Published in final edited form as:

Trends Immunol. 2011 January ; 32(1): 26–33. doi:10.1016/j.it.2010.10.005.

Foxo: in command of T-lymphocyte homeostasis and tolerance

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Abstract

The forkhead box O (Foxo) family of transcription factors consists of the mammalian orthologs of the *Caenorhabditis elegans* longevity protein Daf-16, and has an evolutionarily conserved function in the regulation of nutrient sensing and stress responses. Recent studies have shown that Foxo proteins control expression of immune system-specific genes such as *Il7ra* in naïve T cells and *Foxp3* in regulatory T (Treg) cells, which are crucial regulators of T cell homeostasis and tolerance. These findings reveal that the ancient Foxo pathway has been co-opted to regulate highly specialized T cell activities. The Foxo pathway likely enables a diverse and self-tolerant population of T cells in the steady state, which is an important prerequisite for the establishment of a functional adaptive immune system.

Foxo protein and its ancient biological function

The forkhead box (Fox) family of transcription factors is named after the *Drosophila melanogaster* gene *fh* (fork head), a mutation of which causes developmental defects with a spiked head appearance in adult flies[1]. Characterized by the presence of a winged helix forkhead DNA-binding domain, over 40 structurally related Fox proteins have been identified in mammals, which are further classified into subfamilies on the basis of their sequence homology[2]. There are four “O” subfamily members of Fox (Foxo) proteins in mammals: Foxo1, Foxo3, Foxo4 and Foxo6, whereas a single Foxo ortholog is present in lower organisms including *Drosophila melanogaster* and *Caenorhabditis elegans*.

The best understood mechanism by which Foxo proteins regulate gene transcription is through their binding as monomers to cognate DNA-binding sites with a core consensus motif 5'-TTGTTTAC-3'[3]. In addition, Foxo proteins interact with other transcription factor partners such as nuclear receptors, C/EBP β , STATs, Smads and p300[4], which may enable Foxo proteins to regulate broader transcriptional programs. The activity of Foxo proteins is regulated by their subcellular localization, which is influenced by posttranslational modifications such as phosphorylation[3]. Akt is the most studied kinase capable of modifying Foxo protein, and is activated by growth factors via phosphatidylinositol-3-OH kinase (PI3K). Akt phosphorylation of Foxo1, Foxo3 and Foxo4 at three conserved sites diminishes their DNA-binding activity, and triggers their translocation from nucleus to cytoplasm in a complex with 14-3-3 proteins. Phosphorylation of Foxo1 at Ser249 by CDK2, Ser329 by DYRK1A, and Foxo3 at Ser644 by IKK β also

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promotes their cytoplasmic retention. In contrast, phosphorylation of Foxo4 at Thr447 and Thr451 by JNK, and Foxo3 at Ser207 by MST1 triggers nuclear translocation and activation. The activity of kinases that phosphorylate Foxo proteins is under the control of growth factors and stress signals, which enables Foxo proteins to regulate gene expression in response to various environmental cues (Figure 1).

The *C. elegans* Foxo ortholog Daf-16 is a critical regulator of the formation of a Dauer or “non-aging” larval stage in which the worms halt development and survive for months with a dramatically low metabolic rate under nutrient-depleted conditions[5]. Inactivation of Daf-16 by Akt promotes worms to exit the Dauer larval stage. In addition to Foxo proteins, Akt phosphorylates mammalian target of rapamycin (mTOR) and activates the mTOR complex 1 (mTORC1), a crucial stimulator of cell anabolism and an inhibitor of cell catabolism[6]. A recent study showed that reactive oxygen species (ROS) activates *Drosophila* Foxo (dFoxo) via the activation of JNK. dFoxo induces the expression of *Drosophila* sestrin (dSesn) that functions as a feedback inhibitor of the mTORC1 pathway, and prevents ROS accumulation and the development of age-related pathologies[7]. The insulin-Akt-Foxo pathway is well conserved in mammals[5]. An analogous regulatory circuit of Foxo-Sestrin-mTOR is also present in mammalian cells[8]. These findings reveal an evolutionarily ancient function for Foxo proteins in nutrient sensing and stress responses, which is indispensable for the control of cell metabolism and organismal homeostasis.

Survival of metazoans depends on their ability not only to cope with nutrient availability and cellular stress, but also to defend against microbial invasion. A recent report showed that in *Drosophila* dFoxo directly controls the expression of antimicrobial peptide expression in response to nutrient starvation, revealing an intriguing connection between energy metabolism and innate immunity in flies[9]. Immune responses in mammals are much more sophisticated, and are orchestrated by both the innate and adaptive arms of the immune system. The roles of Foxo proteins in lymphocytes have begun to be elucidated in genetically modified mouse models during the last three years. In this article, we will focus our discussion on the specific functions of Foxo proteins in T cells, the key component of the cellular arm of the adaptive immune system.

Control of T cell trafficking by Foxo proteins

A functional adaptive immune system depends on a diverse and self-tolerant population of T cells that are generated in the thymus, and are further maintained in the peripheral lymphoid organs. Thymocytes undergo selection processes to become mature CD4⁺ or CD8⁺ T cells that are endowed with the capability to emigrate from the thymus. Thymic exit of T cells and their migration into peripheral lymphoid organs depend on the expression of a set of trafficking molecules including Sphingosine-1-phosphate receptor 1 (S1P1, *Edg1*), chemokine receptor CCR7 and adhesion lectin CD62L (*Sell*)[10]. Despite normal T cell differentiation in the thymus, S1P1 deficiency results in compromised T cell thymic emigration and severe T cell lymphopenia in the peripheral lymphoid organs[11]. Although CCR7 is not required for thymic T cell egress, it controls thymocyte migration from cortex to medulla, the migration of circulating T cells to peripheral lymphoid organs[12], and intranodal T cell motility[13]. CD62L is also dispensable for thymocyte emigration, but is important for regulating T cell adhesion to high endothelial venules and lymph node homing.

How the expression of these trafficking molecules is induced in mature T cells has begun to be elucidated. The transcription factor Kruppel-like factor 2 (KLF2) is selectively expressed in mature thymocytes. KLF2 deficiency was first reported to cause a reduction of T cells in the secondary lymphoid organs and the accumulation of T cells in the thymus in chimeras

reconstituted with KLF2-deficient fetal liver cells, which is associated with reduced expression of S1P1, CD62L and CCR7[14]. Although specific deletion of *Klf2* in hematopoietic cells results in similar T cell homing defects as in the fetal liver chimeras, it was proposed that T cell reduction in the secondary lymphoid organs was due to a sequestration of T cells in non-lymphoid tissues as a possible consequence of increased expression of inflammatory chemokine receptors including CCR3, CCR5 and CXCR3[15]. However, more recent studies demonstrated that the upregulation of CXCR3 is not directly caused by KLF2 deficiency, but is due to the increased IL-4 production from PLZF⁺ thymocytes[16,17]. KLF2 over-expression activates *Edg1* and *Sell* promoters[14,18,19], implying *Edg1* and *Sell* as direct target genes of KLF2. Among the three Foxo proteins (Foxo1, Foxo3 and Foxo4) expressed in T cells, Foxo1 is specifically upregulated during T cell maturation[20]. Foxo1 was first reported to control expression of T cell trafficking molecules expression in a study using human T cells. Overexpression of an Akt-insensitive Foxo1 mutant in Jurkat cells induces high expression of CD62L, S1P1 and CCR7[21]. In line with these observations, T cell-specific deletion of the *Foxo1* gene in mice results in reduced CD62L, CCR7 and S1P1 expression on T cells[22–24]. The numbers of T cells from lymph nodes, but not spleens, of 3 week old Foxo1-deficient mice are markedly reduced, whereas the number of mature T cells in the thymus is moderately increased. Compound deletion of *Foxo1* and *Foxo3* genes in T cells does not substantially exacerbate the T cell migration defects, revealing a prominent function for Foxo1 in the control of T cell lymph node homing[25]. KLF2 expression is induced upon Foxo1 overexpression, and is reduced in Foxo1-deficient T cells[21,22]. In ChIP experiments, Foxo1 binds to the *Klf2* promoter, suggesting that *Klf2* is a direct Foxo1 target gene[21]. However, it remains to be determined whether KLF2 down-regulation is solely responsible for the defects of trafficking molecule expression in Foxo1-deficient T cells (Figure 2a).

Foxo proteins, T cell survival and homeostatic proliferation

Naïve T cells are well maintained in the peripheral lymphoid organs by homeostatic processes that are dependent on the common γ -chain cytokine interleukin-7 (IL-7)[26]. IL-7 regulates T cell survival and homeostatic proliferation in part through activation of the JAK-STAT pathway, and induction of the anti-apoptotic protein Bcl-2[27]. As IL-7 is constitutively produced by lymphoid stromal cells, T cell responsiveness to IL-7 is primarily regulated by the expression of IL-7 receptor α -chain (IL-7R α ; also known as CD127)[28]. IL-7R α is expressed at high levels in CD4⁺CD8⁺ thymocytes, downregulated in CD4⁺CD8⁺ T cells, re-expressed in CD4⁺ or CD8⁺ T cells, and maintained at high levels in peripheral naïve T cells. The transcription factor GABP has been reported to induce IL-7R α expression in T cells via its binding to the *Il7r* promoter[29]. In contrast, the transcription repressor GFI1 suppresses IL-7R α expression in T cells through its binding to intronic regulatory elements[30].

A role for Foxo1 in the control of IL-7R α expression was revealed in a gene expression study to identify Foxo1 target genes in naïve T cells[23]. Compared to wild-type T cells, Foxo1-deficient CD4⁺ and CD8⁺ naïve T cells express markedly diminished amounts of *Il7r* mRNA, which is associated with low to undetectable levels of IL-7R α protein expression[22,23]. Acute ablation of Foxo1 in naïve T cells downregulates IL-7R α expression as well[22], revealing a crucial function for Foxo1 in maintaining *Il7r* transcription. Mixed bone-marrow chimera experiments further demonstrate a cell-intrinsic function for Foxo1 in promoting IL-7R α expression[22,23]. Foxo1 associates with an evolutionarily conserved Foxo1-binding site located about 3.6kb upstream of the translation start site of mouse *Il7r* gene, which has been shown to be a major DNase I hypersensitive site in naïve T cells[31]. These findings support *Il7r* as a direct Foxo1 target gene in T cells (Figure 2a). Compromised IL-7R α expression results in blunted responses of Foxo1-

deficient naïve T cells to IL-7 survival signals *in vitro*, and lymphopenia-triggered homeostatic proliferation *in vivo*[22,23]. The number of naïve T cells is reduced in peripheral lymphoid organs of Foxo1-deficient mice, which is associated with reduced Bcl-2 expression[22,23]. Perhaps the best demonstration of Foxo1 control of naïve T cell homeostasis is in T cell receptor (TCR)-transgenic OT-II mice[22,23]. Compared to wild-type mice, Foxo1-deficient mice have approximately 10% naïve OT-II T cells in their peripheral lymphoid organs. Transgenic expression of IL-7R α corrects the Bcl-2 expression defect and largely restores OT-II T cell number in the spleen[23], revealing that diminished IL-7R α expression is a major cause of the naïve T cell homeostasis defects in these mice.

IL-7R α expression is dynamically regulated in peripheral T cells. Acute stimulation of T cells by TCR, co-stimulatory receptor CD28, and cytokines including IL-7 itself, suppresses *Il7r* transcription[22]. A prominent signaling pathway activated by these molecules is PI3K-Akt[26,32]. Akt-mediated phosphorylation inactivates Foxo proteins, providing a negative feedback mechanism to inhibit IL-7R α expression (Figure 2b). Indeed, Foxo1 binding to the *Il7r* locus is reduced in IL-7-stimulated T cells, in addition to the presence of decreased amounts of *Il7r* mRNA[22]. This regulatory circuit likely ensures the survival of maximal numbers of naïve T cells with limited amounts of IL-7, and thereby maintains a diverse repertoire of T cells.

Foxo proteins regulate T cell tolerance

The stochastic process by which the TCR is generated creates an inherent problem of some T cells bearing high-affinity TCRs to self-antigens. Despite thymic negative selection, a proportion of self-reactive T cells are released to peripheral tissues. The “escaped” autoreactive T cells are restrained from provoking autoimmune disease by multiple mechanisms. Studies over the past 15 years have established CD4⁺CD25⁺ regulatory T (Treg) cells as pivotal regulators of peripheral T cell tolerance[33]. Treg cells express the signature forkhead family transcription factor Foxp3. Loss-of-function mutations of Foxp3 cause autoimmune disorders in *Scurfy* mice and in human patients suffering from IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome[34]. Depending on the anatomic sites where Treg cells are produced, they can be divided into two subsets: naturally occurring thymus-derived Treg (nTreg) cells and induced Treg (iTreg) cells that are converted from naïve CD4⁺ T cells in the periphery. The observation that neonatal thymectomy of mice on day 3 substantially diminishes the peripheral Treg cell pool suggests that the thymus is the major site for Treg cell production[35].

A role for Foxo proteins in the control of T cell tolerance was first suggested by *in vitro* studies. The observations that naïve T cells express high levels of Foxo proteins, and that over-expression of an Akt-insensitive Foxo1 mutant impairs human T cell proliferation imply a T cell-intrinsic mechanism for Foxo proteins in the regulation of T cell quiescence[36]. The first *in vivo* evidence supporting a role of Foxo proteins in T cell tolerance came from a study of Foxo3-deficient mice which developed a mild lymphoproliferative syndrome associated with inflammatory lesions in multiple organs[37]. However, a recent study showed that two different strains of Foxo3-deficient mice did not show any signs of spontaneous T cell activation or inflammation. Instead, Foxo3 might constrain inflammatory cytokine production by dendritic cells[38]. Mice with a T cell-specific deletion of the *Foxo1* gene have increased numbers of T cells with an activated CD44^{hi}CD62L^{lo} phenotype[22,23]. Five to six month old Foxo1-deficient mice also develop a mild lymphadenopathy phenotype, and have high titers of dsDNA and nuclear antibody in the circulation[23]. In a bone marrow transfer model, Foxo1 deficiency in T cells triggers the development of inflammatory bowel disease, which is associated with reduced numbers of Treg cells[23]. The fact that mixed chimeras of wild-type and Foxo1-deficient bone

marrow cells do not develop disease suggests that Foxo1 regulation of T cell tolerance might not be intrinsic to effector T cells. However, because Foxo1-deficient naive T cells are less competitive than wild-type T cells in these mice, the definitive mechanisms by which Foxo1 regulates T cell tolerance was not determined.

Foxo3 and *Foxo4* genes are highly expressed in CD4⁺CD8⁺ immature thymocytes[25]. Studies using an inducible model of *Foxo* gene deletion reveal that Foxo1, Foxo3 and Foxo4 proteins have redundant functions in the maintenance of haematopoietic stem cells[39], and in preventing carcinogenesis[40]. To test potential redundant functions for Foxo proteins in T cells and to resolve the afore-mentioned controversy of Foxo3 protein in T cells, mice with T cell-specific deletion of *Foxo1* and *Foxo3* genes have recently been generated[25]. Compared to Foxo1-deficient or Foxo3-deficient mice, mice with compound ablation of Foxo1 and Foxo3 proteins develop a lethal inflammatory disease associated with rampant expansion, activation, and effector differentiation of CD4⁺ T cells[25]. In contrast to the dominant role of Foxo1 in controlling trafficking molecule and IL-7R α expression in mature T cells, Foxo1 and Foxo3 cooperatively regulate nTreg cell differentiation as shown by diminished numbers of thymic and splenic Treg cells in 3-week-old Foxo1-3-deficient mice[25]. Transfer of wild-type Treg cells corrects the lymphadenopathy and inflammatory disorder in these mice, whereas Foxo1-3-deficient Treg cells fail to inhibit *Scurfy* T cell-triggered systemic inflammation or naive T cell-induced colitis in transfer models[25]. These findings reveal that Treg cell defects are the major causes of T cell tolerance loss in Foxo1-3-deficient mice.

Foxo protein regulation of nTreg cell differentiation

An outstanding question in the Treg cell field is how Treg cell lineage is specified during T cell development[41,42]. Treg cells exhibit an “antigen-experienced” phenotype, suggesting that their differentiation is induced or accompanied by exposure to high-affinity self-antigens. Recent studies of transgenic mice expressing TCRs derived from Treg cells demonstrate that nTreg cell differentiation is instructed by TCR specificity[43–45]. In one strain of such TCR-transgenic mice, profound T cell deletion occurs, suggesting that at least some endogenous nTreg cells are differentiated in response to high-affinity self-antigens[44]. nTreg cell differentiation is additionally regulated by costimulatory receptors such as CD28 and cytokines including the common γ -chain cytokine IL-2[35]. How these signals are integrated into the differentiation program of nTreg cells, culminating in the stable expression of Foxp3, is an area of active research.

TCR and CD28 stimulation triggers a signaling cascade resulting in the activation of the transcription factor NF- κ B[46]. Treg cell numbers are markedly reduced in mice deficient in critical components of the NF- κ B signaling pathway, including PKC θ , Bcl10, TAK1, CARMA1, and IKK- β [47–51]. In OT-II or P14 TCR transgenic mice, NF- κ B activation via the expression of a constitutively active form of IKK- β bypasses the requirement for high-affinity TCR signals to induce Foxp3 expression[52]. These observations suggest that NF- κ B is likely the major effector molecule downstream of TCR and CD28 signaling pathways to trigger Treg cell differentiation. Indeed, recent studies have revealed that the c-Rel subunit of NF- κ B is required for the differentiation of nTreg cells[53–58]. In activated T cells, c-Rel is recruited to the NF- κ B-binding sites in the *Foxp3* promoter and intronic regulatory regions, supporting *Foxp3* as a direct NF- κ B target gene[52,53,55].

Nevertheless, mere activation of TCR and CD28 signaling is unlikely to be sufficient for commitment of the nTreg cell lineage. In fact, stimulation of TCR and CD28 activates the PI3K-Akt pathway, which adversely regulates Treg cell development. PI3K deficiency leads to increased numbers of nTreg cells in mice[59], whereas sustained Akt activation inhibits

thymic Treg cell differentiation[60]. In addition, a recent study showed that activation of Akt by the trafficking receptor S1P1 attenuates nTreg cell differentiation[61]. In light of the critical function of Akt in Foxo protein inactivation, the negative Akt effects can be likely explained by a requirement for Foxo1 and Foxo3 in nTreg cell differentiation. Several evolutionarily conserved Foxo protein-binding sites are present in the *Foxp3* promoter and intronic regulatory regions where Foxo proteins are recruited specifically in Treg cells[25,62]. Mutation studies reveal a crucial function for a proximal Foxo-binding element in controlling *Foxp3* promoter activity, thus supporting *Foxp3* as a direct Foxo target gene[25,62].

Because NF- κ B and Foxo protein activities are opposingly regulated by TCR and CD28 stimulation, how can both transcription factors be engaged to turn on *Foxp3* gene expression? It is possible that there are undefined factors that specifically attenuate TCR and CD28-induced Akt activation, and therefore maintain both NF- κ B and Foxo proteins in activated T cells to induce *Foxp3* expression. Alternatively, NF- κ B and Foxo proteins may be sequentially recruited to induce *Foxp3* transcription. Differentiation of nTreg cells occurs mostly late during T cell development in the thymic medulla[63]. A recent study showed that thymocytes are very mobile in the medulla, perhaps to allow antigen scanning[64]. Intriguingly, T cells do not appear to cease migration even after they encounter an agonist antigen that can trigger T cell negative selection[64]. It is conceivable that thymic self-antigens are expressed and presented at different levels. T cells subject to continuous high affinity-antigen stimulation will likely be deleted, whereas T cells with infrequent encounters with self-antigens may differentiate into nTreg cells. Following TCR stimulation by sparsely expressed self-antigens, T cells may undergo a period of resting where Foxo proteins are reactivated, and can cooperate with NF- κ B to induce *Foxp3* expression (Figure 3). Such a “hit and run” model of nTreg cell differentiation is supported by the observation that transient activation of thymic conventional CD4⁺ T cells followed by a resting period triggers robust *Foxp3* expression, which is dependent on Foxo proteins[25]. This model is also in line with the finding that IL-2 stimulation alone is sufficient to induce *Foxp3* expression in thymic CD4⁺ T cells with the activated CD4⁺CD25⁺*Foxp3*[−] phenotype[65].

Foxo proteins and iTreg cell differentiation

Besides thymic production of nTreg cells, peripheral naïve CD4⁺ T cells can acquire *Foxp3* expression, and differentiate into iTreg cells. However, compared to mature thymic CD4⁺ T cells, peripheral T cells are much less efficient in turning on *Foxp3* expression when subject to the same activation-resting protocol [25,66]. These findings imply that additional signals are required to trigger *Foxp3* expression in peripheral T cells. Indeed, the cytokine TGF-B has been shown to be a potent inducer of iTreg cell differentiation in TCR-stimulated T cells[67]. TGF-B is a pleiotropic cytokine with diverse functions in the control of T cell responses[68]. Although TGF-B signaling in T cells was proposed to control *Foxp3* expression in nTreg cells[69], a recent study showed that TGF-B promotes nTreg cell development mostly through its inhibition of T cell negative selection[70]. In addition, deletion of a *Foxp3* gene intronic regulatory element in mice that contains the binding sites for TGF-B-activated Smad transcription factors, impairs iTreg but not nTreg cell development[55]. These findings suggest a specific role of TGF-B in the control of iTreg cell differentiation. Although the c-Rel subunit of NF- κ B is essential for nTreg cell development, it is dispensable for TGF-B-induced *Foxp3* gene expression[54,57]. It is conceivable that other TCR-activated transcription factors such as NFAT may play a more important role in iTreg cell differentiation[71]. Together, these findings suggest that different signaling pathways may be engaged to induce *Foxp3* gene expression in nTreg and iTreg cells.

However, recent studies have revealed that Foxo proteins are also essential for TGF- β -induced *Foxp3* gene expression[25,62]. In line with a crucial function for Foxo proteins in iTreg cell differentiation, expression of an active form of Akt or ablation of negative regulators of Akt such as PTEN and Cbl-b inhibits *Foxp3* induction[60,62,66]. The Akt effect has been shown to be partially dependent on mTOR, as the mTOR inhibitor rapamycin attenuates Akt blockade of TGF- β -induced iTreg cell differentiation[60,66]. mTOR signaling is mediated by mTORC1 and mTORC2 complexes. mTORC1 is a downstream effector of Akt, whereas mTORC2 is an upstream regulator of Akt phosphorylation at Ser473, a crucial modification for Akt inactivation of Foxo proteins[72]. Although mTORC1 is a well-known target of rapamycin, rapamycin can inhibit the assembly and signaling of mTORC2 in some cell types. Recent studies showed that T cells deficient in mTORC2 but not mTORC1 are more prone to differentiating to iTreg cells[73,74], suggesting that the rapamycin effect is likely through its inhibition of mTORC2-dependent Foxo inactivation. Together, these findings demonstrate that Foxo proteins play a critical role in the induction of *Foxp3* expression in both nTreg cells and iTreg cells (Figure 4).

Concluding remarks

Foxo proteins are important regulators of energy metabolism and stress responses that are highly conserved during evolution. Studies in the past few years have started to reveal pleiotropic yet critical roles of Foxo proteins in the mammalian adaptive immune system. The first discoveries regarding Foxo proteins in T cells demonstrate their pivotal functions in the control of T cell migration, survival, and tolerance, functions that are in part mediated by Foxo protein regulation of immune system-specific gene expression. It thus appears that the ancient Foxo pathway has been incorporated into diverse genetic programs that are crucial for the normal functions of T cells. However, it remains incompletely understood how the Foxo pathway has been rewired at the molecular level to regulate T cells. The functions of Foxo proteins in control of T cell responses under disease settings also warrant further investigation. Gaining such additional insights may facilitate targeting of the Foxo pathway to treat a variety of T cell-mediated immune disorders.

Acknowledgments

Work in our laboratory is supported by grants from the National Institute of Arthritis, Musculoskeletal and Skin Diseases (KO1 AR053595 and RO1 AR060723), the Starr Cancer Consortium (13-A123), the Arthritis Foundation and the Rita Allen Foundation.

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Figure 1. Phosphorylation and translocation of Foxo proteins in response to growth signals and stress

Foxo protein phosphorylation at three conserved residuals by growth factor-activated Akt through PI3K-PDK1 and mTORC2 kinases promotes the release of Foxo proteins from DNA, and their subsequent cytoplasmic translocation in a complex with 14-3-3 proteins. By contrast, Foxo protein phosphorylation by stress-activated JNK or Mst1 kinases promotes Foxo protein nuclear translocation and activation even in the presence of Akt-dependent phosphorylation.

Figure 2. Foxo1 regulation of genes involved in T cell trafficking and homeostasis

(a) Foxo1 promotes the expression of trafficking molecules S1P1, CD62L, CCR7, and the homeostasis regulator IL-7R α in naïve T cells. Foxo1 enhances S1P1 and CD62L expression likely in part via its control of transcription factor KLF2 expression. Solid lines depict direct transcriptional activation, whereas dotted lines indicate potential direct regulation. **(b)** A feedback mechanism in the control of *Il7ra* gene expression. Signaling initiated by TCR, CD28, and/or IL-7 stimulation induces Akt activation that inactivates Foxo1 via phosphorylation, resulting down-regulation of *Il7ra* gene transcription.

Figure 3. A “Hit and Run” model for thymic Treg cell differentiation

Thymocytes receiving low-affinity antigen stimulation are positively selected, and develop into conventional T cells. T cells do not express Foxp3 in the absence of nuclear NF- κ B. Thymocytes receiving continuous high-affinity antigen stimulation are deleted by negative selection. T cells fail to express Foxp3 due to the inactivation of Foxo proteins. Thymocytes with infrequent encounters with high-affinity antigens develop into natural Treg cells. High-affinity antigen stimulation in the presence of B7-activated CD28 signals activates NF- κ B via IKK, and inactivates Foxo proteins via Akt. Upon the cessation of antigen stimulation, Foxo proteins translocate back to the nucleus, and cooperate with NF- κ B to induce *Foxp3* gene transcription. Signals from the common γ -chain cytokine IL-2 may also regulate Foxp3 expression.

Figure 4. Molecular control of TGF-β-induced Treg cell differentiation

NFAT and Stat5 activated by TCR and IL-2 signaling pathways respectively, and the Smad complex activated by TGF-β cooperate with Foxo to induce *Foxp3* gene transcription. TCR stimulation inactivates Foxo via Akt-induced phosphorylation, whereas TGF-β signaling likely maintains Foxo activity through undefined mechanisms (depicted as dotted lines). PTEN and Cbl-b promote iTreg cell differentiation through the attenuation of PI3K activation. Although rapamycin inhibits mTORC1, it may also block mTORC2 and the consequent Akt activation to enhance Foxo activity and *Foxp3* expression.