

REVIEW ARTICLE

The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation

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FoxO (forkhead box O; forkhead members of the O class) are transcription factors that function under the control of insulin/insulin-like signalling. FoxO factors have been associated with a multitude of biological processes, including cell-cycle, cell death, DNA repair, metabolism and protection from oxidative stress. Central to the regulation of FoxO factors is a shuttling system, which confines FoxO factors to either the nucleus or the cytosol. Shuttling of FoxO requires protein phosphorylation

within several domains, and association with 14-3-3 proteins and the nuclear transport machinery. Description of the FoxO-shuttling mechanism contributes to the understanding of FoxO function in relation to signalling and gene regulation.

Key words: forkhead, 14-3-3 protein, insulin, protein kinase B (PKB), shuttling.

INTRODUCTION

Initial studies on FoxO (forkhead box O) factors were performed on fusions of PAX3 (paired box 3) [1] or PAX7 [2] to FKHR (forkhead in rhabdomyosarcoma), which results in a transcription factor with altered transcriptional activity [1] implicated in alveolar rhabdomyosarcomas. FKHR has now been renamed to FOXO1, according to the novel nomenclature, and is the first identified member of the FoxO family of transcription factors. Now FoxO proteins have been identified in several different organisms, including *Caenorhabditis elegans*, zebrafish, *Drosophila*, mouse, rat and humans. In the mouse, four different FoxO members have been identified to date: FoxO1, FoxO3, FoxO4 and FoxO6 [3,4] (Figure 1). Over recent years it has become evident that FoxO factors are insulin-sensitive transcription factors with an array of downstream targets and interacting partners. Central to insulin-mediated inhibition of FoxO factors is a shuttling mechanism that regulates FoxO localization to the cytosol, thereby terminating its transcriptional function.

Several intramolecular domains, including phosphorylation sites, are necessary for FoxO factors to shuttle efficiently from nucleus to cytosol. Interestingly, FoxO6, a recently described FoxO family member, displays unique shuttling dynamics, adding information to the mechanism underlying translocation [4]. We will discuss the complex mechanism of intracellular FoxO shuttling in a step-by-step model, by reviewing the upstream FoxO kinases and chaperones involved in FoxO shuttling. In addition, we summarize FoxO mediated transcriptional regulation and *in vivo* function, which are both under direct control of the shuttling system.

SIGNALLING TO FoxO PROTEINS

FoxO factors are regulated by several signal transduction cascades (Figure 2). The main regulator of FoxO function is the PI3K (phosphoinositide 3-kinase) pathway, whereas FoxO function is 'fine-tuned' by the protein kinase CK1 (formerly known as casein kinase 1) and the DYRK1A (dual-specificity regulated kinase 1A) pathway. These kinases regulate the intracellular localization and function of FoxO proteins by phosphorylating FoxO factors within several different intramolecular domains (Figure 3).

Phosphorylation of FoxO proteins through the PI3K–PKB (protein kinase B) pathway

PI3Ks are heterodimers of a catalytic subunit (110 kDa) and a regulatory or adaptor subunit [10,11]. PI3Ks are activated by several proteins, including G-proteins and tyrosine kinases [11]. PI3K signalling is implicated in survival, regulation of the cell cycle, cell differentiation and intracellular traffic processes [10–12]. Recruitment of PI3K to membrane receptors relocates PI3K to its lipid substrates, where it phosphorylates the 3'-hydroxy group of the inositol ring of phosphatidylinositol to generate the phosphoinositide phosphates PIP₂ (phosphatidylinositol 4,5-bisphosphate) and PIP₃ (phosphatidylinositol 3,4,5-trisphosphate) [11]. These two signalling molecules trigger downstream serine/threonine kinases, including PDK1 (phosphoinositide-dependent kinase-1) and PKB [11]. Signalling by PI3K is counterbalanced by the tumour suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome 10). This protein dephosphorylates PIP₂ and PIP₃, and prevents the subsequent activation

Abbreviations used: AR androgen receptor; CBP, CREB-binding protein; C/EBP β , CCAAT/Enhancer binding protein β ; CREB, cAMP-response-element-binding protein; CK1, protein kinase CK1 (formerly known as casein kinase 1); CRM1, chromosomal region maintenance protein 1; dFOXO, *Drosophila* FOXO; dPRL, decidual prolactin; DYRK, dual-specificity regulated kinase; ER, oestrogen receptor; ES, embryonic stem; FoxO, forkhead box O; FKHR, forkhead in rhabdomyosarcoma; G6pc, glucose-6-phosphatase; HNF-4, hepatocyte nuclear factor-4; IGF, insulin-like growth factor; IGFBP-1, IGF-1-binding protein; IRS(1), insulin receptor substrate (1); KIX, kinase-inducible interaction; LMB, leptomycin B; NES, nuclear export sequence; NLS, nuclear localization sequence; PAX(3/7), paired box 3 or 7 respectively; PDK1, phosphoinositide-dependent kinase 1; PGC-1 α , proliferative-activated receptor- γ co-activator 1; PI3K, phosphoinositide 3-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RAR, retinoic acid receptor; RCC1, regulator of chromosome condensation; SGK, serum and glucocorticoid-regulated kinase; SRC, steroid receptor co-activator.

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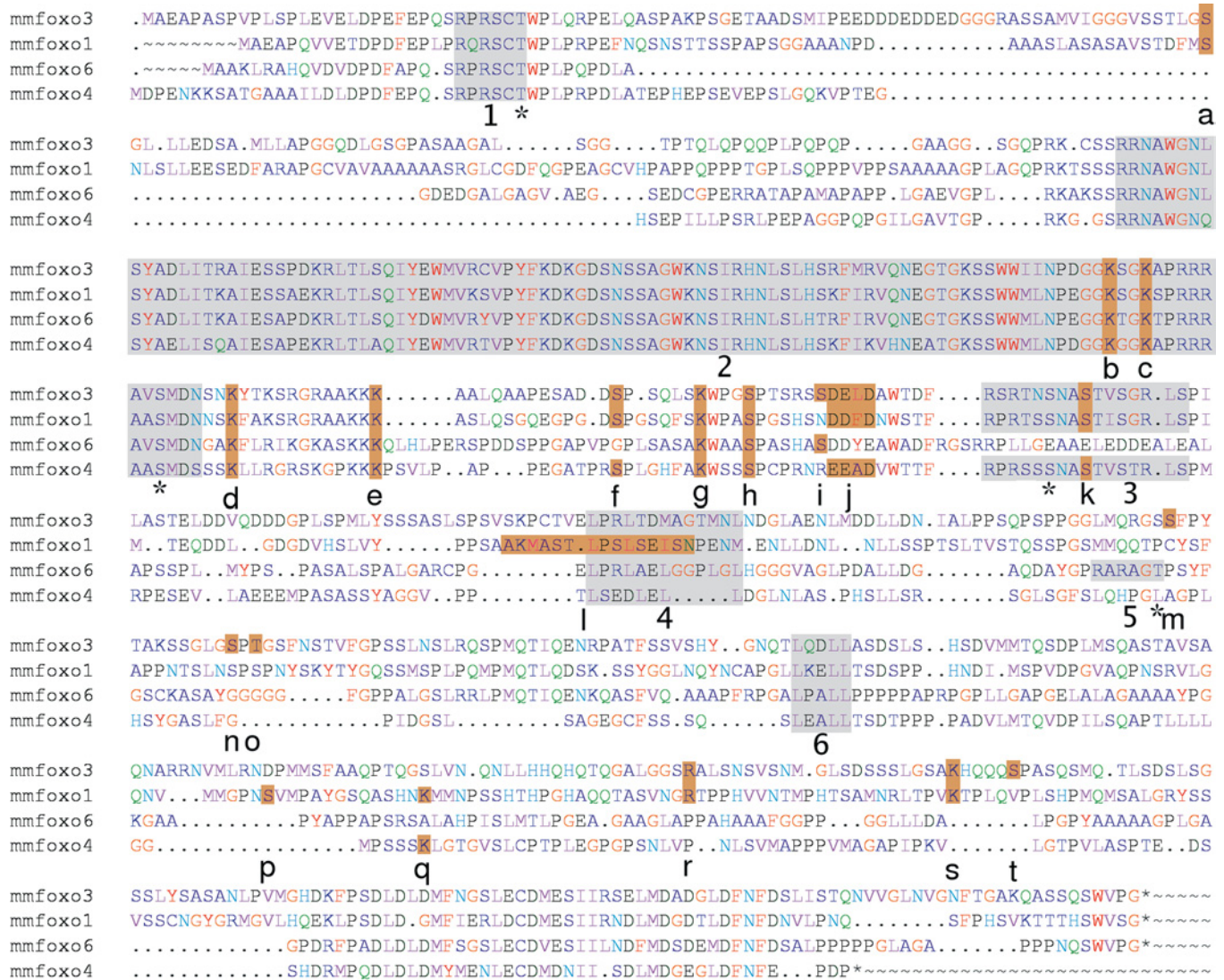


Figure 1 Alignment of all known mouse FoxO proteins

Conserved regions are indicated by boxes, and are numbered accordingly. 1, N-terminal PKB motif: involved in 14-3-3 binding. 2, forkhead domain: mediates interactions with the DNA, containing a PKB motif. Phosphorylation of this site is required for the N- and C-terminal PKB motif to be phosphorylated. This site is also involved in 14-3-3 binding and the regulation of DNA binding. 3, C-terminal PKB motif followed by two CK1 sites and a DYRK1A site, which regulates the speed of nuclear export. This entire stretch is absent in FoxO6. 4, NES. 5, optimal PKB motif in FoxO6 with unknown function. 6, LxxLL motif, possibly involved in the recruitment of nuclear receptors. Asterisks below the sequence show PKB-phosphorylated Ser/Thr residues. a, f, h, i, m, n and t: serine residues, possibly phosphorylated after stress stimuli [5]. o: threonine residue possibly phosphorylated after stress stimuli [5]. b, d, e, g and s, lysine residues possibly acetylated after stress stimuli [5]. b, c and q: lysine residues acetylated by CBP [6]. k, p and l: Residues possibly involved in regulation of transcriptional activity [7]. j: caspase-3-like cleavage motif [8]. r: protease-cleavage site [9].

of serine/threonine protein kinases [10,11]. Recently, glutamatergic activity was linked to PI3K signalling. Activated group I metabotropic glutamate receptors recruit the scaffolding protein Homer and PIKE-L (PI3K enhancer-large). This complex activates PI3K and prevents neuronal apoptosis in response to stress [13].

One of the main downstream mediators of PI3K signalling is PKB, also known as Akt. It is a serine/threonine kinase structurally related to PKA (protein kinase A) and PKC (protein kinase C), and belongs to the AGC family of protein kinases. They share similarity in the catalytic domain and in the mechanism of activation [14]. PKB is extensively studied, partly because it was shown that PKB inactivates proteins of the apoptotic machinery, such as the Bcl-2 family member Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death) [15,16]. There are three widely expressed

isoforms of PKB: PKB α , PKB β and PKB γ . The proteins consist of three functionally distinct regions: an N-terminal pleckstrin homology domain, a catalytic domain and a C-terminal hydrophobic motif [14]. PKB isoforms are recruited to the plasma membrane by PIP₂ and PIP₃, where PKB is phosphorylated on two specific sites required for full catalytic activity [17,14]. At least one of these sites is phosphorylated by PDK1, a major regulator of AGC kinases that is also recruited by phosphoinositides [18]. Activated PKB detaches from the plasma membrane and translocates to the cytosol and nucleus, where it phosphorylates serine or threonine residues within a PKB phosphorylation motif in target proteins [17].

Murine FoxO1, FoxO3, FoxO4 and FoxO6 contain three highly conserved putative PKB recognition motifs (Figure 3; consensus sequence of RXXXS/T, where 'X' denotes any residue [19])

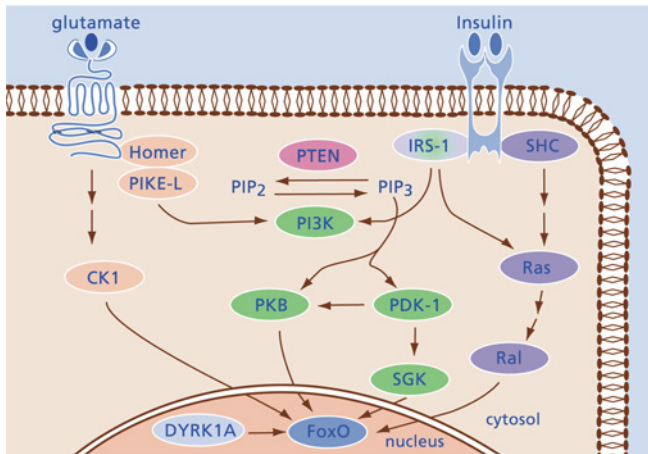


Figure 2 Schematic representation of the events related to the cascade of signalling from the extracellular to the intracellular environment, influencing the activity of FoxO proteins

The PI3K pathway forms the central component of FoxO regulation by acting on PKB and SGK. The signalling is fine-tuned by the CK1 and the Ras–Ral pathway, which converge on FoxOs. The regulation of DYRK1A is unknown, but it is described to phosphorylate FoxOs, thereby influencing their function.

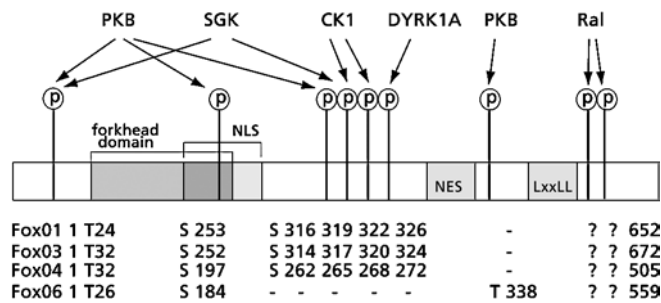


Figure 3 Schematic representation of the primary protein structure of FoxO proteins

The residues that can be phosphorylated by the accompanying kinases are indicated. The exact position of the phospho-residues is indicated below. The total number of residues is indicated on the far right.

[4], two at N- and C-terminal sites respectively, and one located in the forkhead domain. The *C. elegans* FoxO, DAF-16, contains an additional overlapping PKB motif in the forkhead domain. Interestingly, FoxO6 lacks the consensus C-terminal PKB motif. However, an optimal PKB phosphorylation motif is present in the far C-terminus, but it is questionable whether it is an *in vivo* substrate (results not shown). All FoxO proteins have been shown to require the consensus N-terminal PKB site and the PKB site located in the forkhead domain to translocate from nucleus to cytosol [20]. The two phosphorylated residues are essential components for translocation, as they influence the NLS (nuclear localization sequence) function [20] and the association with 14-3-3 proteins (see below) [21]. Besides the binding of 14-3-3, the N-terminal PKB site also regulates the association of FoxO proteins to p300. In the absence of growth factors, p300 binds to the N-terminal part of FOXO3. Growth factor addition and phosphorylation of the N-terminal PKB motif disrupts the interaction of p300 with FOXO3 [22], thereby possibly preventing acetylation of FOXO3 and directly influencing FoxO-mediated transactivation. An important role is reserved for the

C-terminal PKB phosphorylation site. Phosphorylation of this motif accelerates nuclear export [4,23,24]. The importance of this C-terminal PKB site is underlined by FoxO6, which lacks the third conserved PKB phosphorylation site. Differences in subcellular localization are very pronounced, comparing FoxO6 with FoxO1 and FoxO3. Under most conditions, FoxO6 is located in the nucleus, whereas FoxO1 and FoxO3 have a predominant cytosolic localization after PKB activation [4]. The domain surrounding the third PKB site is important for the efficiency of translocation and is a target for additional signalling events. The lack of a third PKB site at the proper position in the protein has therefore clear functional consequences [4].

FoxO phosphorylation by SGK (serum and glucocorticoid-regulated kinase)

A structurally related family member of PKB, which also phosphorylates FoxO factors [24], is SGK. Similar to PKB, SGK is enzymically activated by PI3K and PDK1 [18], although it is not recruited to the plasma membrane by PIP₂ and PIP₃, since it does not contain a pleckstrin homology domain [25]. Serum, glucocorticoids and stressors stimulate the SGK promoter, which results in rapid transcription of the SGK gene and an induction of the protein [26,27]. Since PKB and SGK are able to phosphorylate identical substrate motifs [18], it is at present very difficult to distinguish their activities. Moreover, at this moment there are no specific inhibitors that can discriminate between SGK and PKB. In conclusion, it is suggested that SGK functions complementarily to PKB as an important integrator of cellular signalling [16].

The regulation of nuclear exclusion requires additional kinases besides PKB. In theory, all FoxO putative PKB motifs can be phosphorylated by SGK, but it appears that SGK prefers the third C-terminal PKB motif [24]. The C-terminal PKB site is part of a stretch of four phosphoserine residues, which upon phosphorylation generate an acidic patch [23]. The third PKB site in FOXO1 is not exclusively phosphorylated by SGK [25]. In a mutant cell line able to activate PKB, but unable to activate SGK, the C-terminal PKB site in FOXO1 was still phosphorylated after insulin treatment, as in controls. Therefore PKB may fully compensate for the loss of SGK in this cell system, indicating that the situation *in vivo* is very complex.

FoxO phosphorylation by CK1

CK1 is a serine/threonine kinase, of which at least seven isoforms are identified [28]. CK1 has several physiological substrates, including the M₁ and M₃ muscarinic receptors and FoxO proteins [23,29]. All CK1 members contain a highly homologous kinase domain and a variable N- and C-terminal domain [30], and their kinase activity is negatively regulated by autophosphorylation [31,32]. Importantly, CK1 recognizes and phosphorylates motifs that have been 'primed'; in other words, motifs that already contain a phosphorylated serine or threonine residue [33]. Agonists shown to induce CK1 kinase activity include (S)-3,5-dihydroxyphenylglycine [34] (a mGluR₁ agonist) and DNA damage [35]. Insulin has no apparent direct effect on CK1 activity, but it can be recruited to Nck, a small adaptor protein that is recruited to IRS-1 (insulin receptor substrate 1) after insulin stimulation [36]. Interestingly, it was reported that, after insulin receptor activation, IRS-1 translocates from the membrane to the cytosol and nucleus [37]. Possibly, this complex of IRS-1–Nck and CK1 phosphorylates nuclear proteins.

The second and third serine residues, adjacent to and primed by the C-terminal PKB site, are phosphorylated by CK1 [23].

Phosphorylation of the first CK1 site primes the second CK1 site. Serine-to-alanine mutation of the C-terminal PKB site prevents the phosphorylation of both CK1 residues [23]. This sequential phosphorylation mechanism was studied further in PDK1 ES (embryonic stem) knockout cells, which lack PKB activation. In these cells, no phosphorylation of the PKB and CK1 sites was detected under baseline and IGF-1 (insulin-like growth factor 1)-stimulated conditions [23]. This clearly demonstrates that CK1 phosphorylation of residues 2 and 3 of the serine stretch is dependent on initial phosphorylation of the C-terminal PKB site. This could be substantiated further by introducing a serine-to-aspartate mutant of the C-terminal PKB site into PDK1 ES knockout cells. In this case, the CK1 sites should be phosphorylated, proving PKB dependency on priming and PDK1-independent CK1 activity.

FoxO phosphorylation by DYRK

DYRK1A belongs to the dual-specificity tyrosine-phosphorylated and -regulated kinase group of kinases. The DYRK group consists of DYRK1A/B/C, DYRK2, DYRK3 and DYRK4A/B. DYRK1A and DYRK1B are nuclear, whereas DYRK2 is cytosolic [38]. The intracellular location of DYRK3 and DYRK4A/B is, at present, unknown [38]. DYRKs are serine/threonine kinases that have tyrosine autophosphorylation activity [38]. DYRK kinase activity is dependent on a phosphorylation motif present in its activation loop, suggesting regulation by upstream kinases, which are at present unidentified [39]. Although some DYRK targets have been identified [e.g. CREB (cAMP-response-element-binding protein), eIF (eukaryotic initiation factor) 2B and FoxOs], the intracellular function of DYRK is unclear. A mutant form of DYRK in *Drosophila* results in a 'minibrain phenotype' [40]. These animals have a smaller brain, due to a reduced number of neurons. In humans, DYRK1A has been mapped to the Down's Syndrome critical region. In mice, DYRK1A haploinsufficiency affects viability and causes developmental delay and abnormal brain morphology [41]. Taken together, DYRKs appear to play a role in neuronal proliferation in central nervous system development.

A DYRK1A site, adjacent to the second CK1 site (see above and Figure 3), completes the stretch of serine residues that form an acidic patch upon phosphorylation. This serine residue appears to be constitutively phosphorylated [23]. Inhibitors of PI3K, and serine-to-alanine mutants of the PKB and CK1 residues, do not influence its phosphorylation state [23]. This shows that the phosphorylated residues of PKB and CK1 have no influence on the phosphorylation state of the DYRK1A residue. Mutation of the DYRK1A site serine residue to an alanine does result in an increased nuclear FoxO localization under baseline conditions and increased transactivation of a FoxO reporter construct [39]. This indicates that regulation of the DYRK1A site is involved in the subcellular localization of FoxO proteins, despite the fact that the site is independent of PKB and CK1 activity.

FoxO phosphorylation through the Ras–Ral pathway

Ras is a small GTPase that is activated by growth factors. Recruitment of Ras to growth factor receptors by adaptor proteins results in activation of Ras by loading it with GTP. [43]. Activated Ras has been associated with transcription, DNA synthesis, differentiation and proliferation [43]. It was suggested that Ras activates specific guanine-exchange factors (GEFs), RalGEFs, which activate Ral [44]. Moreover, it was shown that Ral is an important mediator of Ras-induced proliferative signals [45]. Besides Ral activation, Ras activation also regulates activation of the classical MAPK

(mitogen-activated-protein kinase) route and the PI3 kinase route. The Ras–Ral route appears to be an additional route, besides the PI3K and CK1 routes, involved in FoxO phosphorylation [43].

The Ras–Ral signalling pathway influences FoxO transcription factors [43,46]. Although the regulation of FoxO factors by Ral does not alter its intracellular localization, it does influence FoxO transactivational capacity, as was shown for FOXO4 [43]. To date, it is unclear whether or not Ral-mediated phosphorylation of FoxOs is specific for FOXO4 or not, since it has only been documented for FOXO4. Importantly, sequence homology amongst FoxO family members is extremely high, especially in regions encompassing the well-described signalling motifs. The low degree of conservation of the Ral-dependent phosphorylation sites is apparent, comparing human FOXO4 (T⁴⁴⁷PVLT⁴⁵¹) with the corresponding region in mouse FoxO4 (TPVLA). The murine FoxO4 lacks the threonine corresponding to Thr⁴⁵¹ in human FOXO4. This clearly indicates the low degree of conservation of Ral signalling to FoxO proteins in different species.

CELLULAR RELOCATION MECHANISM OF FoxO PROTEINS

Nuclear transport machinery

The transport of FoxO proteins through the nuclear pore is dependent on active-transport mechanisms. The nuclear-pore complex is a large structure spanning the nuclear membrane, and forms a physical barrier between the cytosol and nucleus. It has an estimated molecular mass of 125 MDa. Proteins with a molecular mass up to 50 kDa, or up to 9 nm in diameter, can diffuse freely through the aqueous channels of the nuclear-pore complex. Larger molecules are transported through the nuclear-core complex via active transport. Several variations on nuclear transport have been described previously [47–49]. The basic model is summarized in this review (Figure 4). Transport is regulated via specific adaptor proteins and Ran, a small GTPase of the Ras family that is required for interaction with the nuclear-pore complex [50]. Ran exists in a GDP- or GTP-bound form. The GDP-bound form is mainly present in the cytosol, whereas the GTP-bound form is present in the nucleus. This gradient is the driving force for transport across the nuclear membrane, and is maintained by cytosolic Ran-GAP and nuclear RCC1. In the cytosol, Ran-GAP hydrolyses Ran-GTP to Ran-GDP, whereas in the nucleus RCC1, a chromatin-associated Ran-GEF, converts Ran-GDP into Ran-GTP. Transport across the nuclear-pore complex requires adaptor proteins that mediate either import or export. These adaptors are called importin or exportin receptors respectively [48]. Importins and exportins recognize specific NLSs and NESs (nuclear export sequences) present in the transported protein. Several NESs are recognized by the evolutionary conserved exportin 1 protein, CRM1 (chromosomal region maintenance protein 1). A protein bound to CRM1 is transported through the nuclear-pore complex via an interaction with Ran-GTP. Within the cytosol, the complex containing Ran-GTP is disassembled by Ran-GAP and Ran-binding proteins [47–49]. Nuclear import is mediated via importin receptors. In the nucleus, the importin receptor binds to Ran-GTP, resulting in the release of the transported protein. The dimeric complex of the importin receptor and Ran-GTP is then recycled to the cytosol [49]. The presence of an NLS is a prerequisite for maintaining proteins in the nucleus, whereas a NES maintains proteins in the cytosol. FoxO proteins, however, have both an NLS and an NES. Kinases and interactions with other proteins modulate the effectiveness of these NLSs and NESs, which forms the basis of FoxO shuttling.

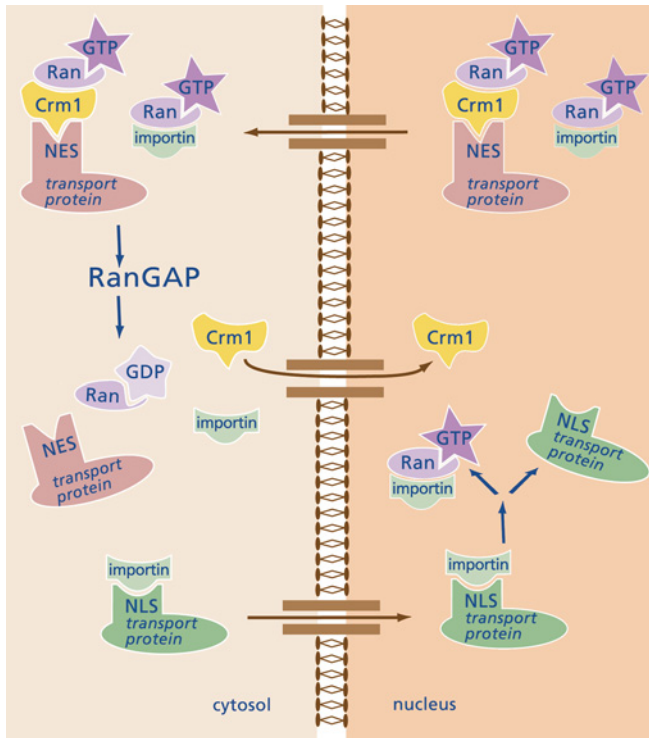


Figure 4 Representation of the molecular mechanisms that play a role in the relocation of proteins

A protein (transport protein) bound to the export receptor Crm1 and Ran-GTP is transported to the cytosol and disassembled by Ran-GAP and other proteins. A protein bound to an importin receptor is translocated to the nucleus, where the complex is disassembled by Ran-GTP binding to the importin receptor. The importin receptor (with bound Ran-GTP) is recycled to the cytosol. 'transport protein' represents the protein that is transported.

Mechanism of FoxO relocation

CRM1

FoxO proteins accumulate in the nucleus, after treatment with LMB (leptomycin B), a herbal fungicide that specifically inhibits CRM1 [4,20]. This links CRM1 function to FoxO export. It was described that FoxO binds to CRM1 [20], although others reported that FoxO–CRM1 interactions could not be detected [23]. The results indicate a direct interaction of FoxO with Ran [23]. The dramatic effect of LMB [20] suggests that an interaction of CRM1 with FoxO is apparent [4]. Moreover, an additional LMB-sensitive NES in FOXO1 has been described [51]. Binding of CRM1 to FoxO proteins is not dependent on the phosphorylation status of the FoxO protein itself, as has been shown for FOXO4. Binding of FOXO4 to CRM1 is phosphorylation-state-independent, as both wild-type FOXO4 and a PKB-phosphorylation deficient FOXO4 mutant bound CRM1. The FOXO4 mutant, unable to be phosphorylated on its PKB sites, was still transported out of the nucleus. This was elegantly shown with the use of cell-fusion assays in which the donor and acceptor nuclei of the fused cells can be distinguished. In this system, export is measured by the accumulation of the FoxO mutant protein in the other nuclei. Since export of FoxO proteins seemed to be independent of phosphorylation, the authors concluded that FoxO shuttling must be controlled through regulation of nuclear import [20]. Importantly, this particular study failed to examine the kinetics of export, as phosphorylation results in a dramatic acceleration of FoxO export. The influence of phosphorylation on the efficiency of FoxO export has now been documented thoroughly [4,52,53].

NLSs in FoxO proteins

All FoxO proteins contain a sequence that conforms to a non-classical NLS. This NLS consists of three arginine residues present in the forkhead domain C-terminus, and three lysine residues located 19 residues downstream of the described arginine residues [20,54]. Within this motif, a PKB phosphorylation motif is present. The arginine residues are part of the RXXRXXS PKB motif [19], in which the serine residue is phosphorylated. The basic region of the NLS is suggested to be essential for its function [20]. Phosphorylation of the serine residue in the PKB motif introduces a negative charge, which might influence the NLS. Moreover, the phosphorylated serine residue might sterically hinder NLS function. Mutation analysis confirmed that phosphorylation of this PKB motif inhibits NLS function, shifting FoxO to a cytoplasmic localization [20]. Besides the blockade of the NLS by PKB-mediated phosphorylation, FoxO proteins require additional factors for cytosolic retention. Recently, it was described that a second functional NLS is present in the N-terminal part of FOXO1 [51].

Relocation by 14-3-3 proteins

Transport of FoxO proteins requires 14-3-3 protein interaction [21]. The name of the latter category of proteins refers to a classification of brain proteins that were separated by DEAE-cellulose chromatography and gel electrophoresis [55]. 14-3-3 proteins have a molecular mass of approx. 30 kDa, and have a U-shaped structure. Within the 'U', 14-3-3 proteins specifically recognize and bind phosphorylated serine or threonine residues. The proteins can form homo- and hetero-dimers with other family members. 14-3-3 proteins control catalytic activity of the bound protein. They regulate interactions between the bound proteins and other molecules through sequestration or modification, and, finally, influence the intracellular localization of bound ligands [55,56]. It was suggested that the N-terminal PKB motif and the PKB motif in the forkhead domain of FoxO proteins are involved in 14-3-3 binding [21]. 14-3-3 recognizes RSXXpSXP and RXXXpSXP motifs, where pS represents a phosphorylated serine residue. FoxO proteins contain only one optimal 14-3-3-binding site, overlapping with the N-terminal PKB motif. An optimal motif, however, is not essential for 14-3-3 binding, as it was demonstrated to bind to degenerated 14-3-3 motifs [55]. Interaction of 14-3-3 proteins with FoxO requires phosphorylation of the N-terminal PKB motif and the PKB motif in the forkhead domain. Phosphorylation of the N- and C-terminal PKB sites depends on the initial phosphorylation of the PKB motif located in the forkhead domain, which functions as a 'gatekeeper' [52]. Serine-to-alanine mutation of the PKB site in the forkhead domain completely abolishes the insulin-induced increase in total FoxO phosphorylation [57], whereas mutation of either of the phosphorylation sites present in the N- and C-terminal parts does not. It has been shown that the amino acids between the second and third PKB motif are responsible for this hierarchical sequence of phosphorylation events [57]. Disruption of the N-terminal PKB site by serine-to-alanine mutation disrupts 14-3-3 binding, and consequently inhibits nuclear export [21,57]. Since the interaction of FoxOs with 14-3-3 proteins depends on phosphorylation, it is logical that disruption of the 'gatekeeper' of phosphorylation completely abolishes all 14-3-3 binding as does disruption of the optimal 14-3-3-binding site itself [55]. Although 14-3-3 monomers are very capable of binding to ligands, even phosphorylation-independent ones [52], it appears that 14-3-3 dimerization is required for optimal phospho-ligand interaction [55]. Optimal regulation of Raf by 14-3-3 requires 14-3-3 dimers,

whereas 14-3-3 monomers, mutated to prevent dimerization, lack optimal Raf regulation [58]. It is suggested that dimerized 14-3-3 binds to ligands with a higher affinity. This was indicated by binding of a synthetic peptide containing a tandem 14-3-3 recognition site. These peptides bound to 14-3-3 proteins with 30-fold-higher affinity compared with peptides with only one 14-3-3 recognition site [55]. It is suggested that a dimeric 14-3-3 protein binds to its ligand via a two-step mechanism [55]. The first step involves the binding of one subunit of the dimeric 14-3-3 protein to a high-affinity binding site. The binding of 14-3-3 to a high-affinity site would function as a 'gatekeeper' of 14-3-3 binding. 14-3-3 binding to a high-affinity site permits the binding of the other subunit to a low-affinity site, which would not bind individually [55]. Recently, it was shown that a FOXO1 mutant relieved of its hierarchical phosphorylation sequence by truncation of the C-terminus required both the N-terminal PKB motif and the PKB motif in the forkhead domain for optimal 14-3-3 binding [51]. Analytical gel-filtration and sedimentation equilibrium experiments indicated that 14-3-3 optimally binds phosphorylated FOXO4 in a 2:1 molar stoichiometry [59]. These studies show that two 14-3-3 proteins bind one FoxO protein, and that this requires phosphorylation of the two PKB sites. Given the nature of 14-3-3 interactions, it is very likely that FoxO proteins themselves have two 14-3-3-binding sites. Possibly, 14-3-3 binds the N-terminal 14-3-3 site first, as it is an optimal binding site, and also given the evidence of direct 14-3-3 binding to this site. Probably, the PKB motif in the forkhead domain defines the low-affinity site.

Influence of phosphorylation on relocation

The phosphorylation of PKB, SGK, CK1 and DYRK1A sites within the FoxO protein is crucial for its localization within the cell. The PKB site located in the forkhead domain acts as a trigger, and the other sites function as 'fine-tuners' of the shuttling behaviour. Disruption of the stretch of four serine residues by mutating the PKB site to an alanine residue, or by inhibition of PI3K with wortmannin, does not inhibit FoxO binding to Ran and CRM1, although it dramatically impairs nuclear export [20,23]. The stretch of four phosphorylated serine residues is suggested to form an acidic patch, facilitating nuclear export [23]. Insertion of the four serine residues from FoxO3 into the corresponding region of FoxO6 rescues the ability of FoxO6 to shuttle efficiently from the nucleus to the cytosol, emphasizing the importance of this region in FoxO relocation [4]. Both wild-type FoxO6 and the FoxO6-4 serine chimaeric protein are exclusively localized in the nucleus under serum-free conditions. This indicates that only the translocation efficiency is augmented by the serine stretch [4].

In summary, translocation from the nucleus to the cytosol can be divided into several sequential steps: (1) Phosphorylation of the PKB site in the forkhead domain leads to disruption of the NLS function and disruption of DNA binding (for details, see below). Phosphorylation leads to accessibility of the N- and C-terminal PKB sites. (2) The N- and C-terminal PKB site are phosphorylated. Phosphorylation of the C-terminal PKB site by SGK or PKB leads to the phosphorylation of a stretch of three serine residues. A DYRK1A site completes the stretch of four phosphoserine residues. (3) The current phosphorylation state induces high-affinity binding of 14-3-3 proteins. (4) The phosphorylated FoxO protein in a complex with Ran and CRM1 is transported through the nuclear pore complex towards the cytosol. In addition, cytosolic and phosphorylated FoxO proteins are degraded by the ubiquitin-proteasome system [60,61], providing the cell with a double negative regulation of FoxO factors [61]. Disruption of

at least one of the above steps results in a disruption of nuclear export, and accumulation of the FoxO protein in the nucleus. The exact mechanism of 14-3-3 binding, and the mechanism through which the phospho-stretch increases nuclear export, are at present unknown.

Shuttling and FoxO protein processing

The FoxO-shuttling system was initially assumed to directly regulate FoxO transcriptional activity by altering its intracellular localization. In summary, this is a two-step model. First, activation of the PI3K pathway leads to translocation of FoxOs to the cytosol by removing the transcription factor from the DNA, and thereby terminating their transcriptional activity. Secondly, transcriptional activity is reinstated by deactivation of the PI3K-PKB pathway by chemical inhibition or removal of growth factors, allowing FoxOs to return towards the nucleus. In fact, transcriptional regulation is partly independent of subcellular localization, and is not only dependent on the shuttling system. FoxO6 transcriptional activity is effectively regulated by growth factors to the same extent as FoxO1 and FoxO3, although FoxO6 is almost completely nuclear [4]. The remaining component of growth-factor-induced cytoplasmic shuttling can never account for the decrease in FoxO6-dependent transcriptional activity. This is also in agreement with results that describe insulin-dependent inhibition of FoxO1 activity in an export-deficient mutant [62]. It is suggested that FoxO6 regulation by growth factors is provided through modulation of DNA binding. Probably, relocation to the cytosol serves additional purposes. Important clues are provided by studies investigating FoxO protein processing by the ubiquitin-proteasome system. It was described that activated PKB results in a decrease in FOXO1 and FOXO3 protein levels [60]. The reduction in FOXO1 and FOXO3 protein levels could be decreased through the application of proteasome inhibitors. This suggests that the proteasome system has a role in the regulation of FoxO activity. In fact, FOXO1 is ubiquitinated and degraded by the proteasome in response to insulin, and this process is PI3K-PKB-dependent [61]. It was shown, through the use of FOXO1 NES/NLS mutants and a constitutive nuclear FOXO1 mutant (a triple PKB-site Ser → Ala mutant), that phosphorylation of the PKB sites and cytosolic localization are required for optimal degradation of FOXO1. The degradation of FoxO factors adds an additional layer of negative regulation of FoxO activity that relies on the FoxO-shuttling system. Since cytosolic localization is required for optimal degradation of FoxO1, this finding has intriguing consequences for FoxO6. Although not investigated, it can be assumed that FoxO6 is not degraded by the proteasome system as efficiently as its family members, making it the most stable protein of its family. Besides processing by the ubiquitin system, FoxOs are processed by proteases, diversifying the processing of FoxOs. FOXO3 is a substrate of caspase-3-like proteases [8]. Cleavage of FOXO3 by caspase-3-like proteases yields two fragments: an N-terminal fragment, which encompasses the first two PKB sites and the forkhead domain, and a C-terminal fragment, which contains the transactivation domain. The C-terminal fragment is always cytosolic, presumably because it contains the NES. The intracellular localization of the N-terminal fragment is still dependent on the PKB-phosphorylation sites. Dephosphorylation of the N-terminal fragment results in translocation to the nucleus, where it possibly functions as a dominant-negative. Interestingly, the sequence identified as the proteolytic-cleavage site appears to be conserved in FOXO1 and FOXO4. An essential aspartic acid of the cleavage motif [63] is, however, not conserved in FoxO6 (DELΔ; position 304) in FOXO3, or the

corresponding region DDYE in FoxO6; see Figure 1, sequence labelled 'j'). A similar proteolytic-cleavage mechanism has been shown for FOXO1. Androgens initiate protease-mediated cleavage (Figure 1, sequence labelled 'r') of a 70 kDa FOXO1 into a 60 kDa N-terminal fragment and an C-terminal 10 kDa fragment [9]. The C-terminal fragment contains the DNA-binding domain and a large portion of the transactivation domain, and functions as a dominant-negative [9]. Via this protease mechanism, androgens are suggested to deregulate FOXO1-mediated processes. In conclusion, negative regulation of transcription by the FoxO-shuttling system is reinforced by proteolytic processing of FoxO factors.

TRANSCRIPTIONAL ACTIVITY OF FoxO PROTEINS

The activity of FoxO proteins can be regulated by repositioning the protein in the cell. Also, direct regulation of transcriptional capacity by protein modification has been reported. These modifications can be found on the DNA-binding interface, as well as in the transactivation domain. Finally, FoxO proteins interact and co-operate with other proteins. These interactions cause changes in FoxO transactivation, but also influence other transcription factors [64].

Modification of the DNA-binding potential

The PKB phosphorylation motif in the forkhead domain is linked to the regulation of DNA binding [54]. Phosphorylation of this serine residue is not sufficient for relocation of the FoxO proteins, although it is part of the NLS sequence and is involved in binding 14-3-3 proteins [51]. Earlier findings from several groups suggested that phosphorylation of this site disrupts transactivation without relocation of the protein [52,62]. Gel-shift experiments have shown that the introduction of a negative charge in the PKB site (Ser → Ala mutation) is sufficient to disrupt DNA binding [54]. Moreover, reporter gene studies showed that phosphorylation limits the transactivating potential of FOXO1 [54]. Interestingly, experiments performed by Obsil et al. [59] indicated that the affinity for DNA is not affected by phosphorylation of the first two PKB sites of FOXO4. However, a clear decrease in the affinity of phosphorylated FOXO4 for DNA is reported after binding of two 14-3-3 proteins. These conflicting results could be that Obsil's group did not perform the studies on 14-3-3 and DNA binding with full-length FOXO4, but with a mutant protein lacking a large portion of the C-terminus. With the use of FoxO6, it was clearly demonstrated that transactivation is limited after phosphorylation by PKB without relocation of the protein from the nucleus to the cytosol [4]. In conclusion, DNA binding is diminished by phosphorylation of the serine residue present in the C-terminus of the forkhead DNA-binding domain.

FoxO-mediated expression of downstream genes can be mediated via direct FoxO binding to FoxO-responsive elements in the target gene. These genes contain the optimal FHRE (forkhead-responsive element)-binding sequence or insulin response unit/sequence. Besides this mechanism, FoxO proteins, however, have the capacity to regulate downstream targets independently of DNA binding. In part, this can be explained by the interactions of FoxO factors with other transcription factors, such as nuclear hormone receptors (discussed below). DNA-independent activity was elegantly shown by comparing expression profiles of two different cell lines, one containing a constitutively nuclear FOXO1 (triple PKB-site-alanine mutant, hereafter designated 'AAA') and one containing the same protein, but which was DNA-binding-deficient (hereafter designated 'HRAAA') [65].

HRAAA was generated by substituting a conserved histidine residue to an arginine in helix 3 of the forkhead domain. It should be noted that this mutant was only tested on binding to, and non-activation of, a 3 × insulin-response sequence reporter. By comparing these two mutant forms of FOXO1, four different groups of downstream targets were classified. The first group of genes was activated by AAA and not by HRAAA. The second group was mainly activated by AAA, and to a lesser extent by HRAAA. A third group encompassed targets activated mainly by HRAAA, and not by AAA; and finally, a fourth group was characterized, with both AAA and HRAAA down-regulated targets. Interestingly, chromatin immunoprecipitations performed on genes from the third and fourth groups still showed binding to HRAAA, suggesting that HRAAA interacts with the promoter independently of an IRS, presumably through interactions with other proteins. Moreover, it is plausible that this is also holds true for wild-type FOXO1. Additionally, it was shown that the down-regulation of genes in group 4 did not depend on the FOXO1-transactivation domain. However, the down-regulation of genes could be reversed by treating the cells with a histone-deacetylase inhibitor, suggesting gene-specific transcriptional repression [65]. In PTEN null LNCaP prostate adenocarcinoma cells [65], AAA induces cell death, but HRAAA does not. In PTEN null 786-O cells [65], AAA and HRAAA inhibit cell cycle progression and suppress tumour formation. In PTEN null U87-MG glioblastoma cells, AAA and HRAAA induce G₁ arrest. This study clearly suggests that only the induction of apoptosis is differentially regulated by AAA and HRAAA. DNA-binding-independent activity of FoxO can have a fundamentally different activity profile compared with DNA-dependent FoxO activity.

Modification of transactivating domains

Studies using the C-terminus of FoxO1 fused to the GAL-4 DNA-binding domain demonstrated that phosphorylation of FoxO1 can directly inhibit the transactivating potential of this domain [7]. Insulin induces phosphorylation of three regions: residues Ser³¹⁹ (Figure 1, label 'k'), Ser⁴⁹⁹ (Figure 1, label 'p') and residues in a stretch of 15 amino acids (at residue positions 350–364; Figure 1, label 'l'), which could explain the results describing the LY-294002-enhanced transcriptional activity in a triple- or quadruple-DAF-16 alanine mutant [66]. This points to other PI3K-dependent residues, besides the classical PKB residues, identified in all FoxO factors. Finally, the phosphorylation of human FOXO4 Thr⁴⁴⁷ and Thr⁴⁵¹ through activity of the Ras–Ral pathway augments transcriptional activity [43]. Taken together, the results suggest direct regulation of the transactivating potential without nuclear exclusion.

Interactions with co-activators

p300/CBP (CREB-binding protein)

Interactions of forkhead proteins and other associated proteins, binding on the promoter of the IGFBP-1 (IGF-1 binding protein) gene are described. This complex was induced by binding to an insulin-responsive element [67]. The FoxO-interacting partner was identified as p300/CBP [22,68]. This protein plays an important role in integrating signalling events to the transcriptional machinery. The primary DNA sequence of the IGFBP-1 promoter provides the integration of glucocorticoid and insulin signalling. The interaction of DAF-16, FOXO1 or FOXO3 with CBP enhances the glucocorticoid-stimulated transcription, through possible interaction with the KIX (kinase-inducible

interaction) and E1A/SRC (steroid receptor co-activator) domains. Interestingly, FOXO4 does stimulate the IGFBP-1 gene, but fails to enhance the glucocorticoid response. The interaction of FOXO4 and CBP is limited to the KIX domain of CBP [67], indicating that the glucocorticoid-stimulated response is established through interaction of the CBP SRC domain. However, the previously described, transcriptionally inactive C-terminal truncation mutant of DAF-16 interacts with the SRC domain, and fails to interact with the KIX domain. The authors conclude that the enhanced glucocorticoid response relies on the interaction with the KIX domain [67]. This conclusion seems to be in conflict with the results derived from the FOXO4 experiments. In conclusion, it seems that both interaction domains of CBP are involved in mediating the enhanced glucocorticoid transcriptional activation.

C/EBP β (CCAAT/enhancer binding protein β)

A combination of *in vivo* and *in vitro* experiments show that FOXO1 interacts with C/EBP β . FOXO1 and C/EBP β bind on a composite element present in the dPRL (decidual prolactin) proximal promoter [69]. Binding of C/EBP β alone stimulates the dPRL promoter. After binding of FOXO1, the stimulation is more than additive. This suggests a functional interaction between these proteins. Experiments using glutathione S-transferase fusion proteins and pull-down assays confirmed the interactions, which are DNA-binding-independent. This demonstrates that the composite element is not required for the interaction. Transient transfection experiments with mutated dPRL promoter constructs showed that deleting either part of the composite element reduces the transactivating potential [69]. This indicates that DNA-binding is important for the co-operative function of FOXO1 and C/EBP β on the dPRL promoter.

DYRK1

In addition to the kinase activity described above, involved in FoxO relocation, DYRK1A and B, but not DYRK2, can interact with FOXO1 and co-operate in activating the G6pc (glucose-6-phosphatase) gene [70]. A DYRK1 catalytically inactive mutant (Lys¹⁸⁸ \rightarrow Arg; [71,72]) was almost equally active in transactivating potential as compared with the wild-type protein. This indicates that kinase activity is not required for the co-operation with FOXO1 in transactivation of the G6pc gene. The functional interaction of these proteins depends on the binding of FOXO1 to its insulin-responsive element. Mutation of the insulin-responsive element in the G6pc promoter abolishes the activation of the promoter by FOXO1/DYRK1. Taken together, the kinases DYRK1A and B can interact with FOXO1 and contribute to its transactivating potential through direct interaction with the G6pc promoter, independent of the kinase activity of DYRK1.

PGC-1 α (proliferative-activated receptor- γ co-activator 1)

PGC-1 α is able to co-operate with FOXO1 in the activation of gluconeogenic liver genes (phosphoenolpyruvate carboxykinase 1 and G6pc) [73]. The activation of these genes by PGC-1 α depends on FOXO1, which was shown by using FOXO1 mutants [73]. Therefore the insulin regulation of these genes depends on the tight regulation of FOXO1, and is based on the co-operation of FOXO1 with PGC-1 α [73,74], independent of DNA binding. Interestingly, the close homologue of PGC-1 α , PGC-1 β , fails to interact with FOXO1, and does not enhance the FOXO1-mediated transcriptional activation of gluconeogenic genes [75]. The comparison of the biochemical properties of these

two homologues might provide more insight into the molecular mechanism of PGC-1 α co-operation with FOXO1.

FoxO–nuclear hormone receptor interactions

Besides regulation of transcription via direct interactions between FoxO and DNA, FoxO factors have a DNA-binding-independent effect on transcription via interactions with nuclear receptors. Nuclear receptors mostly function as ligand-dependent transcription factors. FoxO factors influence nuclear receptor transactivation by repressing or activating transcription, depending on the nuclear receptor involved. On the other hand, it was shown that nuclear receptors interacting with FoxO factors function as inhibitors of FoxO-mediated transcription [65,76,77].

Nuclear receptors interacting with FoxO factors include the ER (oestrogen receptor) [76,77], the progesterone receptor [76], the AR (androgen receptor) [78], the thyroid hormone receptor [76], the glucocorticoid receptor [76], the RAR (retinoic acid receptor) [76], the peroxisome-proliferator-activated receptor [79] and HNF-4 (hepatocyte nuclear factor-4) [80]. Steroid receptors are retained in the cytoplasm, and upon ligand binding they are released from their chaperones, dimerize, enter the nucleus and bind to specific response elements in the regulatory region of target genes [81]. Once bound to the specific response elements, nuclear receptors facilitate the initiation of transcription.

In contrast with steroid receptors, non-steroid receptors are mainly located in the nucleus. In the absence of ligand, they are associated with histone-deacetylase-containing complexes tethered through co-repressors. This process results in chromatin compaction and silencing of the promoter regions of the target genes. Upon ligand binding, the co-repressor-binding interface is destabilized, which leads to their dissociation. Subsequently, nuclear receptors bound to their responsive elements facilitate recruitment of the transcription machinery (the general transcription factors and RNA polymerase II) [81]. All FoxO factors contain an LxxLL domain located in the far C-terminal region of the protein (Figure 1). It is suggested that this domain facilitates interactions with nuclear receptors [82]. The mechanism underlying FoxO regulation of nuclear receptors, and vice versa, is very complex and is influenced by multiple pathways. First, interaction between nuclear receptors and FoxO factors can be dependent or independent of nuclear receptor ligand binding [76]. As a typical example, oestrogen enhances the binding of FOXO1 to the ER [76], whereas binding of FOXO1 to the RAR is ligand-independent [76]. FOXO1 augments transactivation of the ER through an oestrogen-responsive element, whereas the ER represses transactivation of FOXO1 through an insulin-responsive element, indicating a bidirectional function [77]. However, others have described that FOXO1 represses ER-dependent transactivation [76]. The discrepancy between these two studies was explained by experimental differences in cell lines used and in promoters [77]. Secondly, FoxO phosphorylation can influence the interaction with nuclear receptors, as was shown for HNF-4 and FOXO1 [80]. The binding of HNF-4 to FOXO1 is negatively influenced by FOXO1 phosphorylation, and the repression of HNF-4 transactivation by FOXO1 is negatively influenced by insulin [80]. Moreover, repression of HNF-4 transactivation by a FOXO1 mutant in which all PKB sites are substituted for alanine residues is not inhibited by insulin [80]. Since phosphorylation of FoxO factors influences their intracellular localization, it is assumed that FoxO phosphorylation influences the interactions with steroid and non-steroid receptors. The repression of FOXO1 by the AR is, however, independent of FOXO1 phosphorylation [78], demonstrating that the net result on transactivation depends on the particular nuclear receptor involved.

It appears that nuclear receptors somehow recruit FoxO factors to either augment or repress transcription. Whether the functional consequence on transcription is stimulation or repression probably depends on the domains via which nuclear receptors and FoxO factors interact. Interactions between nuclear receptors and FOXO1, FOXO3 and FOXO4 have been documented, but to date nothing is known about the novel member FoxO6. As is true for the other FoxO members, FoxO6 also possesses the LxxLL motif (Figure 1), which is implicated in the interaction with nuclear receptors [82]. Since FoxO6 is mainly nuclear [4], interactions with nuclear receptors could very well differ from the other FoxO proteins. This provides nuclear receptors with a cofactor that is continuously present.

FoxO acetylation

Two independent papers [5,83] have described SIRT1-mediated deacetylation of FoxO factors (where SIRT1 is the human homologue of Sir2, found in *C. elegans*). Sir2 itself positively regulates lifespan in a DAF-16-dependent manner [84], whereas in mammalian cells deacetylation of FoxO factors is a general mechanism that deactivates FoxO transcriptional activity [83]. On the other hand, others suggest that FoxO deacetylation regulates the balance between pro-apoptotic and cell cycle-arresting genes [5]. Surprisingly, deacetylation experiments performed by two different groups led to inconsistent results. Whereas a reduction of Bim by FoxO deacetylation was observed by both groups, a reduction [83] or an increase [5] in p27^{kip} expression was observed. Earlier findings [6] support the latter experimental result regarding p27^{kip}. It was clearly shown that acetylation of FOXO4 by CBP suppresses the activation of p27^{kip} [6]. In addition to this, the FOXO4 lysine residues involved in the acetylation-induced repression have been mapped [6]. Several stress-induced phosphorylation and acetylation sites are also mapped (see Figure 1 and [5]). Functional analysis of these sites was, however, not performed. Interestingly, a possible stress-induced acetylation site was also implicated in CBP-mediated repression of FOXO4. The identified functional acetylated lysine residues are mainly located in the forkhead domain (Figure 1) [6], suggesting that acetylation could affect target DNA binding. Unfortunately, this was not investigated [6]. FoxO deacetylation reduces apoptosis [5,83] and increases G₁ arrest [5]. This suggests that deacetylation can shift the balance from pro-apoptotic processes to cell-cycle arrest and survival. Interestingly, the HRAAA FOXO1 mutant completely abolishes apoptosis, whereas G₁ arrest was unaffected [65] (as described in the Modification of the DNA-binding potential section, above). Although speculative, acetylation and deacetylation may regulate the binding of FoxOs to target DNA, shifting the balance from DNA-dependent to DNA-binding-independent transcription. Interestingly, SIRT1-mediated deacetylation of the tumour suppressor protein p53, thereby suppressing DNA-binding activity [85], parallels the effect of deacetylation of FoxO factors [5,83]. This emphasizes the need to analyse the relationship between FoxO acetylation and DNA-binding activity. Besides acetylation, hydrogen-peroxide-induced stress leads to nuclear accumulation of FOXO3 under growth-factor-rich conditions [5]. This stress-induced translocation does not affect PKB phosphorylation itself or the phosphorylation state of the N-terminal FoxO PKB site. Apparently, stress overrules the PI3K–PKB pathway with respect to FoxO shuttling. It is interesting to examine 14-3-3 binding and the PKB phosphorylation site in the forkhead domain under these conditions, since both can have dramatic effects on the DNA-binding capability of FoxOs.

Table 1 Selection of confirmed FoxO target genes

Cellular function/associated gene	Reference
Survival	
Fas ligand (FasL)	[86–88]
Transforming growth factor β -2 (TGF- β 2)	[89]
Tumour-necrosis-factor-related apoptosis-induced ligand (TRAIL)	[90,91]
Bcl-2-interacting mediator of cell-death (Bim)	[92–96]
Growth-arrest and DNA-damage-response protein 45 (Gadd45)	[97]
Manganese superoxide dismutase (MnSOD)	[98]
Cell cycle	
p27 ^{kip}	[93,99,100]
Polo-like kinase	[101]
Cyclin B	[101]
Cyclin D _{1/2}	[65]
Cyclin G ₂	[65,102]
Metabolism	
IGFBP-1/3	[46,65,103,104]
G6pc	[70,73,74,105]
Phosphoenolpyruvate carboxykinase (Pck1)	[73,74]
Tyrosine aminotransferase	[89,106]
Pyruvate dehydrogenase kinase 4 (PDHK4)	[107]
<i>Drosophila</i> insulin receptor (dlnsR)	[108]
<i>Drosophila</i> prolactin gene	[69]
<i>Drosophila</i> eukaryotic initiation factor 4E-binding protein (d4EBP)	[108–110]
Lipoprotein lipase (LPL)	[111]

Functions of FoxO *in vivo*

As shown in Table 1, FoxO transcription factors have many downstream targets. They are associated with cell-type-specific effects on cell cycle, metabolism, DNA repair, protection against oxidative stress and cell death. Since most of the downstream targets were identified *in vitro*, the *in vivo* extrapolation is problematic. However, data from *C. elegans*, *Drosophila* and the mouse provide valuable information regarding the *in vivo* function of FoxO factors.

FoxO in *C. elegans*

DAF-16 is the FoxO homologue expressed in *C. elegans*, and is regulated by a signalling pathway similar to the mouse insulin–PI3K–PKB pathway. DAF-16 is remarkably similar to mouse FoxO proteins, and can be partially substituted by FoxO3 [112]. Life-span extension, stress resistance and arrest at the dauer diapause stage are accomplished by either inhibition or mutation of the insulin–PI3K–PKB pathway or direct activation of DAF-16 [113]. Besides cell autonomous inputs, DAF-16 also responds to environmental inputs. Starvation, heat and oxidative stress all activate DAF-16, whereas nutrient-rich conditions deactivate DAF-16. In summary, DAF-16 responds to cues of a changing environment to reallocate resources at all stages of life [114].

FoxO in *Drosophila melanogaster*

Recently, a unique FoxO homologue in *Drosophila* was identified and named dFOXO (*Drosophila* FOXO) [108–110]. Ectopic expression of dFOXO results in a marked reduction in body size. This reduction is caused by a decrease in cell number [108–110] and cell size [109,110]. The phenotype observed in starving larvae resembles that of the dFOXO-overexpression mutant [109]. This correlates well with the fact that the insulin–PI3K–PKB signalling cascade [108–110] and nutrients [110] negatively regulate dFOXO. However, dFOXO knock-out flies are viable and of normal size, but are more vulnerable to oxidative stress,

which suggests that dFOXO is not required for proper growth, but does provide protection against oxidative stress [110]. Microarray analysis identified d4E-BP, a translation inhibitor [110], as a dFOXO target, whereas RNase-protection assays identified d4E-BP and the *Drosophila* insulin receptor as dFOXO targets [108]. It is suggested that under stressful conditions such as nutrient deprivation, dFOXO is activated and regulates growth via d4E-BP [108,110]. The *Drosophila* insulin receptor provides this system with negative feedback [108].

FoxO in *Mus musculus*

FoxO1 homozygous null mutants die before birth due to several embryonic defects [115], including incomplete vascular development [116]. Analysis of heterozygote null-mutants indicated that FoxO1 is involved in pancreatic growth, hepatic glucose metabolism and adipocyte differentiation [115,117,118]. A diabetic phenotype, induced by diet or disruption of either the insulin receptor or IRS-2, can be rescued by FoxO1 haploinsufficiency [115,117,118]. Moreover, a FoxO1 gain-of-function mutant induces diabetes [115]. The underlying mechanism involves FoxO1-mediated negative regulation of genes involved in insulin sensitivity in the liver, adipocytes and pancreatic β cells [115,117,118]. Strikingly, FoxO mRNAs are regulated by nutritional and hormonal factors, as was described for mouse liver and skeletal muscle [107,111,119]. Food restriction or glucocorticoid treatment leads to an increase in FoxO1, FoxO3 and FoxO4 mRNA. Refeeding the mice reverses the starvation-induced increase in FoxO mRNA [107,119]. FoxO1 is also induced in skeletal muscle by streptozotocin-induced diabetes and treadmill running [111]. This implicates FoxOs in skeletal muscle energy metabolism. This is strengthened further by the fact that they regulate the expression of pyruvate dehydrogenase kinase 4 and lipoprotein lipase, two enzymes involved energy utilization [107,111].

The mouse FoxO3 knock-out does not have such a dramatic phenotype compared with FoxO1 [116,120], the FoxO4 knock-out does not have any obvious abnormality [116]. Closer inspection of the FoxO3 knock-out reveals haematological abnormalities, a decreased glucose uptake in glucose-tolerance tests [120] and a distinct ovarian phenotype due to premature follicular activation. FoxO3 is thus suggested to function at the early stages of follicular growth as a suppressor of follicular activation [116,120].

Taken together, *in vivo* studies implicate FoxO proteins in the homeostasis of metabolism. In *C. elegans*, *Drosophila* and the mouse, FoxOs respond to nutrients, growth factors and stress in order to 'fine-tune' cellular metabolism and optimally adapt the cell to an ever-changing environment. Intriguing is the fact that FoxO1, FoxO3 and FoxO6 are all expressed in the central nervous system: a role in neuronal metabolism and stress defence is therefore not unlikely, since neurons have to last a lifetime.

CONCLUSIONS

FoxO transcription factors consist of a family with currently four different members in mouse (Figure 1) [3,4]. All FoxO members are regulated by multiple protein kinases, thereby serving as a transcriptional endpoint of several signalling cascades (Figure 2). Central to the regulation of FoxO members is a complex shuttling mechanism that regulates the intracellular FoxO localization (Figures 4 and 5). Phosphorylation at three highly conserved PKB phosphorylation sites results in FoxO sequestration in the cytosol by 14-3-3 proteins. A highly conserved motif that regulates the speed of nuclear export is not conserved in FoxO6 (Figures 1

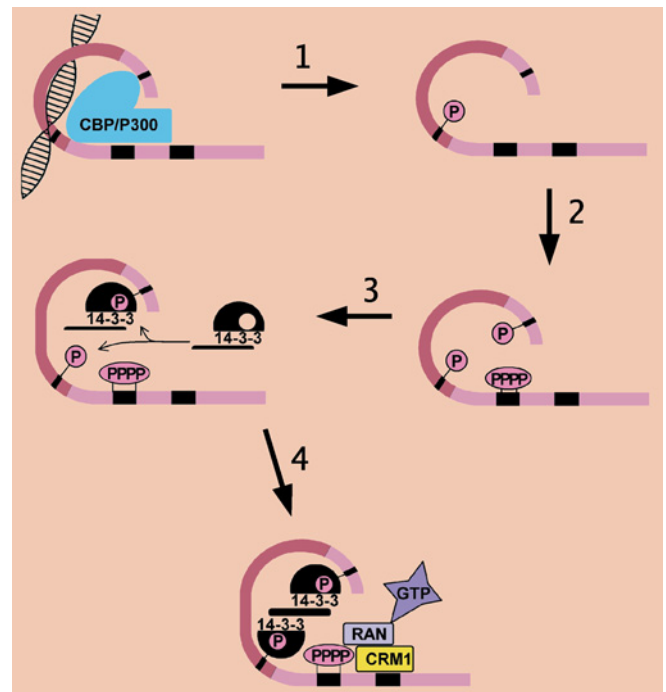


Figure 5 Schematic representation of the sequential phosphorylation events of FoxO proteins triggering nuclear exclusion

1, Under conditions devoid of growth factors, FoxO proteins bind to DNA and are transcriptionally active. FoxO proteins associate with components of the transcription machinery, including CBP/p300. Phosphorylation of the PKB site in the forkhead domain induces dissociation from DNA and blockade of the NLS. 2, First the N- and C-terminal PKB sites are phosphorylated; additionally, two CK1 sites become phosphorylated. The fourth serine, a target of DYRK1A, is also phosphorylated. 3, A 14-3-3 protein binds to the C-terminal PKB motif, allowing subsequent binding of a second 14-3-3 protein, which dimerizes. This second 14-3-3 protein also interacts with the PKB motif in the forkhead domain. 4, CRM1 and Ran-GTP attach to the FoxO protein via interactions with the NES, and possibly the stretch of four phosphorylated serine residues.

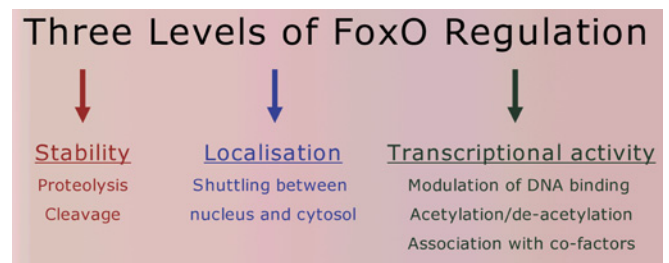


Figure 6 Schematic representation of different levels of FoxO regulation

FoxO factors are regulated by influencing protein stability, localization and their transcriptional activity.

and 3), resulting in a nuclear localization under all conditions tested. Besides the regulation of intracellular localization, growth factors influence FoxO association with the general transcription machinery and DNA binding itself, providing the cell with an additional pathway in the regulation of transcription. FoxO association with nuclear receptors leads to either augmentation or repression of the nuclear receptor target genes, diversifying FoxO-mediated transcriptional regulation. In summary, FoxOs are regulated at several levels and in different cellular compartments (Figure 6). *In vitro* and *in vivo* studies have identified an array of FoxO downstream targets (Table 1). These targets implicate

FoxO as having a functional role in processes such as cell cycle regulation, cell death, metabolism, protection from oxidative stress and survival. *In vivo* studies, ranging from *C. elegans* to the mouse, suggest that FoxO factors are involved in the integration of environmental cues to optimally adapt to changing environmental conditions. The importance of FoxO function is emphasized by the association of the FoxO family members with the development of organs, such as the pancreas [118] and the ovaries [120], and complex diseases, such as diabetes [115]. The functional importance of FoxO1 and FoxO3 *in vivo* suggests important roles for FoxO4 and FoxO6 in cellular metabolism, although the FoxO4 knock-out has no obvious phenotype [116]. Especially interesting is the possible *in vivo* function of FoxO6, since the protein is mainly nuclear and its mRNA is widely expressed in the central nervous system. Therefore a neuron-specific FoxO6 function is conceivable, since neuronal development and maintenance is essential for an organ that must last a lifetime.

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