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Cooperative regulatory events and *Foxp3* expression

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Abstract

The molecular mechanisms that control T_{reg} and T_H17 development and the precise role of transforming growth factor- β in this process are complex and imperfectly understood. New findings indicate that the helix-loop-helix proteins E2A and Id3 are also critically involved in some of these processes.

The transcription factor Foxp3 has a defining role in regulating the development and function of regulatory T cells (T_{reg} cells). Studies have shown that *Foxp3* expression itself is regulated by complex mechanisms, with simultaneous activation of multiple transcription factors by signals through the T cell antigen receptor (TCR) and cytokine receptors, including the receptor for transforming growth factor- β (TGF- β). In this issue of *Nature Immunology*, Maruyama *et al.* demonstrate that complex activities of the helix-loop-helix (HLH) proteins E2A and Id3 and the transcription factor GATA-3 act together in the regulation of *Foxp3* expression¹.

T_{reg} cells suppress excessive immune responses and contribute to the prevention of autoimmune disease caused by potentially hazardous self-reactive T cells. Studies have also shown greater activity of T_{reg} cells associated with tumors. Many laboratories have concentrated on identifying the molecular features of *Foxp3* expression. An emerging picture is that *Foxp3* expression is controlled by four defined regulatory regions, and indeed the binding of many transcription factors in these regions has already been identified (Fig. 1). Notably, the activity of most of the transcription factors identified at present is induced by signaling through either the TCR or cytokine receptors, including the receptor for TGF- β . These transcription factors have cooperative activities that are able to induce and maintain *Foxp3* expression. In addition, transcription factors involved in driving *Foxp3* expression may indirectly regulate development of cells with the T_H17 helper T cell phenotype, because Foxp3 inhibits the function of ROR γ t (encoded by *Rorc*), a key transcription factor needed for the development of T_H17 cells².

Maruyama *et al.* now show that another TGF- β -dependent molecular mechanism is involved in *Foxp3* expression. They examine the development of CD4⁺Foxp3⁺ T_{reg} cells and show that *Foxp3* expression is regulated by cooperation of E2A, Id3 and GATA-3. Not only is Foxp3-dependent T_{reg} cell development impaired in *Id3*^{-/-} mice but also their development of T_H17 cells is greater. As Foxp3 inhibits ROR γ t function², T_H17 cells may be more abundant in these mice as a consequence of the downmodulation of *Foxp3* expression.

Surprisingly, *Rorc* expression is also regulated by E2A. Together these results indicate that T_H17 development is regulated by a balance of E2A and Id3 activity, along with Foxp3-dependent and Foxp3-independent actions.

E2A is a member of the E2A protein family, which includes E47 and E12 and is composed of important factors in the regulation of many developmental pathways³. Maruyama *et al.* observe enrichment of E2A at the *Foxp3* promoter in cells stimulated with TCR plus TGF- β and also in a Foxp3–luciferase reporter assay. Id3 contains an helix-loop-helix domain that can dimerize with E2A proteins but also is known to be able to inhibit DNA binding because of its lack of a basic region³. Because of the inhibitory functions of Foxp3 in differentiation, *Foxp3* expression might be predicted to be upregulated in the absence of Id3; however, Maruyama *et al.* find that *Foxp3* expression is lower in *Id3*^{-/-} mice. At the same time, GATA-3 mediates the inhibition of *Foxp3* expression in cooperation with E2A and Id3. Notably, the activity of both the *Foxp3* and *Il4* promoters is upregulated by E2A. *Gata3* expression is induced in *Id3*^{-/-} mice by signaling through the IL-4 receptor, with IL-4 being upregulated by E2A because of absence of the inhibitory protein Id3. GATA-3 is a key transcription factor for the development of T helper type 2 cells and also functions as a negative regulator of *Foxp3* expression⁴. Indeed, binding of GATA-3 to the *Foxp3* promoter is enhanced in *Id3*^{-/-} mice, and, consequently, *Foxp3* expression is then down-modulated. Foxp3 expression is therefore regulated by an intricate balance of E2A-Id3 and activated signal transducers Smad2 and Smad3, because the regulation of Id3 by TGF- β is itself directly mediated by Smad activation³.

The findings noted above are relevant to previously published observations. For example, mice doubly deficient in Smad2 and Smad3 show considerably inhibited *Foxp3* expression relative to that of mice singly deficient in Smad3 (ref. 5). As Smad3 binds to enhancer 1 of *Foxp3* and regulates *Foxp3* expression⁶, the greater down-modulation of *Foxp3* expression in mice doubly deficient in Smad2 and Smad3 may occur via inactivation of Id3. Notch-mediated *Foxp3* expression has also been studied through the use of pharmacological inhibitors of the Notch pathway (such as γ -secretase inhibitor)⁷. It is therefore possible that Notch also regulates *Foxp3* expression via E2A and Id3, because Notch signaling is able to modulate the transcriptional activity of E2A³.

The regulatory regions for *Foxp3* expression consist of a promoter, two enhancers (1 and 2) and a conserved noncoding sequence region (CNS3) containing a binding site for the c-Rel subunit of the transcription factor NF- κ B (Fig. 1). Mice deficient in enhancer 1 (CNS1), enhancer 2 (CNS2) or CNS3 have been used to characterize each regulatory region⁸. Although c-Rel homodimers bind to the CNS3 region, typical enhancer activity is not detected by Foxp3–luciferase reporter assays⁸, unlike that seen with the enhancer 1 region⁶ and enhancer 2 region⁹. The CNS3 region seems to be responsible for remodeling of the *Foxp3* locus. Studies of the enhancer 1 region have shown that Smad3 and the transcription factor NFAT act together to induce its activity⁶. This enhancer seems to have an important role in the development of induced T_{reg} cells via signals through the TCR and receptor for TGF- β . Enhancer 1 activity has been further shown to involve binding of the transcription activator AP-1 and transcription factor RAR¹⁰. The activity of enhancer 2 is regulated positively by the transcription factors CREB-ATF, STAT5, Foxo1 and Foxo3 but negatively by STAT3. This enhancer seems to maintain *Foxp3* expression. In the *Foxp3* promoter region, E2A, AP-1, NFAT, Sp1, STAT1, Foxo1, Foxo3, RUNX1, RUNX3 and c-Rel bind and positively regulate *Foxp3* expression. However, Id3 and GATA-3 function as negative regulators.

Maruyama and colleagues highlight the cooperative interactions among E2A, Id3 and GATA-3 that help regulate *Foxp3* expression. This complexity suggests that although each

regulatory region seems to have its own special role, transcription factors at these regulatory regions need to act together with each other for full *Foxp3* expression. Indeed, enhancer 1 activity is much lower after individual mutation of the binding region for each transcription factor (AP-1, RAR, NFAT or Smad3)^{6,10}, which suggests that each cooperates in the induction and maintenance of *Foxp3* expression. *Foxp3* promoter activity is also substantially upregulated by enhancer 1 (ref. 6) and enhancer 2 (ref. 9). Transcription factors on the promoter and enhancers clearly influence each other's activity. *Foxp3* expression is regulated not only by binding of transcription factors but also by DNA demethylation of discrete parts of *Foxp3* and chromatin remodeling, which involves defined changes in histone acetylation. Histone acetylation in enhancer 1 (ref. 6) and DNA demethylation in the *Foxp3* promoter and enhancer 2 (ref. 9) are regulated by signaling through the TGF- β receptor. Natural T_{reg} cells might develop by TGF- β -independent pathways, as at least some T_{reg} cells exist in mice doubly deficient in Smad2 and Smad3 (ref. 5). However, histone molecules are considerably acetylated in enhancer 1 (ref. 6), and DNA in the promoter and enhancer 2 (ref. 9) is substantially demethylated in CD4⁺CD25⁺ T_{reg} cells isolated from naive mice. These types of modifications might reflect cooperative processes caused by the binding of transcription factors to the promoter, enhancers and CNS3.

It should also be pointed out that the Foxp3 protein itself could be involved in regulation of *Foxp3* expression. Foxp3 associates with other transcription factors, including NFAT¹¹, to regulate their transcriptional activity, and *Foxp3* expression is regulated by the binding of NFAT to the promoter and enhancer 1. The association of Foxp3 with NFAT or other transcription factors on these regulatory regions might therefore have an important role in modulating gene expression. It should be noted that the histone deacetylases HDAC7 and HDAC9 and several histone acetyl transferases such as Tip60 and p300 associate with Foxp3 (ref. 12), which indicates that this transcription factor might control *Foxp3* expression via histone acetylation or deacetylation at the *Foxp3* locus.

The involvement of multiple transcription factors in *Foxp3* expression patterns is now well established. Notably, *Foxp3* expression is regulated by both positive and negative cooperative processes, which identifies yet another level of complexity that must be elucidated to explain the induction and maintenance of *Foxp3* expression.

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Figure 1.

Transcriptional regulation of *Foxp3*. **(a)** Structure of mouse *Foxp3*. Boxes indicate exons (numbers above). **(b)** Location of the *Foxp3* promoter, enhancers (1 and 2) and CNS3 regions, as well as exons -2a, -2b, -1 and 1. Binding regions for regulators of *Foxp3* transcription are above (negative regulators) and below (positive regulators). This model is based on many publications.