approaches, they demonstrate that the canonical pathway is not required for the effects of OX40 stimulation and that the NF-κB subunit RelA (in the canonical pathway) does not bind the *Il9* promoter. In contrast, the induction of IL-9 by OX40L is dependent on the NF-κB subunit p52, and retroviral transduction of p52 and the NF-κB subunit RelB results in the induction of IL-9 production, but retroviral transduction of p50 and RelA does not. In agreement with those results, RelB is bound to the *Il9* promoter after stimulation with OX40, and coexpression of RelB and p52 activates an *Il9* promoter reporter. Activation of the noncanonical pathway is dependent on the OX40L-induced expression of the transcription factor TRAF6, and transduction of TRAF6-deficient T_H9 cultures to express RelB and p52 restores IL-9 production, which suggests that this represents the main pathway of induction. The results by Xiao *et al.*[7] contrast somewhat with a published report showing that RelA is required for antigen receptor–induced IL-9 (ref. 6), although such differences could be due to distinct stimuli or culture conditions.

Notably, Xiao *et al.* demonstrate that engagement of OX40 *in vivo* promotes inflammation[7]. Mice with transgenic expression of OX40L, which have been shown to develop multiorgan inflammation, have lung inflammation characterized by mucus production, with a concomitant increase in *Il9* mRNA in lung tissue and IL-9-secreting T cells in the lungs. In parallel studies, antibody to OX40 is also shown to promote lung inflammation dependent on endogenous IL-9. The induction of allergic lung inflammation in a model of ovalbumin in alum is dependent on OX40, and OX40 deficiency is associated with less IL-9 in bronchoalveolar lavage fluid, as has been shown before[9,11]. This final set of studies is critical, as it demonstrates the link from OX40 to IL-9 *in vivo* in the absence of transgenic expression of OX40L or injected antibody to OX40.

Both T_H2 and T_H9 cells are probably required for maximal allergic inflammation. The link between OX40 signaling and IL-9 raises the important question of the relative contributions of the OX40-T_H9 pathway and of the previously established OX40-T_H2 pathway to the development of allergic inflammation. In the absence of OX40-OX40L signaling, the production of T_H2 cytokines is diminished, concomitant with the lower abundance of IL-9 observed by Xiao *et al.*[7] in the ovalbumin model of allergic airway disease[8–11]. The conditions in which OX40 would 'preferentially' promote T_H2 or T_H9 development are not clear, and this may depend entirely on the polarizing cytokine milieu. However, the amount of stimulation by OX40L on antigen-presenting cells might also contribute to the target cytokine. The ability of transgenically expressed OX40L to potently induce IL-9 production suggests that higher OX40L expression might favor the induction of IL-9 rather than T_H2 cytokines.

Another key issue is whether OX40-induced IL-9 is transient or whether stimulation induces programming of *Il9*. Xiao *et al.* analyze most of their *in vitro* data after only 3 days of culture, and kinetic data indicate the greatest induction of *Il9* mRNA and IL-9 protein occurs within the first 48 hours, with transcription waning thereafter[7]. Although many members of the TNF superfamily alter the production of helper T cell cytokines, none have been shown to be essential initiators of helper T cell differentiation. Similarly, although NF-κB proteins can induce many cytokines, members of the NF-κB family are not generally thought of as helper T cell lineage-promoting factors. Thus, engagement of OX40 might facilitate a short burst of IL-9 production without resulting in long-term changes in the ability of the stimulated cell to produce IL-9 after subsequent stimulation through the antigen receptor. These issues will need further experimentation to discern both *in vitro* and *in vivo* effects.

Finally, it will be important to determine if the effects of OX40 engagement are restricted to T_H9 cells or are observed in other helper T cell subsets and in non-T cells. Xiao *et al.* note that OX40 promotes IL-9 production when cells are cultured in the presence of TGF-β and IL-4, the T_H9-inducing cytokines, but not in the absence of polarizing cytokines[7]. However, they do not report whether stimulation by OX40 induces IL-9 as it represses expression of Foxp3 or IL-17 in culture conditions that promote regulatory T cells or T_H17 cells, respectively. Engagement of OX40 could explain the ability of several helper T cell subsets to produce IL-9 under some culture conditions. As additional IL-9-producing cell types are identified, such as innate lymphoid cells[12], this pathway could prove to be critical in establishing an IL-9-sufficient environment through many cell types.

COMPETING FINANCIAL INTERESTS
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1. Dardalhon, V. *et al. Nat. Immunol.* **9**, 1347–1355 (2008).
2. Veldhoen, M. *et al. Nat. Immunol.* **9**, 1341–1346 (2008).
3. Chang, H.C. *et al. Nat. Immunol.* **11**, 527–534 (2010).
4. Goswami, R. *et al. J. Immunol.* **188**, 968–975 (2012).
5. Staudt, V. *et al. Immunity* **33**, 192–202 (2010).
6. Jash, A. *et al. J. Biol. Chem.* **287**, 15445–15457 (2012).
7. Xiao, X. *et al. Nat. Immunol.* **13**, 981–990 (2012).
8. Croft, M. *Annu. Rev. Immunol.* **28**, 57–78 (2010).
9. Jember, A.G., Zuberi, R., Liu, F.T. & Croft, M. *J. Exp. Med.* **193**, 387–392 (2001).
10. Ito, T. *et al. J. Exp. Med.* **202**, 1213–1223 (2005).
11. Salek-Ardakani, S. *et al. J. Exp. Med.* **198**, 315–324 (2003).
12. Wilhelm, C. *et al. Nat. Immunol.* **12**, 1071–1077 (2011).

The Foxp3 interactome: a network perspective of T_{reg} cells

Shohei Hori

Foxp3 protein complexes orchestrate the transcriptional network of regulatory T cells. The Foxp3 interactome is now identified and may act as a genetic switch that controls the differentiation of regulatory T cells.

Regulatory T cells (T_{reg} cells) that express the transcription factor Foxp3 are central to the establishment and maintenance of immunological self-tolerance and immunological homeostasis at environmental interfaces. Foxp3 was initially considered the 'master regulator' of T_{reg} cells, but it has become evident that the differentiated state of T_{reg} cells is not determined solely by Foxp3 expression. Two papers in this issue of *Nature Immunology* now provide evidence that the differentiated state of T_{reg} cells is determined by a self-reinforcing transcriptional network that consists of a Foxp3 'interactome'—the protein-protein interaction network of Foxp3 and its partners. Rudra *et al.* identify components of the Foxp3 protein complexes and shows that they form a regulatory circuitry containing multiple feedback loops[1]. By computational network inference

Shohei Hori is with the Research Unit for Immune Homeostasis, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan.
e-mail: shohei@rcai.riken.jp

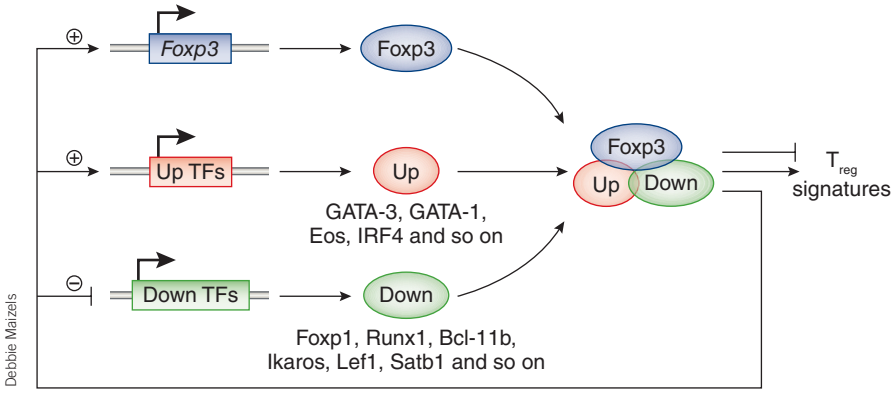

Figure 1 A hypothetical transcriptional circuitry that establishes the T_{reg} cell signature. Foxp3 and its partners cooperatively regulate transcription of genes of the T_{reg} cell signature, including their own transcription, forming multiple positive and negative feedback loops. The Foxp3 complex activates the transcription of genes encoding Foxp3 and some of the partners (Up TFs) while repressing the transcription of genes encoding some other partners (Down TFs).

and experimental confirmation, Fu *et al.* identify five transcription factors, which they designate the 'quintet', that interact with and act together with Foxp3 to elicit much of the characteristic T_{reg} cell transcriptional signature[2]. Fu *et al.* further demonstrate that Foxp3 and the quintet form a transcriptional network with multiple and redundant feedback loops and propose that this circuitry could act as a genetic switch that 'locks in' the self-perpetuating differentiation state of T_{reg} cells.

The initial findings that enforced expression of Foxp3 converts otherwise normal $CD4^+$ T cells into a T_{reg} cell–like phenotype and that loss-of-function mutations in *Foxp3* result in the defective development of functional T_{reg} cells led to the proposal that Foxp3 acts as the 'master regulator' of T_{reg} cells[3]. Naturally, many subsequent studies have focused on the mechanisms by which Foxp3 regulates gene expression in T_{reg} cells. Chromatin immunoprecipitation combined with genome-wide tiling-array analysis has identified many genes that are occupied by Foxp3 and up- or down-regulated in T_{reg} cells, which suggests that Foxp3 acts as both a transcriptional activator and a transcriptional repressor[4,5]. It has also been shown that Foxp3 regulates transcription by interacting with many other transcription factors[3]. It has remained unclear, however, how Foxp3 and its partners orchestrate the transcriptional network of T_{reg} cells.

To identify components of the Foxp3 protein complexes in an unbiased and comprehensive manner, Rudra *et al.* undertake a proteomic approach with a T cell hybridoma that expresses the prokaryotic biotin ligase BirA and Foxp3 protein fused with an *in vivo* biotinylation tag suitable for affinity purification[1]. By purifying the Foxp3 complexes and analyzing them by mass spectrometry, the authors identify as many as 361 proteins in the complexes. They confirm the validity of many of the interactions in *ex vivo*–isolated T_{reg} cells by conventional immunoprecipitation assays. Interestingly, gel-filtration analyses suggest that Foxp3 forms large heterogeneous complexes whose sizes range from 400 kilodaltons to 2,000 kilodaltons. The Foxp3 partners include not only transcription factors but also chromatin regulators, which suggests that Foxp3 recruits different chromatin-modifying and/or chromatin-remodeling complexes to achieve gene activation or repression, depending on the target genes.

The study by Rudra *et al.* also identifies a remarkable feature of the Foxp3 interactome[1]. By referring to data about Foxp3-binding regions obtained by chromatin immunoprecipitation followed by deep sequencing of the whole genome, they find that the group of genes encoding partners of Foxp3 are enriched for direct targets of Foxp3. Although some of these (such as *Gata3* and *Stat3*) are upregulated in T_{reg} cells relative to their expression in $Foxp3^-$ T cells, most of them (such as *Ikzf1* (which encodes Ikaros), *Bcl11b*, *Foxp1* and *Runx1*) are downregulated. Conversely, *Foxp3* transcription itself has been shown to be regulated by some of the partners either positively (GATA-3, Bcl-11b, NFATc2 and Runx1) or negatively (STAT3). These results suggest that Foxp3, its partners and the genes that encode them form a regulatory network with positive and negative feedback loops (**Fig. 1**). Rudra *et al.* further demonstrate that Foxp3 and GATA-3 indeed form a positive feedback loop that increases the transcription of their genes in T_{reg} cells[1].

Despite the essential role of Foxp3 in T_{reg} cells, it has become clear that Foxp3 alone is neither strictly necessary nor sufficient for determining the differentiation of T_{reg} cells. Foxp3 can be promiscuously expressed in activated conventional T cells without inducing a T_{reg} cell phenotype[6]. T cells that are transcribing the *Foxp3* locus but are unable to express functional Foxp3 protein ('T_{reg} cell–like cells') have many features of T_{reg} cells, albeit only partially[7,8]. A meta-analysis of the transcriptome of T_{reg} cells and other Foxp3-expressing cells (such as T cells transduced to express Foxp3) has shown that Foxp3 accounts for only a fraction of the characteristic T_{reg} cell signature[9].

To identify other transcription factors that contribute to the T_{reg} cell signature, Fu *et al.* first make a prediction using a computational 'reverse-engineering' approach and experimentally test some of the predicted regulators[2]. Although ectopic expression of those factors alone has little effect on the T_{reg} cell signature, $CD4^+$ T cells transduced to express both Foxp3 and any of the five transcription factors of the quintet (Eos, IRF4, GATA-1, Lef1 or Satb1) converge toward an identical phenotype in which most genes of the T_{reg} cell signature are elicited. In contrast, the T_{reg} cell signature is not perturbed by genetic inactivation of *Ikzf4* (which encodes Eos) or *Gata1*, which suggests that the quintet of transcription factors acts in a redundant manner. The quintet seems to induce the T_{reg} cell signature by interacting with Foxp3 and by enhancing occupancy by Foxp3 of its genomic targets rather than by spreading the binding of Foxp3 to other targets. Notably, ectopic expression of both Foxp3 and a factor of the quintet leads to upregulation of endogenous *Foxp3*, *Ikzf4* and *Irf4* (which are upregulated in T_{reg} cells) and to downregulation of endogenous *Lef1* and *Satb1* (which are downregulated in T_{reg} cells); this suggests the presence of positive and negative feedback loops among Foxp3, factors of the quintet and genes that encode them (**Fig. 1**).

Those results prompt Fu *et al.* to hypothesize that a molecular circuitry with such multiple and redundant feedback loops would act as a bi-stable switch that transitions between two distinct alternative stable steady states[2]. Fu *et al.* test the plausibility of such a mechanism by constructing a computational simulation model and find that the model indeed shows bi-stable activity when the parameters are set appropriately; overexpression of both Foxp3 and any one of the transcription factors of the quintet elicits the T_{reg} cell signature, and the resulting T_{reg} cell state is self-perpetuating even after withdrawal of the initial triggers. Moreover, the model successfully simulates some experimental observations, including the robustness of the T_{reg} cell signature when faced with removal of any of the transcription factors of the quintet and the partial acquisition of the T_{reg} cell signature in the 'T_{reg} cell–like cells'.

The studies by Rudra *et al.* and Fu *et al.*, although they address different questions by different approaches, converge on the following common view: the differentiated state of T_{reg} cells is not determined solely by individual regulatory components but instead by the collective activity of their transcriptional network as a whole. This network perspective has important implications for several aspects of T_{reg} cell biology and raises many questions for future studies. For example, the bi-stable activity of the T_{reg} cell transcriptional network may underlie the remarkable stability of the differentiation state of T_{reg} cells[6,10]. Many biological networks that have positive feedback or double-negative feedback loops have been shown to display bi-stability, which could account for the irreversibility of biological responses, including cellular differentiation[11]. It should be pointed out, however, that the mere presence of such feedback loops does not guarantee bi-stability as well as irreversibility[11]. Hence, it remains to be determined whether the T_{reg} cell transcriptional network actually acts as a bi-stable switch and confers irreversibility on the course of T_{reg} cell differentiation in the thymus and periphery. In addition, it will be also important to identify the extrinsic signals that 'kick' the bi-stable switch on and to determine whether there is any relationship between that switch mechanism and epigenetic mechanisms (such as demethylation of the *Foxp3* locus)[12] in the control of T_{reg} cell stability. Another aspect of the Foxp3 interactome that remains to be elucidated is its dynamic features. As shown by Rudra *et al.*, the Foxp3–GATA-3 interaction is dependent on stimulation of the T cell antigen receptor. Given that T_{reg} cells are able to change their phenotype and functional modality in response to environmental changes[3], it is conceivable that the Foxp3 complexes incorporate different partners in different immune environments to ensure the adaptability of T_{reg} cells.

In conclusion, the studies by Rudra *et al.* and Fu *et al.* have opened a new era in T_{reg} cell research[1,2]. Further elucidation of the structure and dynamics of the T_{reg} cell transcriptional network will be certainly a next focus of research and will be instrumental for control of the network for therapeutic purposes.

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1. Rudra, D. *et al. Nat. Immunol.* **13**, 1010–1019 (2012).
2. Fu, W. *et al. Nat. Immunol.* **13**, 972–980 (2012).
3. Josefowicz, S.Z., Lu, L.F. & Rudensky, A.Y. *Annu. Rev. Immunol.* **30**, 531–564 (2012).
4. Zheng, Y. *et al. Nature* **445**, 936–940 (2007).
5. Marson, A. *et al. Nature* **445**, 931–935 (2007).
6. Miyao, T. *et al. Immunity* **36**, 262–275 (2012).
7. Gavin, M.A. *et al. Nature* **445**, 771–775 (2007).
8. Lin, W. *et al. Nat. Immunol.* **8**, 359–368 (2007).
9. Hill, J.A. *et al. Immunity* **27**, 786–800 (2007).
10. Rubtsov, Y.P. *et al. Science* **329**, 1667–1671 (2010).
11. Ferrell, J.E. Jr. *Curr. Opin. Cell Biol.* **14**, 140–148 (2002).
12. Huehn, J., Polansky, J.K. & Hamann, A. *Nat. Rev. Immunol.* **9**, 83–89 (2009).