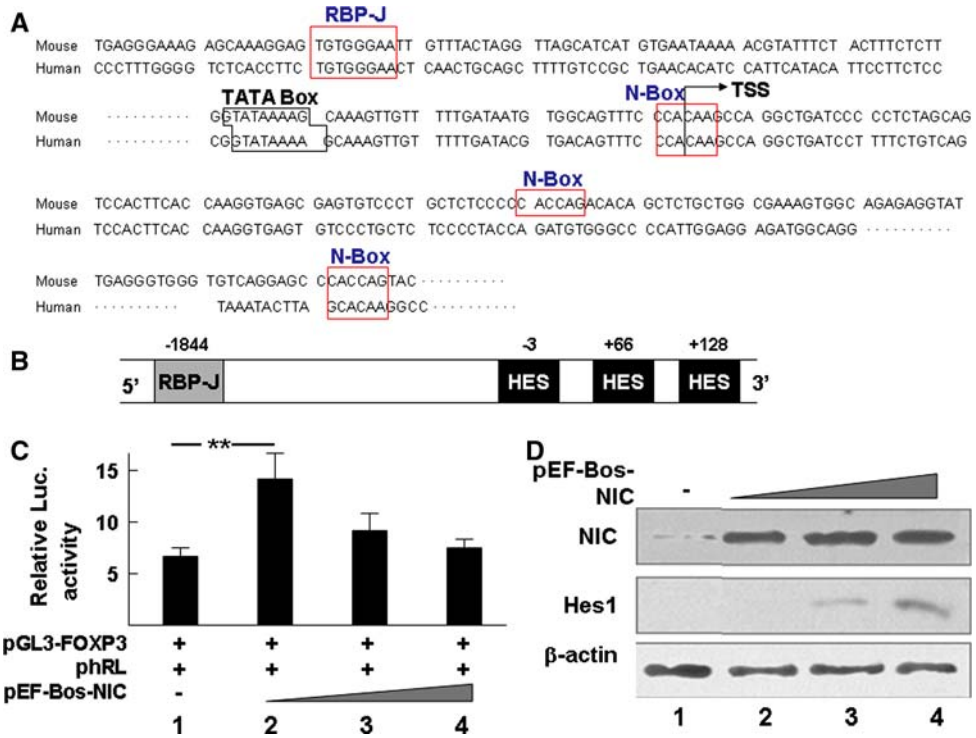

Fig. 1 Ectopic overexpression of NIC regulated FOXP3 promoter activity. (**a**) Sequence alignment of the human and mouse FOXP3 promoters. TATA box, TSS, N-boxes, and a RBP-J-binding site were indicated. (**b**) Scheme of the 5′ region of the mouse FOXP3 promoter, indicating the potential RBP-J and Hes recognition sites. (**c** and **d**) Reporter assays. Hela cells were transfected with pGL3-FOXP3 (100 ng) and gradient doses of NIC (0, 60, 80, and 100 ng) and other plasmids as indicated. The relative luciferase activity (firefly luciferase/renilla luciferase) was analyzed 48 h later. The results were presented as the mean ±S.D. $^{**}P < 0.01$ ($n = 3$) (**c**). NIC and Hes1 proteins in the transfected cells were detected by Western blotting with β-actin as a control for protein loading (**d**)

FoxP3 is not expressed in these cells. Co-transfection with the reporter and an NIC expression vector pEF-Bos-NIC, however, resulted in mild transactivation of the FOXP3 promoter (Fig. 1c, lane 2). Interestingly, with the increasing amount of co-transfected NIC expression vector, notably when Hes1 expression was significantly induced (Fig. 1d, lanes 3 and 4), transactivation of the FOXP3 promoter decreased gradually (Fig. 1c, lanes 3 and 4). These results suggested that Notch signaling might regulate the FOXP3 promoter by repressing the FOXP3 promoter at high magnitude of Notch signaling.

Differential effects of the RBP-J- and Hes1-binding sites on the FOXP3 promoter

In order to understand the function of different parts of the FOXP3 promoter in its transactivation, we first generated a deletion mutant of the FOXP3 promoter, in which the RBP-J-binding site was removed (Fig. 2a). Reporter assay with a construct in which luciferase gene was controlled by the truncated FOXP3 promoter (pGL3-FOXP3-*RBP*) showed that the activation of the FOXP3 promoter by NIC was cancelled (Fig. 2b), suggesting that the RBP-J-binding site in the FOXP3 promoter might be responsible for its transactivation by NIC. This assumption was supported by site-directed mutagenesis of the RBP-J-binding site, which showed similar influence on the transactivation of the FOXP3 promoter by NIC (Fig. 2c).

To look at the function of the N-boxes in the FOXP3 promoter, we disrupted these N-boxes by site-directed mutagenesis, and examined the consequence using the reporter assay (Fig. 3a). As shown in Fig. 3b, mutation of the N-box at position-3, which harbors TSS, resulted in increase of the transactivation of the promoter in the presence of NIC. Disruption of the N-box located at +66 or +128 also increased the transactivation of the promoter by NIC (Fig. 3b). In Jurkat cells, disruption of the N-box located at +66 had no influence on the activity of the promoter, but disruption of the N-box located at +128 increased the transactivation of the promoter (Fig. 3c).

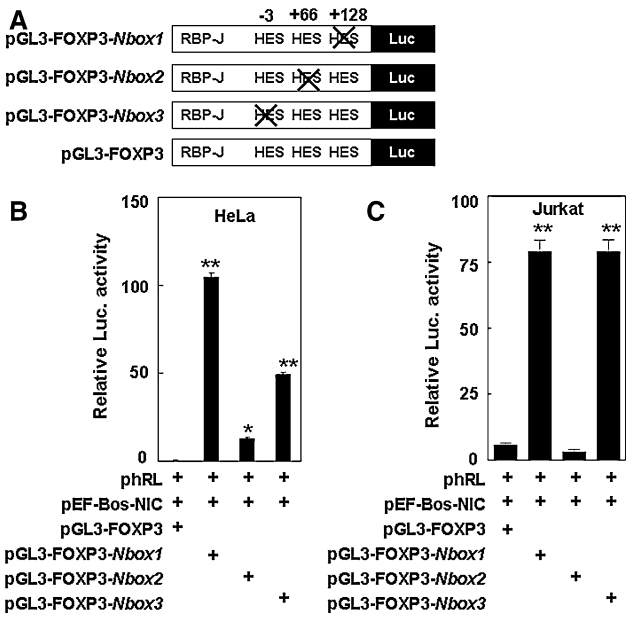

Fig. 3 Effect of the N-boxes in the transactivation of the FOXP3 promoter by NIC. (**a**) Scheme of the mutants of the mouse FOXP3 in which the N-boxes were disrupted. (**b** and **c**) Reporter assays. HeLa cells (**b**) and Jurkat cells (**c**) were transfected independently with 100 ng NIC and 100 ng wild-type or N-box mutants of the FOXP3 reporter constructs and 5 ng phRL-TK, and were assayed as in Fig. 1c

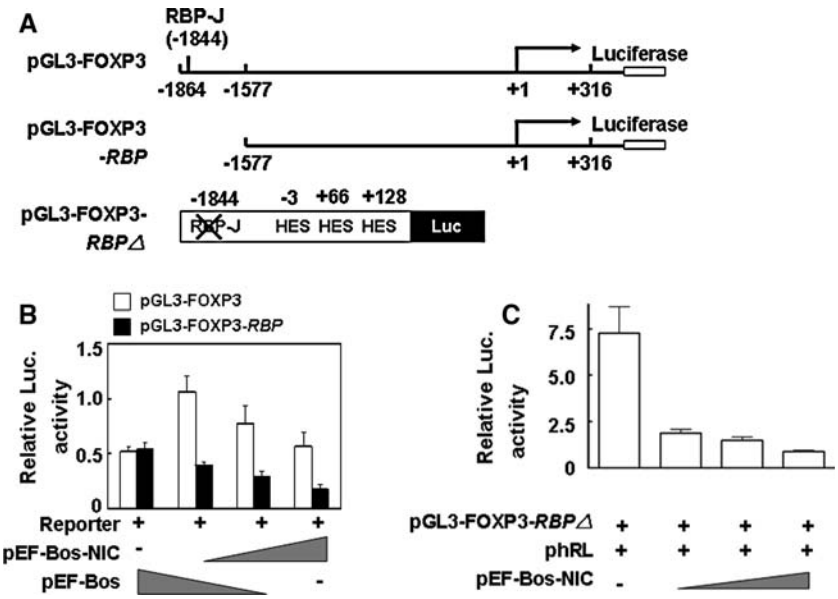

Fig. 2 Effect of the RBP-J-binding site in the transactivation of the FOXP3 promoter by NIC. (**a**) Scheme of the deletion mutants and the site-directed mutant of the mouse FOXP3 in which the RBP-J-binding site was deleted. (**b** and **c**) Reporter assays. HeLa cells were transfected independently with the deletion mutant (**b**) and site-directed mutant (**c**) of the *FOXP3* reporter constructs, and were assayed as in Fig. 1c

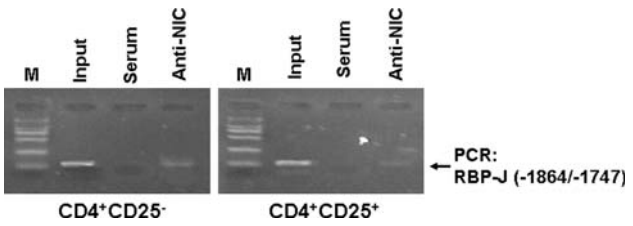

Fig. 4 The NIC-RBP-J complex bound to the FOXP3 promoter in $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T-cells. Chromatin preparations from $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T-cells were immunoprecipitated using anti-NIC, and co-precipitated DNA fragments were amplified by primers specific for the RBP-J-binding site fragment. Normal goat serum was used as a control. Arrows indicated the amplified fragments

These results suggested that Hes1 might repress the transactivation of the FOXP3 promoter by NIC.

NIC binds to the FOXP3 promoter in Tregs and $CD4^+CD25^-$ T-cells

Next we investigated whether NIC could bind to the FOXP3 promoter in freshly isolated mouse $CD4^+CD25^+$ Tregs, which express Foxp3, and conventional $CD4^+CD25^-$ T-cells, by the ChIP assay. Mouse $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T-cells were enriched magnetically, and ChIP assays were performed using anti-NIC. The co-precipitated chromatin DNA fragments were amplified by PCR using primers for the FOXP3 promoter region harboring the RBP-J-binding site (−1864 to −1747). The results showed that the fragment with the RBP-J-binding site was co-precipitated by the anti-NIC antibody in both $CD4^+CD25^+$ Tregs and in $CD4^+CD25^-$ conventional T-cells (Fig. 4).

Discussion

The forkhead transcription factor Foxp3 has been identified as a specific molecular marker of Tregs, and its expression is essential for the programmed development and function of Tregs [2, 22, 23]. Although it has been widely accepted that Foxp3+ Tregs represent a stable population mainly generated as a separate lineage, conclusive data on the molecular mechanisms maintaining stable Foxp3 expression are not available. In the present study, we describe a direct link between Notch signaling and the FOXP3 promoter.

First, we identified a RBP-J-binding site 1844 bp upstream the translation start site and three tandem Hes-binding sites at −3, +66, and +128 positions in the mouse FOXP3 promoter. We also identified binding sites for RBP-J and Hes1 at the similar positions of human FOXP3 genes. This conservation underscored the importance of these motifs as regulatory elements and provided additional novel evidence for the role of Notch signaling in the regulation of the FOXP3 expression. Indeed, using a luciferase reporter assay, we showed that overexpression of NIC in Hela cells resulted in activation of the FOXP3 promoter. ChIP assay using freshly isolated Tregs confirmed the binding of the NIC-RBP-J complex to the FOXP3 promoter regions. Thus, we believed that Notch signaling could regulate Foxp3 expression and influence the immuno-suppressive activity of Tregs. It should be noticed, however, the Notch might not be involved in the lineage determination during Treg development, because the binding of NIC-RBP-J with the FOXP3 promoter could also be detected in conventional $CD4^+CD25^-$ T-cells.

However, our reporter assay also showed that higher dose NIC could down-regulate the FOXP3 promoter activity. To understand the molecular mechanism underlying this phenomenon, we disrupted the RBP-J-binding site and the N-boxes in the FOXP3 promoter by deletion and site-directed mutagenesis. The results confirmed that the NIC-RBP-J complex was a transactivator on one hand, but on the other hand, Hes1 appeared to be a repressor of the FOXP3 promoter. Consistently, in the transfection assay, at higher dose of NIC when the FOXP3 promoter was turned down, Hes1 expression was significantly induced. ChIP assay indicated that in freshly isolated T-cells, while NIC-RBP-J bound to the FOXP3 promoter in both conventional $CD4^+CD25^-$ T-cells and Tregs, Hes1 mainly bound to the FOXP3 promoter in $CD4^+CD25^-$ T-cells. NIC could translocate into the nucleus and bind to RBP-J, then activated transcription of target genes such as Hes1. Hes1 is a transcriptional repressor. Therefore, we assumed that Notch signaling might regulate the FOXP3 promoter in a bi-phasic manner: at low magnitude Notch signaling it activated the FOXP3 promoter through the NIC-RBP-J complex, but at high magnitude Notch signaling it repressed the FOXP3 promoter through Hes1.

Transcription factor network and epigenetic mechanisms are responsible for the Treg differentiation. An evolutionarily conserved region upstream exon-1 of the FOXP3 gene, which was completely demethylated specifically in Tregs, was recently identified associated with stable Foxp3 expression [7]. However, the N-box region examined in this study was not included in the demethylated region. Our results revealed that Hes1 bound to the N-box region of the FOXP3 promoter specifically in $CD4^+CD25^-$ T-cells, but not in $CD4^+CD25^+$ Tregs. In view of the different binding state of Hes1 with FOXP3 gene in $CD25^+$ Tregs and conventional $CD4^+CD25^-$ T-cells and the intensive repressive effect of Hes1 to FOXP3 promoter activity, Hes1 might be an important regulatory factor at the transcriptional level in the lineage determination of Tregs development.

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