Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors

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Abstract
Recently, the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors has been identified as direct targets of phosphoinositide 3-kinase-mediated signal transduction. The AFX (acute-lymphocytic-leukaemia-1 fused gene from chromosome X), FKHR (Forkhead in rhabdomyosarcoma) and FKHR-L1 (FKHR-like 1) transcription factors are directly phosphorylated by protein kinase B, resulting in nuclear export and inhibition of transcription. This signalling pathway was first identified in the nematode worm Caenorhabditis elegans, where it has a role in regulation of the life span of the organism. Studies have shown that this evolutionarily conserved signalling module has a role in regulation of both cell-cycle progression and cell survival in higher eukaryotes. These effects are co-ordinated by FOXO-mediated induction of a variety of specific target genes that are only now beginning to be identified. Interestingly, FOXO transcription factors appear to be able to regulate transcription through both DNA-binding-dependent and -independent mechanisms. Our understanding of the regulation of FOXO activity, and defining specific transcriptional targets, may provide clues to the molecular mechanisms controlling cell fate decisions to divide, differentiate or die.

Introduction
Cellular proliferation that is induced by growth factors or cytokines can occur only in the presence of distinct survival signals. Cells that receive proliferative signals in the absence of survival signals do not proliferate, but rather die by a process termed apoptosis. In this way, apoptosis acts as an important mechanism for the elimination of cells that harbour mutations in cell-cycle regulatory proteins that may otherwise result in uncontrolled cell proliferation. Generally, in addition to exhibiting unrestrained proliferation, tumour cells also need to have adapted mechanisms to overcome apoptosis.

Apoptosis itself is a process characterized by morphologically distinct features, including plasma membrane blebbing, shrinkage of the nucleus and cytoplasm, loss of mitochondrial transmembrane potential, cleavage of intracellular proteins and degradation of chromosomal DNA. The specific cleavage of proteins and resultant disassembly of the cell is carried out by cysteine proteases or ‘caspases’ [1]. Initial caspase activity is induced through the recruitment of caspase pro-enzymes to activator molecules, promoting caspase oligomerization and auto-activation. An important family of proteins involved in the regulation of caspase activation is the Bcl-2 family [2]. Bcl-2 proteins are conserved through evolution and, in the nematode worm Caenorhabditis elegans, the Bcl-2 homologue CED-9 was found to be able to suppress apoptosis by preventing caspase activation [3]. Bcl-2 family members control cell survival by regulating mitochondrial permeability, and thereby also the release of cytochrome c into the cytoplasm, which itself triggers caspase activation. Maintaining an appropriate balance between pro- and anti-apoptotic Bcl-2 family members is a crucial mechanism by which cellular homoeostasis is achieved.

Protein kinase B (PKB) and suppression of programmed cell death
One signalling molecule that has recently received considerable attention as a suppresser of apoptosis is the phosphoinositide 3-kinase (PI3K) effector PKB (also known as c-Akt) [4–6]. Several mechanisms have been proposed by which this serine/threonine kinase mediates cell survival. Firstly, PKB activity can affect the balance of Bcl-2 family members by, for example, transcriptionally up-regulating the anti-apoptotic Mcl-1 [7]. Furthermore, direct phosphorylation of pro-apoptotic Bad (Bcl-2/Bcl-XL-antagonist, causing cell death) by PKB inhibits its activity [8]; however,
Regulation of FOXO transcription factors is conserved between lower and higher organisms

(A) Genetic analysis of *C. elegans* longevity has identified DAF-16 as having a critical role in this process. This transcription factor was found to be regulated by a PI3K-controlled signalling module conserved in mammals. (B) Schematic representation of FOXO showing phosphorylation sites known to be phosphorylated by PKB and/or SGK.

whether or not this is a critical target for PKB-mediated rescue from apoptosis is still controversial [9]. Inhibitory phosphorylation of caspase-9 by PKB is another potential mechanism of inhibiting apoptosis in some systems [10]. Recently, PKB has also been linked to inhibition of death-receptor-induced apoptosis through an increase in c-FLIP expression which inhibits caspase-8 [11]. Finally, PKB has also been shown to inhibit two protein kinases that have been implicated in the induction of apoptosis: apoptosis-signal-regulating kinase-1 (ASK-1) and glycogen synthase kinase-3 (GSK-3). PKB associates with and inhibits ASK-1 activity by direct phosphorylation. This in turn leads to a reduction in JNK (c-Jun N-terminal kinase) activity and inhibition of apoptosis induced by oxidative stress or ASK-1 over-expression [12]. Similarly, activation of GSK-3 has been found to be sufficient to induce apoptosis in neurons [13]. This is inhibited by PKB-mediated phosphorylation of GSK-3, and thus provides another potential mechanism by which PKB may regulate cell survival.

Forkhead transcription factors

Recent studies have revealed that PKB can directly regulate gene expression through control of the Forkhead family of transcription factors. This family of transcription factors consists of approx. 90 members that are evolutionarily conserved [14]. Forkhead transcription factors are classified according to their conserved DNA-binding domain, characterized by a structure of three α-helices and two characteristic large loops or ‘wings’.

Initial clues that PI3K controls Forkhead activity came from studies performed with the nematode worm *C. elegans*. Genetic studies established that PI3K suppresses the function of DAF-16, a Forkhead transcription factor [15,16]. This was found to be critical for proper control of metabolism and life span in this organism (Figure 1A). Analysis of the DAF-16 amino acid sequence revealed three sites that correspond to the consensus PKB phosphorylation site, suggesting that DAF-16 and other Forkhead transcription factors might be direct targets of PKB. Three human orthologues of DAF-16 have been identified, AFX (acute-lymphocytic-leukaemia-1 fused gene from chromosome X), FKHR (Forkhead in rhabdomyosarcoma) and FKHR-L1 (FKHR-like 1). Interestingly these proteins were initially identified at chromosomal break points in several human tumours. Recently, a new nomenclature has been proposed and this subfamily of Forkhead factors are denoted FOXO (Forkhead box, class O) factors [17]. Thus FKHR corresponds to FOXO1, FKHR-L1 to FOXO3a and AFX to FOXO4 respectively. FOXO transcription factors bind as monomers to a consensus DNA-binding sequence, through binding of helix-3 to the major groove of DNA. This optimal core sequence has been determined to be TTGTATTAC [18].

Regulation of FOXO transcriptional activity

How, then, is PKB involved in regulating FOXO activity? Several groups have independently shown that PKB can directly phosphorylate FOXO transcription factors, and thereby inhibit their ability to induce the expression of target genes [19,20]. In fact, FOXO factors are phosphorylated on multiple threonine and serine residues, which are themselves conserved between the different transcription factors. PKB can phosphorylate three of these sites both *in vitro* and *in vivo* on these sites, although with varying stoichiometry (Figure 1B) [19–21].
In the absence of cellular stimulation, FOXOs are localized in the nucleus, where they activate transcription of target genes. However, upon activation of PKB by growth or survival factors, PKB phosphorylates FOXOs at the specific regulatory sites, eliciting their relocalization from the nucleus to the cytoplasm. Several reports have recently indicated that the phosphorylation of FKHR and its homologues may be more complex and involving additional sites on these proteins, mediated by alternative signalling pathways. The serum- and glucocorticoid-induced kinase (SGK) is capable of inactivating FKHR-L1 [22]. SGKs are closely related to PKB and their activation is also dependent upon PI3K/PDK1 (3′-phosphoinositide-dependent kinase-1) activity. Interestingly, SGK and PKB display differences with respect to the efficacy with which they phosphorylate the regulatory sites of FKHR-L1 (Figure 1B) [22]. As the phosphorylation of each regulatory site of FKHR-L1 appears to be critical for the efficient exclusion of FKHR-L1 from the nucleus, it is likely that SGK and PKB co-operate to promote cell survival by co-ordinately regulating FOXO transcription factors.

Furthermore, a role for the small GTPase Ras has been described in the regulation of FOXO [21]. Activated Ras can stimulate the exchange factor, Rap1GEF, for another small GTPase, Rap. Binding of Ras to Rap1GEF results in Rap activation. The Ras/Rap pathway was found to initiate phosphorylation of AFX on two sites that are not phosphorylated by PKB or SGK, thereby augmenting its activity. Surprisingly, when activation of Rap was combined with activation of PKB, by introducing oncogenic Ras into cells, active Ras appeared to enhance the inhibition by PKB. Although the kinase downstream of Rap still has to be identified, this suggests an important role for the Ras/Rap signalling pathway in regulating AFX activity.

### Nuclear import/export of FOXO transcription factors

In the absence of PKB activity, the FOXO transcription factors are predominantly nuclear and are presumed to be active. PKB-mediated phosphorylation of FOXOs on the three conserved residues occurs in the nucleus, and creates docking sites for so-called 14-3-3 proteins. Phosphorylated FOXOs bind to 14-3-3 proteins in the nucleus immediately before FOXOs relocalize to the cytoplasm, and thus 14-3-3 proteins have been postulated to play a direct role in nuclear export. Once in the cytoplasm, FOXOs remain phosphorylated and complexed to 14-3-3 proteins thereby preventing nuclear import (Figure 2). Nuclear import and export of FOXOs is a highly regulated process, which depends on the activity of the export receptor Crm1 and is regulated by the small GTPase Ran [24].

Recently, it has been shown that 14-3-3 proteins may not themselves directly mediate nuclear transport [25]. This study demonstrated that the C-terminal α-helix of 14-3-3, which has been proposed as a nuclear export signal (NES), instead functions in FOXO binding. Efficient nuclear export of FKHR-L1 appears to require both intrinsic NES sequences within FKHR-L1, phosphorylation and the subsequent 14-3-3 protein binding [25]. The binding to 14-3-3 proteins has also been suggested to inhibit DNA binding of FOXO [26]. This implies that phosphorylation by PKB would be required primarily to release FOXOs from the DNA rather than to induce their cytoplasmic retention. Another proposed mechanism is that phosphorylation might disturb the interaction of FOXO with co-factors which are essential to activate transcription. In support of this hypothesis, it has recently been demonstrated that phosphorylation of FKHR/FKHR-L1 inhibits binding to the p300/CBP (cAMP-response-element-binding-protein-binding protein) histone acetylase [27].

### Transcriptional targets of FOXO action

As mentioned above, studies in the *C. elegans* have revealed that DAF-16 is critical in regulating both exit from the dauer larval stage and aging in the adult stage [15,16]. However, the function of FOXO transcription factors in mammalian cells has only recently started to be appreciated (Table 1). Activation of FOXOs in haematopoietic and neuronal cells can result in the induction of apoptosis. In lymphocytes, the induction of apoptosis by FKHR-L1 activation is preceded by a decrease of cells entering the cell cycle [28–30]. In other cell types, activated FOXOs tend to induce cell-cycle arrest, although this may be accompanied by a low level of cell death [31,32]. The most plausible explanation for these observations is that there is cell-type-specific gene regulation of pro- and/or anti-apoptotic genes. Consistent with this, up-regulation of the death gene FasL has been demonstrated to correlate with FOXO nuclear localization [19]. Another pro-apoptotic gene that has been shown to be dramatically up-regulated by FOXO activation in haematopoietic cells is the pro-apoptotic Bcl-2 family member Bim [33]. Bim levels are critical in regulating apoptosis, since Bim (−/−) lymphocytes have an increased resistance to cell death induced by cytokine withdrawal [34]. In several haematopoietic cell lines, cytokine withdrawal has been found to result in a rapid up-regulation of Bim expression, concomitant with induction of the apoptotic programme [29,30,33,35].

### Table 1 | FOXO targets in mammalian cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Functional consequences</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>FasL</td>
<td>Induction of death-receptor-mediated apoptosis</td>
<td>[19]</td>
</tr>
<tr>
<td>p27kip1</td>
<td>Inhibition of proliferation/induction of apoptosis</td>
<td>[28,37]</td>
</tr>
<tr>
<td>Bim</td>
<td>Induction of intrinsic apoptosis pathways</td>
<td>[33]</td>
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<tr>
<td>Bcl-6</td>
<td>Transcriptional repression</td>
<td>[36]</td>
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<tr>
<td>Cyclin B</td>
<td>Completion of cell-cycle progression</td>
<td>[39]</td>
</tr>
<tr>
<td>Plk</td>
<td>Completion of cell-cycle progression</td>
<td>[39]</td>
</tr>
<tr>
<td>Gadd45</td>
<td>Repair of DNA damage</td>
<td>[40]</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Protection from oxidative stress</td>
<td>[41]</td>
</tr>
<tr>
<td>Catalase</td>
<td>Protection from oxidative stress</td>
<td>[42]</td>
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</table>
Figure 2 | Regulation of FOXO transcriptional activity

(A) Addition of growth/survival signals results in activation of PKB, which then translocates into the nucleus. Phosphorylation of FOXO by PKB results in release from DNA, and binding to 14-3-3 proteins. This complex is then transported out of the nucleus, where it remains inactive in the cytoplasm. (B) Upon removal of growth/survival signals, FOXO is dephosphorylated, 14-3-3 is released, and FOXO is transported back into the nucleus where it is transcriptionally active.

FKHR-L1 alone is sufficient to induce Bim expression directly [29,30,33], and there are FOXO consensus binding sites in the Bim promoter (J. Ham, personal communication). FOXOs not only induce pro-apoptotic genes, but can also regulate anti-apoptotic genes. AFX has been demonstrated to strongly up-regulate Bcl-6, a transcriptional repressor. Bcl-6 can directly bind and suppress Bcl-XL promoter activity, an anti-apoptotic Bcl-2 family member [36].

As mentioned above, over-expression of FOXOs causes growth suppression in a variety of cell lines. The FOXO-induced cell-cycle arrest depends on the transcriptional up-regulation and the subsequent increased protein levels of the cell-cycle inhibitor p27KIP1 [28,37] but probably also on up-regulation of the p130 protein, a pRb-like protein associated with quiescence [38]. Interestingly, the expression of mitotic genes, including cyclin B and polo-like kinase, has also been described as being regulated by FOXOs [39]. This suggests that down-regulation of PI3K, and induction of FOXO transcriptional activity, is required for completion of cell-cycle progression.

Recently, a novel role for FOXO transcription factors has been proposed [40]. The growth arrest and DNA damage response gene Gadd45 appears to be a direct target of FKHR-L1. These findings suggest that FKHR-L1 regulates the resistance of cells to stress, by regulating DNA repair. It is suggested that, under low stress conditions, FKHR-L1 may promote DNA repair, whereas at higher levels of cellular stress it may induce programmed cell death. In a similar vein, it has recently been shown that FKHR-L1 can protect quiescent cells from oxidative stress by directly increasing expression of manganese superoxide dismutase (MnSOD) [41]. Cells deficient in MnSOD (Sod2−/−) are no longer protected from oxidant damage by ectopic expression of FOXO, suggesting that this is a critical defence pathway. In agreement with these studies, expression of FKHR-L1 has been recently demonstrated to result in an increase...
in both hydrogen peroxide scavenging and oxidative stress resistance [42]. Taken together, these results are consistent with previous studies demonstrating that DAF-16 can act as a transcriptional activator of several antioxidant scavengers and stress-resistance genes in *C. elegans*. The ability to protect certain cells from damage suggests that, similar to the nematode worm, the evolutionarily conserved FOXO transcription factors may also be able to regulate life span in mammals.

**Cell-type-specific FOXO functions**

Why, then, in some cells does FOXO activation result in a block in proliferation, whereas in haematopoietic cells, for example, programmed cell death is induced? The precise mechanisms that confer cell-type specificity, and determine why some cells become arrested and other cells become apoptotic upon FOXO activation, still have to be completely resolved. Interestingly, while Bim expression is induced by FKHR-L1 activation in lymphocytes [28–30], this does not occur in a human colon carcinoma cell line [38]. Similarly, MnSOD is not induced by FOXO activation in haematopoietic cells (B. Burgering and G. Kops, personal communication), which can act to elicit a survival signal in many other cell types. A recent paper has provided an important clue concerning the mechanism behind these observations [43]. Through a comprehensive gene array analysis, this study revealed that FOXOs can regulate two different subsets of genes: (i) genes that require FOXO DNA binding, and (ii) genes induced independently of FOXO DNA binding. Importantly, a FKHR mutant that could no longer bind DNA, although unable to induce cell death, could still induce cell-cycle arrest. The fact that genes without specific FOXO consensus site can be regulated by this interaction co-factors, should help to understand further the molecular mechanisms underlying cell-fate decisions to divide, differentiate or die.

**References**


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