

# FOXO transcription factors throughout T cell biology

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**Abstract** | The outcome of an infection with any given pathogen varies according to the dosage and route of infection, but, in addition, the physiological state of the host can determine the efficacy of clearance, the severity of infection and the extent of immunopathology. Here we propose that the forkhead box O (FOXO) transcription factor family — which is central to the integration of growth factor signalling, oxidative stress and inflammation — provides connections between physical well-being and the form and magnitude of an immune response. We present a case that FOXO transcription factors guide T cell differentiation and function in a context-driven manner, and might provide a link between metabolism and immunity.

## Tumour suppressor proteins

Proteins that limit the generation of cancer. Many of these proteins regulate scheduled entry to the cell cycle or promote the apoptosis of damaged cells. Loss-of-function mutations in tumour suppressor genes increase susceptibility to cancer.

## Reactive oxygen species

Highly reactive oxygen-containing molecules that can be produced by the mitochondria in eukaryotic cells. Examples include hydrogen peroxide, ions such as hypochlorite, and free radicals such as superoxide and nitric oxide. They can be inactivated by enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase.

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The signal transduction of metazoans seems to be both complex beyond understanding and simple, in that a limited number of pathways, which are conserved across vast evolutionary time, control most cell fate decisions. Many of these signalling pathways were first traced in yeast, worms, flies or dedifferentiated cells in tissue culture, and this has provided a starting point for understanding cellular and organismal responses to environmental change. We imagine that with increasing organismal complexity, new differentiated cell types arose, and the fundamental signalling pathways of single-cell organisms were redirected to integrate new inputs and compute selectable, advantageous results. Thus, similarly rooted pathways in different cell types may or may not serve a common purpose or programme of gene expression.

Forkhead box O (FOXO) transcription factors are central to many aspects of metazoan physiology (BOX 1). They are named for their forkhead domain, a DNA-binding domain of ~100 amino acids, and for their membership of the O subclass<sup>1</sup>. They regulate cell-cycle progression, DNA repair and apoptosis and, as such, are considered to be tumour suppressor proteins<sup>2–5</sup>. They also respond to oxidative stress and regulate ageing, in part through the detoxification of reactive oxygen species and the control of DNA repair pathways<sup>6,7</sup>. Furthermore, they affect metabolism and the anaerobic or aerobic generation of ATP at multiple control points<sup>8</sup>. More recently, FOXO1 was shown to control a programme of pluripotency in human and mouse embryonic stem cells, probably by directly controlling the transcriptional

activity of OCT4 (also known as POU5F1) and SOX2 (REF. 9). It is possible, although not proven, that most if not all cell fates are in some manner regulated by FOXO transcription factors. This great variety of cellular functions seemingly directed by FOXO transcription factors illustrates the principle that individual transcription factors do not determine cell-type specification. Rather, a given transcription factor functions as a scaffold that is post-transcriptionally modified as a result of cellular context, and thus common signalling modules direct different programmes of gene expression. No gene is always activated by a given transcription factor, and conversely there is no circumstance under which a transcription factor activates every one of its target genes<sup>10</sup>. Specifically, the expression of a FOXO transcription factor itself is not predictive of a cellular function or state of differentiation; rather, it enables the amplification of cellular potential. It can provide an integration point for several inputs and induce distinct programmes of gene expression while retaining conserved mechanisms and pathway connections.

As reviewed extensively elsewhere<sup>11–14</sup>, and briefly below, FOXO transcription factors respond to growth factors, oxidative stress, inflammation and nutritional abundance — physiological conditions that influence the magnitude and effectiveness of an immune response. In turn, FOXO transcription factors regulate cell survival, division and energy utilization, and these activities of FOXO transcription factors would be predicted to strongly affect the expansion and contraction of antigen-responsive lymphocyte populations.

## Box 1 | The FOXO transcription factor family

In jawed vertebrates, possibly excluding cartilaginous fish, there are four major forkhead box O (FOXO) paralogues that are orthologous to the single-copy genes found in urochordates, nematodes (in which this gene is known as *daf-16*) and arthropods (in which this gene is known as *foxo*). The four paralogues arose from an initial duplication event that produced the *FOXO1/FOXO4* gene lineage and the *FOXO3/FOXO6* gene lineage, followed by a second pair of duplication events that produced the four separate, unlinked genes: *FOXO1*, *FOXO3*, *FOXO4* and *FOXO6* (REF. 165). *FOXO1*, *FOXO3* and *FOXO4* are widely expressed, and the encoded proteins have many of the same post-translational regulatory mechanisms. By contrast, *FOXO6* is largely expressed in the nervous system and is the most diverged evolutionarily, and the protein it encodes is subject to alternative mechanisms of post-translational regulation<sup>165,166</sup>. In mammals, *FOXO1* is most highly expressed in B cells, T cells and ovaries<sup>66</sup>, although genetic analyses have shown it to have an important function in many tissues<sup>5,167</sup>. *Foxo1*-mutant mice exhibit embryonic lethality at embryonic day 10.5 owing to impaired vascular development<sup>168,169</sup>. *FOXO3* expression is found in most tissues, including lymphocytes and myeloid cells, and *Foxo3*-mutant mice are largely without a noticeable phenotype with the exception that female mice exhibit early depletion of functional ovarian follicles<sup>168,170</sup>. One mouse strain with an insertional mutation in *Foxo3* seemed to develop spontaneous immunopathology<sup>15</sup>; however, this was not apparent in two other *Foxo3*-mutant strains<sup>43,168,170</sup>. *FOXO4* is similarly expressed across human tissues at an apparently low level<sup>66</sup>. Mice with loss-of-function mutations in *Foxo4* have no known phenotypic differences from wild-type mice.

In addition, more recent work has shown that FOXO transcription factors have been co-opted to regulate specialized characteristics of lymphocyte homeostasis, including turnover, homing and differentiation<sup>12,15–17</sup>. In this Review, we address the possibility that FOXO transcription factors programme lymphocytes to respond to diverse physiological changes, some of which are advantageous whereas others may contribute to immune pathology. We also highlight recent work showing that FOXO transcription factors are central to multiple processes of T cell differentiation, including the formation of memory T cells. The study of FOXO transcription factors now affords an opportunity to begin to understand the integration of immunity with the physiological state of an organism, which includes metabolic function, inflammation and oxidative stress.

**Control of FOXO transcriptional activity**

**Cell type-specific expression.** FOXO paralogues are expressed widely, although each has a unique pattern of tissue-specific expression. Several transcription factors have been shown to directly affect the expression of FOXO genes. Such factors include FOXO1, E2F1, p53, E2A, HEB (also known as TCF12) and, interestingly, FOXO1 and FOXO3 themselves, implying a positive-feedback control mechanism<sup>18–20</sup>. Through such feedback, post-translational inhibitory modifications to FOXO transcription factors (as discussed below) could reduce the transcription of FOXO genes. The transcriptional basis for the preferential expression of FOXO1 in lymphocytes is not understood, but signal transducer and activator of transcription 3 (STAT3) seems to bind to the promoters of *Foxo1* and *Foxo3*, and *Stat3*<sup>-/-</sup> naive T cells have low levels of both *Foxo1* and *Foxo3* mRNA<sup>21</sup>. In the determination of B cell fate, E2F1 and E2A have been shown to bind to the *Foxo1*

locus<sup>22</sup>. Currently, the field awaits a comprehensive analysis of the general and lymphocyte-specific promoter and enhancer binding sites that are active in the *Foxo1* and *Foxo3* genes.

**Post-translational modifications.** FOXO transcription factors are subject to extensive and varied post-translational modifications that affect their abundance, localization and transcriptional activity. Together these modifications have been described as a FOXO code<sup>11,13</sup>. Such modifications have been found in various cell types, but, although they are assumed to be generally applicable, they have not been extensively studied in T cells.

A major pathway of FOXO regulation is initiated by growth factors, such as insulin, and results in the activation of phosphoinositide 3-kinase (PI3K) (FIG. 1). The activity of PI3K causes the recruitment of the kinases AKT and SGK1 to the cell membrane and their phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1)<sup>23,24</sup>. AKT and SGK1 can directly phosphorylate FOXO transcription factors on three sites, but to mediate this activity they must also be phosphorylated by mTORC2 (REFS 25–27). Until recently, the activation requirements for mTORC2 were not understood, but a genetic screen in yeast followed by studies in mammalian cell lines have shown that PI3K enhances a (translation-independent) interaction between mTORC2 and the ribosome, and it is this interaction that allows mTORC2 to phosphorylate AKT within its hydrophobic motif (at S473)<sup>28</sup>. The result of the triple phosphorylation of FOXO transcription factors by AKT or SGK1 (at T24, S256 and S319) (FIG. 2) is nuclear export and degradation<sup>7,29,30</sup>, and this basic mechanism is conserved across all the phyla that have been examined<sup>31</sup>. This mechanism is partially mimicked by cyclin-dependent kinase 2 (CDK2), which is important for the transition from G1 phase to S phase in the cell cycle and which has been shown to phosphorylate FOXO1 at S249 to induce nuclear export. This export presumably promotes cell survival during cell-cycle progression, and it is abrogated by DNA damage in a p53-independent manner<sup>32</sup>. The inhibitory mechanism mediated by AKT or SGK1 functions in T cells stimulated through the T cell receptor (TCR), common  $\gamma$ -chain ( $\gamma_c$ ) cytokine receptors or homing receptors<sup>2,3,33–35</sup>.

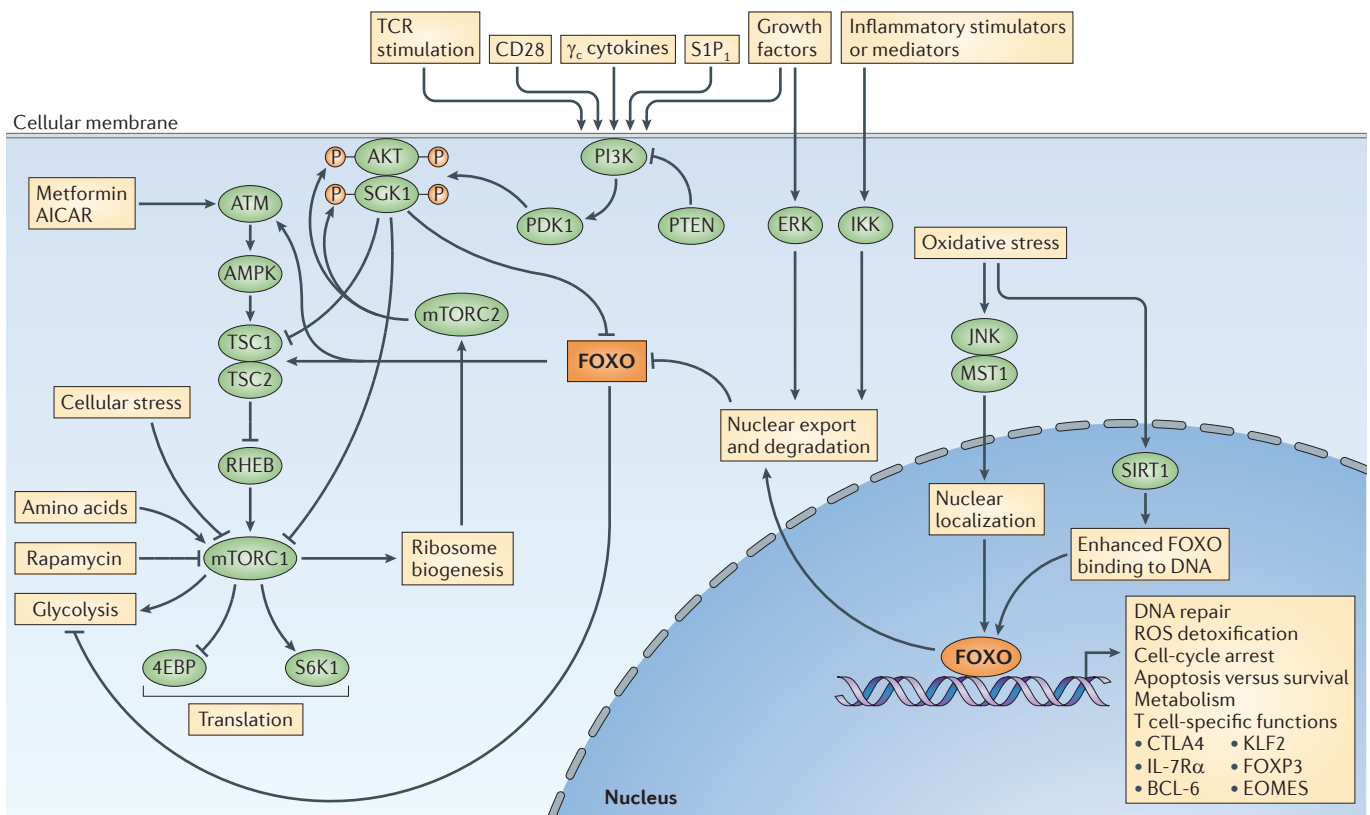
An additional growth factor-controlled inhibitory pathway is mediated through the phosphorylation of FOXO3 at three unique sites by extracellular signal-regulated kinase (ERK) (FIG. 2), and this results in MDM2-mediated ubiquitination and proteosomal degradation of FOXO3 (REF. 36). This pathway also seems to operate in T cells. Under conditions of regulatory T ( $T_{Reg}$ ) cell differentiation, deletion of *Erk2* resulted in a threefold or greater increase in the expression of 20 genes, and three of these genes were direct FOXO1 targets: interleukin-7 receptor subunit- $\alpha$  (*Il7ra*), L-selectin (*Sell*) and Krüppel-like factor 2 (*Klf2*)<sup>37</sup>. Similarly to growth factors, pro-inflammatory cytokines — such as tumour necrosis factor (TNF) — can also affect the

**mTORC2**

(Mammalian target of rapamycin complex 2). A complex consisting of: mammalian target of rapamycin (mTOR); rapamycin-insensitive companion of mTOR (RICTOR); mammalian stress-activated MAP kinase-interacting protein 1 (mSIN1; also known as MAPKAP1); protein observed with RICTOR 1 (PROTOR1); PROTOR2; DEP domain-containing mTOR-interacting protein (DEPTOR); and mLST8 (also known as G $\beta$ L).

**MDM2**

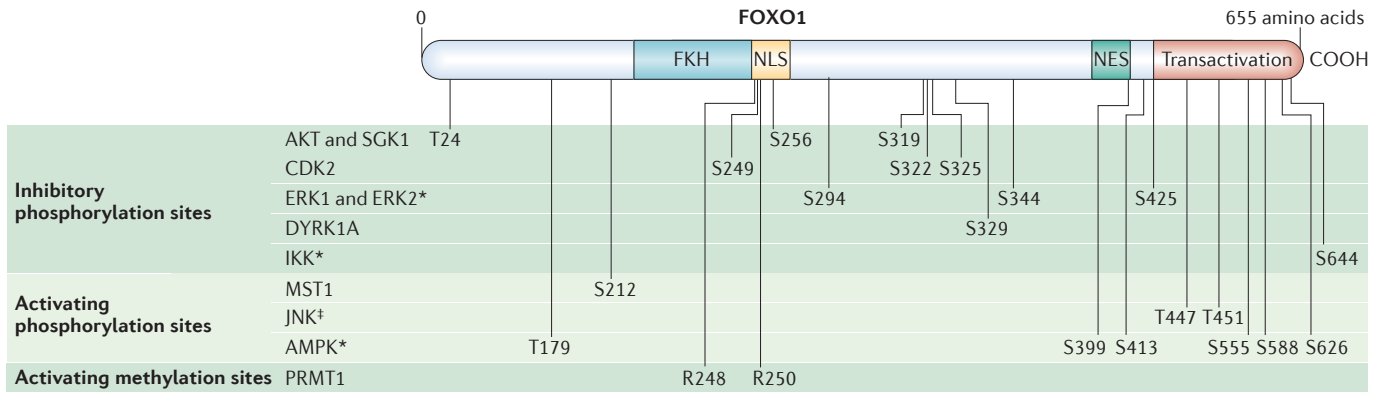
An E3 ubiquitin ligase that can cause the proteasomal degradation of p53 as well as of other tumour suppressor proteins such as FOXO3.



**Figure 1 | A signalling scheme regulating FOXO transcription factors.** Forkhead box O (FOXO) transcription factors make multiple connections with the cellular signalling network. Examples of connecting proteins include: phosphoinositide 3-kinase (PI3K) and AKT<sup>29,30,173–175</sup>; ataxia telangiectasia mutated (ATM)<sup>176,177</sup>; tuberous sclerosis protein 2 (TSC2)<sup>178</sup>; extracellular signal-regulated kinase (ERK)<sup>179</sup>; JUN N-terminal kinase (JNK)<sup>180</sup>; mammalian STE20-like kinase 1 (MST1)<sup>49,181</sup>; IκB kinase (IKK)<sup>42,182</sup>; mammalian target of rapamycin (mTOR)<sup>25</sup>; and CREB-binding protein (CBP)<sup>183</sup>. The mTOR pathway is inhibited in T cells by metformin, 5-aminoimidazole-4-carboxamide riboside (AICAR)<sup>159</sup> and rapamycin<sup>160</sup>. FOXO transcription factors also affect glycolysis<sup>54</sup>. γ<sub>c</sub>, γ-chain; 4EBP, EIF4E-binding protein 1; AMPK, AMP-activated protein kinase; BCL-6, B cell lymphoma 6; CTLA4, cytotoxic T lymphocyte antigen 4; EOMES, eomesodermin; IL-7Rα, interleukin-7 receptor subunit-α; KLF2, Krüppel-like factor 2; mTORC, mTOR complex; PDK1, 3-phosphoinositide-dependent kinase 1; ROS, reactive oxygen species; S1P<sub>1</sub>, sphingosine-1-phosphate receptor 1; S6K1, S6 kinase 1; SIRT1, sirtuin 1; TCR, T cell receptor.

course of an immune response, and a central inflammatory pathway results in the nuclear localization of nuclear factor-κB (NF-κB) through the activity of IκB kinase (IKK)<sup>38,39</sup>. Studies have shown that IKK is the key survival factor for acute myeloid leukaemia (AML) blast cells<sup>40,41</sup>, and it functions through the inactivation of FOXO3 (REF. 42). An implication is that inflammatory processes, however they are induced, inactivate FOXO3 (through increased IKK activity), and this could affect T cell or dendritic cell (DC) function (as described later)<sup>15,43</sup>. Another mode of FOXO turnover may be regulated by GTPases of the immunity-associated protein family (GIMAPs), which are associated with human autoimmunity<sup>44–46</sup>. T cells from mice with a mutation in *Gimap5* displayed a progressive loss of FOXO1, FOXO3 and FOXO4 and a concomitant loss of T<sub>Reg</sub> cell function<sup>47</sup>. The authors speculated that GIMAP5 regulates the turnover of FOXO transcription factors through an increase in the expression of S-phase kinase-associated protein 2 (SKP2) and thus proteasomal degradation.

In opposition to these FOXO-inhibitory pathways is oxidative stress, the effects of which are propagated through several post-translational FOXO modifications. These include alternative phosphorylations by JUN N-terminal kinase (JNK) or mammalian STE20-like kinase 1 (MST1); both prevent the interaction of 14-3-3 scaffold proteins with FOXO transcription factors, thereby promoting FOXO nuclear localization<sup>48,49</sup>. The JNK phosphorylation sites have been identified for FOXO4 but are not conserved in other paralogues; however, JNK also phosphorylates 14-3-3 proteins. As this too interferes with FOXO binding, one possibility is that JNK indirectly regulates the nuclear localization of all FOXO transcription factors<sup>50</sup>. In addition, arginine methylation of FOXO transcription factors by protein arginine N-methyltransferase 1 (PRMT1) inhibits AKT-mediated phosphorylation at S256, thereby preventing AKT-mediated nuclear exclusion<sup>51</sup>. Oxidative stress can also affect p300-mediated acetylation and sirtuin 1 (SIRT1)-mediated deacetylation of FOXO transcription factors in a temporally complex manner<sup>52,53</sup>. In some



**Figure 2 | Inhibitory and activating post-translational modifications of FOXO1 transcription factors mapped onto a schematic diagram of the functional domains.** Unless indicated the post-translational modifications were demonstrated for forkhead box O1 (FOXO1). See the FIG. 1 legend for references with the exception of dual-specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A)<sup>184</sup>. AMPK, AMP-activated protein kinase; CDK2, cyclin-dependent kinase 2; ERK, extracellular signal-regulated kinase; FKH, forkhead domain; IKK, IκB kinase; JNK, JUN N-terminal kinase; MST1, mammalian STE20-like kinase 1; NES, nuclear export signal; NLS, nuclear localization signal; PRMT1, protein arginine N-methyltransferase 1; transactivation, transcriptional activation domain. \*Shown for FOXO3. ‡Shown for FOXO4.

cases, these modifications can supersede growth factor-mediated inhibition of FOXO transcriptional activity; however, we note that not all of these mechanisms have been verified in T cells.

FOXO transcription factors in the liver are also regulated by nutrient levels and metabolic control mechanisms<sup>54</sup>. The energy sensor AMP-activated protein kinase (AMPK) enhances the transcriptional activity of FOXO3 directly through phosphorylation at six unique sites<sup>55</sup>, and indirectly through inhibition of the mTORC2 pathway<sup>56</sup>. In response to nutrient availability, FOXO transcription factors are also modified and activated by O-linked glycosylation with N-acetylglucosamine (GlcNAc), which is catalysed by a single enzyme, O-GlcNAc transferase. This modification functions as an energy sensor because it is highly sensitive to the concentration of the donor sugar, UDP-GlcNAc, which is the end product of the hexosamine biosynthetic pathway<sup>57-59</sup>. As the amount of UDP-GlcNAc is regulated by nearly every metabolic pathway, a prediction is that FOXO activity is responsive to the overall metabolic state of the cell. These modifications by AMPK and O-GlcNAc transferase have been described in liver, muscle and fat, but we still do not know how they control FOXO transcription factors in lymphocytes, macrophages or DCs. We also do not yet know how FOXO transcription factors integrate metabolism with T cell responses or the overall state of inflammation.

**miRNAs.** FOXO transcription factors are also subject to post-transcriptional control by multiple microRNAs (miRNAs). For example, miR-27a, miR-96 and miR-182 decrease FOXO1 mRNA expression in breast cancer cells<sup>60</sup>. A contemporaneous report showed that melanoma metastasis depends on miR-182-mediated inhibition of FOXO3 expression<sup>61</sup>, whereas the expression of FOXO1 in endometrial cancer cells seems to be regulated by as many as five miRNAs<sup>62</sup>. In antigen- or mitogen-stimulated mouse T cells, miR-182 is induced by IL-2

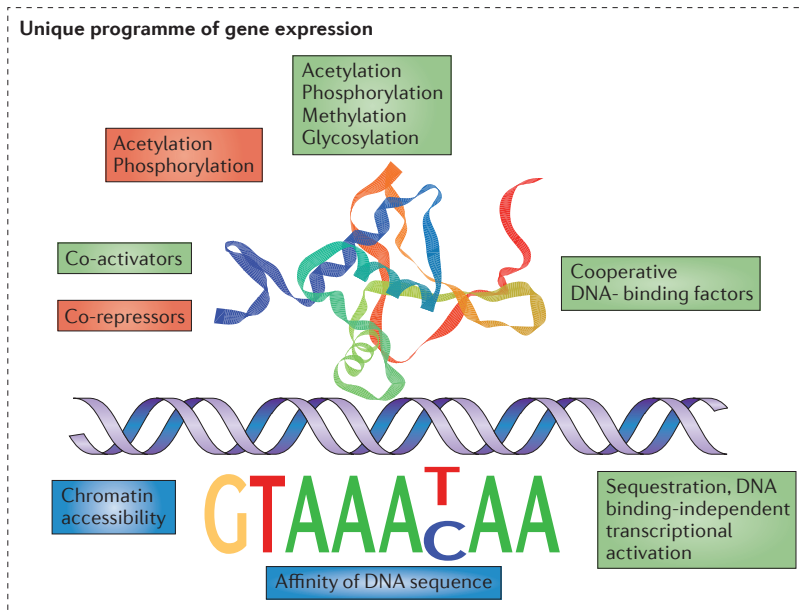
and decreases FOXO1 expression, allowing T cell proliferation to continue even after growth factor-induced, AKT-mediated inhibition of FOXO transcription factor activity wanes<sup>63</sup>. Conversely, inhibition of miR-182 causes increased levels of Foxo1 mRNA and decreased T cell proliferation secondary to increased cell death.

Another miRNA that potentially inhibits FOXO expression is miR-155. Studies have identified miR-155 as a signature of natural T<sub>Reg</sub> cells and have shown it to be important for targeting mRNAs encoding suppressor of cytokine signalling 1 (SOCS1), PU.1, MAF, activation-induced cytidine deaminase (AID), SH2 domain-containing inositol 5'-phosphatase 1 (SHIP1) and interferon-γ (IFNγ)<sup>64</sup>. In addition, miR-155 targets Foxo3 transcripts in a T cell line and in mouse T cells<sup>65</sup>. As genetic studies have shown that the dominant FOXO transcription factor in T cells is FOXO1 (REF. 66), we predict that dysregulation of FOXO3 expression in T cells through gain or loss of miR-155 would not be immediately discernable<sup>43</sup>. However, a recent report showed that loss of Foxo3 caused a twofold increase in the proliferation of CD8<sup>+</sup> T cells in some tissues in response to infection with lymphocytic choriomeningitis virus<sup>67</sup>. This suggests that miR-155 could contribute to the regulation of T cell clonal expansion and memory. As FOXO1 and FOXO3 additively control the differentiation of T<sub>Reg</sub> cells<sup>68,69</sup>, another possibility is that the balance between conventional T cells and T<sub>Reg</sub> cells is determined, in part, by miRNA-mediated post-transcriptional regulation.

**Targets of FOXO transcriptional control**

**Direct DNA-binding transcriptional activity.** The forkhead DNA-binding domain (also known as the winged-helix domain) of FOXO transcription factors is evolutionarily conserved and recognizes the sequence motif 5'-GTAAA(T/C)AA-3' (or 5'-TT(A/G)TTTAC-3'), which is known as the DAF-16 binding element (DBE)<sup>70</sup> (FIG. 3). The actual recognition sequence is somewhat broader, however, as FOXO1 has a fivefold

**MicroRNAs** (miRNAs). Non-coding RNA molecules that provide recognition for the RNA-induced silencing complex (RISC), which has inhibitory effects on transcription and/or translation. This large, multisubunit, nucleoprotein complex includes DICER, which is essential for processing precursor RNA molecules, and Argonaute, which is central to silencing.



**Figure 3 | Context-dependent FOXO transcriptional activity.** The figure shows a schematic representation of the forkhead box O1 (FOXO1) DNA-binding domain (amino acids 150–249) bound to the FOXO consensus DNA sequence (the structural data were obtained from Protein Data Bank entry 3C06 (REF. 71)). The transcriptional activity depends on recruited cofactors, post-translational modifications and cooperative binding, which can have positive (green) or negative (red) effects. FOXO transcription factors bind with differing affinities to DNA sequences related to the consensus site. FOXO factors can also affect chromatin conformation, thereby acting as gateway factors to make gene loci accessible for further regulation. The expectation is that FOXO factors offer unique programmes of gene expression in each distinct cell type.

higher affinity for a DBE sequence that includes T-rich 3' flanking sequences<sup>71</sup>. FOXO proteins also bind with lower affinity to the insulin response element (IRE), which has the sequence 5'-(C/A)(A/C)AAA(C/T)AA-3' (REFS 71,72). Specific acetylation or phosphorylation of FOXO1 was shown to inhibit or entirely preclude DNA binding, directly demonstrating that post-translational modifications affect the transcriptional function of FOXO transcription factors in addition to their intracellular localization<sup>72</sup>.

FOXO transcription factors that are bound to DNA function as independent transcriptional activators, as deduced from their roles in oncogenesis<sup>73,74</sup>. FOXO1 was originally identified as part of a fusion protein with paired box protein 3 (PAX3) resulting from a chromosomal translocation that is responsible for alveolar rhabdomyosarcoma<sup>75</sup>. The DNA-binding domain and thus target gene specificity was derived from PAX3, whereas the transcriptional activation domain came from FOXO1 (the reverse, which might enhance the gene expression programme of the FOXO1 tumour suppressor, would not be expected to lead to oncogenesis). Both FOXO3 and FOXO4 are also targets of chromosomal translocations in mixed-lineage leukaemias<sup>76</sup>; the fusions mainly occur within and disrupt the FOXO DNA-binding domain, resulting in chimeric proteins that derive transcriptional activity from the FOXO factors. It would seem that the FOXO transactivation domains are particularly potent.

T follicular helper cells ( $T_{FH}$  cells).  $CD4^+$  T cells that migrate to the B cell-rich follicles in an active immune response and provide helper functions that promote the differentiation of B cells into antibody-producing cells. They are variously described as a separate T cell subset or a further differentiation of  $T_H1$ ,  $T_H2$  and  $T_H17$  cells.

**Other forms of transcriptional activity.** FOXO transcription factors might also regulate transcription in a DNA-binding-independent manner, as mutant FOXO variants with inactivated DNA-binding domains were still able to regulate a subset of FOXO target genes<sup>77</sup>. A caveat is that these results were derived using an overexpressed and constitutively nuclear form of FOXO1 in PTEN-deficient cells. FOXO transcription factors also have chromatin binding and remodeling functions, endowing them with the ability to modulate active chromatin states<sup>78</sup>. In this regard, the transcriptional activity of FOXO transcription factors is often or always achieved by association with other transcriptional activators or repressors. FOXO transcription factors have been shown to associate with SMAD proteins, histone deacetylases and acetyltransferases, many nuclear hormones,  $\beta$ -catenin and other transcriptional regulators<sup>79</sup>. The mechanisms of co-regulation include direct binding interactions, sequestration and cooperative promoter binding sites (FIG. 3). FOXO transcription factors in collaboration with other factors are thus able to integrate extrinsic information from many different pathways to coordinately initiate or facilitate varied programmes of transcriptional regulation. As stated above, the target genes of FOXO factors might be widely disparate in different tissues owing to differences in chromatin accessibility, available cofactors and post-translational modifications. Thus, our ability to extrapolate specific roles for FOXO transcription factors between different tissues is likely to be limited.

**Control of T cell gene expression.** The identification of FOXO target genes in T cells has depended on multiple avenues of investigation (BOX 2). Published studies show that several direct FOXO gene targets are actively transcribed in T cells. One important target is *Il7ra*<sup>66,68,80</sup>, which encodes IL-7R $\alpha$ , a subunit of the primary survival receptor for naive T cells<sup>81</sup>. FOXO1 binds to an enhancer approximately 3 kb upstream of the *Il7ra* start site, and acute deletion of *Foxo1* causes the loss of *Il7ra* expression in naive T cells<sup>66</sup>. A second important target is *Klf2*, which in turn controls the expression of *Sell* (which encodes L-selectin) and sphingosine-1-phosphate receptor 1 (*S1pr1*, which encodes S1P<sub>1</sub>). These receptors are important for lymphocyte entry into and exit from lymphatic tissues<sup>66,82,83</sup>. In addition, FOXO transcription factors control *Klf4*, a tumour suppressor gene that is particularly active in B cells<sup>84,85</sup>. As discussed below, FOXO transcription factors also directly regulate the expression of *Foxp3* (REFS 86,87) and cytotoxic T lymphocyte antigen 4 (*Ctla4*)<sup>69</sup>, which are essential for the differentiation and function of  $T_{Reg}$  cells. Furthermore, there is evidence that FOXO transcription factors directly control the expression of B cell lymphoma 6 (*BCL6*)<sup>88–90</sup>, which encodes a transcriptional repressor that is required for the differentiation of  $CD4^+$  T follicular helper cells ( $T_{FH}$  cells), B cells,  $T_{Reg}$  cells,  $CD8^+$  T cells and natural killer T (NKT) cells<sup>91–95</sup>. In each case, *BCL6*-expressing cells have a tendency to localize to lymphoid follicles, and one of

**Box 2 | Analysis of FOXO transcription factor target genes**

Several criteria for identifying forkhead box O (FOXO) target genes can be used to assess the participation of FOXO transcription factors in lymphocyte-specific gene expression. One is the evolutionarily conserved presence of a FOXO-binding consensus motif in the promoter or enhancer regions of a gene of interest. A second criterion is the specific binding of FOXO transcription factors to such a site in T cells, as shown by electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP) or ChIP followed by sequencing (ChIP-seq) experiments. A third is the demonstration that a sequence containing the FOXO-binding consensus motif can function as an active regulatory region in T cell-specific reporter experiments. A fourth means of identifying a role for FOXO transcription factors in the transcriptional regulation of a particular gene is to show that acute genetic deletion of one or more FOXO genes results in the loss of target gene expression. The fifth commonly presented form of evidence is that constitutively nuclear mutant forms of FOXO transcription factors induce the expression of a given gene. Combinations of one or more of these criteria have identified several important FOXO target genes in T cells, but new technologies will undoubtedly establish target genes more definitively. Such an analysis would consist of global gene expression analyses (using a microarray or mRNA sequencing) with and without the acute deletion of a FOXO gene, ChIP-seq to identify FOXO-bound regulatory sequences (enhancers and silencers), and 3C techniques to identify the physical associations between regulatory sequences and 5' proximal promoters<sup>171,172</sup>.

the roles of BCL-6 is to direct B and T cells into close proximity as part of the multifaceted germinal centre response. More recently, FOXO transcription factors were shown to bind to a promoter site in the eomesodermin (*Eomes*) gene and to control its expression in differentiated CD8<sup>+</sup> T cells<sup>96</sup>. Together, the known cell type-specific FOXO target genes profoundly affect the survival, homing, proliferation and differentiation of T cells. By targeting these genes, together with numerous other, more widely expressed genes, FOXO transcription factors control the immune system in varied and diverse ways<sup>14</sup> (TABLE 1).

**Role in T cell survival and cell-cycle progression**

One of the characteristic features of FOXO transcription factors is their control of the cell cycle and apoptosis. Specifically, evidence has been presented that FOXO transcription factors (mainly FOXO3) positively control the expression of several cyclin-dependent kinase inhibitors, including p15 (also known as INK4B), p19 (also known as INK4D), p21 (also known as CIP1) and p27 (also known as KIP1), as well as that of the pro-apoptotic molecules BIM (also known as BCL2L11), PUMA (also known as BBC3) and FAS ligand (also known as CD95L)<sup>3,4,29,97–100</sup>. Most of these studies were carried out in non-lymphoid cell lines and so their relevance to T cells is not clear, although FOXO3 was shown to induce the expression of p27 and BIM in the CTLL cell line<sup>3</sup> and that of PUMA in T cells following the withdrawal of IL-2 (REF. 98). The concept is that signalling through growth-inducing receptors (such as the TCR or cytokine receptors) transiently activates the PI3K pathway, which inactivates FOXO transcription factors, and this is the primary signal promoting cell division and survival<sup>101</sup>. A caveat is that constitutively active AKT (as a result of a PTEN deficiency) does not completely rescue the loss of viability following IL-2 withdrawal<sup>98</sup>, and thus there may be redundant death pathways operative

in T cells. In fact, FOXO1- and FOXO3-deficient naive T cells do not spontaneously enter the cell cycle, and mice with a haematopoietic deletion of *Foxo1*, *Foxo3* and *Foxo4* do not develop lymphomas until 22 weeks of age, with almost 75% of the mice remaining disease-free for 75 weeks<sup>5</sup>.

A further complication is that FOXO1 seems to have a role distinct from that of FOXO3. FOXO1 promotes the survival of naive T cells through the expression of IL-7R $\alpha$  and thus BCL-2 (REFS 66,68). Furthermore, CD4<sup>+</sup> but not CD8<sup>+</sup> T cells require FOXO1 for antigen- or mitogen-stimulated population expansion, both in culture and *in vivo*. In particular, following the acute deletion of *Foxo1* and cell activation through CD3 and CD28, CD4<sup>+</sup> T cells progress through multiple rounds of cell division, but undergo apoptosis at a very high rate (Y. Kerdiles and E.L.S., unpublished data). This defect in survival was not rescued by the transgenic expression of *Il7ra*, and the mechanism underlying a requirement for FOXO1 in T cell survival is currently unknown. Similar experiments with FOXO3-mutant T cells stimulated *in vitro* revealed no differences compared with wild-type T cells<sup>43</sup>. The unique roles for FOXO1 and FOXO3 in T cell survival and division have so far not been resolved; however, these studies suggest that FOXO1 expression is required for naive T cell survival and that its nuclear expression is attenuated, but not ablated, following T cell activation. By contrast, increased FOXO3 activity induced by a growth factor deficiency is pro-apoptotic. The underlying basis for these intricately controlled and opposing functions is presently unknown.

Changes in FOXO3 expression are apparent in T cells from HIV-infected patients. HIV induces the apoptosis of primary CD4<sup>+</sup> T cells, and the presence of the HIV protein Tat alone is sufficient to recapitulate this effect. Tat was proposed to function through the activation of PTEN and FOXO3, and, given its propensity to cross the plasma membrane, it may not act in a cell-autonomous manner<sup>102–104</sup>. In addition, CD4<sup>+</sup> memory T cells from HIV-infected individuals who control HIV replication (elite controllers) have increased amounts of phosphorylated FOXO3, and this is correlated with the increased survival of memory CD4<sup>+</sup> T cells<sup>105</sup>. Another study showed that preventing FOXO3 phosphorylation in CD4<sup>+</sup> central memory T cells increased apoptosis<sup>106</sup>. The proposed model is that an increase in FOXO3 activity, possibly owing to Tat signalling in patients with HIV, results in T cell apoptosis, whereas attenuation of FOXO3 through phosphorylation (as occurs in elite controllers) results in enhanced memory T cell survival. From this we conclude that the amount, localization, activity and specificity of FOXO transcription factors, as regulated by some or all of the mechanisms already described, are important in determining the outcome of an immune response. In particular, the balance between persistent pathogen infections and clearance may be influenced by the precise physiological state of the infected individual as interpreted by FOXO transcription factors.

**CTLL cell line**

A T cell line that grows indefinitely in the presence of interleukin-2 (IL-2) with no requirement for stimulation through the T cell receptor (TCR). This is not a feature of freshly explanted T cells, which require a cycle of TCR and IL-2 stimulation followed by rest before re-stimulation.

**Electrophoretic mobility shift assay**

(EMSA). An assay used to measure DNA-protein interactions. Short stretches of double-stranded, radio-labelled DNA are mixed with nuclear extracts and subjected to sizing by gel electrophoresis. In the presence of bound proteins, the labelled DNA will migrate more slowly. To determine the identity of the bound proteins, specific antibodies can be added to see whether the migration of the complex is altered — either becoming even slower ('supershifted') or being prevented altogether.

Table 1 | Cell type-specific phenotypes of *Foxo1*- and *Foxo3*-knockout cells

Cell type	<i>Foxo1</i> knockout	<i>Foxo3</i> knockout	Notes	Refs
Thymic $T_{Reg}$ cells	Strongly depleted	No effect	FOXO1 may control part of the $T_{Reg}$ cell gene expression programme, including <i>Ctla4</i>	69,86
$T_{Reg}$ cells in secondary lymphoid organs	Present with Ki67 <sup>+</sup> phenotype	No effect	In <i>Foxo1</i> -knockout mice, the small $T_{Reg}$ cell population that develops may homeostatically expand	69,86
Induced $T_{Reg}$ cells (in culture)	No induction, $T_H1$ cells result	Reduced induction	FOXO1 is required for the TGF $\beta$ -induced downregulation of T-bet expression	69,86,87
CD4 <sup>+</sup> T cells	Decreased numbers of naive T cells; increased numbers of activated CD4 <sup>+</sup> T cells	No effect	The reduction in naive T cells is due to the loss of IL-7R $\alpha$ and homing receptor expression; the increase in activated T cells is secondary to a loss of functional $T_{Reg}$ cells	66,68,83
$T_{FH}$ cells	High numbers of $T_{FH}$ cells and spontaneous germinal centre formation	No effect	The effects in <i>Foxo1</i> -knockout mice may depend on the loss of $T_{Reg}$ cells; FOXO transcription factors may regulate BCL-6, which is necessary for $T_{FH}$ cell differentiation, although the direction of regulation is the reverse of that expected	69
CD8 <sup>+</sup> T cells	Decreased numbers of naive CD8 <sup>+</sup> T cells (owing to a loss of IL-7R $\alpha$ , L-selectin and S1P <sub>1</sub> expression); no increase in spontaneously activated CD8 <sup>+</sup> T cells	No effect	Activation of FOXO1-deficient naive CD8 <sup>+</sup> T cells progresses normally, in contrast to that of CD4 <sup>+</sup> T cells	66,68,83
CD8 <sup>+</sup> effector T cells	FOXO1 inhibits T-bet expression and thus the CD8 <sup>+</sup> T cell effector molecules IFN $\gamma$ and granzyme B	Increased approximately twofold owing to enhanced survival	IL-12 promotes T-bet expression through the inactivation of FOXO1 in culture	67,96
CD8 <sup>+</sup> memory T cells	Unknown	Increased approximately twofold	FOXO1 inhibits T-bet expression and promotes eomesodermin expression and hallmarks of memory cell formation in culture	67,96,164

BCL-6, B cell lymphoma 6; CTLA4, cytotoxic T lymphocyte antigen 4; FOXO, forkhead box O; IFN $\gamma$ , interferon- $\gamma$ ; IL, interleukin; IL-7R $\alpha$ , IL-7 receptor subunit- $\alpha$ ; S1P<sub>1</sub>, sphingosine-1-phosphate receptor 1; TGF $\beta$ , transforming growth factor- $\beta$ ;  $T_{FH}$ , T follicular helper;  $T_H1$ , T helper 1;  $T_{Reg}$ , regulatory T.

### $T_{Reg}$ cell development and function

**Natural  $T_{Reg}$  cell development.** In addition to having defects in naive T cell survival and homing (owing to decreased expression of *Il7Ra* and *Klf2*)<sup>66,68,83</sup>, mice with a T cell-specific conditional mutation in *Foxo1* have substantial immunopathology that is not cell autonomous but rather is associated with decreased numbers of FOXP3<sup>+</sup> natural  $T_{Reg}$  cells in the thymus<sup>69,86</sup>. This diminution in numbers is even more pronounced when FOXO1-deficient T cell precursors have to compete with wild-type precursors in a mixed bone marrow chimaera<sup>69</sup>. Furthermore, mice lacking both FOXO1 and FOXO3 in T cells have few natural  $T_{Reg}$  cells in the thymus and decreased  $T_{Reg}$  cell numbers in secondary lymphoid organs when at a young age but have normal numbers of FOXP3<sup>+</sup> T cells as adults. Mortality from excessive immunopathology was seen starting at 8 weeks of age, and experiments showed that FOXO1-deficient bone marrow cells were unable to complement a loss of FOXP3 in mixed bone marrow chimaera. The conclusion drawn from these studies is that both the development and function of natural  $T_{Reg}$  cells are retarded in the absence of FOXO1, with an additive contribution from FOXO3 (REFS 66,86,87).

These results highlight the suggestion that FOXP3, although essential for  $T_{Reg}$  cell function, is not a lineage specification factor. Studies have shown that it is only one of the essential transcription factors required for the development of a  $T_{Reg}$  cell phenotype<sup>107–111</sup>. In

fact, an analysis of genes expressed by various  $T_{Reg}$  cells showed that FOXP3 expression positively correlated with the expression of only a small number of  $T_{Reg}$  cell signature genes<sup>108</sup>. Additional studies indicate that the AKT–mTOR axis might regulate a substantial portion of the  $T_{Reg}$  cell gene signature independently of FOXP3 (REFS 112,113). A conclusion of these studies was that a higher level of regulation, upstream of FOXP3, determines the  $T_{Reg}$  cell lineage<sup>108</sup>, and one possibility is that FOXO transcription factors, which are inhibited by AKT, constitute an essential aspect of this higher level of control.

**Induced  $T_{Reg}$  cell development.** Mature naive CD4<sup>+</sup> FOXP3<sup>-</sup> T cells can give rise to induced  $T_{Reg}$  cells in the presence of transforming growth factor- $\beta$  (TGF $\beta$ )<sup>114</sup>. When naive CD4<sup>+</sup> T cells lacking FOXO1 or both FOXO1 and FOXO3 were cultured in this manner, few FOXP3<sup>+</sup> induced  $T_{Reg}$  cells developed<sup>69,86,87</sup>. FOXO3-deficient CD4<sup>+</sup> T cells also showed a decreased ability to develop into induced  $T_{Reg}$  cells, although the effect was less pronounced than for FOXO1 deficiency<sup>87</sup>.

Although there are some discrepancies, in general conditions that favour activation of the PI3K–AKT pathway retard the differentiation of induced  $T_{Reg}$  cells, whereas inhibition of the PI3K–AKT pathway promotes  $T_{Reg}$  cell differentiation<sup>87,112,115–119</sup>. We thus propose that inhibition of PI3K–AKT signalling is specifically

**Chromatin immunoprecipitation (ChIP).** An assay used to determine whether specific transcription factors are bound to chromatin. DNA–protein complexes are stabilized by reversible crosslinking, the DNA is sheared to an average size of about 500 bp, an antibody specific for a suspected chromatin-associated factor is used to carry out immunoprecipitation, and the complexes are isolated. Following dissolution of the crosslinks and protein digestion, PCR is used to determine whether specific DNA sequences were co-isolated. A positive signal using appropriate controls indicates that a given factor is within proximity (about 500 bp) of the primers used to amplify the DNA.

ChIP-seq

An assay similar to chromatin immunoprecipitation (ChIP) with the exception that the immunoprecipitated DNA is modified by the addition of coded oligonucleotides, and the resulting libraries of DNA are sequenced using massively parallel sequencing techniques.

mRNA sequencing

In this technique, poly(A)-containing mRNA isolated by hybridization to oligo-dT columns may be fragmented and is then converted to complementary DNA (cDNA) using the enzyme reverse transcriptase. The cDNA is then prepared for parallel sequencing. The number of sequencing reads specific for each gene correlates with mRNA abundance. Information can also be obtained pertaining to alternative splicing or transcriptional start isoforms of each gene. This technique yields accurate and abundant data, and is rapidly superseding microarray technologies.

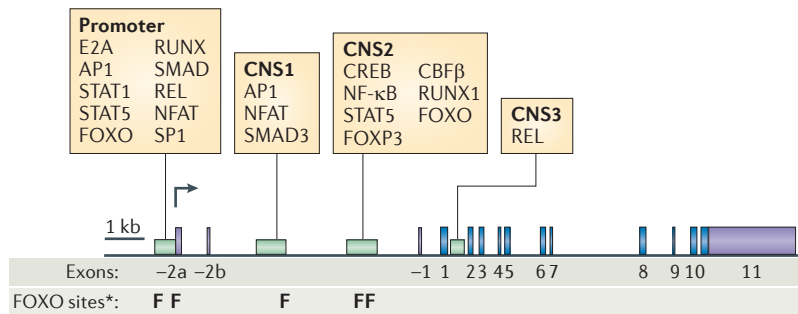
required for the nuclear localization and activity of FOXO transcription factors<sup>69</sup>. This is also in accord with the activity of vitamin A metabolites and vitamin D, both of which have been shown to induce nuclear localization of FOXO transcription factors in myeloid leukaemia and squamous carcinoma cells<sup>116,120</sup> and, in separate studies, the induction of T<sub>Reg</sub> cells<sup>121-123</sup>.

At the heart of these effects is the mTOR pathway — the master regulator of protein synthesis<sup>124,125</sup>. mTOR forms two distinct kinase complexes, mTORC1 and mTORC2, and these complexes are intricately related in terms of their activity as well as their structure<sup>126</sup>. mTORC1 is activated downstream of AKT by the inhibitory phosphorylation of the mTORC1 inhibitor, tuberous sclerosis protein 2 (TSC2). Activated mTORC1 promotes protein translation in several ways, including through an increase in ribosome biogenesis (FIG. 1). We can therefore deduce that this would sequentially activate mTORC2, stabilize AKT and thus lead to inactivating phosphorylations of FOXO transcription factors.

Genetic or pharmacological inhibition of mTOR leads to the generation of induced T<sub>Reg</sub> cells in the absence of added cytokines, and naive T cells in which mTOR is inhibited do not differentiate into T helper 1 (T<sub>H1</sub>), T<sub>H2</sub> or T<sub>H17</sub> cells<sup>127</sup>. In particular, rapamycin — which inhibits mTORC1 and, with prolonged application, mTORC2 — causes activated T cells to differentiate into T<sub>Reg</sub> cells<sup>113,128-130</sup>. Likewise, a T cell-specific deletion of the gene encoding mTOR causes activated T cells to assume a T<sub>Reg</sub> cell phenotype<sup>131</sup>. Furthermore, mTOR signalling is decreased when the availability of essential amino acids is limited, and this decrease synergizes with TGFβ in promoting the differentiation of induced T<sub>Reg</sub> cells<sup>132</sup>. Genetic ablations leading to the individual loss of mTORC1 or mTORC2 are not sufficient to cause T<sub>Reg</sub> cell differentiation, implying that both pathways have to be abrogated for T<sub>Reg</sub> cell differentiation to occur<sup>131</sup>. We conclude that T<sub>Reg</sub> cell differentiation requires the inhibition of mTOR signalling, and we propose that this is mediated in part through an increase in the nuclear localization and activity of FOXO transcription factors.

**Mechanisms of FOXO transcription factors in the function of T<sub>Reg</sub> cells.** The mechanisms underlying the requirement for FOXO transcription factors in T<sub>Reg</sub> cell differentiation are not yet understood. The simplest idea is that FOXO transcription factors are required for the transcription of *Foxp3*, as studies have shown that FOXO transcription factors can bind to and transcriptionally activate a previously mapped *Foxp3* promoter sequence<sup>86,87</sup> (FIG. 4). However, we found that T<sub>Reg</sub> cells are present in the secondary lymphoid organs of adult *Foxo1*<sup>-/-</sup>*Foxo3*<sup>-/-</sup> mice and have only slightly decreased FOXP3 expression<sup>69</sup>. Nonetheless, these cells are not sufficient to prevent immunopathology<sup>69,86</sup>. As T<sub>Reg</sub> cells are all but absent in the thymus in the absence of *Foxo1* and *Foxo3*, we deduce that a small number of FOXP3<sup>+</sup> T<sub>Reg</sub> cells proliferate over time to produce T<sub>Reg</sub> cells in normal numbers. One proposal is that FOXO transcription factors together with REL (which binds to the CNS3 enhancer in *Foxp3*) facilitate chromosome accessibility within the *Foxp3* locus<sup>133</sup> to promote the eventual accumulation of other transcription factors<sup>133</sup> (FIG. 4). Such factors include STAT1 (REF. 134), STAT5 (REF. 135), Helios<sup>136</sup> and, notably, FOXP3 itself, which implies self-reinforcing gene expression<sup>18,133</sup>. Without REL or FOXO factors, the *Foxp3* locus might be relatively inaccessible, but over time it could achieve close to full activity owing to this self-reinforcing mechanism.

Given the presence of FOXP3<sup>+</sup> T cells in secondary lymphoid organs, what is the basis for the immunopathology seen in mice deficient for FOXO1 and FOXO3? It does not seem to be intrinsic to the expanded populations of effector T cells, but rather results from a lack of T<sub>Reg</sub> cell function. Thus, one possibility is that a small diminution of FOXP3 expression is sufficient to handicap the extant T<sub>Reg</sub> cells. Another possibility is that, in the absence of FOXO transcription factors, FOXP3<sup>+</sup> T<sub>Reg</sub> cells lack the suppressive function required for immune quiescence. Consistent with this idea, active AKT prevents FOXP3<sup>+</sup> T<sub>Reg</sub> cell differentiation, whereas it does not affect established FOXP3 expression in T<sub>Reg</sub> cells<sup>112</sup>. Another study has shown that enforced expression of an active allele of AKT in human CD4<sup>+</sup>CD25<sup>+</sup> T cells (which is presumed to constrain FOXO activity) inhibits their suppressive function<sup>137</sup>. In addition, FOXP3<sup>+</sup> T<sub>Reg</sub> cells that are deficient in both FOXO1 and FOXO3 are less efficient than wild-type cells in a suppression assay in cell culture<sup>86</sup>. One key feature of T<sub>Reg</sub> cells is their expression of CTLA4, an indispensable co-receptor for T<sub>Reg</sub> cell function<sup>138-140</sup>. We found that FOXO1 is required for CTLA4 expression (whether directly or indirectly) in all T cells tested, and it binds to a DBE located 193 base pairs upstream of the *Ctla4* transcriptional start site<sup>69</sup>. Although there are likely to be other genes crucial for T<sub>Reg</sub> cell function that are under the control of FOXO transcription factors, the requirement for FOXO1 in CTLA4 expression would itself be sufficient to explain the immunopathology seen in both *Foxo1*<sup>-/-</sup> and *Foxo1*<sup>-/-</sup>*Foxo3*<sup>-/-</sup> mutant mice.



**Figure 4 | The *Foxp3* locus.** The forkhead box P3 (*Foxp3*) promoter and enhancers may be controlled by activator protein 1 (AP1), forkhead box O1 (FOXO1), nuclear factor of activated T cells (NFAT), SMAD3 and signal transducer and activator of transcription 5 (STAT5), as well as by other factors identified through evolutionarily conserved consensus sites<sup>185</sup>. The enhancer nomenclature for *Foxp3* is from REF. 133. CBFβ, core-binding factor-β; CREB, cAMP-responsive element-binding protein; NF-κB, nuclear factor-κB. \*Evolutionarily conserved DAF-16 binding elements.



**3C techniques**

Chromosome conformation capture (3C) is used to determine whether a distal enhancer sequence is in proximity to a promoter in a given state of a particular cell type. The basic concept is that DNA–protein and protein–protein interactions in the nucleus are reversibly crosslinked to stabilize interacting regions of DNA. The DNA is digested to completion with a restriction enzyme, and intramolecular ligation is carried out to link promoter and enhancer sequences. The resulting complex can be analysed by sequencing in several ways to identify known or unknown interacting regulatory elements.

**mTORC1**

(Mammalian target of rapamycin complex 1). A complex consisting of: mammalian target of rapamycin (mTOR), which is a serine/threonine kinase; regulatory-associated protein of mTOR (RAPTOR); proline-rich AKT substrate of 40 kDa (PRAS40), which is an mTORC1 inhibitor; mLST8 (also known as GβL), which is of unknown function; and DEP domain-containing mTOR-interacting protein (DEPTOR), which is an mTOR inhibitor.

**CNS3 enhancer**

One of four DNA regulatory regions in the *Foxp3* gene (together with the promoter, CNS1 and CNS2) that was initially defined by histone modifications that are permissive for transcription. CNS1, CNS2 and CNS3 were then analysed for activity by generating mice with deletions spanning each region of the chromosome.

***Cd4Cre Foxo1<sup>fl/fl</sup>* mice**

Mice in which both alleles of the *Foxo1* gene are modified to include *loxP* sites flanking exon 2 and in which the Cre recombinase gene from the P1 bacteriophage is expressed from a transgene using control elements of the *Cd4* gene. In such mice, the *Foxo1* gene is inactivated in the T cell lineage during the CD4<sup>+</sup>CD8<sup>+</sup> stage of thymic development.

**T<sub>H</sub>1 cell differentiation**

Recently, the mTOR–FOXO pathway has emerged as a key pathway regulating T<sub>H</sub>1 cell versus T<sub>Reg</sub> cell differentiation. S1P<sub>1</sub> (which is encoded by *S1pr1*) activates AKT and also activates S6 kinases downstream of the mTORC1 pathway. Experiments have shown that a loss-of-function mutation in *S1pr1* promotes T<sub>Reg</sub> cell development, whereas an *S1pr1* gain-of-function mutation inhibits the differentiation of induced T<sub>Reg</sub> cells. Instead, under conditions that promote T<sub>Reg</sub> cell induction in wild-type T cells (such as the presence of TGFβ), the presence of an *S1pr1* transgene diverted naive T cell differentiation towards the generation of T<sub>H</sub>1 cells<sup>141,142</sup>. In particular, the ability of TGFβ to maintain the phosphorylation of SMAD3 for between 24 and 48 hours and to induce T<sub>Reg</sub> cells was lost in the presence of the *S1pr1* transgene. A conclusion based on these results could be that S1P<sub>1</sub> signalling interferes directly with the TGFβ–SMAD3 pathway, which is an important component of T<sub>Reg</sub> cell differentiation. An alternative explanation could involve FOXO1. Naive FOXO1-deficient CD4<sup>+</sup> T cells that are activated with antibodies specific for CD3 and CD28 in the presence of TGFβ do not differentiate into FOXP3<sup>+</sup> T<sub>Reg</sub> cells, as would be expected for wild-type T cells, but instead become T-bet<sup>+</sup>IFNγ<sup>+</sup> T<sub>H</sub>1 cells<sup>69</sup>. Consistent with this result, FOXO1 is required for the TGFβ-induced inhibition of the expression of T-bet (which is encoded by *Tbx21*) that occurs in wild-type T cells. However, FOXO1 deficiency does not simply inhibit TGFβ signalling, as *Foxo1*<sup>-/-</sup> cells have normal levels of SMAD3 phosphorylation at early time points, and activation of FOXO1-deficient cells in the absence of TGFβ does not induce T<sub>H</sub>1 cell differentiation<sup>69</sup>. As SMAD proteins form transcriptional complexes with FOXO transcription factors<sup>4</sup>, the loss of sustained SMAD3 phosphorylation at later times may be indirect and result from the inactivation of FOXO1 through S1P<sub>1</sub> signalling. We conclude that, under some conditions, T<sub>H</sub>1 cells and T<sub>Reg</sub> cells constitute alternative fates that depend on S1P<sub>1</sub> signalling, mTOR activation and FOXO transcription factor activity.

**T follicular helper cells**

FOXO transcription factors clearly have a role in regulating the differentiation of CD4<sup>+</sup> T cells into T<sub>Reg</sub> cells and T<sub>H</sub>1 cells; however, the potential role of FOXO transcription factors in regulating the differentiation of other T cell subsets is just beginning to be studied. Mice with a T cell-specific deletion of *Foxo1* spontaneously accumulate a large number of CXCR5<sup>+</sup>PD1<sup>+</sup> T<sub>FH</sub> cells, and this corresponds with the appearance of germinal centres, class-switched B cells and DNA-specific antibodies<sup>69</sup>. The differentiation of T<sub>FH</sub> cells from naive CD4<sup>+</sup> T cells normally depends on signalling through the co-stimulatory molecule inducible T cell co-stimulator (ICOS) and on sequential antigen presentation by DCs and B cells<sup>143–146</sup>. Signalling through ICOS is dependent on PI3K, such that a mutation in the cytoplasmic tail of ICOS that prevents PI3K recruitment decreases T<sub>FH</sub> cell development<sup>147</sup>. Furthermore, mice with a T cell-specific deletion of the gene encoding the

p110γ subunit of PI3K have greatly decreased numbers of T<sub>FH</sub> cells after immunization with foreign proteins adsorbed to alum adjuvant<sup>148</sup>. These studies clearly establish the role of the ICOS and PI3K pathway in the differentiation or survival of T<sub>FH</sub> cells and, on the basis of the emergence of T<sub>FH</sub> cells in *Cd4Cre Foxo1<sup>fl/fl</sup>* mice, we speculate that a role of ICOS signalling is to inactivate FOXO transcription factors. However, as described above, FOXO3 has been shown to positively regulate the expression of *BCL6*, which encodes the essential transcription factor for T<sub>FH</sub> cell differentiation<sup>91–93</sup>. Thus, the enigma is that in some cells FOXO3 positively regulates BCL-6, even though ICOS signalling (which is required for T<sub>FH</sub> cell differentiation) would be predicted to negatively regulate all FOXO transcription factors. A recent study showed that strong IL-2 signalling inhibits the expression of a profile of T<sub>FH</sub> cell-associated genes, including *Bcl6*, and this was correlated with decreased binding of FOXO1 to the *Bcl6* promoter<sup>149</sup>. The manner by which FOXO1 regulates BCL-6 expression, and how this relates to ICOS signalling and T<sub>FH</sub> cell differentiation, requires further study.

**CD8<sup>+</sup> T cell differentiation and function**

Following host infection with an intracellular pathogen, antigen-specific naive CD8<sup>+</sup> T cells accumulate in draining lymphatic organs, proliferate and differentiate into effector T cells. The expanding T cell population diversifies, such that a small proportion of cells acquires characteristics of memory cell precursors, whereas most cells maintain an effector phenotype. At the peak of the expansion phase (usually around 8 days after infection in mouse models of acutely infectious agents), this T cell diversity is evident on the basis of the differential expression of key cell-surface molecules. Memory precursor effector cells have a KLRG1<sup>low</sup>CD127<sup>hi</sup> phenotype, whereas effector cells have a KLRG1<sup>hi</sup>CD127<sup>low</sup> phenotype<sup>150,151</sup>. The differentiation of activated T cells into T cell memory precursors and then long-lived memory T cells is thought to be based on a progressive and self-reinforcing programme of gene expression that may originate with stochastic changes in the expression of a few genes<sup>150–153</sup>. One part of this gene expression programme is the transcription factor EOMES<sup>154,155</sup>, and EOMES expression depends on WNT-mediated conversion of the transcription factor TCF7 (also known as TCF1) into an active complex with β-catenin<sup>156,157</sup>.

As described earlier, FOXO1 regulates programmes of homing, self-renewal, cell-cycle entry and progression, and cell survival, all of which are essential components in the control of memory versus effector T cell differentiation. In accord with this, recent work has shown that FOXO1 and FOXO3 might each have a role in CD8<sup>+</sup> T cell differentiation. CD8<sup>+</sup> T cells acquire some of the hallmarks of effector function (such as a KLRG1<sup>hi</sup> phenotype) through the expression of *Tbx21* (which encodes T-bet), and this is amplified by antigen-induced stimulation in the presence of IL-12 (REF. 152). Reminiscent of the role of FOXO1 in the TGFβ-mediated inhibition of T-bet<sup>69</sup>, a recent study showed that IL-12 promotes *Tbx21* expression by inactivating FOXO1,

and *Tbx21* expression can be inhibited indirectly by FOXO1 overexpression<sup>96</sup>. Furthermore, FOXO1 seems to directly target *Eomes*, and thus constitutes a second pathway that converges on *Eomes* expression in order to promote a memory cell phenotype. T cells that were adoptively transferred following *in vitro* activation and knockdown of *Foxo1* expression survived poorly after 40 days and showed no ability to be reactivated *in vivo*<sup>96</sup>. Thus, FOXO1 activity may be at least one deciding factor in effector versus memory precursor CD8<sup>+</sup> T cell differentiation. This is consistent with a report describing the effects of sustained AKT activation in CD8<sup>+</sup> T cells, a condition that would induce the inactivation of FOXO1. Indeed, these cells had decreased levels of EOMES and TCF7 and a reduced potential to survive and adopt a memory phenotype compared with control cells<sup>158</sup>. These studies may help to explain the mechanism underlying the ability of metformin or rapamycin to promote CD8<sup>+</sup> memory T cell formation<sup>159,160</sup>. Both drugs inhibit the mTORC1 and mTORC2 pathways<sup>161–163</sup> and, as such, they might function in part or even entirely through their ability to promote FOXO transcription factor activity.

Other studies have shown that the maintenance of a memory T cell phenotype is an active process dependent on T cell interactions with DCs, and these interactions are mediated through CD27 and 4-1BB<sup>164</sup>. CD27 signalling correlates with decreased FOXO1 activity, leading to the conclusion that inhibition of FOXO1 is required for the maintenance of a memory T cell phenotype. Although this might seem to be inconsistent with the role of FOXO1 inactivation in inducing an effector T cell phenotype, these studies probe different stages in T cell differentiation. The exact amount of nuclear FOXO1, its available cofactors and its post-translational modifications might differ between newly emerging activated CD8<sup>+</sup> T cells and long-term memory T cells.

Experiments also support a role for FOXO3 in CD8<sup>+</sup> T cell proliferation. In our studies, there was a clear increase in the expansion of CD8<sup>+</sup> T cell populations in FOXO3-deficient mice, but at early time points we found the increase to originate with the DC population. In the absence of FOXO3, DCs produced excess IL-6, TNF and CC-chemokine ligand 2 (CCL2; also known as MCP1), and the additional IL-6 was sufficient to cause

higher than normal levels of T cell population expansion owing to an increase in survival<sup>43</sup>. However, a more recent study showed that in mice with a T cell-specific deletion of *Foxo3*, there was up to a twofold increase in the number of antigen-specific T cells present in the spleen at day 8 after virus infection<sup>67</sup>. Furthermore, one possibility is that FOXO1 and FOXO3 have overlapping, but possibly distinct, functions in CD8<sup>+</sup> T cell proliferation and differentiation, and future studies will elucidate the specific programmes of gene expression mediated by these two paralogs.

### Conclusion

In almost every aspect of T cell biology so far examined, there is a role for FOXO transcription factors. They respond to a wide range of extrinsic signals to fundamentally alter the trajectory of a T cell-dependent immune response. The programmes of gene expression affected include cell type-specific genes involved in differentiated functions, as well as genes that control the essential aspects of general cellular physiology, such as cell division, survival and metabolism. The challenge will be to isolate the direct effects of FOXO transcriptional regulation from the indirect effects that ripple and echo throughout the signalling network of the cell. Given the right antibodies or tagged versions of FOXO transcription factors, the technology is now available to definitively characterize the FOXO1 and FOXO3 gene targets at each stage of T cell differentiation.

To what end? In addition to providing a map of gene connections, we contend that such an analysis will unveil the relationships between inflammation, metabolism and oxidative stress, and the manner by which these conditions affect the strength and effectiveness of an immune response. How does hyperinsulinaemia affect the course of an acute, persistent or latent infection? Do pathogens that provoke an oxidative burst experience a different response from those that elicit high levels of inflammation? Do these physiological changes act directly on T cells, or are the effects on DCs and macrophages more important? We think that a broad analysis of the part played by FOXO transcription factors in the immune system will illuminate and eventually bring resolution to these issues of medical significance.

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#### Competing interests statement

The authors declare no competing financial interests.

#### FURTHER INFORMATION

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