Title: Control of regulatory T-cell differentiation and function by T-cell receptor signalling and Foxp3 transcription factor complexes

Author: Masahiro Ono
Affiliation: Department of Life Sciences, Imperial College London
Correspondence author's contact details:
Sir Alexander Fleming Building
Imperial College London
London SW7 2AZ

Summary

The transcription factor Foxp3 controls the differentiation and function of regulatory T-cells (Treg). Studies in the past decades identified numerous Foxp3-interacting protein partners. However, it is still not clear how Foxp3 produces the Treg-type transcriptomic landscape through cooperating with its partners. Here I show the current understanding of how Foxp3 transcription factor complexes regulate the differentiation, maintenance, and functional maturation of Treg. Importantly, T-cell receptor (TCR) signalling plays central roles in Treg differentiation and Foxp3-mediated gene regulation. Thus, differentiating Treg will have recognised their cognate antigens and received TCR signals before initiating *Foxp3* transcription, which is triggered by TCR induced transcription factors including NFAT, AP-1, and NF-κB. Once expressed, Foxp3 seizes TCR signal-induced transcriptional and epigenetic mechanisms through interacting with is AML1/Runx1 and NFAT. Thus Foxp3 modifies gene expression dynamics of TCR-induced genes, which constitute cardinal mechanisms for Treg-mediated immune suppression. Next, I discuss the following key topics, proposing new mechanistic models for Foxp3-mediated gene regulation: (1) how Foxp3 transcription is induced and maintained by the Foxp3-inducing enhanceosome and the Foxp3 autoregulatory transcription factor complex; (2) molecular mechanisms for effector Treg differentiation (i.e. Treg maturation); (3) how Foxp3 activates or represses its target genes through recruiting coactivators and corepressors; (4) the 'decision-making' Foxp3-containing transcription factor complex for Th17 and Treg differentiation; and (5) the roles of post-translational modification in Foxp3 regulation. Thus, this article provides cutting-edge understanding of molecular biology of Foxp3 and Treg, integrating findings by biochemical and genomic studies.

Running title: Foxp3 transcription factor complexes

Abbreviations: Treg, regulatory T-cells; TCR, T-cell receptor; IPEX, Immune dysregulation, polyendocrinopathy, enteropathy, X-linked; eTreg, effector Treg; APC, antigen presenting cell; activator protein-1, AP-1; IL, interleukin; SP, single positive; Tocky, Timer of Cell Kinetics and Activity; AML1, Acute Myeloid Leukaemia-1; CNS, conserved non-coding sequences; ChIP, Chromatin immunoprecipitation; TNFRSF, TNF receptor superfamily; TRAF, TNF receptor associated factor; PI(3)K, Phosphoinositide 3-kinase; TSDR, Treg-specific demethylation region; iTreg, induced Treg; HAT, histone acetyltransferase; CBP, CREB-binding protein; H3K27ac, histone H3 lysine 27 acetylation; H3K27me3, histone H3 lysine 27 trimethylation

Key words: Foxp3, AML1/Runx1, protein-protein interaction, regulatory T-cells, transcription factor, transcriptional regulation, TCR signalling, NFAT, NF-κB, p300

Introduction

Upon recognising antigens, T-cells receive T cell receptor (TCR) signals, thereby get activated and differentiate into effector T-cells, and thus induce immune responses. In inflammatory environments, in which antigen presenting cells (APCs) are matured [1], T-cells effectively receive CD28 signals from its ligands CD80/CD86 on APCs when TCRs engage with peptide-MHC complex. These two signals induce T-cell activation and proliferation [2, 3]. Then, activated T-cells produce interleukin (IL)-2 and CD25 (IL-2 receptor α chain), which forms the high-affinity IL-2 receptor with β and γ chains. Activated T-cells efficiently receive IL-2 signals, which promote their own survival and differentiation. Thus, a positive feedback loop for T-cell activation is established[4, 5]. T-cell activation also induces molecules for negative feedback regulation in any T-cells, including CTLA-4, which binds to and inhibits CD80/CD86 on APCs and thereby blocks CD28 signalling, inducing T-cell quiescence [6].

However, upon recognising antigens, a minor subset of $CD4^+$ T-cells do not induce immune responses but suppress the activities of other T-cells. These cells are designated as regulatory T-cells (Treg), or natural Treg[7, 8]. Treg specifically express the transcription factor Foxp3, which controls their differentiation and function[9-11]. Notably, Treg do not produce IL-2 by TCR stimulation[12], and the majority of them have an activated memory phenotype ($CD25^+CD45RB^{low}$) [13, 14]. $Foxp3^+$ Treg constitute 10-15% of $CD4^+$ T-cells in the periphery and 5-6% of CD4-single positive (SP) cells in the thymus of healthy mice [15, 16].

Although the mechanism of Treg-mediate suppression is still debated, Foxp3 represses *Il2* transcription, upregulates the expression of CD25 and other Treg markers, and confers suppressive activities to $CD4^+$ T-cells [9]. Mutations of the *Foxp3* gene impair Treg function and lead to autoimmune diseases in mice (Scurfy disease [17, 18]) and humans (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked [IPEX] syndrome [19]). The depletion of $Foxp3^+$ Treg can induce autoinflammation [20, 21] and can enhance anti-cancer immunity [22]. On the other hand, cell transfer of ex-vivo isolated Treg or Foxp3-transduced T-cells can suppress the activities of autoinflammatory T-cells [23]. Collectively, these indicate that the regulation of Foxp3 expression is critically important for controlling immune responses.

The unique gene expression dynamics of TCR-induced genes constitute key mechanisms for the suppressive activities of Treg. Treg highly express CD25 and do not produce IL-2, and thus, they can absorb IL-2 proteins in microenvironments and prevent effector T-cells from proliferating and surviving[24]. In addition, Treg highly express CTLA-4, and thus, when inflammation occurs, Treg can immediately prevent other T-cells from receiving CD28 signalling[25].

Foxp3 has a DNA-binding domain (forkhead domain) and binds DNA as homodimer or heterodimer with Foxp1 [26]. Foxp3 interacts with key transcription factors for T-cell function, including NFAT [27], NF-κB [28], and AML1/Runx1 (hereafter Runx1)[29]. The Foxp3 transcription factor complex further associates with transcriptional repressors and activators to regulate transcriptional activities of its target genes in a context-dependent manner. Studies in the past 16 years identified more than 300 proteins as Foxp3-interacting partners, although many of these interactions still need further investigations.

In this article, I will show the up-to-date understanding of how Foxp3 regulates the differentiation, maintenance, and functional maturation of Treg through interacting with other transcription factors. To this end, I will firstly discuss the key evidence for the current understanding of Treg, highlighting the importance of Foxp3 expression dynamics.

From Treg classification to Foxp3 expression dynamics

Treg are heterogeneous. The identification of functional Treg subsets is key to understand Treg function. Treg are often classified into thymic and peripheral Treg, depending on whether they become Treg in the thymus or the periphery, respectively [30]. In addition, both Treg subsets can become activated and show enhanced suppressive activities in vivo as *effector Treg* (eTreg).

Thymic Treg and peripheral Treg

Thymic Treg (tTreg) are defined as the Treg that differentiate in the thymus [30]. In addition, $Foxp3^{-}CD4^{+}$ T-cells (designated as conventional T-cells) can become Treg in the periphery (peripheral Treg, pTreg), especially in the intestinal system [31] and the placenta [32]. Food antigens and commensal bacteria induce Foxp3 expression in T-cells in the small intestine and the colon, respectively, generating pTreg [33, 34]. Intriguingly, recent thymic emigrant $CD4^{+}$ T-cells have higher capacities to initiate Foxp3 expression in the periphery [35]. The majority of natural Treg are believed to be tTreg [36], although quantitative data are not yet available.

While Helios (*Ikzf2*) and neuropilin-1 (Nrp1) are often used for markers for tTreg, their expression can be changed depending on experimental conditions [36]. The majority of Treg in the colon express RORγt (also known as a Th17 transcription factor). $ROR\gamma t^{+}$ Treg are considered to be pTreg, as they do not express Helios and are induced by gut microbiota, while $Helios^{+}$ Treg are regarded as tTreg. However, it is debated how specific these markers are, and

a recent single cell study showed that conventional T-cells can generate both Helios$^+$ and RORγt$^+$ Treg in the colon [37].

By definition, tTreg must have TCRs that recoginse self-antigens presented in the thymus, because Treg differentiation requires the recognition of cognate antigens [38, 39]. On the other hand, the TCR repertoire of pTreg may be enriched with those for commensal and food antigens [40, 41]. However, investigations are hampered by the lack of methods to specifically identify tTreg and pTreg [42].

Understanding effector Treg and Treg maturation by Foxp3 expression dynamics

Regardless of in which organ Foxp3 expression is initiated, functional Treg have the common characteristics: high Foxp3 expression. Foxp3 expression is primarily controlled by transcriptional and epigenetic regulation. A recent technological advancement to analyse temporal dynamics of *Foxp3* transcription is providing new insights into Treg differentiation and function [43]. Notably, highly sustained *Foxp3* transcription over time induce the differentiation of eTreg, which is also known as Treg maturation [44]. On the other hand, once Foxp3 expression is completely lost, Treg can become memory-phenotype T-cells during homeostasis [45] or fully function as effector T-cells that promote inflammation(also known as 'ex-Treg') [46]. Interestingly, Nrp1 expression is high in resting Treg but is substantially reduced in eTreg with persistent *Foxp3* transcription [44]. Interestingly, in a skin allergy model, both eTreg differentiation and de novo Foxp3 expression in effector T-cells are promoted [44]. This suggests that new Foxp3 induction and Treg maturation use a common mechanism, which most likely involves TCR-induced transcription factors.

Foxp3 expression can be induced just by TCR stimulation in most of CD4$^+$ T-cells in humans, and by the combination of TGF-β and IL-2, and TCR stimulation in mice (induced Treg, iTreg) [47]. Since these in vitro induced Foxp3 expression is not 'stable' over time, the induction of Foxp3 has been dismissed as 'transient'. However, 'transient' Foxp3 expression has cell-intrinsic roles to suppress TCR-induced activation [48-50], and may have large impacts on T-cell regulation, considering that any acute inflammation is also 'transient'. In humans, notably, the majority of Foxp3$^+$ T-cells are low Foxp3 expressors that produce pro-inflammatory cytokines and are not suppressive in vitro, whereas Foxp3^{high} CD25^{high}CD45RO$^+$CD4$^+$ T-cells are suppressive and designated as eTreg [51, 52].

The difference of Foxp3 dynamics between humans and mice can be explained by epigenetic regulation. Importantly, *Foxp3* promoter is TCR-responsive [53]. CpG islands in the human *Foxp3* promoter are demethylated in naïve T-cells [54], whereas those in mouse *Foxp3* promoter

are only partially demethylated in naïve T-cells and fully demethylated by TGF-β signals [53]. These can explain why human T-cells can express Foxp3 just by TCR signals, while mouse T-cells generally require TCR and TGF-β signals for efficient induction of Foxp3. On the other hand, the demethylation of CpG islands in Treg-specific demethylation region (TSDR) is associated with 'stable' Foxp3 expression. TSDR is methylated in conventional T-cells and iTreg cells in mice and humans, and human Foxp3^{low} T-cells, while it is fully demethylated in mouse Treg and human Foxp3^{high}CD25^{high}CD45RO^{+} eTreg[52, 55].

Collectively, Treg function is dependent of Foxp3 expression level in mice and humans. Both mouse and human Treg have an activated memory phenotype.

TCR signalling downstream is continuously active in Treg due to infrequent but regular antigen recognition

This section shows that Treg have a constitutive active status, which is induced by TCR signals independently from Foxp3, and that Foxp3 exploits TCR-induced mechanisms to produce Treg phenotype and function. To understand these, it is important to analyse temporal dynamics of TCR signalling and Treg differentiation in vivo.

A recent breakthrough was made through the new technology *Timer of Cell Kinetics and Activity* (Tocky). Tocky uses as a reporter gene Fluorescent Timer protein that spontaneously and irreversibly changes its emission spectrum from blue to red within 4 hours on average [56]. Using the TCR downstream gene *Nr4a3*, Nr4a3-Tocky allows analysis of real-time dynamics of TCR signal downstream activities at the single cell level. In addition, Foxp3-Tocky enables analysis of temporal dynamics of *Foxp3* transcription, and thereby reveals temporal orders of Treg differentiation events in the thymus and the periphery [56].

Importantly, when Foxp3 is expressed in differentiating or mature Treg, TCR-signal induced transcription factors will have been already active. Thus, once expressed, Foxp3 can immediately make complex with these transcription factors during Treg differentiation and homeostasis. This is a non-obvious fact but can be safely concluded from the following three lines of evidence.

Firstly, TCR signals always come first before Foxp3 expression. Foxp3 is a TCR signal downstream gene as discussed above. This means that, when Treg differentiate, they will have been already activated by TCR signals [56, 57]. Treg differentiation is triggered and controlled by TCR signals in the thymus and the periphery. When differentiating CD4-SP cells receive strong

TCR signals, they upregulate CD25 expression, becoming Treg precursors. If these precursor cells continue to receive persistent TCR signals across time, they initiate *Foxp3* transcription and become Treg [56]. In the periphery, $CD4^+$ conventional T-cells can differentiate into pTreg by recognising food and microbial antigens as discussed above. Furthermore, new *Foxp3* transcription can be initiated in effector T-cells upon antigen recognition during inflammation [44].

Secondly, Treg receive infrequent-yet-regular TCR signals in the periphery. Moran et al developed Nr4a1-GFP reporter mice and showed that Treg have higher GFP, suggesting that Treg received more TCR signals, although their temporal dynamics were not known [58]. Nr4a3-Tocky analysis showed that the majority of Treg spontaneously recognized their cognate antigen and received TCR signals approximately once a few days [44]. Thus, Treg regularly recognise their cognate antigens and receive TCR signals. Since T-cells specific to tissue antigens or microbiota can differentiate into Treg (either as tTreg or as pTreg), it is not surprising that Treg regularly encounter with their cognate antigens while circulating the body. This also indicates that the relative 'short-life' of pTreg may be due to the removal of their cognate antigens from the system [50], especially when the antigens are exogenous.

Thirdly, genes that are stimulated downstream of TCR signaling are actively transcribed in Tregs. Our multidimensional transcriptome analysis showed that Treg were as activated as tissue-infiltrating effector T-cells and memory phenotype T-cells at the transcriptome level [59]. Furthermore, upon the deletion of TCRα in the periphery, Treg downregulate Treg markers [60] and lose the transcriptional signature of activated T-cells [59].

Summarising, Foxp3 is induced in T-cells that have received strong and persistent TCR signals, and Foxp3 expression is maintained in a unique type of activated T-cells, which regularly encounter with their cognate antigens. In other words, as we previously proposed, whether in tTreg or pTreg, Foxp3 is induced and maintained as a negative feedback on cognate antigen-induced activation processes, and that Treg are activated T-cells in which negative regulatory mechanisms are actively operating[50]. Accordingly, transcription factors downstream of TCR signalling are always available to Foxp3 protein in Treg.

In the following sections, I highlight the following key Foxp3-interacting transcription factors as central players for Treg differentiation and function: (1) TCR signal-induced transcription factors (e.g. NFAT); (2) Runx and cofactors; (3) Foxp3 autoregulatory transcription factor complex; (4) transcription factors for eTreg maturation; and (5) Foxp3-containing transcription factor complex for fate bifurcation (e.g. Foxp3/RORγt/Hif1a) (**Table 1**).

Foxp3 interacts with TCR signalling downstream transcription factors

Foxp3 interacts with TCR-induced transcription factors, which are activated through signalling cascades initiated by TCR and CD28 costimulatory signals. TCR-MHC engagement activates proximal signalling molecules including PLCγ1 and PKC, which subsequently activate calcium signalling and NF-κB and Ras/MAPK pathways [61]. Calcium influx activates and translocates NFAT into the nucleus [62]. TCR and CD28 costimulatory signals cooperatively activate MAPK and NF-κB pathway and efficiently activate AP-1 transcription factors [63] and NF-κB [64]. Notably, NFAT and AP-1 make a complex and binds to and activates the IL-2 promoter [62].

NFAT and AP-1

AP-1 transcription factors are formed by heterodimerization of bZIP proteins including Jun (e.g c-Jun, JunB, and JunD), Fos (e.g. c-Fos), and ATF (e.g. ATF2, BATF) [65]. Particularly, Fos and Jun regulate TCR signal downstream genes [65]. Foxp3 is proposed to bind c-Jun and inhibit AP-1 activities [66], although the evidence is limited.

Foxp3 binds NFAT and inhibits its transcriptional activities [27, 28]. Wu et al analysed crystal structure of NFATc2 (NFAT1), the forkhead domain of FOXP2 (which is considerably similar to that of FOXP3), and a 19 base-pair-DNA sequence from the *IL2* promoter (namely, ARRE2), and characterised the NFATc2:FOXP2:DNA complex. Interestingly, when making a complex with NFATc2, the forkhead domain bound the DNA sequence that are occupied by Fos and Jun when they bind NFAT, suggesting that Foxp3 competitively inhibit the NFATc2:Fos:Jun complex by binding NFATc2 and replacing Fos and Jun [27]. However, this view is challenged, as another Foxp member, Foxp1, is constitutively expressed by $CD4^+$ T-cells [67], which means that Foxp1 may constitutively make complex with NFAT in the absence of Foxp3.

Functional significance of the Foxp3-NFAT interaction is still not fully clear. Wu et al showed that Foxp3 mutants that did not bind NFATc2 were defective in mediating *Il2* repression, the upregulaton of Treg-associated surface proteins (e.g. CD25), and suppressive activities [27], suggesting that the Foxp3-NFATc2 complex regulates transcriptional activities of a wide range of genes. The Foxp3-binding domain of NFATc2 is conserved with other NFAT isoforms (i.e. NFAT2, NFAT3, and NFAT4) [27]. However, it is not known whether different NFAT isoforms play different roles in regulating Foxp3 function.

NF-κB

Immunoprecipitation experiments showed that Foxp3 physically interacted with RelA (p65), an NF-κB subunit [28, 68, 69], although another study failed to confirm this [70]. Camperio et al showed that Foxp3 and RelA cooperatively activated the transcription of the promoter of *Il2ra* (CD25) by a reporter assay [68], while Foxp3 rather repressed NF-κB reporters in other studies [70, 71]. Loizou et al showed that Foxp3 interacted with the DNA binding domain of another NF-κB component, c-Rel, and repressed NF-κB transcriptional activities although the evidence is limited to overexpression and reporter assays [71]. These conflicting results suggest that the interaction between Foxp3 and NF-κB may be indirect through NFAT, which can make a complex with RelA [72] and c-Rel [73], or through Runx1, which interacts with the NF-κB subunit p50 [74].

Collectively, Foxp3 participates in TCR-induced transcription factor complexes and modulates their activities. This is considered to be critically important to modulate the gene expression dynamics of TCR-induced genes in Treg, which constitute cardinal mechanisms for Treg-mediated immune suppression.

Foxp3 seizes Runx transcription factor complex for shaping the Treg-type transcriptome

Foxp3 interacts with Runx1 and modulates its transcriptional regulation in Treg. Thus, this section briefly discusses the roles of Runx1 in non-regulatory T cells, and thereafter shows the Foxp3-Runx1 complex-mediated regulation of Treg differentiation and function.

Roles of Runx1 in CD4$^+$ T-cells

Runx1 directly bind to specific DNA sequences through its N-terminal DNA-binding domain (Runt domain) by making a complex with the cofactor CBF-β (**Fig. 1**). As its original name (Acute Myeloid Leukaemia-1) implies, Runx1 is a frequent target of chromosomal translocation and fusion protein formation in acute leukaemia [75]. In normal cells, Runx1 mediates DNA-sequence-specific transcriptional regulation and plays key roles in multiple stages of haematopoietic cell development, from haematopoietic stem cells to peripheral T-cells [76]. Importantly, Runx1 controls the differentiation and homeostasis of CD4$^+$ T-cells, although underlying mechanisms are not clear. Deletion of Runx1 in thymic T-cells results in the blockade of CD4-SP maturation, and CD4$^+$ T-cells are reduced in the periphery [77]. On the other hand, transgenic expression of *Bcl2* in CD4$^+$ T-cells restores the number of CD4$^+$ T-cells in the Runx1-deficient background [78], suggesting that Runx1 inhibits the activities of pro-apoptotic Bcl-2 family proteins.

The Foxp3- Runx1 interaction

Foxp3 physically interacts with Runx1 and thereby modifies Runx1-mediated gene regulation. The Runx1-binding region of Foxp3 is the inter-domain region between the forkhead domain and the leucine zipper motif, which does not have other protein interaction or any known function [29] (**Fig. 1**). Ono et al showed that the Foxp3 mutants that did not bind Runx1 lacked the abilities to produce Treg function and phenotype, including *Il2* repression, Treg marker expression (including CD25 and GITR), and suppressive activities [29]. Treg-specific deletion of Runx1 or CBF-β induces the proliferation of $CD4^+$ T-cells and the development of autoimmune gastritis, indicating that Runx1 is required for Treg's suppressive function [79].

In the absence of Foxp3, Runx1 binds *Il2* enhancers in $CD4^+$ T-cells and is poised to activate *Il2* transcription [29]. Although Runx1 is dispensable for initiating *Il2* transcription [80], Upon receiving TCR signals, Runx1 enhances *Il2* transcription in non-Treg, and the amounts of Runx1 protein are correlated with *Il2* transcriptional activities [29, 81]. Retroviral gene transduction using murine conventional T-cells showed that Runx1 binds to the *Il2* promoter and enhances IL-2 production, which is suppressed by Foxp3 [29]. Importantly, IL-2 production is markedly increased in *Cbfb*-deficient Treg [79], indicating that Foxp3 cannot repress *Il2* in the absence of the Runx1-CBF-β complex. Thus, it is likely that Runx1 is required for the optimal expression dynamics of IL-2, orchestrating NFAT, AP-1, and NF-κB to control temporal dynamics of *Il2* transcriptional activities. However, once Foxp3 is expressed, the Foxp3-Runx1 complex functions as a repressor for *Il2* transcription.

The transcription factor Ets1 interacts and cooperates with Runx1, promoting the binding of Runx1 to DNA [82], activating transcription of their target genes [83] (**Fig. 1**). *Ets1*$^{-/-}$ mice show B-cell activation, and have reduced numbers of T-cells, which are also highly activated. This phenotype can be rescued by adoptive transfer of WT Treg, indicating the functional impairment of *Ets1*$^{-/-}$Treg [84]. Interestingly, Kwon et al showed Chromatin immunoprecipitation (ChIP) peak data that Ets1 was excluded from Foxp3-binding sites specifically in Foxp3-repressed genes [69].

Runx-guided control of active enhancers and Treg transcriptome

Samstein et al showed that Foxp3-binding sites were significantly enriched with Runx, Ets, and forkhead motif sequences [85]. Importantly, nucleosome-free genomic regions are largely overlapped between Treg and activated conventional T-cells, and are enriched with AP-1 motif [85]. These regions are likely to be opened and maintained by the cooperation of NFAT and AP-1, because they lose chromatin accessibility in Treg-specific knock-out of calcineurin B (*Cnb1*), which is required for the nuclear translocation of NFAT upon calcium influx [85]. Interestingly, another ChIP-seq analysis of H3K4me2 showed that primed enhancers in activated $CD4^+$ T-cells

were enriched with the motifs for Runx1, Ets1, IRF4, NF-κB/Rel, and AP-1 [86]. These studies highlight the central role of Runx1 in regulating enhancers in conventional T-cells and Treg.

Importantly Treg transcriptomes are largely similar to memory-phenotype T-cell transcriptomes, and their shared feature is TCR-induced T-cell activation [59]. As demonstrated by multidimensional transcriptome analysis, Runx1 enhances transcription of some of the TCR-induced genes in memory-phenotype T-cells, which are mostly repressed by Foxp3. On the other hand, Foxp3 largely sustains the expression of Runx1-independent TCR-induced genes [59]. Thus, Runx1 enhances transcription of genes downstream of TCR signalling, while Foxp3 binds Runx1 and modifies the transcriptional regulation of their common target genes.

Accordingly, here I propose a model for Runx1-guided control of transcriptional programme for Treg differentiation (**Fig. 2**). In the absence of Foxp3, Runx1 binds to a wide range of primed or poised enhancers of the genes for T-cell differentiation and response (**Fig. 2a**). Upon antigen recognition, TCR-induced transcription factors (including NFAT, AP-1 and NF-κB) are recruited to Runx1-bound genes, and their cooperation control gene expression dynamics of TCR-induced genes (including IL-2 and CD25) (**Fig. 2b**). However, once Foxp3 expression is induced, Foxp3 binds to the primed and active enhancers through interacting with Runx1 and NFAT (**Fig. 2c**). Subsequently, Foxp3 will further recruit cofactors, modifying the composition of pre-existing Runx1-containing complex. When acting as a transcriptional repressor complex, Foxp3 may recruit corepressors (**Fig. 2c**). On the other hand, when acting as a transcriptional activator, Foxp3 recruits p300 and other coactivators, stabilising TCR-induced enhanceosomes, and thereby maintains constitutively active transcription of its target genes (**Fig. 2d**).

Foxp3-inducing transcription factors and Foxp3 autoregulatory transcriptional circuit

When *Foxp3* transcription is newly induced in differentiating thymic CD4-SP and peripheral non-Treg $CD4^+$ T-cells, transcription factors downstream of TCR signalling and IL-2 signalling play key roles. However, once *Foxp3* transcription is sustained and becomes stabilized, it is regulated by the Foxp3 autoregulatory transcription factor complexes that are bound to the key regulatory regions of the *Foxp3* gene. This section discusses these two layers of mechanisms for regulating Foxp3 expression.

Transcription factors for the induction of Foxp3 expression

There are two significantly conserved sequences between species in the first intron of the *Foxp3* gene, and these are designated as the conserved non-coding sequences (CNS) 1-2. In addition, the sequence just downstream of the first coding exon is designated as CNS3 [87]. The genetic

deletion of each CNS showed that CNS1 and CNS3 controls the induction of Foxp3 expression in the periphery and the thymus, respectively [87].

Foxp3 transcription is initiated after thymic CD4-SP T-cells receive strong and persistent TCR signals and upregulate the expression of CD25 and GITR [56]. In addition, *Foxp3* transcription is controlled by CD28, IL-2, and TGF-β signals in vivo [88]. Importantly, the genetic deletion of either c-Rel or RelA (p65) markedly reduces both $Foxp3^{+}$ Treg and $CD25^{+}GITR^{high}Foxp3^{-}$ Treg precursors in the thymus [89, 90], indicating that NF-κB promotes the differentiation of Treg precursors (**Fig. 3a**). ChIP PCR experiments showed that c-Rel bound to the *Foxp3* promoter [91] and CNS3 [87, 91]. NF-κB activities are further enhanced by TNF receptor superfamily (TNFRSF) molecules (including TNFR2, OX40, and GITR) through TNF receptor associated factors (TRAFs) and promote thymic Treg differentiation [92].

CD28 signalling enhances TCR-triggered NF-κB activities, and is required for thymic Foxp3 expression [93]. However, CD28 signals can also inhibit *Foxp3* transcription by activating the cascade of signalling proteins, including Phosphoinositide 3-kinase (PI(3)K), Akt, and mTOR [94, 95]. Given that Foxo1/Foxo3 promotes Treg differentiation [96, 97], the inhibitory effect of CD28 is by activating PI(3)K and Akt, which phosphorylates and inhibits Foxo1/Foxo3 by nuclear exclusion.

In addition to NF-κB (c-Rel), the *Foxp3* promoter is bound by TCR-induced transcription factors including NFAT, AP-1 [98], and Nr4a proteins [99] (**Fig. 3a**). Among these factors, Nr4a may play non-redundant roles in mediating TCR-induced Foxp3 expression, as *Nr4a1*$^{-/-}$*Nr4a3*$^{-/-}$ double knockout mice have markedly reduced $Foxp3^{+}$ T-cells [99]. In addition, CNS1 enhances TCR- and TGF-β-induced *Foxp3* expression [87]. In fact, NFAT can activate the *Foxp3* promoter and also binds to CNS1 [100]. CNS1 is also bound by Smad3, which activates *Foxp3* transcription in a TGF-β and TCR dependent manner [100].

The enhancer regions of the *Foxp3* gene also participate in the initiation of *Foxp3* transcription. The CNS0 enhancer, which is 8.5 kb upstream of the transcription start site, controls the initiation of *Foxp3* transcription in the thymus and the periphery. CNS0 is bound by the histone methyltransferase MLL4 which promotes *Foxp3* transcription by increasing the histone modification H3K27me1, which is commonly found in primed and active enhancers [101]. CNS0 is also bound by the genome organiser Satb1, which promotes Foxp3 expression in the thymus [102]. In addition, the CNS1 enhancer promotes Treg differentiation in the periphery, and is bound by NFAT and Smad3 [103] (**Fig. 3a**). The key enhancers (CNS0-3) can interact with the *Foxp3* promoter by forming chromatin loops [101, 104]. TCR-induced transcription factors may

thus interact with transcription factors in the distal enhancers to form a Foxp3-inducing enhanceosome.

In addition, the transcription factor Bach2 indirectly promotes Foxp3 expression in thymic and peripheral T-cells by repressing effector T-cell genes [105, 106]. However, given that effector T-cell genes can be highly expressed by eTreg [44] and other differentiated Treg subsets (e.g. Th1-Treg, Th2-Treg) [50], the repression of effector T-cell genes per se does not explain the promotion of Treg differentiation. Further studies are expected to elucidate new mechanisms for Foxp3 induction through Bach2.

Foxp3 autoregulatory transcription factor complex for the maintenance of Foxp3 expression

TSDR is a part of CNS2. CNS2-deficient Treg cannot maintain Foxp3 expression after cell divisions [107], indicating that CNS2 is required for the maintenance of Foxp3 expression. Importantly, CNS2 is bound by Foxp3 [87], Runx1-CBFβ [87], Ets-1 [108], and Stat5 [107] (**Fig. 3b**). The binding of Stat5 to CNS2 is compatible with the finding that IL-2 signalling is dispensable for Foxp3 induction but is required for maintaining Foxp3 expression [109, 110].

Runx1 can directly activate *Foxp3* transcription, as the deletion of Runx1 or CBF-β reduces Foxp3 protein expression levels in peripheral Treg [79, 111]. Bruno et al showed that Runx proteins bind the *Foxp3* promoter and activates *Foxp3* transcription, and thus, proposed a feedforward regulation in which Runx proteins control Foxp3 expression and subsequently make transcription factor complexes with Foxp3 to control target genes [112].

Using Foxp3-Tocky, we recently showed that the demethylation of CNS2 occurs after *Foxp3* transcription was initiated and sustained in CD4-SP cells for some time, most probably for a day [44]. Thus, it is likely that Foxp3 binds to CNS2 and coordinates its DNA demethylation process. Intriguingly, sustained *Foxp3* transcription requires Foxp3 protein. Using Foxp3 mutant (scurfy) carrying Foxp3-Tocky, we recently showed that Foxp3 protein deficient T-cells with Foxp3 transcription (‘want-to-be Treg’) could not sustain *Foxp3* transcription, and that each Foxp3 transcription was short and prematurely terminated [44]. Thus, Foxp3 enhances its own transcription by forming the Foxp3 autoregulatory transcription circuit, which likely involves the Foxp3-Runx1 interaction, since both Foxp3 and Runx1 bind CNS2 [87] (**Fig. 3c**).

Summarising, TCR-induced transcription factors form ‘Foxp3-inducing enhanceosome’ across CNS0-1-3, integrating TCR and costimulatory signalling pathways to initiate *Foxp3* transcription. However, once *Foxp3* transcription is sustained over time, the Foxp3 autoregulatory

transcriptional circuit is established and maintains *Foxp3* transcription through consolidating epigenetic modifications in CNS2.

Foxp3 transcription factor complex for Treg maturation

Functionally mature Treg can be found in the $CD44^{high}CD62L^{low}$ Treg fraction, which is activated at the transcriptome level in a TCR dependent manner[59]. eTreg highly express CD25, coinhibitory molecules including CTLA-4, Tigit, ICOS, and Klrg1 [113], and migrate to lymph nodes and infiltrate inflamed tissues in which they suppress immune responses [114]. $CD44^{high}CD62L^{low}$ Treg are more dependent on TCR signals than resting Treg, as shown by the induced deletion of *Tcra* [115]. TCR signal dynamics for inducing eTreg differentiation must be different to *infrequent-yet-regular* TCR signals (see above) from self-antigens in non-inflammatory conditions [56].

Treg maturation is considered to be promoted by cognate antigen signalling together with CD28, TNFRSF, and cytokine signals, which are generally enhanced in inflammatory environments. Transcription factors for Treg maturation include Myb[116], IRF4[70, 117], Blimp1[117], JunB [118], CARMA1[119], and the NF-κB subunit RelA [120]. TCR signalling by high-affinity antigens induces the expression of IRF4, which binds and cooperates with AP-1 transcription factors [121]. Notably, IRF4 activates *Prdm1* (Blimp-1) transcription [122]. Interestingly, IRF4 may physically interact with Foxp3 [70], although the evidence is limited. CARMA1 is required for TCR signal-induced NF-κB activation [123]. Although the mechanism of Myb activation in T-cells is less clear, Myb expression is induced by IL-2 signalling and PI(3)K [124], which activities are induced by TCR and CD28 signals.

Another cardinal hallmark of eTreg is their temporally sustained *Foxp3* transcriptional activities [44], which maintains Foxp3 protein levels for inducing Tregs' suppressive activities while inhibiting their own differentiation into effector T-cells. Human studies also indicate that the high FOXP3 protein expression is key for the suppressive activities of Treg, as shown by the analysis of $FOXP3^{high}$ $CD45RO^{+}$ ($CD45RA^{-}$) $CD4^{+}$ eTreg in autoimmunity [52] and in cancer [51, 125]. Foxp3 proteins may participate in TCR-induced transcription factor complex in eTreg. Further studies are required.

Collectively all the findings above indicate that TCR signalling triggers and promotes Treg maturation, and that NF-κB activating signals (e.g. TNFRSF signals) can have synergistic effects. These signals also induce persistent *Foxp3* transcription and thereby promote eTreg differentiation.

Mechanisms for controlling transcriptional activities of Foxp3 transcription factor complex

Early studies characterised Foxp3 as a transcriptional repressor that inhibits NFAT-mediated *Il2* transcription [126]. However, Foxp3 can also activate transcription of TCR/CD28-induced genes, particularly *Il2ra* (CD25) and other surface proteins [29, 127]. It is still unclear how Foxp3 switches between activator and repressor, but a clue was obtained by the most common IPEX mutation, A384T.

The A384T mutation disrupts the interaction between Foxp3 and the HAT TIP60 (Kat5), suggesting that the Foxp3-TIP60 interaction is required for Treg suppression [128]. In addition, pharmacological inhibition of p300 impairs the suppressive function of Treg while less affecting effector T cell responses [129]. While it is widely believed that HATs mediate their effects on Treg through acetylating and increasing the stability of Foxp3 protein [130, 131] (see below), p300 is a potent transcriptional coactivator [132] and may directly support the Treg-type transcriptional regulation.

The HATs p300 and CREB-binding protein (CBP) are potent transcriptional coactivator and may play central roles in activating transcription of Foxp3 target genes [132]. CBP/p300 can bind and assemble NF-κB, AP-1 and other major transcription factors to form the enhanceosome complex that associates with RNA polymerase II and enhance transcription [133]. Liu et al showed that pharmacological inhibition or conditional deletion of p300 impaired Treg's suppressive function [129]. Importantly, CBP and p300 interact with Runx1 and activate Runx1-mediated transcription as coactivators [134]. Thus, it is plausible that Foxp3, Runx1, and CBP/p300 cooperate to activate transcriptional activities of Foxp3 target genes. However, the presence of p300 in an enhancer is not sufficient for its activity, as p300 can occupy both active and poised enhancers, the latter of which is distinguished by the absence of histone H3 lysine 27 acetylation (H3K27ac), enrichment of histone H3 lysine 27 trimethylation (H3K27me3), and are linked to transcriptionally inactive genes [135] (**Fig. 2**). Although the upstream signal of p300 in Treg is not known, the MAPK/ERK pathway is known to activate p300 activity [136], which suggests that TCR signals play key roles.

When Foxp3 functions as a repressor, Foxp3 may recruit transcriptional corepressors. Eos (Ikzf4) and HDAC3 contribute to *Il2* repression, and both bind the proline-rich N-terminal domain of Foxp3 protein (**Fig. 1**). Eos and HDAC3 can recruit the transcriptional corepressors CtBp1 [137] and NCoR1/NCoR2 [138], respectively, although roles of these corepressors in the Foxp3 transcription factor complex is yet to be elucidated. The histone methyltransferase Ezh2 also has

significant roles in the maintenance of Treg transcriptome [139]. Ezh2 is a functional subunit of the Polycomb Repressive Complex 2 (PRC2), which mediates heritable transcriptional silencing [140]. Arvey et al showed that Ezh2 protein co-immunoprecipitated with Foxp3 in activated Treg, and that histones in Foxp3-bound genomic sites were more marked with H3K27me3, suggesting the recruitment of Ezh2 to Foxp3-bound regions [141]. However, PRC2 preferentially binds unmethylated CpG islands, which have high CG content, unique DNA conformations in transcriptionally inactive genes [142]. Therefore, it is not clear whether the association of Foxp3-Ezh2 is the cause or the consequence of transcriptional repression. Further studies are required to elucidate mechanisms for the roles of PRC2 in Foxp3-mediated gene regulation.

Summarising, Foxp3 functions as a transcriptional activator by recruiting transcriptional coactivators including CBP/p300, which can interact and cooperate with Runx1. When functioning as a repressor, Foxp3 can recruit corepressors. Further studies are required to understand the coordination between Foxp3, corepressors, and epigenetic factors.

Roles of bivalent Foxp3-containing transcription factor complexes for bifurcation of Treg and Teff differentiation

It is a common regulation that two lineage-specific transcription factors compete with each other for the lineage choice. Notably, mechanisms for the differentiation of Th17 and Treg are closely related. Th17 cells produce Th17-cytokines (IL-17, IL-21) and play key roles in autoimmune inflammation. Importantly, when stimulated by TCR ligation and TGF-β, $CD4^+$ T-cells can express not only Foxp3 but also ROR-γt [143]. Subsequently, IL-2 promotes Treg differentiation by enhancing Foxp3 expression while repressing ROR-γt expression, whereas IL-6 promotes Th17 differentiation and inhibits Treg differentiation [144]. In these differentiating T-cells, Foxp3 interacts with Runx1 and ROR-γt [145]. Thus, Runx1 provides a platform for the competitive interaction between Foxp3 and ROR-γt.

Hif1a plays important roles for the choice between Th17 and Treg differentiation. Hypoxia stabilizes Hif1a, which enhances Th17 differentiation by making a complex with ROR-γt and p300. Hif1a binds and promotes the degradation of Foxp3 [146]. On the other hand, the ubiquitin E3 ligase Deltex1 promotes the degradation of Hif1a regulates the Foxp3-ROR-γt complex [147].

The bivalent Foxp3-containing transcription factor may be further controlled by TCR signals. Molinero et al showed that low concentrations of anti-CD3 antibody (~0.5 μg/ml) together with IL-2 and TGF-β promoted Treg differentiation, while high concentrations (~5 μg/ml) rather suppressed Foxp3 expression and induced Th17 differentiation [148]. Ashouri et al used Nur77-

GFP reporter in an arthritis-prone background and showed that Nur77-GFP^{high} cells are enriched with arthritogenic Th17 cells [149]. These results suggest that TCR-induced transcription factors may control the Th17-Treg bivalent transcription factor complex.

Collectively, Foxp3 and Th17-inducing factors make transcriptional complex, which works as a decision-making complex for the choice of Treg and Th17 differentiation.

Roles of Foxp3 post-translational modifications in the activities of Foxp3 transcription factor complex

Post-translational modifications (PTMs) regulate Foxp3 protein stability and thereby control Foxp3 transcription factor complex. Major PTMs for Foxp3 include ubiquitination, phosphorylation, and acetylation.

Foxp3 is degraded by the ubiquitin-proteasome-system (UPS). The molecular chaperon Hsp70 interacts with Foxp3 and recruits the E3 ubiquitin ligase Stub1, which promotes polyubiquitination and proteasome-dependent degradation of Foxp3 [150]. On the other hand, the deubiquitinase USP7 can interact with Foxp3 protein, removing ubiquitin and thereby increasing the stability of Foxp3 protein [151]. Intriguingly, the proinflammatory cytokine IL-6 upregulates Stub1 and decreases USP7 expression, and thus induces Foxp3 degradation [150, 151]. Importantly, IL-6 represses *Foxp3* transcription as well in a Stat3 dependent manner[152]. In addition, Morawski et al showed that Cdk2 phosphorylated and enhanced the stability of Foxp3 [153], although the evidence is largely from the analysis of a Foxp3 mutant.

The other types of phosphorylation and acetylation can stabilise Foxp3 protein. Nemo-like kinase (NLK) phosphorylates Foxp3 and prevents Stub1 from interacting with and ubiquitinating Foxp3 [154]. Fleskens et al showed that TCR and costimulatory signals induce Foxp3 phosphorylation through the activation of NLK in a TGFβ-activated kinase 1 (TAK1) dependent manner [154]. TAK1 is a part of the MAPK cascade [155], and also, activates IκB kinase (IKK) and NF-κB [156]. Thus, TCR and other NF-κB inducing signals phosphorylate and stabilises Foxp3 protein, which may promote TCR-induced *Foxp3* expression (see above). Meanwhile, van Loosdregt et al showed that the HAT p300 interacts with and acetylates Foxp3, and thereby prevents proteasome-dependent degradation of Foxp3 [157]. This suggests that p300-containing Foxp3 transcription factor complex can stably regulate transcription of its target genes.

Summarising, PTMs of Foxp3 regulate the expression level of Foxp3 protein, which may have significant impacts in the fate of each Foxp3+ cell. This may be particularly relevant for

differentiating Treg, Foxp3+ non-suppressive T-cells in humans, and proliferating Treg, in which Foxp3 protein expression level can determine their fate. In addition, Foxp3 PTMs may play roles in context-dependent gene regulation by changing the stability of each Foxp3 transcription factor complex.

Perspectives

Outstanding questions include (1) how Foxp3 transcription factor complex switches between transcriptional repressor and activator; (2) how signals from cytokines and antigen recognition can change the compositions and activities of Foxp3 transcription factor complex; (3) whether and how Foxp3 transcription factor complex regulates epigenetic modifications. The current challenge is to obtain mechanistic understanding by integrating molecular, immunological, and genomic data including epigenomic and single cell data. Bioinformatics analysis is becoming more and more important. Tocky is useful for analysing the temporal aspects of these cellular and molecular processes. These new technologies will make breakthroughs in the understanding of the molecular mechanisms of Foxp3-mediated immune suppression, which may well lead to the development of next-generation immunotherapy for cancer and autoimmunity in the future. Learning from the history of Treg biology and immunotherapy [43], it is needed to obtain both mechanistic understanding of molecular and cellular mechanisms and systemic view on the entire T-cell system and the immune system.

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Disclosures

The author has no conflicts of interest to declare.

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Figure legends

Fig. 1. Structure of Foxp3 and AML1/Runx1 proteins.

Foxp3 binds DNA through the forkhead (FH) domain, which directly interacts with NFAT. Foxp3 makes homodimer or heterodimer with Foxp1 through its Leucin zipper (LZ) domain. Foxp3 directly interacts with AML1/Runx1 through the inter-domain region between FH and LZ domains [29], which is distinct from the interacting domains for other key partners including NFAT [27], Foxp3/Foxp1 [26], HDAC7 and TIP60/Kat5 [158]. AML1/Runx1 binds DNA through the Runt domain by making a complex with CBF-β. AML1/Runx1 interacts with Foxp3 through its inhibition domain (ID) [29]. AML1/Runx1 can associate with key transcription factors for T-cell activities including Stat5, Smads, AP-1, Ets1 and NF-κB. In addition, AML1/Runx1 can recruit and interact with major transcriptional activators and repressors including p300, Kat6a/Myst3/Moz and TEL [75, 159, 160]. ZF, zinc finger; LZ, leucine zipper; FH, forkhead; AD, transactivation domain.

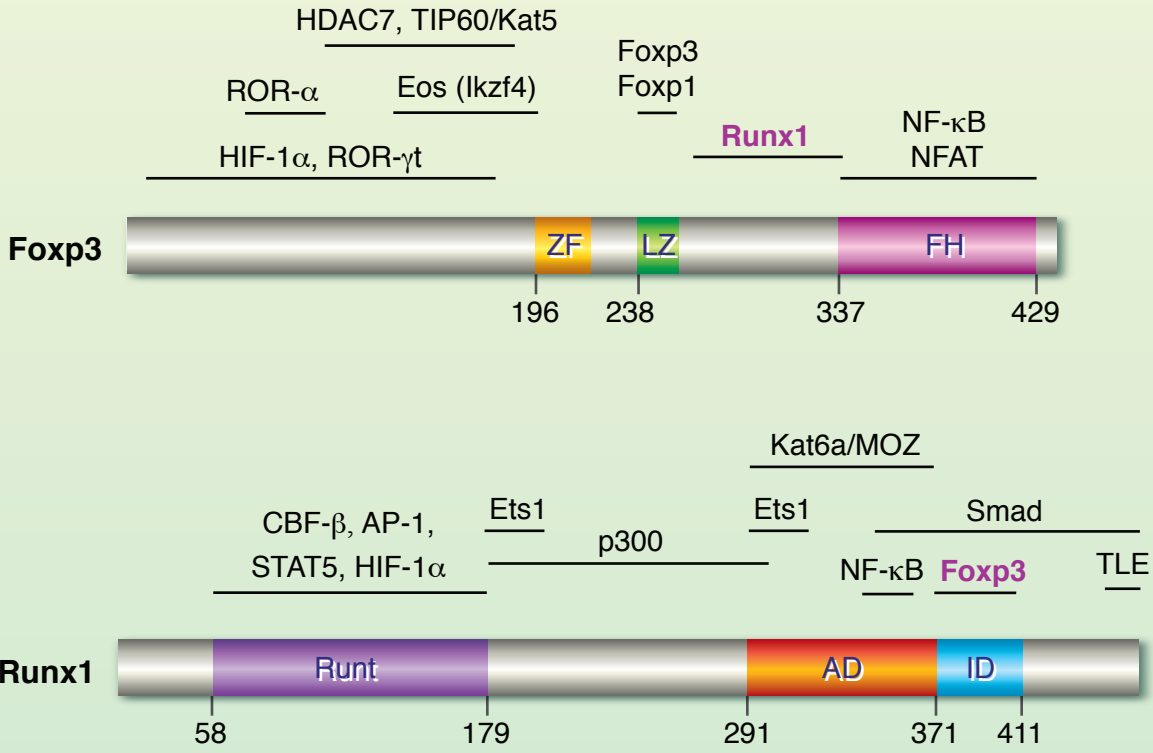
Fig. 2. A model for Runx1-guided control of Treg-type transcriptome.

AML1/Runx1 has different roles in Foxp3$^-$ T-cells (conventional T-cells) (**a**, **b**) and Foxp3$^+$ Treg (**c, d**). (**a**) When T-cells are matured, the AML1/Runx1-CBF-β complex may bind primed and poised enhancers, which are transcriptionally inactive, and AML1/Runx1 is poised to execute its function for T-cell differentiation and response. Primed enhancers are marked by H3K4me1 but not by H3K27me3. Poised enhancers are similar to active enhancers in p300 binding and nucleosome depletion but are marked by H3K27me3 in addition to H3K4me1. Histones at the promoter are marked by H3K4me3, which is the feature of active promoter. In poised enhancer, AML1/Runx1 presumably interacts with p300, while PRC2 and HDACs actively suppress p300-mediated histone acetylation and prevent transcriptional activation. (**b**) Upon cognate antigen recognition, TCR signals activate its downstream transcription factors, including NF-κB, NFAT, and AP-1, which are recruited to promoters and enhancers of AML1/Runx1-bound genes. This will form a p300-containing enhanceosome, which acetylates histones together with other HATs such as Kat2b and TIP60, increasing H3K27Ac, and enhances RNA polymerase II (Pol II)-mediated transcription at the transcription start site (TSS). (**c**) In Treg, Foxp3 is highly expressed and TCR signals are regularly conveyed. When Foxp3 expression is induced, whether in the thymus or in the periphery, T-cells have received strong TCR signals. Some genes such as *Il2* have repressed enhancers, to which the Foxp3-AML1/Runx1 complex binds. Foxp3 and AML1/Runx1 may further recruit transcriptional corepressors such as NCoR1/NCoR2 (through HDAC3) and TLE, respectively (Fig. 1). (**d**) Treg highly express activation-induced proteins including CD25 and coinhibitory molecules, which transcriptional activities are sustained with the help of Foxp3 and infrequent-but-regular TCR signals. Foxp3 is considered to stabilise TCR signal-induced enhanceosomes by interacting with AML1/Runx1 and TCR signal downstream transcription factors.

Fig. 3. A model for dynamic regulation of Foxp3 expression in Treg precursors and differentiated Treg

(a) The signalling and transcriptional landscape of Treg precursors. Before *Foxp3* transcription is initiated, Treg precursors receive signals from TCR, CD28, and TNFRSF. These signals induce transcription factors required for the initiation of Foxp3, including NFAT, c-Rel, and AP-1, which bind the promoter and CNS3 of the *Foxp3* gene in thymic T-cells. Here prolonged CD28 signals can also inhibit *Foxp3* transcription through activating Akt and mTOR and thereby inactivating Foxo proteins, which have roles in activating *Foxp3* transcription. TNFRSF molecules such as GITR, OX40, and TNFR2 are expressed in both mature Treg and differentiating Treg including Foxp3- Treg precursors, and activate the NF-κB pathway.

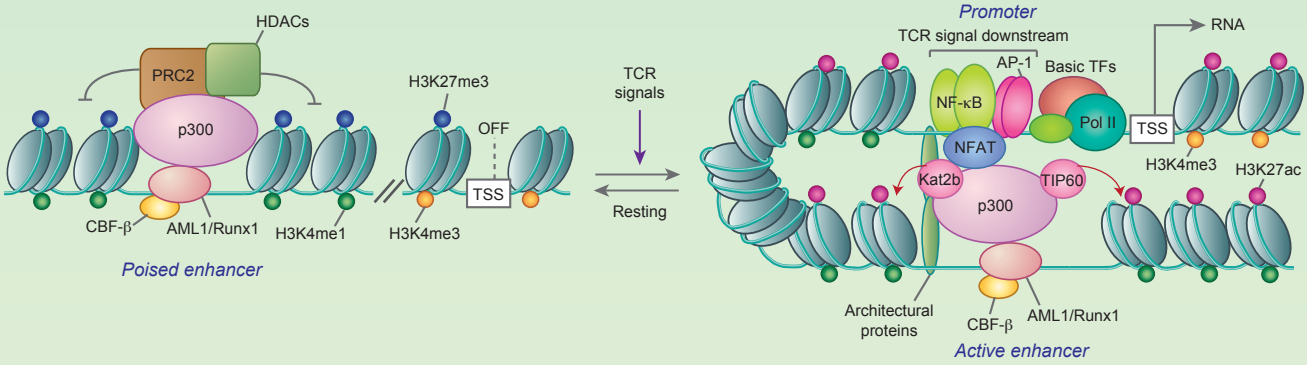
(b) Maintenance of *Foxp3* transcription through Foxp3 autoregulatory transcriptional circuit in mature Treg. Once Foxp3 protein is translated, it will join the regulation of Foxp3 transcription, establishing Foxp3 autoregulatory transcriptional loop. CNS2 plays key roles in the maintenance of Foxp3 expression, and is bound by both AML1/Runx1 and Foxp3 in mature Treg. Thus, it is considered that Foxp3 interacts with AML1/Runx1 in CNS2, which triggers DNA demethylation of the region, which is also known as TSDR. In addition, IL-2 signalling may consolidate Foxp3 transcription through STAT5 binding to CNS2.


HDAC7, TIP60/Kat5
ROR-α
Eos (Ikzf4)
Foxp3
Foxp1
Runx1
NF-κB
NFAT
HIF-1α, ROR-γt
Foxp3
ZF
LZ
FH
196
238
337
429
Kat6a/MOZ
CBF-β, AP-1,
STAT5, HIF-1α
Ets1
p300
Ets1
Smad
NF-κB
Foxp3
TLE
AML1/Runx1
Runt
AD
ID
58
179
291
371
411

Foxp3^{-} T cells (conventional T cells)

(a) **AML1/Runx1 binds primed and poised enhancers**

(b) **TCR signals transiently induce active enhancers (e.g. *Il2*)**

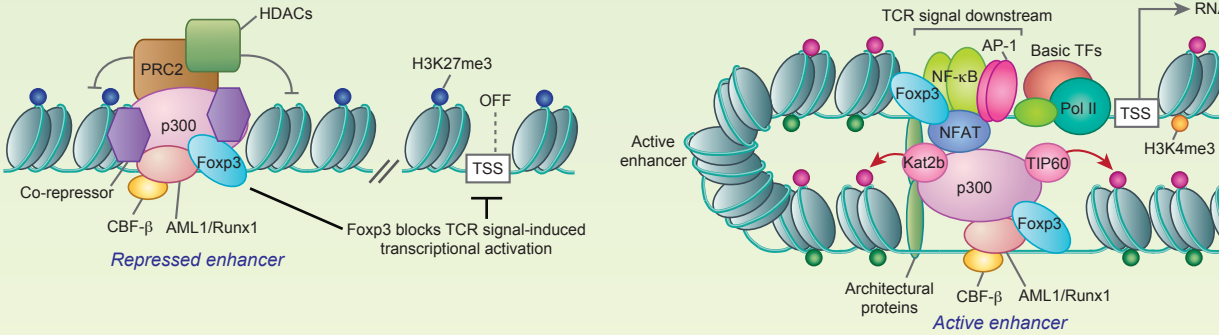


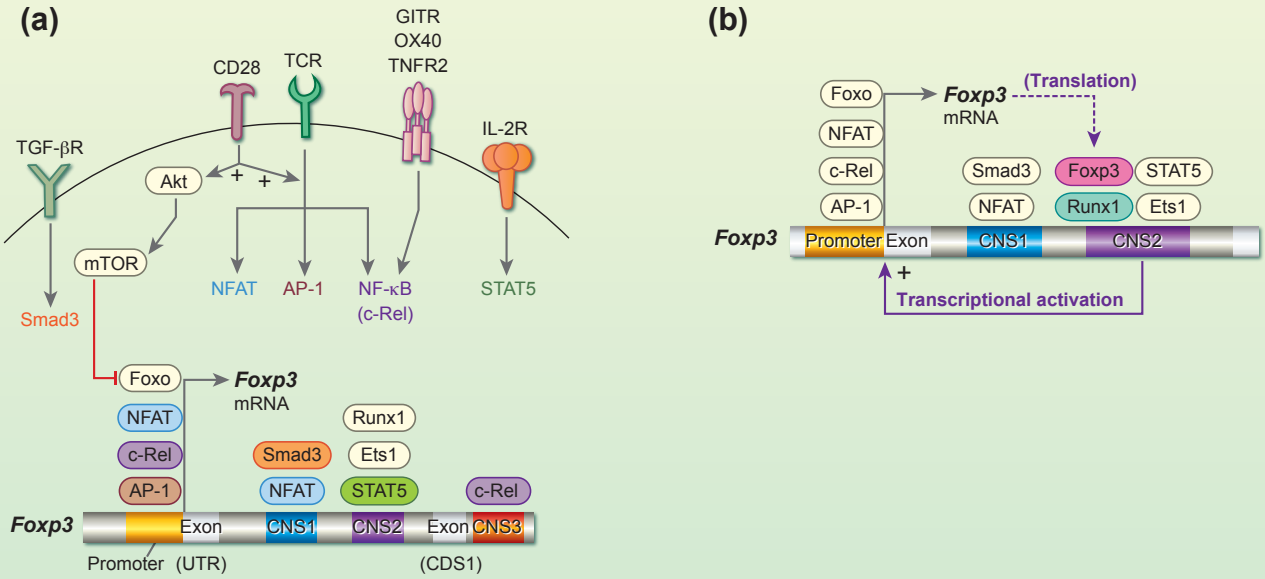
Foxp3^{+} Treg

Foxp3 + TCR signals

(c) **Repressed enhancer by Foxp3-AML1/Runx1 (e.g. *Il2*)**

(d) **'Constitutive' active enhancer by Foxp3-AML1/Runx1 (e.g. *Il2ra*)**




(a)
GITR
OX40
TNFR2
CD28
TCR
IL-2R
TGF-βR
Akt
+
+
mTOR
Smad3
NFAT
AP-1
NF-κB
(c-Rel)
STAT5
Foxo
NFAT
c-Rel
AP-1
Foxp3
mRNA
Runx1
Smad3
Ets1
NFAT
STAT5
c-Rel
Foxp3
Exon
CNS1
CNS2
Exon
CNS3
Promoter
(UTR)
(CDS1)
(b)
Foxo
NFAT
c-Rel
AP-1
Foxp3
mRNA
(Translation)
Smad3
Foxp3
STAT5
NFAT
Runx1
Ets1
Foxp3
Promoter
Exon
CNS1
CNS2
+
Transcriptional activation