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BRIEF REVIEWS

FOXO Transcription Factors as Regulators of Immune Homeostasis: Molecules to Die for?

Kim U. Birkenkamp and Paul J. Coffer

Regulation of phosphatidylinositol 3-kinase (PI3K) activity has been demonstrated to be critical for correct lymphocyte function. The molecular targets of this lipid kinase have been the subject of extensive research, and many functional effects of PI3K activation are thought to be mediated by the serine-threonine kinase protein kinase B (PKB/c-akt). Genetic analyses in the nematode worm Caenorhabditis elegans have identified a novel PI3K-regulated signaling pathway that regulates organism lifespan through inhibition of a Forkhead (FOX) transcription factor, DAF-16. Recent studies have subsequently revealed an evolutionarily conserved signaling module in higher eukaryotes in which PKB can directly phosphorylate and inactive a family of Forkhead box class O (FOXO) transcription factors. Phosphorylation results in nuclear exclusion and inhibition of transcription. FOXO transcription factors have been found to play critical roles in regulation of proliferation, apoptosis and control of oxidative stress. This occurs through both activation and repression of target gene expression by multiple mechanisms. Here the regulation and function of these transcription factors is discussed with specific relevance to immune homeostasis. A greater understanding of the regulation and function of this signaling pathway in lymphocytes may provide novel therapeutic opportunities for immune diseases.

Stimulation of cells by cytokines, growth factors, and hormones results in the phosphorylation of plasma membrane-associated inositol lipids and the subsequent initiation of a cascade of events that can result in cell survival, division, or differentiation. The molecular mechanisms by which inositol lipid phosphorylation results in dramatic changes in cellular homeostasis have been the subject of extensive studies over the last decade. Pivotal in understanding the mechanisms regulating these processes was the discovery of the inositol lipid kinase, phosphatidylinositol 3-kinase (PI3K) (1). PI3Ks phosphorylate the D3 position of the inositol head group of the phosphoinositide lipid PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3. The generation of these phosphorylated lipid products in the plasma membrane results in the relocalisation and assembly of complexes of intracellular signaling molecules containing so-called PH-domains (1) (Fig. 1). One of these, the protein serine-threonine kinase protein kinase B (PKB/c-Akt), has been the subject of much attention as it appears to mediate many of the cellular functions previously ascribed to PI3K (2). Critical in attenuation of PI3K signaling is dephosphorylation of the lipid products generated by PI3K by phophatase and tensin homolog deleted on chromosome ten (PTEN), a phosphoinositide phosphatase.

The PI3K family contains various isoforms with diverse functions and has been subdivided into several classes (1). The class IA PI3Ks are composed of a regulatory subunit and a catalytic subunit, of which currently four isoforms (p110α, β, γ, δ) have been identified in mammalian cells. p110α, p110β, and p110δ, associate with a regulatory molecule which can include p85α, p85β, or p55γ, all generated by alternative splicing of the same gene. The specific functions of potential heterodimers of these catalytic and regulatory subunits remain unclear, although this class of PI3K isoforms tend to be activated by receptors with intrinsic or associated tyrosine kinase activity. In contrast, the class IB PI3Ks are regulated by G-protein coupled receptors, and the sole catalytic subunit, p110γ, associates with a p101 adapter protein.

The specific role of the different PI3K isoforms in immune cell function have been analyzed using specific, targeted gene deletions (3). Studies in mice that were deficient in all the class IA regulatory subunits demonstrated that these animals have impaired B cell development and reduced numbers of peripheral mature B cells. The B cells that are present show decreased proliferative responses. Surprisingly, no apparent defects in T cell activation were found in p85-deficient cells. However, studies with mutants of the different catalytic subunits demonstrated that PI3K does play an important role in T cell proliferation and survival. p110α and p110β knockout mice were found to be embryonic lethal thus hampering analysis of their individual roles on immune function. But, p110δ loss of function mutants demonstrated impaired T cell proliferation and function, and a reduced number of T cells, with a more naive phenotype. This suggests that T cells develop normally, but fail to mature or survive in the periphery. p110γ knockout mice showed major defects in functions of innate immune cells, a decreased number of splenic CD4^+ T cells, proliferation of T

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2 Abbreviations used in this paper: PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; FOXO, Forkhead box class O; FKHR, Forkhead in rhabdomyosarcoma; SGK, serum- and glucocorticoid-induced kinase; NLS, nuclear localization signal; CDK, cyclin-dependent kinase; CKI, CDK inhibitor.
cells was reduced and the thymocytes exhibited enhanced apoptosis. These data, together with the fact that mice ectopically expressing an active PI3K variant show enhanced T cell viability and reduced susceptibility to Fas-mediated apoptosis, demonstrate that PI3K plays a central role in lymphocyte survival, proliferation, and function.

While a role for PI3K in lymphocyte homeostasis is clear from the studies outlined above, the importance of PKB has only recently been investigated. In transgenic mice that express a constitutively active variant of PKB (gagPKB) specifically in T cells, lymphocytic infiltration was observed in several organs, and mature T cells were resistant to Fas-induced apoptosis (4). In another similar study, mice developed thymomas at an early age, primarily due to inappropriate cell cycle control (5). One mechanism by which PI3K-PKB may regulate lymphocyte survival is through the transcriptional regulation of pro- or anti-apoptotic molecules. For example, in gagPKB transgenic mice, anti-apoptotic Bcl-XL protein levels were increased in thymocytes and T cells (4). Indeed the hunt for transcription factor targets which are directly regulated by PKB has now become critical to understanding how this kinase can modulate lymphocyte homeostasis.

Of worms and FOXes: a novel family of PKB-regulated transcription factors

Initial investigations leading to identification of Forkhead transcription factors as key effectors of PI3K-PKB signaling began in the nematode worm Caenorhabditis elegans. This soil dwelling organism feeds on bacteria and usually lives for a few days. However, when environmental conditions are adverse, worms can enter a so-called Dauer or “non-aging” larval stage where they can survive for months. This is regulated by a neuroendocrine pathway which has been extensively studied by genetic analysis (Fig. 1, inset). Regulation of a C. elegans PI3K (age-1) signal transduction pathway was found to be critical in modulation of diapause entry. Critically, mutations in the Forkhead transcription factor DAF-16 completely suppress the dauer arrest, metabolic shift and longevity observed in age-1 mutants (6, 7).

Members of the Forkhead superfamily are characterized by the presence of a conserved 110 aa DNA binding domain, called the “forkhead” domain or “winged helix” domain (8). Recently, a new nomenclature for these factors has been adopted and they are now denoted Forkhead box (FOX) factors. Within the larger family of FOX factors, a subfamily has recently been identified with homology to DAF-16: the Forkhead box class O (FOXO) factors. Three mammalian proteins belong to this subfamily: Forkhead in rhabdomyosarcoma (FKHR), FKHR-like 1, and acute-lymphocytic-leukemia-1 fused gene from chromosome X (AFX). FKHR is now known as FOXO1, FKHR-like 1 is known as FOXO3a, and AFX is known as FOXO4, respectively. These FOXO proteins interact preferentially with a core consensus recognition motif 5’T-TTGTTTAC-3’, although the residues flanking this core contribute to the DNA binding specificity. Interestingly, the FOXO factors were initially identified at chromosomal breakpoints in several human tumors. These genetic alterations of FOXOs in human cancers strongly suggested that they may play a role in the regulation of proliferation, survival or differentiation in higher organisms.

Regulation of FOXO activity

Functional consequences of phosphorylation and localization of FOXOs. As discussed, mechanistic evidence suggesting that FOXO isoforms are regulated by PKB was initially obtained from genetic studies in the nematode worm C. elegans (6, 7). Analysis of the DAF-16 sequence revealed that this transcription factor contains four putative consensus sequences for PKB phosphorylation (RXRXXS/T), suggesting the possibility

![Figure 1](http://www.jimmunol.org/DownloadedFrom)
of direct phosphorylation of DAF-16 by PKB. Importantly, three of these residues are conserved in the mammalian FOXO isoforms (Fig. 2).

Although the concept that FOXO function could be mediated by direct PKB phosphorylation was proposed by C. elegans researchers several years ago, it was work by the groups of Burg-erling and Greenberg (9, 10) that initially demonstrated this in mammalian systems. Subsequently FOXOs have been found to be phosphorylated in vivo on multiple threonine (T1, T2) and serine residues (S1, S2, S3, S4, S5) (Fig. 2). Three of these phosphorylated residues, one N-terminal threonine and two C-terminal serines (T1, S1 and S2) lie within a consensus motif for phosphorylation by PKB. Indeed, FOXO proteins can be phosphorylated by PKB on these sites, both in vitro and in vivo, although with varying stoichiometry (9–12).

Several reports have recently indicated that phosphorylation of FOXOs may be more complex and involve additional sites on these proteins. For example, the serum- and glucocorticoid-induced kinase (SGK) is also capable of inactivating FOXO3a (13). SGKs are closely related to PKB and their activation is also dependent upon PI3K-activity. Interestingly, SGK and PKB display differences with respect to the efficacy with which they phosphorylate the regulatory sites of FOXO3a. As the phosphorylation of each regulatory site of FOXO3a appears to be critical for the efficient exclusion from the nucleus (see below), it is likely that SGK and PKB cooperate in coordinately regulating FOXO transcription factors.

Recently, two further kinases have been identified that can phosphorylate FOXOs on additional sites. Phosphorylation by the dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) decreases the ability of FOXO1 to stimulate transcription, and reduces the proportion in the nucleus (14). In addition, PKB-catalyzed phosphorylation of the residue equivalent to S2 in FOXO1 creates a recognition motif for Crm1 and this is critical for the inhibition of FOXO transcription (15).

Phosphorylation of S5 (15).

PKB-mediated phosphorylation of FOXOs occurs in the nucleus and creates docking sites for so-called 14-3-3 proteins. 14-3-3s are abundant, ubiquitously expressed molecules that preferentially bind to specific motifs containing phosphorylated serine and threonine residues. In the nucleus, phosphorylated FOXOs bind to 14-3-3 proteins immediately before they relocalise to the cytoplasm, and thus 14-3-3 proteins have been postulated to play a direct role in nuclear export (17). Once in the cytoplasm, phosphorylated FOXOs remain complexed to 14-3-3 proteins preventing nuclear import. It has been proposed that 14-3-3 proteins obscure the NLS or nuclear export signal of the protein to which they bind, because they impart no specific information concerning subcellular targeting themselves (17, 18). In this way, 14-3-3 proteins would affect subcellular localization of their binding partners by sterically interfering with the association of transport receptors inhibiting import receptor binding.

Recently it has been suggested that phosphorylation may also regulate FOXO transcriptional activity through an alternative mechanism. The phosphorylation of FOXOs has been proposed to directly inhibit DNA binding (19, 20). This might imply that signaling by PKB would be primarily required to release FOXOs from the DNA rather than to induce their cytoplasmic retention. In all three FOXO isoforms the NLS is located in the DNA-binding domain. In FOXO1, part of this putative NLS lies within the PKB phosphorylation motif, suggesting that phosphorylation contributes to nuclear exclusion by masking the NLS (21). In addition, phosphorylation of FOXO1 on the two CK1-specific sites has been proposed to accelerate nuclear export and/or disrupt nuclear retention (15). In summary it is likely that phosphorylation and 14-3-3 binding involves multiple events resulting in release of FOXOs from DNA and subsequent nuclear-cytoplasmic shuttling.

Transcriptional regulation through multiple mechanisms: FOXO’s partners in crime. The interaction of FOXOs with accessory proteins or cofactors might modulate transcriptional activity as well as determine which target genes are regulated by specific FOXOs (Fig. 3). A recent paper has shed some light on the potential role of such cofactors in regulating FOXO activity (22). Using a comprehensive gene array analysis, it was demonstrated that FOXOs can activate two different subsets of genes: (1) genes that require FOXO DNA binding, and, intriguingly, (2) genes regulated independently of FOXO DNA binding. For example, a down-regulation of D-type cyclins was observed by a FOXO1 mutant that can no longer bind DNA (22). This suggests that FOXOs interact with other transcription factors, thereby modulating their activity.

**FIGURE 2.** Regulation and function of FOXO phosphorylation. A schematic representation of FOXO transcription factors and their potential sites of phosphorylation. The corresponding amino acids are indicated where phosphorylation has been reported in vitro and/or in vivo. NES, nuclear export sequence; Ral-dK, Ral GTPase—dependent kinase.
Transcriptional Activation

- Direct binding of FOXO to promoter, activating transcription
- FOXO co-operates with other TFs to activate transcription

Transcriptional Repression

- FOXO tethers away co-factor(s) to repress transcription
- FOXO tethers away specific transcription factors resulting in repression of specific TF-dependent target genes

FIGURE 3. Potential mechanisms of transcriptional regulation by FOXOs.

In support of this, several recent studies have demonstrated that FOXOs can indeed interact with different binding partners. For instance, it has been shown that FOXO1 can interact with both the transcriptional coactivator p300/CREB-binding protein, an acetyl transferase that modulates the activity of its targets by acetylation, and also as the steroid receptor coactivator protein (23). Furthermore, FOXO1 was shown to interact with both steroid and nonsteroid nuclear receptors. FOXO1 differentially regulated the transcription mediated by these different nuclear receptors, either as a coactivator or corepressor, depending on the receptor and cell type (24, 25). In differentiating human endometrial stromal cells it was shown that FOXO1 directly interacts and transcriptionally cooperates with C/EBPβ, a transcription factor which has been shown to play a role in differentiation (26). Finally, roles for FOXOs in modulating HNF-4 and STAT3-mediated transcription have very recently been reported (27, 28). Interestingly, FOXO1 increased STAT3 expression but did not negatively regulate the transactivation mediated by these different nuclear receptors, either as a coactivator or corepressor, depending on the receptor and cell type (24, 25).

The ability of the different FOXO isoforms to specifically interact with transcriptional cofactors predicts distinct biological functions for these proteins (Fig. 3). This is born out by the distinct subset of genes modulated by DNA-binding deficient FOXO1 relative to wild-type FOXO1 (22). In fact this mutant also had the ability to suppress tumor formation in nude mice, suggesting that interaction of cofactors is in fact critical in modulating the functional effects of FOXO isoforms.

Functional consequences of FOXO activation

The PI3K/PKB/FOXO signaling module appears to be remarkably conserved during evolution. While the regulation and function of FOXO activity has been resolved in detail through genetic analysis in C. elegans, in mammalian cells FOXO function has only recently started to be explored.

Regulation of lymphocyte survival. The regulation of programmed cell death plays a critical role in the maintenance of self-tolerance in the immune system. The number of T cells that leave the thymus and enter the peripheral T cell pool is a minor fraction of the number initially generated. Thymocytes that express receptors binding to self-peptide-complexes with high affinity, are negatively selected and die. The negative selection of thymocytes is clearly mediated by apoptosis and the key mediators of this process are just starting to be identified. The innate immune system must also be able to adapt to sudden changes in the production of blood cells, such as during microbial infection. Therefore, the size of each hematopoietic subpopulation is controlled by continually counterbalancing stem cell renewal and differentiation with apoptotic cell death. A plethora of lineage specific survival factors result in PI3K/PKB-mediated phosphorylation and inhibition of FOXOs in these cell types. For instance, IL-2 and IL-3 which play important roles in the proliferation and survival of lymphocytes inactivate FOXO3a via PI3K/PKB-mediated phosphorylation (30, 34).

Although FOXO3a expression has been shown to be regulated by a variety of receptors present on immune cells, Greenberg and coworkers (10) were the first to describe a role for FOXO3a in the modulation of programmed cell death. Here, a constitutively active FOXO3a mutant was found to induce apoptosis of Jurkat T cells in a Fas-dependent manner (Fig. 4). However this appears to be a cell type-dependent effect.
because Fas-independent mechanisms of apoptosis have been observed in other cell lines (35).

While Fas has been clearly implicated in regulating lymphocyte death, there are alternative pathways resulting in the same cellular fate. In contrast to Fas, Bcl-2 regulates a separate pathway leading to T cell death (36). The Bcl-2 family of apoptotic regulators consists of pro- and anti-apoptotic members whose individual levels determine whether a cell will survive or die. An abundance of pro-apoptotic Bcl-2 proteins results in a breakdown of mitochondrial integrity, leakage of cytochrome c into the cytoplasm and caspase activation. The pro-apoptotic Bcl-2 family member Bim has been shown to be dramatically up-regulated by FOXO transcription factors (34, 35, 37). Withdrawal of cytokines from survival factor-dependent lymphocyte cell lines results in an up-regulation of Bim expression, concomitant with induction of the apoptotic program (Fig. 4). Specific activation of FOXO3a alone was found to be sufficient to induce Bim expression, and could recapitulate all known elements of the apoptotic program normally induced by cytokine withdrawal (34, 35, 37). In the immune system Bim has been critically implicated in modulating lymphocyte homeostasis. Bim<sup>-/-</sup> mice succumb to autoimmune kidney disease, accumulation of lymphoid and myeloid cells and perturbed T cell development (38). In addition, lymphocytes were refractory to apoptotic stimuli and showed impaired negative selection. Because Bim is a direct target of FOXOs, (34, 35, 37), this suggests that FOXOs play an important role in regulating T cell selection through regulation of pro-apoptotic Bcl-2 family members.

**Oxidative stress and organismal lifespan.** In *C. elegans*, worms deficient in components of the PI3K-PKB pathway have enhanced DAF-16 activity resulting in enhanced lifespan, or entry into the Dauer larval stage. These mutants develop a resistance to cellular stress that may play a role in development of the observed phenotypes. FOXOs have recently also been demonstrated to play a role in the stress response of mammalian cells, thereby providing protection from oxidative stress. Intracellular reactive oxygen species are thought to contribute to aging in a wide spectrum of organisms, although the precise mechanism remains elusive. Following activation, lymphocytes produce increased levels of reactive oxygen species, which may serve as an intracellular second messenger (39). Antioxidants can block MHC/Ag activation-induced death of specific T cell hybridomas and thus a redox imbalance may play a role in regulating programmed cell death.

It was recently shown that FOXO3a can protect quiescent cells from oxidative stress by directly increasing expression of manganese superoxide dismutase (MnSOD), an anti-oxidant enzyme (40). Cells deficient in MnSOD were no longer protected from oxidant damage by ectopic expression of FOXO, suggesting that this is a critical defense pathway. These results are consistent with previous studies in *C. elegans* demonstrating...
that life-extending mutations in the DAF-16 pathway requires the presence of a gene that encodes a cytosolic catalase, which degrades hydrogen peroxide (41).

The growth arrest and DNA damage response gene Gadd45a was also shown to be a direct target of FOXO3a (42) indicating that FOXOs can play a role in the resistance of cells to stress by regulating DNA repair. It is suggested that under low stress conditions, FOXOs may promote DNA repair, whereas at higher levels of cellular stress, programmed cell death is induced. The ability to protect certain cells from damage suggests that, similar to the nematode worm, the evolutionary conserved FOXO transcription factors may also regulate life span in mammalian cells.

**Perspectives**

In conclusion, activation of FOXOs causes growth suppression in a variety of cell types, while in cells of the immune system, programmed cell death is often induced. These different effects can most probably be ascribed to the fact that FOXO activation results in cell type-specific gene regulation, which can be regulated by interaction of FOXOs with other transcription factors or accessory proteins (Fig. 3). What is then the benefit for the organism of this type of regulation? In the immune system lymphocytes undergo a process of selection where large number of cells must be eliminated. Cells of the innate immune system are produced daily in relatively large numbers in the bone marrow, and thus they must also be readily eliminated. In cell systems in which a rapid turnover of cells is required for the well being of the organism, the stress-induced activation of FOXOs results in cell death. In contrast, in cells that are not continuously produced, but rather are still required by the organism, stress-induced FOXO activity results in cell cycle arrest, letting them survive the adverse conditions. These quiescent cells are kept alive through the FOXO-mediated resistance to stress, for example by up-regulation of oxidant scavenging proteins. This is similar to observations made in *C. elegans* where, in the absence of nutrients, DAF-16 is activated, resulting in Dauer formation, and longevity as opposed to programmed cell death. This observation concerning the ability of FOXO transcription factors to affect the nematode worm, the evolutionary conserved FOXO transcription factors may also regulate life span in mammalian cells.

The broad expression of FOXO transcription factors in cells of the immune system suggests that they could be critical in modulating immune homostasis and lymphocyte selection. That the functions of this family of transcription factors relies heavily on cell-type specific interactions with cofactors, suggests that it might be possible to generate cell-type specific FOXO inhibitors or activators. While this is an attractive idea, we will have to await further research on this new family of Forkhead proteins to determine whether their functions can be exploited therapeutically.

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