# Forkhead box transcription factors as context-dependent regulators of lymphocyte homeostasis

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Abstract | Lymphocytes have evolved to react rapidly and robustly to changes in their local environment by using transient adaptations and by regulating their terminal differentiation programmes. Forkhead box transcription factors (FTFs) can direct leukocyte-specific responses, and their functional diversification promotes a high degree of context-dependent specification. Many, often antagonistic, FTFs have overlapping expression patterns and can thereby compete for binding to the same chromosomal target sequences. Multiple molecular mechanisms also connect extracellular signals to the expression and functionality of specific FTFs and, in this way, fine-tune their activity. Through these diverse mechanisms, FTFs can function as context-dependent rheostats responding to diverse environmental stimuli. Focusing on the various mechanisms by which their functional activity is modulated, as well as on their mechanisms of action, we discuss how specific FTFs control lymphocyte function, allowing for the establishment and maintenance of immune homeostasis.

Forkhead box transcription factors (FTFs) constitute a highly conserved family of proteins that share a DNA-binding module — the forkhead domain — and bind shared DNA consensus sequences throughout the genome[1]. By mediating both the activation and repression of transcription, as well as through their interaction with multiple cofactors, FTFs are involved in a wide range of cellular processes, including cellular differentiation, metabolism, cell cycle progression, apoptosis and autophagy, as well as in protection against cellular stress[2]. During development, FTFs regulate tissue formation and homeostasis through multiple mechanisms[3]. Thereby, expression of specific FTFs can guide irreversible cell fate decisions, or FTFs can act as rheostats, adapting cellular responsiveness to changing microenvironments[4].

Few cell types react so rapidly and change their phenotype so fundamentally in response to external challenges as lymphocytes during infection and inflammation. The selection for effective immune mechanisms has included the incorporation of FTFs, presumably for their ability to rapidly respond to changing extrinsic conditions[1,5,6]. Similarly to their contribution during embryonic development and tissue homeostasis, FTFs contribute in two fundamentally different ways to controlling immune responses. First, FTFs can direct generally irreversible transcriptional events, ensuring terminal differentiation of a specific leukocyte population and thereby imposing a long-term transcriptional programme that is required for immune cell function. Second, FTFs can function as rheostats to environmental stimuli, allowing for a rapid and transient adaptation to changing environmental challenges, thereby optimizing immune responses while maintaining immune homeostasis. In this way, local environmental signals, such as cytokines or nutritional status, can rapidly influence both the expression levels and the functionality of FTFs in lymphocytes. The differential expression and functionality of FTFs in turn regulate the differentiation, phenotype and functionality of specific leukocyte subsets, regulating the magnitude and effectiveness of immune responses (see TABLE 1). In this way, FTFs play a central role in regulating immune responses and immune homeostasis.

In this Review, we discuss the various mechanisms through which specific FTFs control the differentiation programmes of distinct leukocyte populations and contrast these with the mechanisms FTFs use to mediate a transient adaptation of lymphocytes to changing local environments. Finally, we discuss the molecular mechanisms that regulate the expression and functionality of FTFs.

## FTFs as regulators of leukocyte differentiation

***Cell fate imposition.*** Transcription factors are often segregated into those that can directly bind and open chromatin, the 'pioneers', and those that can only regulate transcription at open chromatin, the 'settlers'. FTFs fall into both of these classes, and this affects their functional diversity, as discussed below. During

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https://doi.org/10.1038/s41577-018-0048-9

Table 1 | **Immune functions of FTFs**

| FTF | Functions | Refs |
|---|---|---|
| FOXA1 | Directs the lineage differentiation and immunosuppressive properties of a distinct $T_{reg}$ cell population | 15 |
| FOXD1 | Enforces T cell quiescence | 46 |
| FOXJ1 | Controls egress from the thymus | 47 |
| | Enforces quiescence in B cells and T cells by blocking NF-κB | 48,49 |
| FOXO1 | Cooperates with FOXO3 to promote FOXP3 expression in $T_{reg}$ cells | 28,37,76 |
| | Enhances $T_{reg}$ cell function | 38 |
| | Regulates $T_{reg}$ cell homing to non-lymphoid organs | 44 |
| | Promotes memory $CD8^+$ T cell differentiation | 51,54–58 |
| | Regulates development, homing and survival of naive T cells | 28,29,40 |
| | Blocks NK cell maturation | 31 |
| | Blocks $T_{FH}$ cell differentiation | 32 |
| | Blocks $T_H17$ cell differentiation | 34 |
| | Regulates RAG expression during B cell maturation | 65,66 |
| | Regulates germinal centre reaction | 68–70 |
| FOXO3 | Diminishes memory $CD8^+$ T cell formation via the regulation of pro-apoptotic protein expression | 63,64 |
| | Cooperates with FOXO1 to promote FOXP3 expression in $T_{reg}$ cells | 37,76,77 |
| FOXP1 | Maintains T cell quiescence in naive and differentiating $CD8^+$ T cells | 23,24 |
| | Prevents $T_{FH}$ cell differentiation | 27 |
| | Transcriptional regulator of early B cell development | 19 |
| | Contributes to B cell homeostasis by activating NF-κB and by suppressing pro-apoptotic genes | 20 |
| | Blocks B cell activation and germinal centre formation | 21 |
| | Inhibits plasma cell differentiation | 22 |
| FOXP3 | Directs the lineage differentiation and immunosuppressive properties of $T_{reg}$ cells | 10 |

FOX, forkhead box protein; FTF, forkhead box transcription factor; NF-κB, nuclear factor-κB; NK, natural killer; RAG, recombination-activation gene; $T_H17$ cell, T helper 17 cell; $T_{FH}$ cell, T follicular helper cell; $T_{reg}$ cell, regulatory T cell.

embryonic development, FTF expression is sufficient to impose cell type-specific transcriptional programmes, and, in a similar manner, the expression of a number of FTFs is sufficient to direct cell fate decisions in a variety of haematopoietic lineages directing immune cell production. For lymphocytes, perhaps the best-characterized example of how a single FTF can direct cell type-specific function is the expression of forkhead box protein P3 (FOXP3) in regulatory T ($T_{reg}$) cells[7–10]. Once stably expressed in $CD4^+$ T cells, FOXP3 is able to maintain its own expression through a positive feedback loop, and through specific transcriptional changes it ensures terminal differentiation of $T_{reg}$ cells[10]. Although FOXP3 cooperates with several additional transcription factors in the differentiation of $T_{reg}$ cells[11,12], the pure ectopic expression of FOXP3 in $CD4^+$ T cells is sufficient to impose a regulatory T cell phenotype[10,13,14].

Another example of FTF-driven terminal differentiation is highlighted by FOXA1 expression in T cells[15]. While FOXP3 is considered a settler transcription factor requiring the cooperative action of additional transcription factors (as described in more detail below), FOXA1 is considered to be one of a small family of pioneer transcription factors[16], having the ability to directly bind to nucleosomes, induce nucleosomal rearrangement and alter chromatin structure, resulting in the activation of specific transcriptional programmes[17]. One role of FOXA1 is to induce hepatocyte differentiation in developing tissues[18]. In naive $CD4^+$ T cells, IFNβ-induced expression of FOXA1 expression is sufficient to induce their stable differentiation into a programmed cell death 1 ligand 1 (PDL1)-expressing suppressive $CD4^+$ T cell population, which directly contributes to suppression of inflammation, mainly in the central nervous system[15]. The ectopic expression of FOXA1 in $CD4^+$ T cells is also sufficient to induce this suppressive capacity[15].

***Functional road block in differentiation.*** Another mechanism by which FTFs control immune homeostasis is by acting as roadblocks, actively preventing the activation and differentiation of specific leukocyte subsets. Expression of such FTFs is not in itself sufficient to impose a specific differentiation programme, but, instead, their temporary inactivation provides a window of opportunity to progress through a specific developmental checkpoint, allowing cells to fully differentiate (TABLE 1). FOXP1 is a good example of this. For instance, it critically regulates several distinct stages of T cell and B cell differentiation (FIGS 1,2). During B cell maturation, FOXP1 coordinates the transition from the pro-B cell stage to the pre-B cell stage by regulating the expression of the recombination-activating genes (RAGs) and initiating the rearrangement of the B cell receptor (BCR)[19]. In mature B cells, FOXP1 expression promotes B cell survival[20], and during the activation of follicular B cells, FOXP1 acts as a transcriptional activator and repressor of several genes involved in germinal centre (GC) reactions. FOXP1 expression inversely correlates with B cell lymphoma 6 protein (BCL-6) expression, and FOXP1 expression is required to be downregulated in B cells in order to initiate GC formation[21]. However, following the resolution of the GC reaction, FOXP1 expression is re-initiated in memory B cells in order to prevent B lymphocyte-induced maturation protein 1 (BLIMP1, encoded by *PRDM1*) expression and their differentiation into plasma cells[22].

In a similar manner to its role in B cell development, FOXP1 expression also regulates several stages in the differentiation of T cells (FIG. 2). FOXP1 is an essential transcriptional regulator during thymic development of T cells, and genetic ablation of *Foxp1* results in the premature activation of single positive thymocytes that acquire effector functions[23]. Naive $CD8^+$ T cells that lack FOXP1 expression proliferate and gain an activated and/or memory phenotype[24,25]. Epigenomic profiling of human $CD4^+$ T cell subsets further supported these findings. These studies revealed a gradual global loss of DNA methylation in heterochromatic parts of the genome during the differentiation of $CD4^+$

memory T cells, a process paralleled by transcriptome reprogramming. FOXP1 was thereby found to be a 'naive-keeping' checkpoint regulator of $CD4^+$ memory T cell differentiation[26]. Furthermore, genetic ablation of *Foxp1* expression specifically in T cells results in preferential differentiation of naive cells into T follicular helper ($T_{FH}$) cells[27]. This roadblock function is exerted through FOXP1-mediated inhibition of IL-21 expression and the dampening of T cell receptor (TCR) signalling by FOXP1-mediated reduction of the expression of the co-stimulatory molecule inducible T cell co-stimulator (ICOS)[26]. By contrast, in $CD8^+$ T cells, FOXP1 expression prevents their premature differentiation by dampening the expression of IL-7R[24]. FOXO1 is required to disengage FOXP1 from the enhancer driving IL-7R expression, thereby overcoming FOXP1-induced quiescence and regulating the health and lifespan of naive T cells[24,28,29]. These observations highlight the complexity underlying context-dependent functionality of a single FTF within individual T cell subsets.

Similarly to FOXP1, the FOXO FTF family members also regulate the differentiation of leukocyte subtypes. Although the regulation of FTF expression levels can drive or inhibit selective differentiation programmes, the FOXO FTF family is generally regulated by cycles of activation and deactivation rather than through control of protein levels[30]. FOXOs can directly suppress the expression of several master transcription factors, which subsequently, upon FOXO inactivation, promotes the terminal differentiation of specific cell types. FOXO1, for example, regulates the expression of T-bet in natural killer (NK) cells[31] and BCL-6 in naive $CD4^+$ T cells[32]; these master transcription factors are critical for the differentiation, maturation and function of NK cells and the differentiation of $T_{FH}$ cells, respectively. The activity of FOXO proteins is regulated by protein kinase B (PKB; also known as AKT)-mediated phosphorylation, which leads to nuclear exclusion and degradation of FOXO proteins[2]. Cytokine receptor signalling, for example, induced by IL-15 or IL-12 in NK cells or ICOS signalling in $T_{FH}$ cells, induces the transient deactivation of FOXO1, allowing expression of T-bet or BCL-6 and thereby enabling the functional maturation of immature NK cells[31] or the differentiation of naive $CD4^+$ T cells into $T_{FH}$ cells[32].

In a similar manner, FOXO1 functions as a negative regulator of the T helper 17 ($T_H17$) cell programme by directly blocking RORγt, the master transcription factor essential for the differentiation and function of $T_H17$ cells[33]. Reducing FOXO1 expression in $CD4^+$ T cells results in increased RORγt activity and higher numbers of $T_H17$ cells in vivo. Treating naive $CD4^+$ T cells in vitro with pharmacological inhibitors of the PKB pathway, preventing the inactivation of FOXO1, reduces the in vitro differentiation of naive CD4 T cells into $T_H17$ cells[34,35].

FOXO transcription factors also play complex, critical roles in regulating the expression and activity of other FTF members. For example, FOXO transcription factors also play a key role in regulating *FOXP3* expression. Efficient *FOXP3* induction requires expression of proteins that limit the output of the phosphoinositide 3-kinase (PI3K)–PKB axis[36], and, in line with this, FOXO transcription factors are able to directly bind to

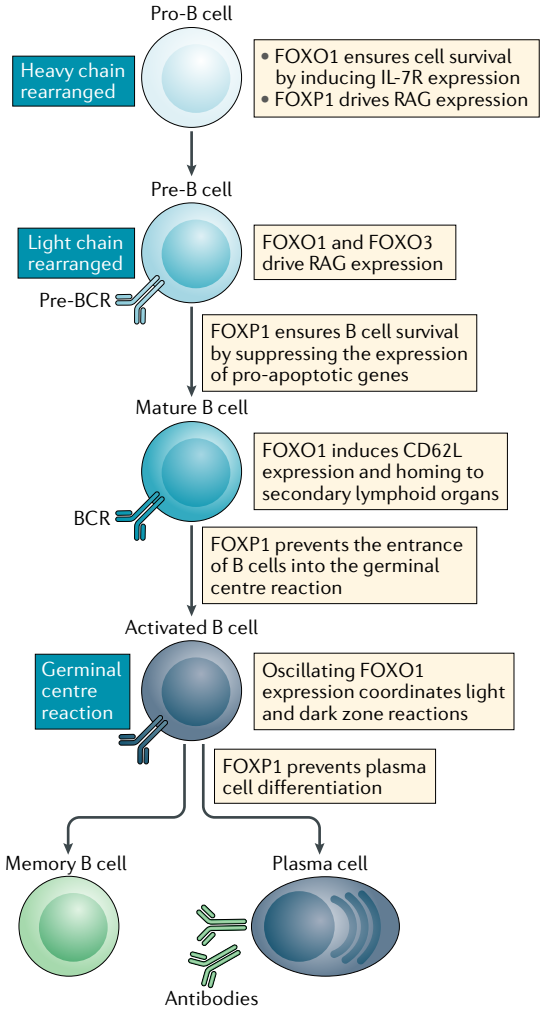


Fig. 1 | **Role of FTFs during B cell maturation and functioning.** In immature B cells, forkhead box protein O1 (FOXO1) sustains survival by inducing IL-7R expression and contributes together with FOXO3 to the rearrangement of the B cell receptor (BCR) gene locus. FOXP1 also contributes to the transcriptional regulation of B cell maturation and ensures the survival of naive B cells, but blocks the activation of naive B cells and has to be degraded for the initiation of an efficient germinal centre reaction and plasma cell differentiation. By contrast, FOXO1 is essential for B cell homing to secondary lymphoid organs, and its controlled expression during germinal centre reactions ensures isotype switching and efficient affinity maturation. FTFs, forkhead box transcription factors; RAG, recombination-activating gene.

the *FOXP3* promoter, thereby increasing its expression[37]. Despite the fact that in the thymus, FOXP3-expressing T cells concomitantly express higher levels of FOXO1 (REF.[38]), ablation of FOXO FTFs does not affect the development of thymus-derived $T_{reg}$ ($tT_{reg}$) cells, also known as natural $T_{reg}$ cells, but instead results in a marked reduction of FOXP3 expression in transforming growth factor-β (TGFβ)-dependent peripherally induced $T_{reg}$ ($pT_{reg}$) cells, also known as induced $T_{reg}$ cells[37]. However, FOXO1 and FOXO3 do show some redundancy in the development of FOXP3 regulatory cells; loss of *Foxo1* alone is sufficient to limit $tT_{reg}$ cell development

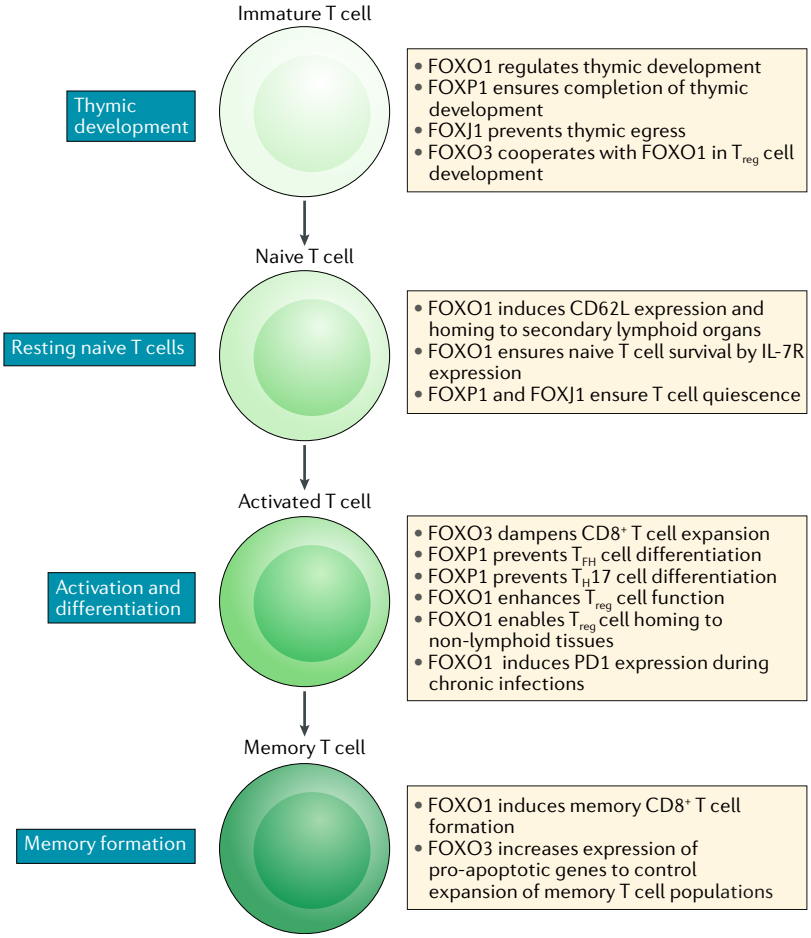


Fig. 2 | **Role of FTFs during T cell maturation and functioning.** During thymic selection, several forkhead box transcription factors (FTFs) cooperate. Forkhead box protein O1 (FOXO1) ensures full T cell development and cooperates with FOXO3 in regulatory T cell development, FOXP1 prevents the premature activation of T cells and FOXJ1 prevents the premature egress of T cells from the thymus. In a similar manner, in naive T cells, FOXO1 ensures T cell survival and homing to the secondary lymphoid organs, while FOXP1 and FOXJ1 prevent premature T cell activation. During T cell activation, FTFs control the expansion rate, the differentiation of activated T cells and memory formation. FOXO1 expression enhances regulatory T ($T_{reg}$) cell function, while T cell receptor (TCR)-mediated degradation of FOXO1 leads to a decline of retention signals for $T_{reg}$ cells in lymph nodes and allows their egress into the periphery. FOXP1 prevents the differentiation of $CD4^+$ T cells into T helper 17 ($T_H17$) and T follicular helper ($T_{FH}$) cells, while FOXO3 limits the excessive expansion of $CD8^+$ T cell populations and, by the induction of pro-apoptotic proteins, limits the expansion of memory T cell populations. During chronic infections, FOXO1 induces programmed cell death 1 (PD1) expression.

and results in a slowly developing autoimmune state. Nevertheless, while a single allele of *Foxo1* or *Foxo3* is sufficient for normal numbers of both $tT_{reg}$ and $pT_{reg}$ cells, complete loss of either gene abrogates inducible *Foxp3* expression and lowers levels of $tT_{reg}$ cells during the first 3 weeks of life[39]. These studies clearly demonstrate a dynamic interaction between FOXO and FOXP3 family members, with a dependence on FOXO proteins to transcriptionally upregulate FOXP3.

Taken together, these examples demonstrate that the immune system has adopted evolutionary and developmentally conserved mechanisms of FTF-mediated and FTF-regulated cell differentiation to control the terminal differentiation of leukocyte populations.

## FTFs and adaptation to changing environments

In addition to regulating the terminal differentiation programmes of lymphocytes, the activation of FTFs in response to changing local environmental conditions can transiently alter the functionality of lymphocyte populations. The FOXO subfamily of FTFs in particular dynamically regulates lymphocyte functions and their differentiation in response to environmental cues, as discussed below.

***Regulation of T cell homing.*** Naive T cells emigrate from the blood to peripheral lymph nodes in a multistep process consisting of tethering and rolling, followed by transendothelial migration. FOXO1 can potentially play an important role during all stages of this complex process. First, FOXO1 can regulate the expression of the adhesion molecule L-selectin on naive T cells[29,40], promoting their tethering to high endothelial venules (HEVs). FOXO1 also induces the expression of CC-chemokine receptor 7 (CCR7), which allows T cells to exit from tissues and home to secondary lymphoid tissues[29,41,42]. Finally, FOXO1 can induce the expression of sphingosine-1-phosphate receptors (S1PRs) on T cells[40], which allows for the egress of T cells from the secondary lymphoid tissues. Thus, TCR-induced phosphorylation and deactivation of FOXO1 reduces the expression of these lymph node-homing receptors and allows for the migration of antigen-activated T cells into inflamed peripheral tissues[29] (FIG. 3). Such a mechanism of T cell homing is well established for naive T cells and central memory T cells[43]. Nevertheless, it has recently also been described for $T_{reg}$ cells[44]. In order for $T_{reg}$ cells to gain their full suppressive capacity, they cycle between inflamed peripheral tissues and lymphoid tissues[45]. In secondary lymphoid organs, $T_{reg}$ cells have a resting phenotype, in contrast to an activated phenotype in non-lymphoid tissue[44]. High FOXO1 expression in $T_{reg}$ cells directs a transcriptional programme that favours lymphoid organ homing, and their activation-induced inhibition of the FOXO1-mediated transcriptional programmes allows for the migration to the site of inflammation[44]. In line with this, $T_{reg}$ cell-specific expression of a PKB-insensitive mutant form of FOXO1 was found to impede $T_{reg}$ cell homing to non-lymphoid organs, resulting in $CD8^+$ T cell-mediated autoimmune diseases[44]. These findings suggest a 'goldilocks' model for the effects of FOXO1 activity on T cell functionality and homing.

A similar TCR-mediated homing regulation might exist between FOXD1 and FOXJ1. FOXD1 is expressed in naive $CD4^+$ T cells and is rapidly downregulated upon TCR stimulation[46]. FOXD1 controls FOXJ1 expression, and the transgenic *Foxj1* overexpression was found to inhibit the ability of single positive thymocytes to emigrate from the thymus, causing peripheral T cell lymphopenia[47]. Nevertheless, FOXJ1 not only controls the egress of thymocytes from the thymus but also regulates nuclear factor-κB (NF-κB) inhibitor-β (NFKBIB) expression and, in this way, keeps NF-κB transcriptional responses in check. As a consequence, *Foxj1* deletion, similarly to *Foxd1* deletion[46], leads to rapid systemic, multi-organ inflammation[48,49]. Thus, FTFs comprise an integrated transcriptional network in T cells that can

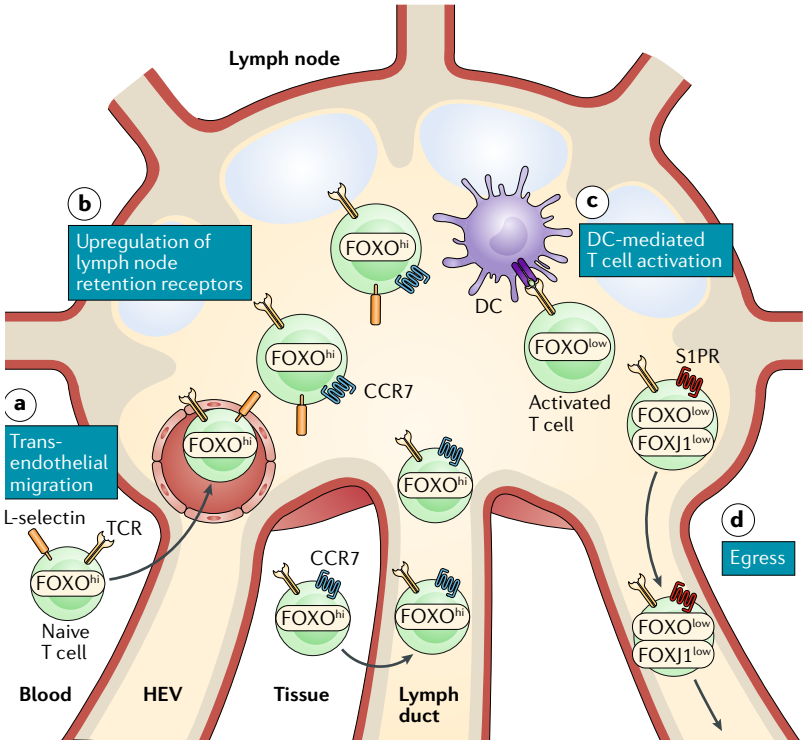


Fig. 3 | **FOXO-mediated regulation of T cell homing.** Naive T cells emigrate from the blood to peripheral lymph nodes in a multistep process consisting of tethering and rolling, followed by transendothelial migration. **a** | Forkhead box protein O1 (FOXO1) upregulates the expression of the adhesion molecule L-selectin on naive T cells, tethering them to high endothelial venules (HEVs). FOXO1 can also induce the expression of CC-chemokine receptor 7 (CCR7), which promotes the homing to and retention of T cells in secondary lymphoid tissues. **b,c** | During dendritic cell (DC)-mediated activation of T cells, T cell receptor (TCR) signalling induces the phosphorylation and deactivation of FOXO1, which reduces T cell expression of lymph node homing and retention receptors. **d** | The reduced expression of these receptors on T cells, as well as FOXO1 induction of sphingosine-1-phopshate receptors (S1PRs), allows for the egress of specific antigen-activated T cells, which can then migrate to inflamed peripheral tissues. Low FOXJ1 expression is also required for this to occur.

control T cell functionality and homing. Establishing the precise level of FTF expression and transcriptional output can therefore exquisitely modulate the capacity of T cell populations to home to tissues and remain there.

***Control of CD8⁺ memory T cell differentiation.*** Within days of pathogen exposure, naive antigen-specific CD8⁺ T cells undergo rapid population expansion and acquire effector functions. In addition, they form precursors to memory T cells capable of self-renewal and indefinite survival. The transition of naive CD8⁺ T cells into effector and memory cells is a well-characterized process in which the regulated activation and inactivation of FTFs play a central role (FIG. 4). FOXO1 drives IL-7Rα expression in naive T cells, which is critical for their survival[29,50]. During effector CD8⁺ T cell differentiation, FOXO1 is inactivated, which is then associated with a transient downregulation of IL-7Rα expression[51]. However, CD8⁺ T cells require IL-7Rα expression in order to transit into memory T cells[52]. During the effector phase, two CD8⁺ T cell populations can be distinguished, one comprising short-lived effector T cells (SLECs) and the other consisting of memory precursor T cells (MPECs)[53]. Compared with SLECs, MPECs express higher levels of FOXO1, promoting the generation of memory T cells[54–56]. FOXO1 also suppresses the expression of the transcription factor T-bet, and this is essential for the differentiation of naive CD8⁺ T cells to SLECs[51,57]. At the same time, FOXO1 enhances the expression of transcription factor 7 (TCF7) and eomesodermin (EOMES), both of which are essential transcription factors that drive CD8⁺ T cell memory formation, and it induces metabolic reprogramming in T cells, leading to the upregulation of fatty acid oxidation[58]. Furthermore, continuous activity of FOXO1 is required to prevent anergy and maintain the memory phenotype of CD8⁺ T cells[59,60]. FOXO1 transcriptional activity is modulated by post-translational modifications (PTMs) that are induced by extrinsic cellular signals (see sections below), allowing dynamic control over the state of CD8⁺ T cell differentiation and generation of memory cells depending on the changing environment of a responding CD8⁺ T cell (FIG. 4).

The strength of mechanistic target of rapamycin complex 2 (mTORC2) signalling has been proposed to play an additional role in controlling FOXO1-mediated CD8⁺ memory T cell differentiation[58]. Activation of mTORC2 results in PKB activation and thereby subsequent FOXO1 inactivation. This shifts the balance towards T-bet expression and away from TCF7 and EOMES, allowing for the expansion of effector T cell populations[51,54–56,58]. If this balance is disrupted, for example, by treatment with rapamycin to inhibit mTOR complexes, then FOXO1 activation results in a higher frequency of CD8⁺ memory T cells[61,62]. Interestingly, this FOXO1-mediated effect is counterbalanced by FOXO3A activity in effector CD8⁺ T cells[63]. Here, FOXO3A induces the expression of the pro-apoptotic factors BCL-2-interacting mediator of cell death (BIM; also known as BCL2L11) and p53 upregulated modulator of apoptosis (PUMA; also known as BBC3), reducing the number of memory precursor effector cells and memory subsets[64]. A higher number of memory precursor effector cells and memory subsets are observed in FOXO3A-deficient mice[63,64], and FOXO3A-deficient memory CD8⁺ T cells show enhanced survival when transferred to RAG1-deficient recipients, suggesting that FOXO3A acts in a cell-intrinsic manner to regulate the survival of primed CD8⁺ T cells[64]. Therefore, by working together, FOXO1 and FOXO3A help to regulate the rapid population expansion of effector CD8⁺ T cells and the eventual survival of CD8⁺ memory T cell subsets during infection.

***Regulation of germinal centre reaction.*** Several distinct stages of B cell differentiation are regulated by both FOXP1 and FOXO1 activity. FOXO1 has a central role in regulating B cell proliferation, isotype switching and efficient affinity maturation. GCs are specialized environments where mature B cells undergo repeated rounds of clonal expansion and secondary diversification of their immunoglobulin genes. *Ig* gene recombination is mediated by RAG1 and RAG2, which are both expressed in pro-B and pre-B cells, where their expression is regulated

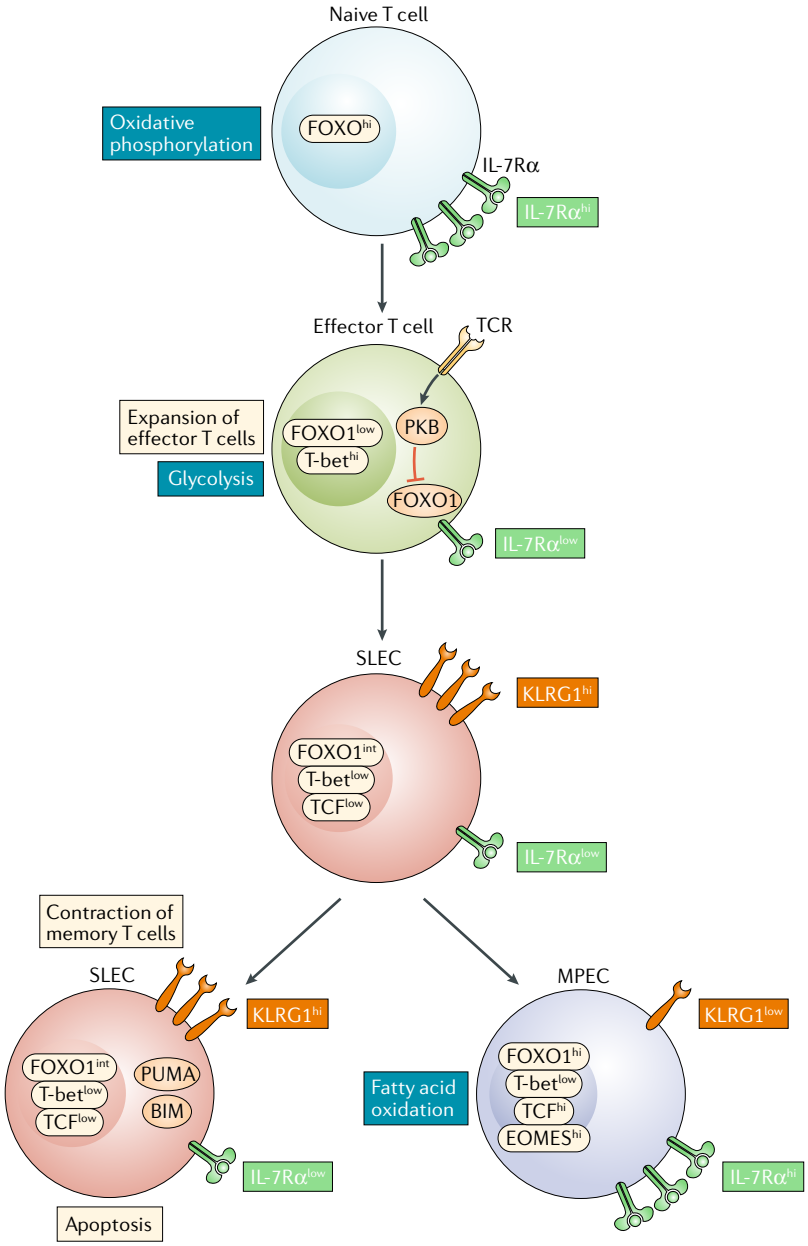


Fig. 4 | **Control of memory CD8$^+$ T cell generation by FOXO1.** Naive CD8$^+$ T cells express high levels of forkhead box protein O1 (FOXO1) and are metabolically characterized by the use of oxidative phosphorylation to generate energy in their resting state. During effector CD8$^+$ T cell differentiation and population expansion, FOXO1 is inactivated by T cell receptor (TCR)-mediated phosphorylation, resulting in transient downregulation of IL-7Rα expression and a switch to a glycolytic metabolism, which is required to balance both energy and metabolic demand expansion. During the effector phase of a CD8$^+$ T cell response, two distinct CD8$^+$ T cell populations can be distinguished: one comprising short-lived effector T cells (SLECs) and the other consisting of memory precursor T cells (MPECs). Compared with SLECs, MPECs express higher levels of FOXO1, promoting the generation of memory T cells. FOXO1 inhibits expression of T-bet, which is essential for the differentiation of naive CD8$^+$ T cells to SLECs. At the same time, FOXO1 enhances the expression of transcription factor 7 (TCF7) and eomesodermin (EOMES), both essential transcription factors leading to CD8$^+$ T cell memory formation, and induces metabolic reprogramming to fatty acid oxidation. FOXOA3 can induce the expression of BCL-2-interacting mediator of cell death (BIM) and p53 upregulated modulator of apoptosis (PUMA), reducing the number of MPECs and memory CD8$^+$ T cells (not depicted in figure). KLRG1, killer cell lectin-like receptor subfamily G member 1; PKB, protein kinase B.

by FOXO1 (REFS[65,66]). The purpose of the GC reaction is the generation of B cells that express high-affinity BCRs, and that can further differentiate into memory B cells and plasma cells to mediate adaptive immune response. Activated B cells migrate to the dark zone of GCs, where they undergo rounds of proliferation and mutation of their antigen receptor before returning to the light zone where they undergo antigen-dependent selection for high-affinity receptors[67]. FOXO1 is highly expressed and localized within the nucleus of B cells located in the dark zone of the GC, while in the light zone of the GC, in which B cells are subjected to antigen-specific activation and receive CD4$^+$ T cell-mediated help, PI3K activity is enhanced, resulting in reduced FOXO1 nuclear localization[68,69]. FOXO1 drives the expression of dark zone genes and, in collaboration with BCL-6, suppresses the expression of genes that lead to plasma cell differentiation, such as BLIMP1. As a consequence, genetic deletion of *Foxo1* using a GC-specific promoter leads to a loss of dark zone cells and a lack of antibody maturation[68,69]. Basic leucine zipper transcriptional factor ATF-like (BATF) is transiently induced in light zone GC B cells in a FOXO1-dependent manner, and deletion of BATF similarly leads to GC disruption, suggesting a direct involvement of FOXO1-induced BATF expression in the switch from light zone to dark zone programmes[70].

Taken together, over the past decade, it has become clear that FTFs play critical roles in various aspects of the development, activation and function of lymphocyte subsets. In the section below, the general molecular mechanisms by which FTFs can regulate transcriptional output are discussed in more detail.

## FTF-mediated immune homeostasis

FTFs have been described to both enhance the expression of specific sets of immunologically relevant genes and to repress the expression of others. This is exemplified by FOXO1, which transcriptionally activates expression of IL-7Rα and of CCR7 and L-selectin in naive T cells as well as during CD8$^+$ T cell memory formation[28,29,51,55,56]. FOXO1 also directly suppresses the transcription of other genes, for example, *Tbx21*, *RORγt*, *T-bet* and *Bcl-6*, that are involved in the regulation of NK cell, $T_H17$ cell and $T_{FH}$ cell development[31,32,34]. Recent genome-wide systematic analyses revealed that most FTFs function as so-called transcriptional settlers, which bind to already open chromatin and regulate gene expression through direct transcriptional initiation or suppression[71–73]. This allows FTFs to rapidly respond to pre-existing as well as changing chromatin landscapes and environmental changes by altering gene expression programmes.

***Cooperation and competition between FTFs.*** FTFs generally function in multiprotein complexes containing a variety of transcriptional modulators. For example, mass spectrometric analysis of FOXP3-binding partners in $T_{reg}$ cells has revealed that FOXP3 forms multiprotein complexes of 400–800 kDa containing more than 350 proteins[74]. In this way, FTFs can form dynamic complexes with a wide variety of cofactors, including other FTFs (FIG. 5).

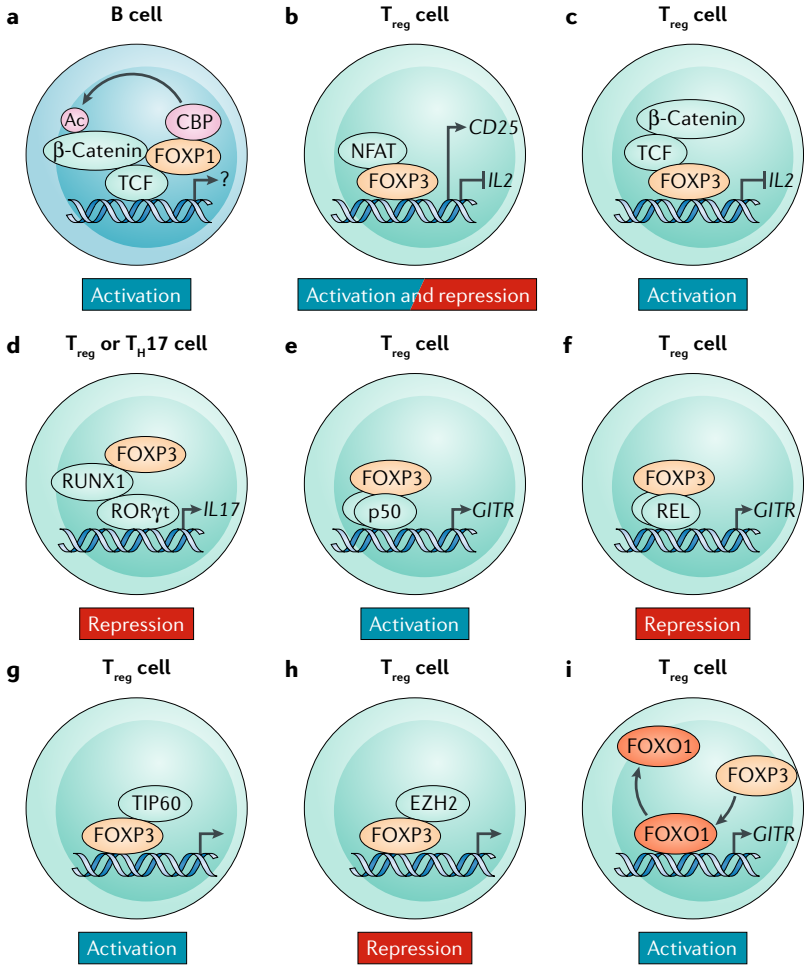


Fig. 5 | **Interactions between FTFs and cofactors in lymphocytes.** Forkhead box transcription factors (FTFs) can act as both transcriptional activators and repressors depending on cellular context. They have the capacity to interact with multiple transcriptional cofactors that can modify their function in either a positive or negative manner. Additionally, they are themselves able to modulate the activity of other transcriptional complexes by associating with them. Examples of FTF interactions are depicted in the figure. Such interactions can promote or inhibit lymphocyte differentiation or function. **a** | Association of forkhead box protein P1 (FOXP1) with the acetyltransferase CREB-binding protein (CBP) promotes the acetylation of β-catenin-promoting transcription. **b** | Association between FOXP3 and nuclear factor of activated T cells (NFAT) is critical in both positively and negatively modulating regulatory T ($T_{reg}$) cell-dependent transcriptional programmes. **c** | Association between FOXP3 and transcription factor TCF–β-catenin negatively regulates FOXP3-mediated transcription. **d** | FOXP3 forms a transcriptional complex with both runt-related transcription factor 1 (RUNX1) and RORγt, regulating the differentiation of IL-17-producing T cells. **e,f** | FOXP3 stabilizes the binding of the nuclear factor-κB (NF-κB) transcription factors p50 and proto-oncogene REL, enabling local accumulation of transcriptional complexes important for $T_{reg}$ cell differentiation. **g** | FOXP3 associated with 60 kDa Tat-interactive protein (TIP60) is found to be active and primarily in complexes activating transcription. **h** | FOXP3 poises its targets for repression through association with histone-lysine *N*-methyltransferase (EZH2), facilitating the formation of repressive chromatin in $T_{reg}$ cells in response to inflammatory signals. **i** | FOXP3 exploits a FOXO1-dependent pre-existent enhancer landscape to drive $T_{reg}$ cell differentiation. Ac, acetylated; $T_H17$ cell, T helper 17 cell.

Despite the fact that FTFs can compete for binding to similar consensus sequences[75], there also exists a high level of cooperation. Comparing FOXP3-target and FOXO1-target genes in $T_{reg}$ cells, there are unique populations of gene expression patterns, with less than 10% overlap between these FTFs[73]. Nevertheless, in mature $T_{reg}$ cells, FOXO1 appears to cooperate with FOXP3 as a transcriptional repressor, and in *Foxo1*-deficient $T_{reg}$ cells, pro-inflammatory cytokines such as IFNγ are inappropriately and highly expressed[76]. This is further emphasized by the observation that *Foxo1*$^{-/-}$*Foxo3*$^{-/-}$ $T_{reg}$ cells produce $T_H1$ and $T_H17$ cell signature cytokines at steady state despite normal expression of FOXP3 (REF.[38]). Such findings suggest that one FTF, such as FOXP3, can cooperate with another FTF, in this case FOXO1, at genomic loci encoding genes regulating cell type-specific function, in this case $T_{reg}$ cell-mediated suppression. In accordance with such cooperativity between FTFs, FOXO1 has also been found to act as a transcriptional 'predecessor', as it is associated with many FOXP3-binding sites in $T_{reg}$ precursor cells. Nevertheless, once FOXP3 is bound, FOXO1 is subsequently displaced, resulting in the downregulation of proximal genes[38,73]. In this way, FTFs can act by exploiting a pre-established chromatin landscape consisting of accessible enhancers that are maintained in a poised state by cofactors[77].

***FTF-binding partners.*** Fairly well-characterized binding partners of FTFs include the transcriptional activator TCF7 and its cofactor β-catenin, both of which are critical modulators of the WNT signalling pathway[78–80]. An example of such an interaction between FTFs and the WNT signalling pathway is the role FOXP1 plays in regulating B cell development. As described earlier, *Foxp1*-deficient lymphoid stem cells fail to differentiate beyond the pro-B cell stage[19], and it was observed that FOXP1 potentiates β-catenin-dependent gene expression[81]. Gain-of-function and loss-of-function studies revealed that FOXP1 acts as a general enhancer of WNT signalling, forming a chromatin-bound complex with β-catenin. FOXP1 thereby promotes the acetylation of β-catenin, which is required for potentiation of transcriptional activity[81].

Furthermore, WNT signalling has been shown to modulate $T_{reg}$ cell function. Activation of WNT signalling abrogates $T_{reg}$ cell-mediated suppression both in vitro and in vivo[80]. This is mediated by an association between FOXP3 and TCF7–β-catenin, and co-binding of target sites at the chromatin level. CD3-mediated and CD28-mediated activation of effector T cells can also result in an increase in WNT3A expression, which dampens $T_{reg}$ cell function under inflammatory conditions. In accordance with this model, effector T cells isolated from the synovial fluid of patients with juvenile idiopathic arthritis are resistant to $T_{reg}$ cell-mediated suppression[82], and inhibition of WNT secretion in these effector T cells was sufficient to improve the suppressive capacity of $T_{reg}$ cells[80]. These data demonstrate how inflammation can dampen $T_{reg}$ cell-mediated suppression through the local production of WNT3A at the site of inflammation. These findings highlight the capacity of the local environment to modulate immune homeostasis by controlling FTF functionality.

The REL-domain family, which includes the calcium-regulated nuclear factor of activated T cells (NFAT) transcription factor family (NFAT1–NFAT4) and the NF-κB family, also undergoes well-characterized interactions with FTFs. All REL family members have a

structurally highly conserved DNA-binding domain, and the crystal structure of an NFAT–FOXP2–DNA complex has revealed the exact interaction interface between the DNA-binding domain of a REL-domain protein and that of an FTF[83]. As the DNA-binding domains of both families are highly conserved within their respective members, this crystal structure provides a general understanding of how members of these transcription factor families can cooperate with each other to regulate gene expression. In this respect, a relevant example is the interaction between NFAT and FOXP3. In effector T cells, NFATs form cooperative complexes with the activator protein 1 (AP-1) family of transcription factors and regulate T cell activation-associated genes. In $T_{reg}$ cells, however, NFAT directly associates with FOXP3 (REF.[83]). This complex then binds to gene loci of $T_{reg}$ cell signature genes and thereby represses the expression of specific genes, such as IL-2, while upregulating the expression of $T_{reg}$ cell markers, such as cytotoxic T lymphocyte antigen 4 (CTLA4) and CD25 (REF.[83]). Specifically, disrupting the interaction between FOXP3 and NFAT reduced the production of in vitro-generated $T_{reg}$ ($iT_{reg}$) cells and improved antitumour responses in vivo[84].

Members of the NF-κB family of transcription factors have also been found to associate with other FTFs, including FOXO3A[85] and FOXP3 (REF.[86]), allowing post-translational fine-tuning of immune responses. The family of NF-κB transcription factors serves a diverse range of functions in lymphocytes, most prominently the control of pro-inflammatory gene activation. Nevertheless, this transcription factor family is also involved in $tT_{reg}$ cell development by regulating the expression of the *FOXP3* gene[87–89]. The fact that NF-κB is involved in both pro-inflammatory and immune suppressive transcriptional programmes may seem somewhat paradoxical. However, direct interaction with FOXP3 provides the skewing of NF-κB transcriptional activity in $T_{reg}$ cells specifically. For example, at the *Gitr* promoter, FOXP3 has been proposed to stabilize NF-κB p50 binding by anchoring it to the enhancer, thereby enabling local accumulation of transcriptional complexes containing other members of the NF-κB and IκB families[90]. These findings also help to explain mechanistically how FOXP3 can activate expression of certain genes while suppressing others.

Another example of how the interaction of FTFs with transcription factors can skew immune responses is the interaction between FOXP3 and runt-related transcription factor 1 (RUNX1). Both transcription factors are induced in TCR-stimulated T cells exposed to TGFβ. RUNX1 induces the expression of RORγt, and FOXP3 can directly associate with both RUNX1 and RORγt, thereby inhibiting their activity. As a consequence, RORγt-induced $T_H17$ cell differentiation can effectively be prevented by FOXP3 expression[91,92]. *Foxp3* expression, however, is itself negatively regulated by pro-inflammatory cytokines, including IL-6, IL-21 and IL-23 (REFS[92,93]). As the levels of FOXP3 and RORγt are crucial for both $T_H17$ and $pT_{reg}$ cell differentiation, the fate of TGFβ-exposed T cells will be directly reliant on the local inflammatory environment. Thus, the complex three-way interactome comprising RORγt, RUNX1 and FOXP3 is critical for determining and directing the immune responses of pro-inflammatory $T_H17$ cells as well as anti-inflammatory $T_{reg}$ cells according to environmental conditions. Taken together, FOXP3 is able to usurp normally active transcriptional complexes, whether NFAT, NF-κB, RORγt or RUNX1, and thereby repress transcriptional activation of pro-inflammatory cytokines (FIG. 5).

## Post-translational regulation of FTF function

The most immediate way that lymphocytes can adapt to changing environmental conditions is to modify already expressed proteins, thereby activating or deactivating their functionality. PTMs, such as ubiquitylation, phosphorylation and acetylation, have been described for several FTFs, which may lead to their activation, inactivation, cellular translocation or degradation. It appears that this form of regulation is a common feature for FTFs, and many examples of FTF PTMs have been described. Our current knowledge of how PTMs of FTFs regulate leukocyte function is primarily exemplified by studies focusing on FOXO and FOXP3, and this is discussed in detail below (FIG. 6).

***Ubiquitylation.*** Ubiquitylation-induced proteasome-mediated degradation of FTFs can regulate cell function and is exemplified by the control of FOXP3 stability in $T_{reg}$ cells. Complimentary studies initially described a ubiquitylation–deubiquitylation cycle in $T_{reg}$ cells that can modulate FOXP3 protein half-life[93,94]. Both studies made the initial observations that FOXP3 is an inherently unstable protein and that inflammation-associated stress signals can act to destabilize its protein expression. In vitro experiments in $T_{reg}$ cells delineated the proteasome-dependent degradation pathway. It was observed that FOXP3 associated with heat shock protein 70 (HSP70) and that this resulted in the recruitment of the STUB1 ubiquitin E3-ligase, leading to K48-linked polyubiquitylation and degradation of FOXP3 (REFS[93,94]). In parallel work, the ubiquitin carboxyl-terminal hydrolase 7 (USP7) deubiquitinase (DUB) was identified as a FOXP3-binding partner[94]. Binding of USP7 to FOXP3 could stabilize the FOXP3 protein both in vitro and in vivo. Both groups employed variants of an adoptive transfer model of colitis in their studies, validating the relevance of FOXP3 protein stability in $T_{reg}$ cell function. The addition of pro-inflammatory stimuli, including IL-6, lipopolysaccharide (LPS) and heat shock, had the cooperative effect of both increasing STUB1 expression and association with FOXP3 and decreasing the association and expression of USP7, the net result of which is an increase in proteasome-mediated degradation of FOXP3 (REF.[95]). Thus, taken together, by regulating the stability of FOXP3 within $T_{reg}$ cells, the state of inflammation can regulate the suppressive capacity of the local $T_{reg}$ cell population. This work on ubiquitylation-mediated FOXP3 destabilization has been expanded on by the identification of CBLB, an E3 ubiquitin ligase that also targets FOXP3 for degradation, thereby regulating thymic-derived $CD4^+CD25^+$ $T_{reg}$ cell development[96]. CD28 co-stimulation is essential

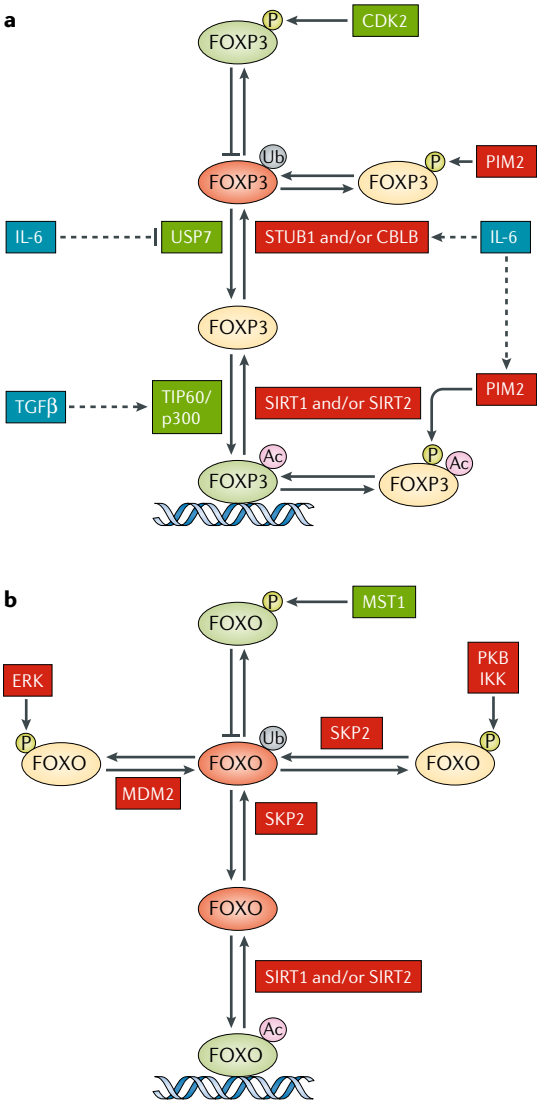


Fig. 6 | **Post-translational modifications control FTF transcriptional output.** Phosphorylation, acetylation and ubiquitylation can control forkhead box transcription factor (FTF) function as exemplified by forkhead box protein P3 (FOXP3) (part **a**) and FOXO (part **b**) transcription factors. Here, the various modifying enzymes are depicted. A positive role in FTF activation is shown in green, while a negative role is shown in red. The transcriptional activity of FOXP3 and FOXO is indicated as green for active and red for inactive. Competition for acetylation and ubiquitylation of FTFs on lysine residues provides dynamic control of FTF functionality. **a** | FOXP3 activity can be controlled through regulation of protein stability. Inflammatory cues induce the ubiquitylation and degradation of FOXP3 protein. Acetylation of FOXP3 counteracts this, resulting in protein accumulation through inhibition of inactivating ubiquitylation. In a similar manner, FOXP3 phosphorylation impinges on these events, stabilizing FOXP3 and increasing transcriptional activity in a so-far undefined manner. **b** | FOXO transcription factors are excluded from the nucleus by protein kinase B (PKB)-mediated phosphorylation, and this can also result in protein degradation. By contrast, phosphorylation of FOXOs by mammalian STE20-like protein kinase 1 (MST1) results in increased stability and nuclear translocation. Both S phase kinase-associated protein (SKP2) and MDM2 E3-ligases have been shown to ubiquitylate FOXOs, resulting in degradation, and this can be promoted by prior phosphorylation of FOXOs. Deacetylation of FOXOs by sirtuin 1 (SIRT1) and/or SIRT2 leads to SKP2-mediated FOXO ubiquitylation and degradation. Taken together, these post-translational modifications allow for the rapid and transient regulation of T cell function, often by controlling the levels of nuclear FTF protein. Ac, acetylation; CBLB, E3 ubiquitin-protein ligase CBLB; CDK2, cyclin-dependent kinase 2; ERK, extracellular-signal-regulated kinase; IKK, IκB kinase; P, phosphorylation; p300, histone acetyltransferase p300; PIM2, serine/threonine-protein kinase PIM2; STUB1, E3 ubiquitin-protein ligase CHIP; TGFβ, transforming growth factor-β; TIP60, 60 kDa Tat-interactive protein; Ub, ubiquitylation; USP7, ubiquitin carboxyl-terminal hydrolase 7.

for $T_{reg}$ cell development, and loss of CBLB was found to partially rescue the defective development of $tT_{reg}$ cells in CD28-deficient animals, suggesting that CD28-induced signals counteract CBLB-induced FOXP3 degradation[96].

Control of FTF stability by ubiquitylation is not restricted to FOXP3, but, for instance, the stability of FOXO transcription factors is also well established to be regulated by proteasomal degradation[2]. FOXO3A can be phosphorylated by extracellular-signal-regulated kinase (ERK), resulting in E3 ubiquitin-protein ligase MDM2-mediated ubiquitylation and proteasomal degradation[97]. Furthermore, pro-inflammatory signals, such as tumour necrosis factor (TNFα), are able to inactivate FOXO proteins. Here, activation of IκB kinase (IKK) can phosphorylate and inhibit FOXO proteins, with the potential to modify T cell function[98]. PKB-mediated phosphorylation can also result in FOXO transcription factor degradation[99]. The ubiquitin ligase S phase kinase-associated protein 2 (SKP2) was found to mediate this process[100], and inhibition of SKP2 converted pathogenic autoreactive T cells into FOXP3-expressing $T_{reg}$ cells[101]. Here, reduction of SKP2 levels in diabetogenic $CD4^+FOXP3^-$ T cells induced the upregulation of FOXO1 and FOXO3A protein levels, which induced the potent suppressive phenotype of these cells both in vitro and in vivo. Taken together, ubiquitylation of FTFs should be considered as a rapid, reversible mechanism for controlling lymphocyte fate.

Besides polyubiquitylation, proteins can also be monoubiquitylated. While polyubiquitylation induces proteasomal degradation, monoubiquitylation modifies the functionality of the tagged protein[102], and monoubiquitylation of FTFs has frequently been reported. For instance, the monoubiquitylation of FOXP3 (REF.[94]) and the stress-induced monoubiquitylation of FOXO4, which result in nuclear translocation and increased transcriptional activity[103], have both been observed. At this stage, however, the functional relevance of FOXO monoubiquitylation for lymphocyte homeostasis and thus the role of regulating FOXO and FOXP3 ubiquitylation in tandem by controlling of USP7 activity remain unclear and to be determined.

***Phosphorylation.*** The concept of controlling FTFs through the regulation of their protein stability was first explored for members of the FOXO family. FOXO transcription factors can respond to changes in a variety of

physiological conditions, including activation by lymphocyte antigen receptors, exposure to cytokines and growth factors, nutrient abundance and oxidative stress by PKB-mediated phosphorylation, resulting in subcellular re-localization from the nucleus to cytoplasm[2]. This translocation of FOXO protein has major modifying effects on the homeostasis and differentiation of lymphocytes, and these effects on the immune system have been detailed extensively elsewhere[6]. Nevertheless, a number of additional pathways have been revealed, which are discussed in more detail here.

One prominent example is the regulation of FOXP3 by cyclin-dependent kinase 2 (CDK2). Having previously described a role for CDK2 as a negative regulator of $T_{reg}$ cell function[104], Wells and colleagues subsequently identified multiple CDK2 phosphorylation sites within the FOXP3 protein[105]. In vitro kinase assays identified FOXP3 Ser19 and Thr175 as bona fide CDK2 phosphorylation sites, and mutating Ser19 led to an increase in FOXP3 protein stability and half-life in transfected cells, which was accompanied by increased FOXP3-mediated transcriptional output and increased suppressive capacity both in vitro and in vivo in transduced $CD4^+CD25^-$ lymphocytes. In an adoptive transfer model of inflammatory bowel disease, mice receiving mutant CDK2-expressing $T_{reg}$ cells had fewer outward signs of morbidity, less pathology and an elevated frequency of FOXP3-expressing donor cells in the mesenteric lymph nodes. These data suggest that the CDK motifs in FOXP3 are important for controlling its stability and functions.

In addition to CDK2-mediated phosphorylation of FOXP3, a number of other pathways and kinases have been described, such as TNFα-regulated phosphorylation of FOXP3 at Ser418 (REF.[106]) or its phosphorylation by the PIM kinases. PIM serine/threonine kinases have been shown to play essential roles in T cell development and function[107], and FOXP3-mediated induction of *PIM2* expression in $T_{reg}$ cells has been shown to be essential for $T_{reg}$ cell expansion[108]. PIM2 directly phosphorylates FOXP3 on multiple sites within the amino-terminal domain, negatively regulating $T_{reg}$ cell suppressive function in vitro[109]. Mice deficient in PIM2 have increased FOXP3 and CD25 expression, suggesting that PIM2 phosphorylation influences protein stability[109].

Another kinase that regulates immune homeostasis by targeting FTFs is mammalian STE20-like protein kinase 1 (MST1; also known as STK4). Human MST1 deficiency causes a primary immunodeficiency syndrome with autoimmune manifestations, and MST1-deficient mice are prone to autoimmune disease in a $T_{reg}$ cell-dependent manner[110,111]. MST1 is required for induction of FOXP3 expression, apparently through its ability to directly phosphorylate FOXO1 and FOXO3 and prevent their proteasome-mediated degradation and also by indirectly attenuating TCR-induced PKB activation[110,111]. This concordant inhibition of PKB activity and the stabilization of FOXO proteins promoted FOXP3 expression. Consequently, in MST1-deficient T cells, TCR-induced increases in *Foxp3* expression were diminished owing to reduced FOXO stability. Thus, upon TCR stimulation, MST1-mediated phosphorylation and stabilization of FOXO proteins are required for the efficient induction of FOXP3 expression. FOXOs have been shown to be critical mediators of haematopoietic stem cell resistance to physiological oxidative stress, and this has been borne out in many non-haematopoietic systems[112]. Loss of MST1 in peripheral T cells results in decreased FOXO1 and FOXO3 activation and protein levels, resulting in decreased superoxide dismutase 2 (SOD2) and catalase expression[113]. The consequence of this is increased intracellular reactive oxygen species and induction of apoptosis. These data support a critical role for FOXO transcription factors in the maintenance of naive T cell homeostasis in the periphery.

***Acetylation.*** Protein acetylation is a tightly controlled and highly dynamic process reciprocally regulated by lysine acetyl transferases (KATs; also known as histone acetyl transferases) and lysine deacetylases (KDACs; also known as histone deacetylases). A number of leukocyte FTFs have been described to be acetylated; the most well explored is FOXP3, revealing that $T_{reg}$ cell function is closely regulated by acetylation and deacetylation[95]. FOXP3 has been reported to directly associate with the KATs 60 kDa Tat-interactive protein (TIP60; also known as KAT5) and histone acetyltransferase p300 to promote acetylation and increase protein stability[114,115]. Conditional deletion and pharmacological inhibition of the p300 KAT in FOXP3-expressing $T_{reg}$ cells impaired their suppressive capacity and blocked the induction of $pT_{reg}$ cells[116]. p300 deletion did not affect $CD4^+$ $T_{reg}$ cell numbers or *Foxp3* expression under basal conditions, and suppressive function was only modestly affected. However, transfer of p300-deficient $T_{reg}$ cells to immunodeficient mice revealed that they were poor suppressors of homeostatic effector T cell proliferation[105]. Importantly, p300 deletion promoted antitumour immunity in lung adenocarcinoma-bearing animals that correlated with lower levels of both total and acetylated FOXP3 protein. This directly supports a model whereby acetylation of FOXP3 is required to maintain protein expression and the suppressive capacity of $T_{reg}$ cells[116]. The sirtuins (SIRT1–SIRT7) are NAD-dependent protein deacetylases that regulate many transcription factors, including FOXOs and FOXP3. Ectopic expression of SIRT1 decreases FOXP3 acetylation and concomitantly increases K48-linked ubiquitylation, reducing protein levels[117]. In addition, pharmacological inhibition of SIRT activity by treatment with NAD or the SIRT1 inhibitor EX-527, as well as gene deletion specifically in $T_{reg}$ cells, had the inverse effect, resulting in increased *Foxp3* expression and enhanced suppressive capacity[118–120]. Sirtuins are likely not the only class of KDACs that can modulate FOXP3 acetylation, as broad-spectrum KDAC inhibitors can increase $T_{reg}$ cell-mediated suppression in vitro[110,114]. Treatment with KDAC inhibitors has also been shown to reduce colitis and increase cardiac island allograft survival in in vivo mouse models[118,121]. Furthermore, the SIRT inhibitor EX-527 was also found to replace or synergize with low doses of TGFβ in the in vitro generation and in vivo maintenance of $pT_{reg}$ cells[119]. SIRT1 expression has been found to be markedly downregulated in terminally differentiated $CD8^+CD28^-$ memory T cells[122], a lymphocyte population that increases with age[123]. Decreased SIRT1

levels resulted in loss of FOXO1 levels, causing metabolic reprogramming, enhanced granzyme B production and unwarranted cytotoxicity. This suggests that the SIRT1–FOXO1 axis represents a therapeutic target to reprogramme terminally differentiated memory T cells, thereby delaying immune ageing. As SIRT1 has been shown to regulate FOXP3 activity, these observations may also have implications for $T_{reg}$ cell functionality during ageing.

Mechanistically, a number of different possibilities may explain why acetylation influences the stability and functionality of FOXP3. The crystal structure of the FOXP3 coiled-coil domain that is critical for FOXP3 homodimerization and also for its heterodimerization with FOXP1 has identified key lysine residues for conformational change and stability of FOXP3 (REF.[124]). Acetylation of these residues may be important to maintain FOXP3 homodimerization and suggests that modifications of acetylation status explain the sensitivity of $T_{reg}$ cells to changes in acetylation activity. Nevertheless, both ubiquitylation and acetylation are restricted to ε-lysine residues, and, therefore, a competition model for lysine modification can be readily envisaged. Here, the balance of signals regulating KAT–KDAC and E3-ligase–DUB activities will define the functional level of FOXP3 protein and thereby the suppressive capacity of $T_{reg}$ cells (FIG. 6). In a similar mechanism of PTM regulation, FOXO1 can be methylated by protein arginine *N*-methyltransferase 1 (PRMT1), and this can directly block PKB-mediated phosphorylation, promoting prolonged FOXO1 activation[125]. Acetylation modulates the functionality of not only FOXP3 but also a number of other FTFs, such as FOXOs, upon induction of cellular stress[126]. In addition, deacetylation of FOXO3 by SIRT1 or SIRT2 has been demonstrated to result in SKP2-mediated FOXO3 ubiquitylation and degradation[127]. Similarly, SIRT1 also mediates FOXA2 degradation by deacetylation in a nutrient-dependent manner[128]. Here, the association of SIRT1 and FOXA2 is reduced in times of nutrient deprivation, resulting in increased acetylation and protein stabilization.

Taken together, these interactions clearly underscore the possibility for multiple PTMs to fine-tune FTF stability in response to changing environmental conditions.

## Challenges on the road ahead

Over the past decade, the relevance of the FTF family for immune homeostasis has become increasingly clear. For example, the discovery of FOXP3-expressing $T_{reg}$ cells and the capacity for FOXP3 to induce a regulatory cell phenotype in naive $CD4^+$ cells constituted a central paradigm shift in immunology. This finding has helped to further highlight the relevance of the FTF family in general for the immune system. The diverse functions of FTFs allow for adaptation to changing environments and thus context-dependent control of immune homeostasis. This can be in the form of a transcriptional roadblock, as observed for FOXP1, or by inducing terminal differentiation, as in FOXA and FOXP3. Furthermore, regulation of PTMs can lead to rapid changes in FTF output, as observed for FOXOs.

Conditional knockout animals have helped to uncover the functions of individual FTFs in a variety of leukocyte subsets, as well as to define the transcriptional target genes of these FTFs (TABLE 1). Technical advances have also made it possible to identify genes directly regulated by FTFs. However, such studies have not yet been thoroughly performed in individual immune cell subsets. Therefore, it is not always clear whether transcriptional targets are directly or indirectly regulated by specific FTFs. Another challenge is in understanding the composition of FTF-associated transcriptional complexes, and there are very few studies that have attempted to evaluate this in primary immune cells. These issues are hampered by the often small numbers of primary cells that are available for analyses. Understanding protein–protein interactions between FTFs and cofactors might allow the development of interaction-specific inhibitors that would target only FTF complexes in specific leukocyte subtypes. In the same vein, identification and characterization of PTMs that regulate FTF function in immune cells have remained challenging. Work addressing the function of such PTMs has relied on tractable in vitro systems, which do not duplicate the complex microenvironments that immune cells often find themselves in. In vivo genetic approaches where single amino acids are altered to either prevent or mimic specific PTMs have so far not been performed for the FTFs described here and may prove difficult to evaluate in practice owing to potential redundancy in PTM functionality. This has made it difficult to be able to truly understand the function and relevance of specific FTF PTMs in vivo.

Understanding the mechanisms regulating FTF function will not only further reveal the underlying complexity behind immune responses but also increase our understanding of the pathogenesis of autoimmunity. The FOXP3 mutations responsible for the development of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome are a clear example of this, and it is likely that single nucleotide polymorphisms affecting cell type-specific enhancers regulating FTF expression levels also have causal roles under certain inflammatory conditions. The next decade of FTF research will hopefully lead to pharmacological targeting of these proteins with the possibility to manipulate or correct inappropriate immune responses.

Published online: 03 September 2018


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**Acknowledgements**
We would like to apologise to any authors whose work could not be cited due to space contstraints. The Zaiss laboratory is supported by the Medical Research Council, grant MR/M011755/1, and the European Union, grant CIG-631413 ("EGF-R for Immunity"). The Coffer laboratory is supported by grants from the Dutch Cancer Society (UU 2015-7838) and Dutch Reumatology Foundation (16-1-301)

**Author contributions**
Both authors contributed to the researching of data, discussion of content and writing, reviewing and editing of the manuscript.

**Competing interests**
The authors declare no competing interests.

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**Reviewer information**
*Nature Reviews Immunology* thanks M. Suresh and the other anonymous reviewer(s) for their help with the peer review of this manuscript.