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Affiliation

*Inflammation, Autoimmunity and
Transplantation Research,
Roche Palo Alto, LLC
3431 Hillview Ave, Mailstop
R7-101, Palo Alto, CA 94304,
USA
Tel.: +1 650 855 5649
Fax: +1 650 855 5501
stanford.peng@roche.com*

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Interactions of Fox proteins with inflammatory transcription-factor pathways

Stanford L Peng

The forkhead (Fox) protein family includes a wide diversity of 'winged helix' transcription factors, now known to play critical roles in immune homeostasis. Although Foxp3 remains the most widely studied member for its role in the development and/or function of regulatory T cells, which extrinsically modulate the activity of effector leukocytes, other family members, such as Foxj1, Foxo3a and Foxd1, have received growing attention for their importance in the cell-intrinsic regulation of immune homeostasis, especially in T cells, by antagonizing the activity of proinflammatory transcription-factor pathways, such as the nuclear factor- κ B or nuclear factor of activated T-cell families. This review summarizes a present understanding of these factors in this context.

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The forkhead (Fox) protein family comprises transcription factors that share a 'winged helix' DNA-binding domain consisting of three α helices flanked by two 'wings' of β strands and loops. Fox domains have been found in over 100 proteins, comprising at least 17 subclasses, FoxA through FoxQ, which play critical roles in multiple developmental, metabolic, aging and oncological processes [1,2]. Recently, roles for several Fox family members have begun to be elucidated in the immune system, with Foxp3 receiving the most attention for its role in regulatory T (T_{reg}) cells [3,4]. However, a growing number of non-Foxp3 Fox transcription factors have received attention for their essential roles in establishing immune homeostasis, particularly in T cells, by antagonizing the activity of inflammatory transcription-factor pathways and, thereby, forming a network of intracellular immunoregulatory interactions (TABLES 1 & 2; FIGURE 1). This review presents a current understanding of these factors and their mechanisms of action. For a general discussion of the Fox family, the reader is directed to several reviews in both immunology and other publications [1–5].

Immunologically relevant Foxes & their interactions with inflammatory transcription-factor pathways

Foxj1

Outside of the immune system, Foxj1 (hepatocyte nuclear factor forkhead homolog [HNF]-4, FKHL-13) plays critical roles in the development of ciliated epithelium, such as in the lung, choroid plexus and reproductive tract [6–9]. Foxj1-deficient mice lack cilia, resulting in multiple developmental abnormalities, such as heterotaxy and hydrocephalus, resulting in lethality *in utero* or soon postpartum.

A role for Foxj1 in the immune system was initiated by microarray studies to identify novel transcription factors in autoimmunity [10]. There, lymphocytes from lupus-prone mice were found to have significantly reduced expression of Foxj1 compared with nonautoimmune mice [11], suggesting that loss of function in Foxj1 might predispose, or otherwise contribute to, systemic autoimmunity. Indeed, animals with Foxj1-deficient lymphoid systems, generated through fetal liver chimerization in recombination-activating gene (Rag)-deficient animals, developed a spontaneous, multisystemic autoinflammatory

syndrome involving the lung, liver, kidney and salivary gland, associated with both T- and B-cell hyperactivation and autoreactivity [11,12]. *Foxj1*-deficient T cells, for instance, proliferated and secreted cytokines more vigorously in response to T-cell receptor (TCR) or costimulatory CD28 ligation and proliferated vigorously in autologous mixed lymphocyte reactions, indicating the presence of autoreactivity. Foxj1 deficiency also conferred a B cell-intrinsic hyperactivity and autoimmunity, associated with the spontaneous formation of germinal centers and high-affinity autoantibodies, as well as the autoimmunity-linked cytokine interleukin (IL)-6.

Such phenotypes appear to reflect a requirement for Foxj1 in naïve T and B cells to restrain spontaneous nuclear factor (NF)- κ B activation through the inhibitory subunit inhibitor of NF- κ B (I κ B) β . *Foxj1*-deficient T and B cells possess elevated spontaneous NF- κ B activity, the inhibition of which by antisense or decoy oligonucleotides abrogates these cells' hyperactive and autoreactive state. Foxj1 deficiency results in an I κ B β deficiency, because Foxj1 is required, at least in these cell types, for I κ B β transcription: Foxj1 is bound to, and can transactivate, the *I κ B β* promoter *in vivo*, and *Foxj1*-deficient T and B cells have significantly reduced I κ B β levels [PENG SL, UNPUBLISHED DATA] [11,12]. Therefore, Foxj1 interacts with the NF- κ B pathway at least by suppressing its activity through the promotion of transcription of the inhibitory I κ B β subunit.

Interactions for Foxj1 with additional transcription-factor pathways in immunity almost certainly exist as suggested by the ability of Foxj1 to prevent egress of thymocytes in an apparently NF- κ B-independent mechanism [13]. Furthermore, in respiratory systems, the sex-determining region on the Y chromosome (Sry)-related high-mobility group (HMG) box transcription-factor Sry-like HMG-box gene (*Sox17*) can

enhance *Foxj1* promoter activity [14]. Therefore, additional interactions beyond only the NF- κ B transcription-factor pathway seem probable both up- and down-stream of *Foxj1* in immunity, but these remain to be elucidated fully.

Foxo3a

The Foxo family includes at least Foxo1 (forkhead in rhabdomyosarcoma [FKHR]), Foxo3a (FKHR-like [FKHRL]-1) and Foxo4 (AFX, mixed lineage-leukemia [trithorax homolog] translocated to 7 homolog [Mllt7]), which are all mammalian homologs of the *Caenorhabditis elegans* dauer-formation mutant (*DAF*)-16 gene that plays critical roles in insulin signaling, metabolism, life span and fertility. Accordingly, mammalian Foxo proteins regulate apoptosis, cell cycle, metabolism and resistance to oxidative stress: Foxo1 deficiency results in abnormalities of insulin sensitivity, adipocyte differentiation and angiogenesis, Foxo3a deficiency results in abnormalities of ovarian development and fertility, whereas Foxo4 deficiency appears to be largely without obvious deficits [15–21].

Interest in the Foxo transcription factor in the immune system has been long pursued based upon multiple potential roles, for example, in the regulation of apoptosis or cell cycle. However, it was the ability of Foxo3a to inhibit NF- κ B activity *in vitro*, in studies inspired by attempts to identify Foxj1-like Fox family members, that prompted investigations into its potential role in autoimmunity [22]. Analogous to Foxj1, Foxo activity was diminished in lymphocytes from lupus-prone compared with nonautoimmune mice, and Foxo3a deficiency resulted in mild systemic autoimmunity, characterized by lymphadenopathy and splenomegaly, as well as inflammation of the salivary gland, lung and kidney. As in

Table 1. Effects of the known immunologically relevant Fox family members on transcription-factor pathways.

Fox gene	Affected pathway	Effect	Mechanism	Known affected cell lineages	Ref.
<i>Foxd1</i>	Foxj1	Activation	<i>Foxj1</i> gene transactivation	T cell	[39]
	NFAT	Inhibition	CK1 gene transactivation	T cell	[39]
	NF- κ B	Inhibition	I κ B β transactivation via Foxj1	T cell	[39]
	Smad1	Inhibition	BMP-4 suppression?	Renal capsule	[40]
<i>Foxj1</i>	NF- κ B	Inhibition	I κ B β gene transactivation	T cell, B cell	[11,12]
<i>Foxo3a</i>	NF- κ B	Inhibition	I κ B β and I κ B ϵ expression (indirect in part via Foxj1)?	T cell	[22]
	ER	Activation	ER expression	Breast cancer	[33]
<i>Foxp3</i>	NFAT	Inhibition	Physical interaction	T cell	[41,43,44]
			Competition for binding site?		
	NF- κ B	Inhibition	Physical interaction	T cell	[42,43]
	CREB	Inhibition	?	T cell	[42]

BMP: Bone morphogenic protein; cAMP: Cyclic adenosine monophosphate; CK: Casein kinase; CREB: cAMP-responsive element-binding protein; ER: Estrogen receptor; Fox: Forkhead; I κ B: Inhibitor of NF- κ B; NF: Nuclear factor; NFAT: NF of activated T cells; Smad: Mothers against decapentaplegic homolog.

Table 2. Transcription-factor pathway regulators of Fox transcription factors.

Fox target	Pathway	Transcription factor	Effect	Cell lineage demonstrated	Ref.
Foxj1	?	Foxd1	Transactivation	T cell	[39]
	?	Foxo3a	Upregulation	T cell	[22]
	?	Sox17	Transactivation	Respiratory epithelium	[14]
Foxo3a	?	Runx3	Co-activation	Gastric cancer	[30,31]
	NF- κ B		IKK-mediated phosphorylation	Breast cancer	[23]
	CBP		Sir2-mediated deacetylation	Multiple cancers	[24–28]
	DNA damage	p53	SGK1-induced phosphorylation	Fibroblasts	[32]
Foxn1	Wnt	TCF/LEF	Transactivation?	Epithelial	[52]
	BMP-4	Smad	Transactivation?	Thymic stroma	[53]
Foxp3	TCR	NFAT, AP-1	Transactivation	Effector T cell	[45]
	TGF- β	Smad	Transactivation	T cell	[46]
	ER	ER	Transactivation	T cell	[47]

BMP: Bone morphogenic protein; cAMP: Cyclic adenosine monophosphate; CBP: CREB-binding protein; CREB: cAMP responsive element-binding protein; ER: Estrogen receptor; Fox: Forkhead; HMG: High-mobility group; IKK: I κ B kinase; LEF: Lymphoid enhancer factor; Runx: Runt-related; SGK: Serum- and glucocorticoid-inducible kinase; Sir: Silent information regulator; Smad: Mothers against decapentaplegic homolog; Sox: Sry-like HMG-box gene; Sry: Sex-determining region on the Y-chromosome; TCF: T-cell factor; TCR: T-cell receptor; Wnt: Wingless.

Foxj1 deficiency, Foxo3a deficiency similarly resulted in T-cell hyperactivation, including accentuated TCR-mediated proliferation and cytokine production, as well as autoreactivity as demonstrated by proliferation in autologous mixed lymphocyte reactions.

These parallels between Foxo3a deficiency and Foxj1 deficiency appear to reflect a requirement for Foxo3a to regulate the expression of *Foxj1* in T cells, since *Foxo3a*-deficient T cells are deficient in Foxj1. Indeed, as is the case with Foxj1 deficiency, Foxo3a deficiency results in elevated spontaneous NF- κ B activity, the inhibition of which by decoy NF- κ B oligonucleotides abrogates the hyperactivity of *Foxo3a*-deficient T cells, associated with a deficiency in the Foxj1 target gene *I κ B β* . Alternatively, though, *Foxo3a*-deficient T cells are also deficient in the inhibitory NF- κ B subunit I κ B ϵ , and *Foxo3a*-deficient B cells contain normal levels of Foxj1 [PENG SL, UNPUBLISHED DATA] [22]. In this sense, it is interesting to note that the NF- κ B pathway may itself interfere with Foxo3a, as the NF- κ B-activating kinase I κ B kinase may phosphorylate and inhibit Foxo3a, at least in some cell types [23], and the activity of Foxo family members, in general, can be modulated by post-translational acetylation, as regulated by the transcriptional co-activator cyclic adenosine monophosphate (cAMP)-response element-binding protein (CREB)-binding protein (CBP) and the nicotinamide adenine dinucleotide-dependent histone deacetylase silent information regulator (Sir)-2, again at least in some cell types [24–28]. Furthermore, runt-related (Runx)3, one of the Runx family of transcription factors heavily implicated in the regulation of immunity and

autoimmunity [29], can interact with and enhance the transcriptional activity of Foxo3a in gastric cancer cells [30,31], p53 activation is known to be capable of leading to the inhibition of Foxo3a through phosphorylation [32], and Foxo3a itself can promote the expression of the estrogen receptor [33]. Therefore, although a major pathway by which Foxo3a regulates T-cell homeostasis appears to be the restraint of spontaneous NF- κ B activity through Foxj1 and I κ B β , Foxo3a is clearly capable of additional, as yet incompletely elucidated, interactions with the NF- κ B and probably other transcriptional pathways.

Foxd1

Foxd1 (BF-2, Hfh10, FREAC4, FKHL-8) was described initially for its critical role in renal morphogenesis, where *Foxd1*-deficient mice exhibit impaired renal differentiation, at least in part owing to a requirement for Foxd1 in the transcription of placental growth factor [34,35]. Recent studies have also revealed a role for Foxd1 in the development of the optic chiasm [36].

Prior interest in Foxd1 as a possible regulator of immune homeostasis might be inferred from previous studies demonstrating the ability of Foxd1 to modulate cAMP responsiveness through the regulation of the RI α subunit of cAMP-dependent protein kinase (PK)A in Sertoli cells, and the modestly reduced sensitivity of T cells from mice deficient in the related Fox family member *Foxd2* to cAMP-mediated inhibition of proliferation [37,38]. However, direct investigation of Foxd1 in immunity *in vivo* was prompted by a search to identify potential Fox tran-

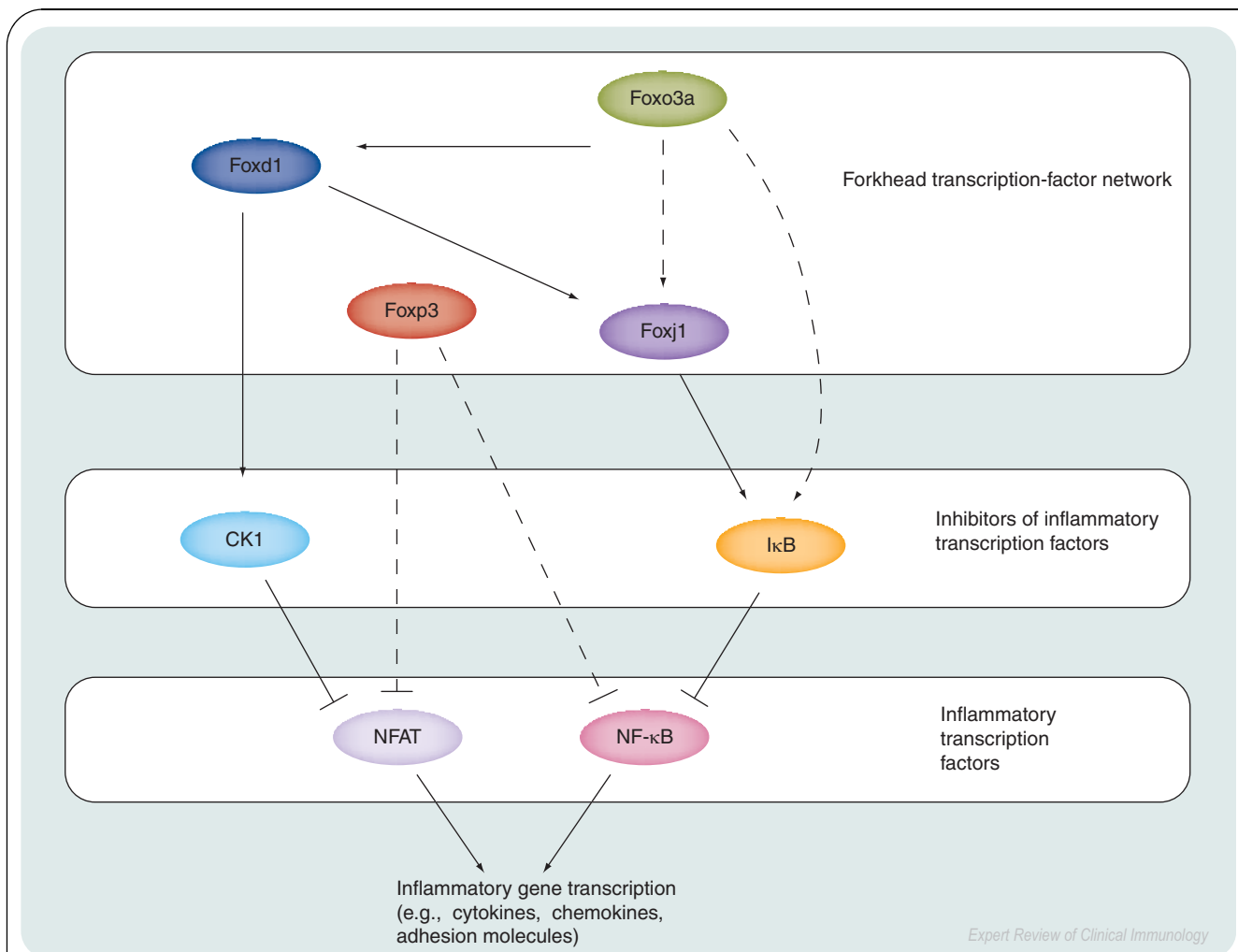


Figure 1. Immune homeostasis by Fox-mediated regulation of inflammatory transcription-factor activities. The activity of immediate-early inflammatory transcription factors NF- κ B and NFAT are kept in check in naïve T cells by inhibitory components of their pathways, such as I κ B family members and NFAT kinases (e.g., CK1), respectively. The expression of these inhibitory proteins require the coordinated activity of Fox transcription factors, particularly Foxd1, Foxj1 and Foxo3a. Solid lines indicate interactions demonstrated *in vivo*; dotted lines indicate possible points of interaction, as demonstrated by *in vitro* studies: Foxo3a may regulate Foxj1 and I κ B independently of Foxd1; also, Foxp3 inhibits the activity NFAT and NF- κ B proteins through direct physical binding. See text for further details.

CK: Casein kinase; Fox: Forkhead; I κ B: Inhibitor of NF- κ B family; NF: Nuclear factor; NFAT: NF of activated T cells.

scriptional regulators of Foxj1, during which Foxd1 was found to be capable of transactivating and binding to a *Foxj1* promoter *in vitro* and *in vivo* [39]. Accordingly, animals with *Foxd1*-deficient lymphoid systems, generated through fetal liver chimerization in *Rag*-deficient animals, strongly resembled Foxj1 deficiency, with spontaneous systemic autoimmunity, T-cell hyperactivation and autoreactivity as demonstrated by positive autologous mixed lymphocyte reactions, all associated with deficient *Foxj1* expression levels in T cells.

Not surprisingly, *Foxd1*-deficient T cells possess elevated spontaneous NF- κ B activity, as well as diminished I κ B β expression, in accordance with a secondary Foxj1 deficiency. However, here elevated NF- κ B activity alone was unable to account for T-cell hyperactivity, as decoy NF- κ B oligonucleotides were unable to abrogate their hyperactive phenotype, in contrast to Foxj1 or Foxo3a deficiency. By contrast, Foxd1

was also found to be required to regulate the activity of NFAT in an analogous fashion to NF- κ B by regulating the expression of the NFAT inhibitory kinase casein kinase (CK)1: *Foxd1*-deficient T cells possessed elevated spontaneous NFAT activity and lacked CK1 expression; Foxd1 could transactivate and bind to a CK1 promoter; and combined inhibition of NF- κ B and NFAT, through decoy NF- κ B oligonucleotides and the calcineurin inhibitors cyclosporine A or FK506, was sufficient to abrogate the hyperactivity of *Foxd1*-deficient T cells. Furthermore, in *Foxd1*-deficient T cells, *Foxj1* overexpression restored I κ B β expression and normalized NF- κ B, but not NFAT activity, whereas CK1 overexpression normalized NFAT, but not NF- κ B activity. Therefore, Foxd1, quite interestingly, plays critical roles in T-cell homeostasis by interacting with at least two proinflammatory transcription-factor pathways: NF- κ B, through Foxj1 and the

inhibitory subunit I κ B β ; and NFAT, through the inhibitory kinase CK1. Nonetheless, it is interesting to speculate upon additional roles for Foxd1, as, for example, Foxd1 deficiency causes ectopic bone morphogenetic protein (BMP)-4 signaling and Smad1 activation, at least in the renal system [40]. Therefore, as for Foxj1 and Foxo3a, Foxd1 probably interacts both up- and down-stream of additional transcription-factor pathways in immunity.

Foxp3

Foxp3 (scurfin [sf], JM2) remains an important regulator of autoimmunity through its role in the development and/or function of T_{reg} cells [5]. Although the mechanisms by which Foxp3 contribute to T_{reg} cell activity remain incompletely understood, emerging evidence indicates that it, similar to the aforementioned Fox proteins, is also capable of inhibiting the activity of inflammatory transcription factors, including both NF- κ B and NFAT, as well as possibly the cAMP–CREB pathway [41–44]. In contrast to Foxj1, Foxo3a and Foxd1, however, Foxp3 appears to mediate such effects largely by physically binding to target transcription factors, such as NF- κ B and NFAT, inhibiting the latter's ability to transactivate target genes, such as *IL*-2, -4 and interferon- γ ; and, in the case of NFAT, promoting a T_{reg}-cell-differentiation pathway [44]. Interestingly, NFAT and possibly AP-1 transcription factors themselves may regulate the expression of *Foxp3*, at least in some human effector T cells [45], and other transcription factors have been proposed to regulate *Foxp3* expression during the derivation of mouse T_{reg} cells, such as those in the TGF- β /Smad [46] or estrogen receptor [47] pathways. Still, the overall relevance of such findings to T_{reg}-cell biology remains incompletely defined: the lack of significant disruption of T_{reg}-cell function in TGF- β 1- or estrogen receptor-deficient mice suggests that those pathways may not be critical to the regulation of Foxp3 itself [48]; and although Foxp3-mediated inhibition of transcriptional pathways, such as NF- κ B and NFAT, probably explain at least part of the T_{reg}-like phenotype induced by Foxp3 when expressed ectopically in effector

T cells, whether or not such interactions impact substantially upon endogenous T_{reg}-cell biology *in vivo* remain undetermined, as effector T cells generally lack *Foxp3* expression in physiological settings.

Expert commentary

Fox transcription-factor biology has evolved into an exciting research area, not only because of the diversity of transcription factors and their individual roles in the immune system, but also because of their diversity of interactions with other transcription-factor families. However, it is important to note that the majority of these data remains confined to the mouse system, and direct parallels between mouse and human biology cannot be guaranteed: indeed, Foxp3, the most well documented of these factors, more tightly correlates with T_{reg} cell activity and/or phenotype in mouse than human T cells [5]. Nonetheless, the now multiple instances by which Fox proteins interact with inflammatory transcription-factor pathways, especially NF- κ B and NFAT, strongly suggest a general importance of Fox transcription factors in immune homeostasis through such interactions. Indeed, it is encouraging to observe that human mutations in *Foxp3* result in the immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy [IPEX], autoimmunity-allergic dysregulation syndrome [XLAAD]; Online Mendelian Inheritance in Man [OMIM] #304790 [101]) disorder, which strongly resembles mouse Foxp3 deficiency; moreover, reduced levels of Foxp3 have been found recently in autoimmune diseases, such as multiple sclerosis [49], and *Foxj1* polymorphisms have been associated with allergic rhinitis [50]. Furthermore, the interactions of Fox proteins with NF- κ B and NFAT is consistent with the long-established efficacies of the NFAT inhibitors cyclosporine A and FK506, as well as other immunosuppressive and anti-inflammatory therapies with potency against NF- κ B, such as salicylates, glucocorticoids, leflunomide and sulfasalazine [51]. Therefore, it is highly probable that the importance of Fox transcription factors will extend to the

Key issues

- The forkhead (Fox) family consists of several immunoregulatory transcription factors, many of which remain uncharacterized immunologically.
- Specific Fox genes, including *Foxj1*, *Foxd1* and *Foxo3a*, are required to maintain immune homeostasis by antagonizing the activity of inflammatory transcription factors, such as the nuclear factor (NF)- κ B and nuclear factor of activated T-cell (NFAT) families, particularly in T cells, in a cell-intrinsic manner.
- Other Fox family members are known to play critical roles in immunity and interact with inflammatory transcription-factor pathways, however, the physiological relevance of such interactions remains incompletely understood. Foxp3, for instance, plays critical roles in the development and/or activity of regulatory T cells and can suppress the activity of NF- κ B and NFAT, but these two functions may not be causative of each other, especially as Foxp3 is largely considered to regulate immune homeostasis indirectly through regulatory T cells, which in turn regulate effector cells.
- Future studies will hopefully establish the relevance of these largely mouse-based findings in humans and translate these mechanisms into therapeutic interventions.

homeostasis of human immunity, although the specifics of the mechanisms might not surprisingly differ somewhat compared with mice or other species.

Five-year view

Future work on Fox proteins appears, therefore, to elucidate further the multitude and importance of interactions between these and other Fox transcription factors and inflammatory transcription-factor pathways. Foxn1 (HNF-11, FKHL20, Whn, nude [nu]), for instance, is responsible for the rodent and human *nu* mutations, which result in defective hair and T-cell development, and is induced by wingless (Wnt) glycoproteins (implicating interactions with T-cell factor), lymphoid enhancer factor-1 transcriptional regulators [52], as well as bone morphogenic protein BMP-4 (implicating Smad transcription factors) [53]. Its relative Foxn3 has been shown recently to interact with Ski-interacting protein (SKIP) NCoA-62, a transcriptional coregulator with predominantly repressor functions [54]. Finally, the relevance of these pathways to human autoimmune diseases is likely to be elucidated as the expression and activity patterns of the Foxes are correlated

with disease activity and therapy, as has begun with observations of the upregulation of *Foxp3* during epitope-specific immunotherapy of rheumatoid arthritis with dnaJp1 in humans [55], or the activation of *Foxo3a* by CTLA4 (CD152) inhibition in mice [56,57]. Additional confidence will be gained through genetic studies to continue to associate polymorphisms in Fox proteins with disease susceptibility and manifestations. Furthermore, it will probably be quite difficult to translate such findings directly to drug therapies for inflammatory diseases, since transcription factors remain in general challenging drug targets, despite some recent progress in targeting them through modulation of their protein–protein or protein–DNA interactions, or pathways in general [58,59]. Nonetheless, the relevance of the Foxes' inhibitory interactions with other transcription factors would and should, therefore, be based on detailed mechanistic studies in human diseased and nondiseased cells analogous to the murine studies.

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Affiliation

- Stanford L Peng, MD PhD
 Director, Arthritis Research
 Inflammation, Autoimmunity and
 Transplantation Research
 Roche Palo Alto, LLC
 3431 Hillview Ave, Mailstop R7–101
 Palo Alto, CA 94304, USA
 Tel.: +1 650 855 5649
 Fax: +1 650 855 5501
stanford.peng@roche.com