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REVIEW

FOXO-binding partners: it takes two to tango

KE van der Vos[1] and PJ Coffer[1,2]

[1]Molecular Immunology Lab, Department of Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands and [2]Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands

Modulation FOXO transcription factor activities can lead to a variety of cellular outputs resulting in changes in proliferation, apoptosis, differentiation and metabolic responses. Although FOXO proteins all contain an identical DNA-binding domain their cellular functions appear to be distinct, as exemplified by differences in the phenotype of Foxo1, Foxo3 and Foxo4 null mutant mice. While some of these differences may be attributable to the differential expression patterns of these transcription factors, many cells and tissues express several FOXO isoforms. Recently it has become clear that FOXO proteins can regulate transcriptional responses independently of direct DNA-binding. It has been demonstrated that FOXOs can associate with a variety of unrelated transcription factors, regulating activation or repression of diverse target genes. The complement of transcription factors expressed in a particular cell type is thus critical in determining the functional end point of FOXO activity. These interactions greatly expand the possibilities for FOXO-mediated regulation of transcriptional programmes. This review details currently described FOXO-binding partners and examines the role of these interactions in regulating cell fate decisions.
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Introduction

Over the past few years the mammalian DAF-16-like transcription factors FOXO1, FOXO3 and FOXO4 have been demonstrated to play crucial roles in a plethora of cellular processes including proliferation, apoptosis, differentiation, stress resistance and metabolic responses (Birkenkamp and Coffer, 2003; van der Horst and Burgering, 2007). To ensure that the correct, cell type-specific effect is initiated by these widely expressed factors, FOXOs utilize a wide range of binding partners allowing for a much broader transcriptional response. The consequences of such physical associations are perhaps best highlighted by oncogenic FOXO fusion proteins responsible for mixed lineage leukaemia and alveolar rhabdomyosarcoma (Barr, 2001; So and Cleary, 2003). Both FOXO3 and FOXO4 are frequent targets of chromosomal translocations in human leukemias, resulting in fusions to mixed lineage leukaemia. Fusion tends to occur in the middle of the forkhead-binding domain resulting in chimeric proteins harbouring the transcriptional activation domains of the respective forkhead proteins. Similarly PAX3-FOXO1 fusions found in alveolar rhabdomyosarcoma exhibit a similar chimeric structure containing the FOXO transactivation domain and PAX3 DNA-binding domains. The FOXO1 transactivation domain has more robust transcriptional activation potential than that of PAX3 and can thus more strongly activate PAX3-mediated transcription. This has led to the hypothesis that fusion with FOXO1 drives oncogenesis through enhanced transcriptional activation of PAX3 target genes. These 'physical interactions' demonstrate that functional association of FOXO proteins with other transcription factors can have dramatic consequences on transcriptional programmes which may even lead to cellular transformation.

The first real evidence that association of FOXOs with accessory proteins played a critical role in transcriptional regulation came from work by Sellers and coworkers (Ramaswamy *et al*., 2002). Using transcriptional profiling, chromatin immunoprecipitation and functional experiments to identify FOXO target genes, Ramaswamy *et al*. demonstrated that a FOXO mutant, in which DNA-binding was abolished, was still able to effectively regulate a specific subset of these genes. The authors proposed that DNA-binding was not required for FOXO-dependent tumour suppression and cell-cycle regulation. This surprising result could be explained by a possible 'altered' DNA-binding specificity of the FOXO mutant utilized, but more likely is that FOXO regulates a subset of target genes through interaction with other transcription factors. Indeed it has become apparent that FOXO proteins are able to associate with a wide variety of diverse transcription factor partners resulting in a far broader spectrum of gene regulation (Table 1). Here, we will discuss the various associating proteins and their implications for regulating cellular responses to FOXO activation.

Correspondence: Professor PJ Coffer, Molecular Immunology Lab, Department of Immunology, Wilhelmina Children's Hospital, University Medical Center, Lundlaan 6, 3584 EA Utrecht, The Netherlands.
E-mail: p.j.coffer@umcutrecht.nl

Table 1 FOXO transcription factor-binding partners

Androgen receptor (AR)	(Li *et al*., 2003; Fan *et al*., 2007)
β-catenin	(Essers *et al*., 2005; Almeida *et al*., 2007)
Constitutive androstane receptor (CAR)	(Kodama *et al*., 2004)
Cs1	(Kitamura *et al*., 2007)
C/EBPα	(Qiao and Shao, 2006; Sekine *et al*., 2007)
C/EBPβ	(Christian *et al*., 2002; Gomis *et al*., 2006a)
Estrogen receptor (ER)	(Schuur *et al*., 2001)
FoxG1	(Seoane *et al*., 2004)
Follicle stimulating hormone receptor (FSHR)	(Nechamen *et al*., 2007)
Hepatic nuclear factor-4 (HNF4)	(Hirota *et al*., 2003)
HOXA5	(Foucher *et al*., 2002)
HOXA10	(Kim *et al*., 2003)
Myocardin	(Liu *et al*., 2005)
PGC-1α	(Puigserver *et al*., 2003)
PPARα	(Qu *et al*., 2007)
PPARγ	(Dowell *et al*., 2003)
Pregnane X receptor (PXR)	(Kodama *et al*., 2004)
Progesterone receptor (PR)	(Kim *et al*., 2005; Rudd *et al*., 2007)
Retinoic acid receptor (RAR)	(Zhao *et al*., 2001)
RUNX3	(Yamamura *et al*., 2006)
Smad3	(Seoane *et al*., 2004)
Smad4	(Seoane *et al*., 2004)
STAT3	(Kortylewski *et al*., 2003)
Thyroid hormone receptor (TR)	(Zhao *et al*., 2001)

Integration of PI3K/FOXO and TGFβ/Smad pathways

In the nematode worm *Caenorhabditis elegans* the FOXO transcription factor DAF-16 regulates metabolism, development and longevity (Gottlieb and Ruvkun, 1994; Larsen *et al*., 1995). Many of these effects are mediated through DAF-2, a homologue of the mammalian insulin receptor and AGE-1, the nematode PI3-kinase. Null mutations in *daf-16* were found to suppress the effects of mutations in *daf-2* or *age-1*, whereas genetic ablation of *daf-16* bypasses the need for insulin receptor-like signalling (Ogg *et al*., 1997). A parallel TGFβ/DAF-7 and Smad/DAF-3 pathway also regulates *C. elegans* metabolism and development (Ren *et al*., 1996; Patterson *et al*., 1997). In mammals, TGFβ activates a receptor serine/threonine kinase complex that phosphorylates Smad2 and Smad3 (Shi and Massague, 2003). Once activated, Smads translocate to the nucleus where they form transcriptional complexes with Smad4 plus additional coactivators and repressors. In the nematode worm *daf-3* acts in the TGFβ pathway in an analogous manner to *daf-16* in the insulin-like pathway, and DAF-3 activity is negatively regulated by upstream TGFβ signalling. Importantly, the DAF-2 pathway was found to exhibit genetic synergy with the nematode DAF-7/DAF-3 pathway, suggesting that DAF-16 can cooperate with nematode SMAD proteins in regulating the transcription of key metabolic and developmental control genes (Ogg *et al*., 1997).

On the basis of these findings, Ruvkun and coworkers proposed that DAF-16 and DAF-3 might form heteromers that repress the expression of key genes regulating metabolism and reproductive development (Ogg *et al*., 1997). This can be extrapolated to mammals where it can be envisaged that FOXO proteins might functionally interact with SMAD transcription factors. This hypothesis was further investigated in studies analysing the regulation of neuroepithelial and glioblastoma cell proliferation by TGFβ (Seoane *et al*., 2004). TGFβ delivers cytostatic signals to epithelial, neuronal and immune cells and its subversion can contribute to tumour development. Transcriptional activation of *p21Cip1* and *p15Ink4b*, cell cycle inhibitors and repression of growth promoting *Id1* and *c-myc* are critical for this TGFβ driven cytostatic programme. In support of the genetic analysis performed in *C. elegans*, Massagué and coworkers identified FOXO proteins as key partners of Smad3 and Smad4 in TGFβ-dependent formation of a *p21Cip1* transactivation complex (Seoane *et al*., 2004). TGFβ results in the reorganization of a transcriptional complex on the *p21Cip1* promoter with removal of c-Myc and binding of Smad2/3 and Smad4. The binding of Smads to the *p21Cip1* promoter overlaps with a consensus forkhead-binding element and mutation of this site was found to abrogate the TGFβ response of this promoter. FOXO-mediated induction of *p21Cip1* promoter activity in TGFβ-treated cells was also increased by Smad overexpression. The cooperation of these distinct transcription factor families suggested that they may form a TGFβ-regulated complex. Indeed coimmunoprecipitation experiments confirmed that FoxO1, FoxO3 and FoxO4 can all bind to Smad3 and Smad4 in a TGFβ-dependent manner. By generation of mutant proteins it was demonstrated that the FOXO transactivation domain was critical for TGFβ-mediated $p21^{Cip1}$ expression, and that this was not due to general sequestration of Smads, but formation of a specific complex at the *p21Cip1* promoter. As previously proposed by work in the nematode worm, these data suggest that insulin and TGFβ signalling could be integrated at the level of FOXOs. Indeed inhibition of PI3-kinase signalling potentiated the induction of *p21Cip1* by suboptimal concentrations of TGFβ. This model of integration of insulin and TGFβ signalling is further complicated by the identification of FoxG1 as an antagonist of FOXOs (Seoane *et al*., 2004). FoxG1 is a transcriptional repressor whose function is to protect neuroepithelial progenitor cells from cytostatic signals (Hanashima *et al*., 2004). FoxG1 overexpression inhibits *p21Cip* induction by TGFβ, and it could also be coimmunoprecipitated with FOXOs. An analysis of glioblastomas revealed high PI3K activity and FOXG1 expression with an inability of cells to increase *p21Cip1* levels after TGFβ treatment. Thus FOXOs can be considered a nodal point for the integration of Smad-, PI3K- and FoxG1-signalling modules. In support of this it has recently been demonstrated that in human keratinocytes, FOXOs are essential for 11 of 115 immediate gene activation responses to TGFβ (Gomis *et al*., 2006a). Taken together, these results suggest that

formation of a FOXO and Smad transcription factor complex is critical in the control of cell growth and proliferation, and that perturbation of the association or function of this complex could contribute to neoplasia.

β-catenin and FOXOs fighting stress together

As already alluded to, the *C. elegans* FOXO transcription factor DAF-16 increases longevity in the nematode worm by inducing entry into the dauer diapause, an alternative larval stage. Essers and coworkers found that in animals lacking the β-catenin gene, *bar-1*, dauer development and nematode life-span are perturbed (Essers *et al*., 2005). Further genetic analysis has revealed that BAR-1 is required for DAF-16 function (when DAF-16 activity is limiting), raising the intriguing possibility that β-catenin may also be required for FOXO function in mammalian cells. Indeed coexpression of these proteins resulted in increased transcription from several FOXO promoter reporters and they were also found to physically associate. In *C. elegans*, and in mammalian cells, oxidative stress activates FOXO transcriptional activity by stimulating nuclear relocalization (Brunet *et al*., 2004; Essers *et al*., 2004). Increasing levels of oxidative stress by hydrogen peroxide treatment of cells resulted in increased association of β-catenin and FOXO4. Importantly, in the nematode worm DAF-16-induced transcriptional responses to oxidative stress require BAR-1.

The best characterized β-catenin-binding partner is TCF, a transcription factor required for a variety of developmental processes (Arce *et al*., 2006). One consequence of oxidative stress-induced association of FOXOs and β-catenin is that this would divert a limited pool of intracellular β-catenin away from TCF. β-catenin/TCF-mediated transcription is required for osteoblast differentiation, and hydrogen peroxide induces reciprocal changes in FOXO- and TCF-mediated transcription in osteoblastic cells (Almeida *et al*., 2007; Glass and Karsenty, 2007). Increased levels of reactive oxygen species are also able to suppress osteoblast differentiation affecting skeletal homeostasis. Thus it appears that levels of oxidative stress, which is thought to increase during the age of an organism, will result in increased β-catenin/FOXO association at the expense of TCF. This could be an important pathogenic factor in the development of skeletal involution which is associated with old age.

CCAAT/enhancer-binding protein interactions

Massagué and coworkers characterized a subset of FOXO/Smad-dependent TGFβ gene responses which were found to additionally require the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) (Gomis *et al*., 2006a). C/EBPβ was found to be essential for TGFβ induction of the cell-cycle inhibitor *p15INK4b* by a FOXO-Smad complex and repression of *c-MYC* by an E2F4/5-Smad complex in human epithelial cells (Gomis *et al*., 2006b). The molecular mechanisms underlying C/EBPβ-mediated effects have not been completely resolved but diverse configurations of FOXO and Smad-binding elements in the promoters of target genes were identified. C/EBP transcription factors exhibit a broad expression pattern with tissue-specific transcriptional responses controlling diverse cellular processes including hematopoiesis, adipocyte differentiation and gluconeogenesis in the liver (Nerlov, 2007). More recently, their position at the crossroads between proliferation and differentiation has made them strong candidate regulators of tumorigenesis, and C/EBPs have been described as both tumour promoters and tumour suppressors.

One such process where C/EBPs play a role is decidualization of uterine endometrial stroma (ES). This is characterized by the morphological and biochemical transformation of the ES in which the stromal fibroblasts differentiate to become rounded, secretory decidual cells. It is regulated by ovarian estrodiol and progesterone and appears to require elevated cAMP levels and sustained activation of protein kinase A (PKA) (Brar *et al*., 1997). C/EBPβ expression is upregulated during ES cell differentiation and this is cAMP-inducible (Pohnke *et al*., 1999). Expression of decidual prolactin (dPRL) by ES cells is a widely used biochemical marker of decidual differentiation and C/EBPβ forms part of a transcriptional complex binding to the dPRL promoter upon PKA activation. Treatment of primary ES cell cultures with cAMP was found to induce the sustained expression of nuclear localized FOXO1 (Christian *et al*., 2002). Ectopic expression of FOXO1 was found to regulate expression of several decidualization-specific genes such as dPRL and, in the absence of exogenous hormones, also results in a noticeable change in stromal cell shape (Buzzio *et al*., 2006). FOXO1 was found to transcriptionally activate the dPRL promoter and mutation of C/EBP-binding sites in the dPRL promoter abolished this effect (Christian *et al*., 2002). This suggests a physical interaction between C/EBPβ and FOXO1, and this has been subsequently confirmed by *in vitro*-binding assays. Furthermore, attenuation of FOXO1 levels in hormone-treated ES cells by RNAi resulted in the dramatic inhibition of expression of marker genes associated with decidualization (Grinius *et al*., 2006). FOXO1 is therefore an important effector of the decidual response in part through interaction with C/EBPβ. These studies also reveal a novel functional interaction with the PKA/cAMP signal transduction module. The cooperative action of FOXO transcription factors in endometrial decidualization is not only restricted to C/EBPβ. The homeobox (HOX) protein HOXA10 also follows similar patterns of expression to FOXO1 during different stages of the baboon menstrual cycle and pregnancy (Kim *et al*., 2003). HOX proteins are developmentally regulated transcription factors that are important for spatial identity and differentiation of tissues in the developing embryo. HOXA10 null mutant mice exhibit infertility due to compromised endometrial

decidualization during blastocyst implantation (Benson *et al*., 1996). Kim *et al*. were able to demonstrate a direct *in vitro* association between HOXA10 and FOXO1 as well as cooperative transactivation of the insulin-like growth factor-binding protein-1 (IGFBP-1) promoter (Kim *et al*., 2003). During pregnancy IGFBP-1 is expressed in decidualized stromal cells where it is thought to play a role during blastocyst implantation (Giudice *et al*., 1993). In a human fibroblast cell line (HuF) FOXO1 was found to cooperatively regulate IGFBP-1 expression together with another HOX protein, HOXA5 (Foucher *et al*., 2002). However, in the same study it was demonstrated that in the HepG2 cell line, HOXA5 actually represses FOXO1-induced IGFBP-1 transcription. Interestingly, these observations suggest that association between FOXO proteins and other transcription factors will have cell context-dependent effects.

FOXO1 has also recently been demonstrated to link insulin signalling to another C/EBP family member, C/EBPα and can thereby regulate gluconeogenesis in the liver (Sekine *et al*., 2007). During mammalian development the liver progresses from a major site of hematopoiesis in the foetus to a central metabolic tissue in the adult. Newborns have to rapidly cope with the loss of maternal nutrient feeding after birth and adaptation in the liver is reflected by transcriptional changes. C/EBPα is critical for regulation of glucose metabolism during this adaptation phase and genetic ablation of this transcription factor leads to low blood glucose levels and subsequent neonatal death (Wang *et al*., 1995). Although glucose metabolism in the liver is precisely controlled by insulin which represses gluconeogenesis, it has remained unclear until recently how this was linked to C/EBPα function. C/EBPα is expressed in the fetal liver and this expression does not change dramatically after birth. In contrast, FOXO1 expression is low in early fetal liver, but increases dramatically during development (Sekine *et al*., 2007). Functional interaction between FOXO1 and C/EBPα were observed when analysing their coordinated ability to regulate the phosphoenolpyruvate carboxykinase (PEPCK) promoter, a gluconeogenic gene. Coimmunoprecipitation experiments, utilizing neonatal liver, demonstrated that C/EBPα and FOXO1 physically interact. Recruitment of C/EBPα-FOXO1 complexes to the PEPCK promoter *in vivo*, was confirmed using chromatin immunoprecipitation (ChIP) assays with neonatal liver extracts. Critically, using C/EBPα (−/−) cells it was shown that FOXO1 promoter association requires C/EBPα. Since insulin treatment results in PKB-mediated FOXO phosphorylation and nuclear exclusion, this suggests a simple model whereby insulin suppresses the expression of gluconeogenic genes. Indeed suppression of PEPCK expression is observed when cultured fetal liver cells are treated with insulin (Sekine *et al*., 2007). The synergistic activity and physical interaction between FOXO1 and C/EBPα has been further confirmed in differentiated 3T3-L1 adipocytes (Qiao and Shao, 2006). FoxO1 expression is induced early during adipocyte differentiation and FoxO1 haploinsufficiency leads to significant reduction of adiponectin gene expression in adipose tissue (Nakae *et al*., 2003). Adiponectin enhances insulin sensitivity, improves fatty acid oxidation in skeletal muscle and suppresses hepatic gluconeogenesis (Berg *et al*., 2001). FOXO-binding sites in the adiponectin promoter were found to bind a transcriptional complex containing FOXO1 and C/EBPα (Qiao and Shao, 2006). Furthermore, the association of FOXO1 and C/EBPα was found to be regulated by SIRT1 activity, an NAD + -dependent protein deacetylase that is also involved in adipogenesis (Picard *et al*., 2004). SIRT1 deacetylates three lysine residues in the FOXO1 forkhead domain, which is the region that interacts with C/EBPα (Brunet *et al*., 2004). This suggests the interesting possibility that the post-translational modification status of FOXOs regulates their ability to interact with other cofactors.

FOXO partners regulating muscle homeostasis

Smooth muscle cells (SMCs) are unique in that they exhibit phenotypic plasticity and can transition between a quiescent contractile phenotype and a proliferative phenotype (Owens *et al*., 2004). This is critical in response to vascular injury where they are induced to dedifferentiate and proliferate. The PI3-kinase signalling pathway has been demonstrated to stimulate SMC differentiation (Hayashi *et al*., 1999). Based on this observation, Olson and coworkers investigated the role of Foxo4 on phenotypic modulation of vascular smooth muscle cells (Liu *et al*., 2005). Several critical observations were initially made: (i) IGF-1 promotes SMC differentiation in a PI3-kinase-dependent manner, (ii) ectopically expressed Foxo4 was found to inhibit SMC differentiation and (iii) Foxo4 siRNA promotes expression of SM contractile genes. Importantly the inhibitory effect of Foxo4 on SMC differentiation was independent of DNA-binding; however, Foxo4 was found to associate with promoters of SMC marker genes *in vivo*. This suggests that Foxo4 must be associating with additional transcription factors or cofactors to regulate promoter activity of these genes. Expression of the transcription factor myocardin is sufficient to activate a programme of SM differentiation in fibroblasts, and was identified as a direct Foxo4-binding partner through coimmunoprecipitation and GST pull-down assays. Myocardin itself associates with, and is a potent co-factor for, serum response factor (SRF) (Wang *et al*., 2004). SRF also associates with Foxo4, and the interaction of Foxo4 with myocardin is enhanced in the presence of SRF (Liu *et al*., 2005). Taken together this suggests that Foxo4 forms a ternary complex with myocardin and SRF. While Foxo4 was found to repress the transcriptional activity of myocardin, and this was dependent on physical association, Foxo1 and Foxo3 were unable to recapitulate these effects. In response to insulin or IGF-1 stimulation, SMCs adopt a differentiated phenotype while mitogenic stimulation or injury, results in dedifferentiation and enhanced proliferation of SMCs. Foxo4 therefore represents a link between

these mitogenic effects and regulation of myocardin transcriptional activity. But how precisely does Foxo4 inhibit myocardin-dependent transcription? It doesn't appear to be due to displacement of SRF, since myocardin-SRF-FOXO4 was found to be associated in a ternary complex. The current mechanism is unknown but it could simply involve Foxo4-mediated recruitment of conventional corepressors, such as HDACs, to target promoters. Since Foxo4 activation has been reported to result in cell-cycle arrest (Medema *et al*., 2000), it is perhaps rather surprising that nuclear Foxo4 is associated with SMC proliferation. A possible explanation is that the unique transcriptional programme initiated by Foxo4-myocardin overrides any direct effects modulated by Foxo4 itself. In contrast, Foxo1 and Foxo3, which do not bind myocardin, may have an anti-proliferative role in SMCs. This is supported by the finding that overexpression of Foxo3a in SMCs of rat carotid artery results in smooth muscle cell-cycle arrest (Park *et al*., 2005).

It is not only in SMCs where FOXO transcription play a role in regulating myogenesis, it has recently been demonstrated that Foxo1 can also regulate myogenic differentiation in skeletal muscle (Kitamura *et al*., 2007). The Notch pathway plays a critical role in muscle differentiation during embryogenesis (Luo *et al*., 2005). After ligand-induced cleavage, the intracellular domain of the Notch receptor translocates to the nucleus where it interacts with the DNA-binding protein Cs1 to generate an active transcriptional complex. Accili and coworkers made the connection that Foxo gain-of-function has similar effects on myoblast differentiation as Notch1 activation, while Foxo1 ablation in mice has a similar phenotype to Notch1 (−/−) animals (Krebs *et al*., 2000; Hosaka *et al*., 2004). In C2C12 cells, growth factor withdrawal results in myogenic conversion and ectopic expression of a constitutively active Foxo1 mutant, blocked this effect in a DNA-binding independent manner (Kitamura *et al*., 2007). Constitutively active Notch1 had identical effects in blocking myoblast differentiation, and Foxo siRNA rescued the inhibition. These data indicate that Foxo1 and Notch1 signalling are functionally connected. Demonstration that Foxo1 directly interacts with Cs1 using *in vitro* association assays, coimmunoprecipitation and chromatin immunoprecipitation provided a molecular mechanism by which Foxo1 could modulate Notch1 signalling. Interaction of Foxo1 and Cs1 was required for Notch1-mediated induction of the transcriptional target *Hes1* and this was independently of Foxo1 transcriptional function. Instead, it appears that Foxo1 acts to aid displacement of Cs1-associated corepressors (NcoR/Smrt) allowing association of coactivators (Maml1). These findings provide a molecular mechanism by which two distinct signalling modules, PI3K and Notch, can coordinately and synergistically regulate muscle differentiation. The ability of Notch/Foxo1 to functionally interact may allow the integration of diverse environmental cues (through Notch) and metabolic cues (through Foxo1) to regulate progenitor cell maintenance and differentiation in multiple cellular contexts. Acilli *et al*. suggest that this might allow committed progenitor cells to avoid differentiation in response to developmental cues when Foxo1 is active, for example in the absence of growth factors.

FOXOs, steroid hormone receptors and cancer

FOXO proteins have been shown to interact with multiple members of the nuclear hormone receptor (NHR) family, leading to changes in the transcriptional activity of both proteins (Figure 1). NHRs have a

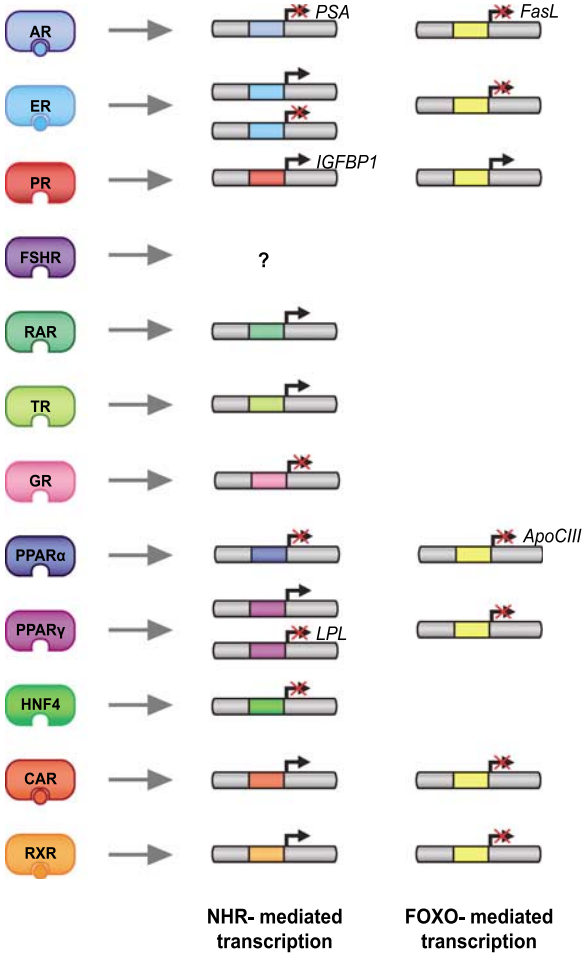

Figure 1 FOXO interaction partners: the nuclear hormone receptors. FOXOs have been shown to interact with a large number of nuclear hormone receptors, resulting in changes in transcriptional activity of both proteins. FOXO interacts in a ligand-dependent manner with the androgen receptor (AR), the oestrogen receptor (ER), constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Interaction with the progesterone receptor (PR), the follicle stimulating hormone receptor (FSHR), the thyroid hormone receptor (TR), the retinoid acid receptor (RAR), the glucocorticoid receptor (GR), peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor γ (PPARγ) and hepatic nuclear factor-4 (HNF4) is independent of the presence of a nuclear hormone receptor (NHR) ligand. In most cases, phosphorylation of FOXOs leads to a disruption of the complex. Increased transcriptional activity is indicated by an arrow, while a red cross indicates decreased activity of either FOXO or the associated NHR. The affected target genes, which have been described are indicated in italic.

modular structure with two domains that can act independently: a ligand binding domain, a central hinge region and a DNA-binding domain. Binding of the cognate ligand induces conformational changes leading to dimerization, recruitment of coactivator complexes and binding to hormone response elements located in target genes (Pardee *et al*., 2004; Biggins and Koh, 2007).

The association of FOXOs with steroid receptors has been shown to either inhibit or enhance their transcriptional activity. These interactions could potentially play a role in the development of steroid-dependent cancers, such as prostate cancer, breast cancer and ovarian cancer. The first hint of a functional link between FOXOs and steroid hormone receptors came from the observation that androgen protects prostate cancer cells from PTEN-induced apoptosis (Li *et al*., 2001). The androgen receptor (AR) belongs to the subfamily of steroid receptors and its ligands include testosterone and 5α-dihydrotestoterone (5α-DHT). AR-dependent gene expression in androgen target tissues, including prostate, skeletal muscle, liver and central nervous system is responsible for male sexual differentiation and male pubertal changes (Gao *et al*., 2005). In addition, functional androgen receptor signalling is necessary for the development and maintenance of prostate cancer and antagonists are currently used for therapy (Gao *et al*., 2005). The ability of androgens to inhibit apoptosis in both normal and malignant prostatic cells has been well documented. However, the underlying molecular mechanisms are understood poorly. Li *et al*. observed that inhibition of PI3-kinase was able to inhibit the transcriptional activity of AR resulting in decreased androgen-induced proliferation (Li *et al*., 2001). FOXO1 was found to directly associate with the androgen receptor, and thereby inhibit its transcriptional activity (Li *et al*., 2003; Fan *et al*., 2007). Utilizing transcription reporter assays it was shown that AR, in a ligand-dependent manner, could also reciprocally inhibit both FOXO1 and FOXO3 activity. This effect is not due to altered FOXO phosphorylation status since a FOXO1 null phosphorylation mutant was still potently inhibited by AR (Li *et al*., 2003). Interaction with AR decreased FOXO1 DNA binding and importantly rescued prostate cancer cells from FOXO1-induced cell death. Alternative mechanisms might also be relevant since it was also observed that addition of androgens can lead to proteolysis of FOXO1 by acidic cysteine proteases (Huang *et al*., 2004). The importance of the FOXO1-AR interaction is strengthened by the observation that expression of FOXO1 in prostate cancer cells is reduced when compared to normal prostates (Dong *et al*., 2006). Hemizygous deletion of *FOXO1A* was detected in 31% of primary prostate cancers, while ectopic expression of FOXO1 in two prostate cancer cell lines inhibited their proliferation. These results suggest that FOXO1 can be considered as a tumour suppressor in prostate cancer. It is likely that in the prostate a tight balance between androgen receptor signalling and FOXO signalling ensures an equilibrium between cell proliferation and death. FOXO1 inactivation will lead to enhanced androgen receptor activities and the development and progression of prostate cancer. These effects are not unique to FOXO1 since FOXO3 is also expressed in prostate tissue and it has been reported that binding of the androgen receptor to FOXO3 can inhibit its transcriptional activity (Li *et al*., 2003, 2007).

A second steroid receptor implicated in the development and maintenance of cancer cells is the oestrogen receptor (ER). This receptor is mainly expressed in mammary gland tissue, ovarian tissue and the uterus. Binding of oestrogen leads to homodimerization and transcription of oestrogen-responsive genes, which stimulate cell proliferation, invasion, metastasis and angiogenesis while they inhibit apoptosis (Deroo and Korach, 2006). The receptor is frequently overexpressed in breast cancer cells and the cumulative exposure of breast epithelium to oestrogen has been associated with the development of breast cancer. Ablation of the ERα gene delays the onset of tumour development in mouse models, indicating that oestrogen receptor-mediated signalling indeed plays an important role in breast cancer (Bocchinfuso and Korach, 1997). FOXO1 was found to interact with ERα in a ligand-dependent manner, however there are conflicting reports as to the effect of this interaction on ER transcriptional activity (Schuur *et al*., 2001; Zhao *et al*., 2001). Schuur *et al*. have also reported that ERα reciprocally repressed FOXO1-mediated promoter transactivation, while cell cycle arrest induced by FOXO1 in MCF7 cells was abrogated by estradiol (Schuur *et al*., 2001). The physiological relevance of this interaction was tested in oestrogen-dependent human breast cancer cells, where overexpression of FOXO1 inhibits proliferation (Zhao *et al*., 2001). However, it remains unclear whether the FOXO1-mediated inhibition of proliferation is specifically dependent on its interaction with ERα.

It has been suggested that FOXOs may interact with NHRs through a LxxLL motif located C-terminal of the Forkhead DNA-binding domain (Zhao *et al*., 2001). The LxxLL motif is present in critical coactivators and corepressors that interact with NHRs, for example the histone acetyl transferase p300 (Plevin *et al*., 2005). All four FOXO family members contain a LxxLL motif but it is not present in the *Drosophila melanogaster* dFOXO protein, or in the *C. elegans* homologue DAF16 (Table 2). Regions flanking the LxxLL motif differ between FOXOs and might play a role in NHR selectivity.

Table 2 LxxLL motif in FOXOs

FOXO1	APGL***LKELL***TSDSPPHNDIMT
FOXO3	GNQT***LQDLL***TSDS-LSHSDVMMT
FOXO4	SSGA***LEALL***TSDTPPPPADVLMT
FOXO6	PPGA***LPALL***PPP-PPAP
DFOXO	TTTMSPAYPNSEPSSDSLNTYSN
dAF16	QIKQESKPIKTEPIAPPPSYHEL
FOXA1	GPGALASVPPAS

All mammalian FOXO isoforms contain an LxxLL motif that is postulated to interact with nuclear hormone receptors. This sequence is absent in non-vertebrate FOXOs and other forkhead transcription factors (Zhao *et al*., 2001).

These findings provide an important link between cell surface signalling mechanisms that act through the PI3-kinase pathway and nuclear hormone receptors. Furthermore, they provide an alternative mechanism of steroid hormone action in responsive cells. It suggests that one of the oncogenic properties of steroid hormones might result from inhibition of FOXO activity, and supports a role for FOXO transcription factors as tumour suppressors.

Regulation of glucose and fatty acid metabolism by FOXO-interactions

Hepatic gluconeogenesis is a requirement for survival during prolonged fasting or starvation but is inappropriately activated in diabetes mellitus. Glucocorticoids and glucagon have potent gluconeogenic actions in the liver, while insulin suppresses this. FOXO1 is the most abundant FOXO isoform in insulin-responsive tissue, and negatively regulates insulin insensitivity in liver, adipose tissue and pancreas, by mediating insulin-induced changes in gluconeogenic enzymes (Nakae *et al*., 2001; Barthel *et al*., 2005). It has been demonstrated that FOXO1 haploinsufficiency restores insulin insensitivity and rescues diabetic phenotype in insulin resistant mice by reducing expression of gluconeogenic enzymes in the liver (Nakae *et al*., 2002). In contrast, targeting a gain-of function FOXO1 mutant to liver and pancreas results in promotion of diabetes. These effects of FOXO1 on insulin sensitivity can in part be explained by the observations that FOXOs can interact with peroxisome proliferator-activated receptors (PPARs). This group of nuclear receptors is involved in nutrient sensing and regulation of carbohydrate and lipid metabolism. For example, PPARγ can increase insulin sensitivity by regulating adipocytes hormones and cytokines (Fievet *et al*., 2006).

Differentiated adipocytes secrete a variety of cytokines that affect adiposity and insulin resistance. It has been suggested that insulin resistance in adipocytes is the first metabolic manifestation leading to development of type 2 diabetes (Pilch and Bergenhem, 2006). Foxo1 expression is induced in early stages of adipocyte differentiation, but activation is delayed until the end of the clonal expansion phase (Nakae *et al*., 2003). Expressing an active Foxo1 mutant in preadipocytes inhibits differentiation, while an inhibitory Foxo1 mutant is able to restore differentiation in fibroblasts from insulin receptor deficient mice. PPARγ is an important regular of adipocyte differentiation and the observation that FOXO1 binding to PPARγ antagonized PPARγ function could explain the FOXO1-mediated differentiation block. One proposed mechanism by which FOXO1 could inhibit PPARγ function is through disrupting formation of a PPARγ/RXR complex resulting in loss of DNA binding (Dowell *et al*., 2003). Reducing transcription of the glucose reporter GLUT4 by PPARγ can lead to a decrease in insulin sensitivity in adipocytes (Armoni *et al*., 2003). Thus in addition to inhibiting differentiation, FOXO1 activation leads to upregulated GLUT4 levels and a further increase in cellular insulin sensitivity (Armoni *et al*., 2006, 2007). Evidence also suggests that FOXO1 might function as a coactivator of PPARα in myocytes. FOXO1 was found to enhance expression of LPL (lipoprotein lipase), a PPARα target gene, in a myocyte cell line (Kamei *et al*., 2003). LPL plays a role in lipid usage in muscle cells by hydrolyzing plasma triglycerides into fatty acids, and is upregulated during fasting, exercise and diabetes. FOXO1-induced LPL levels increased even further in the presence of PPARα ligand; however, whether this was due to a direct interaction between PPARα and FOXO1 needs to be determined (Kamei *et al*., 2003). Adding further complexity, chromatin immunoprecipitations have revealed that PPARα can inhibit FOXO1 transcriptional activity by decreasing the DNA binding capacity (Qu *et al*., 2007).

PPARγ coactivator-1 (PGC-1α) interacts with several transcription factors and plays important roles in regulation of mitochondrial biogenesis, respiration, thermogenesis and hepatic gluconeogenesis (Finck and Kelly, 2006). Spiegelman *et al*. have shown that the binding of PGC-1α results in co-activation of Foxo1 (Puigserver *et al*., 2003). Furthermore expression of an inhibitory mutant of Foxo1 *in vivo* revealed that Foxo1 is required for the PGC-1α induced increase in glucose-6-phosphatase (G6Pase) levels in murine liver cells. Increased expression of G6pase contributes to the increasing production of glucose by the liver that occurs in individuals with diabetes. The authors propose a model in which the direct interaction of PGC-1α with Foxo1 leads to increased binding of Foxo1 to the promoter of G6Pase (Puigserver *et al*., 2003). However, a recent report has suggested that the synergism between PGC1α and Foxo1 is not the consequence of a direct Foxo1 PGC-1α interaction, but rather results from the presence of both Foxo1 and nuclear receptor-binding sites in the G6Pase promoter (Schilling *et al*., 2006). In this experimental setup, mutation of FOXO-binding sites did not decrease the ability of PGC1α to increase G6Pase expression, whereas mutating the nuclear receptor-binding site did (Schilling *et al*., 2006). However, although these *in vitro* experiments show that the synergism between Foxo1 and PGC-1α can result from the presence of multiple-binding sites in the promoter, they do not exclude effects of Foxo1 on PGC-1α activity. In conclusion the interplay between PGC-1α and Foxo1 plays an important role in regulating the transcription of genes involved in gluconeogenesis. Whether Foxo1 might function as a true PGC-1α coactivator, thereby explaining the negative effect on G6Pase expression in mice expressing an inhibitory Foxo1 mutant, requires further research.

In muscle, maintaining size and fibre composition requires contractile activity. This in turn stimulates the expression of PGC-1α, which promotes fibre-switching from glycolytic toward more oxidative fibres. Upon fasting, and in many systemic diseases, muscles undergo atrophy and FOXO proteins have been implicated in this loss of muscle mass (Sandri *et al*., 2004). In contrast

to liver, it has been reported that in skeletal muscle that PGC-1α expression inhibits Foxo3-dependent transcription (Sandri *et al*., 2006). Transgenic expression of PGC-1α alters the expression of key atrophy-specific genes and reducing the ability of Foxo3 to cause muscle atrophy. However, it remains inconclusive whether this effect is due to a direct interaction between Foxo3 and PGC-1α.

The regulatory role of FOXO1 in inhibiting insulin sensitivity in diabetic mice, makes it a promising target for therapeutic intervention. Indeed in support of this, targeted reduction of Foxo1 levels by antisense oligonucleotides decreased expression of G6Pase, lowered plasma glucose concentration and improved insulin sensitivity in diabetic mice (Samuel *et al*., 2006).

Concluding remarks

FOXO transcription factors have a similar, if not identical, DNA-binding domain; however, ablation of Foxo1, Foxo3 and Foxo4 in mice has overlapping but distinct effects (Hosaka *et al*., 2004). As previously discussed, studies by Sellers and coworkers demonstrated that FOXO proteins can induce transcriptional responses independently of DNA-binding (Ramaswamy *et al*., 2002). The studies highlighted in this review demonstrate that direct association of FOXO proteins with diverse transcription factor families can mediate the regulation of a plethora of cellular processes independently of FOXO DNA-binding (Figure 2). Furthermore, it suggests a mechanism by which specific FOXO isoforms can uniquely regulate transcriptional

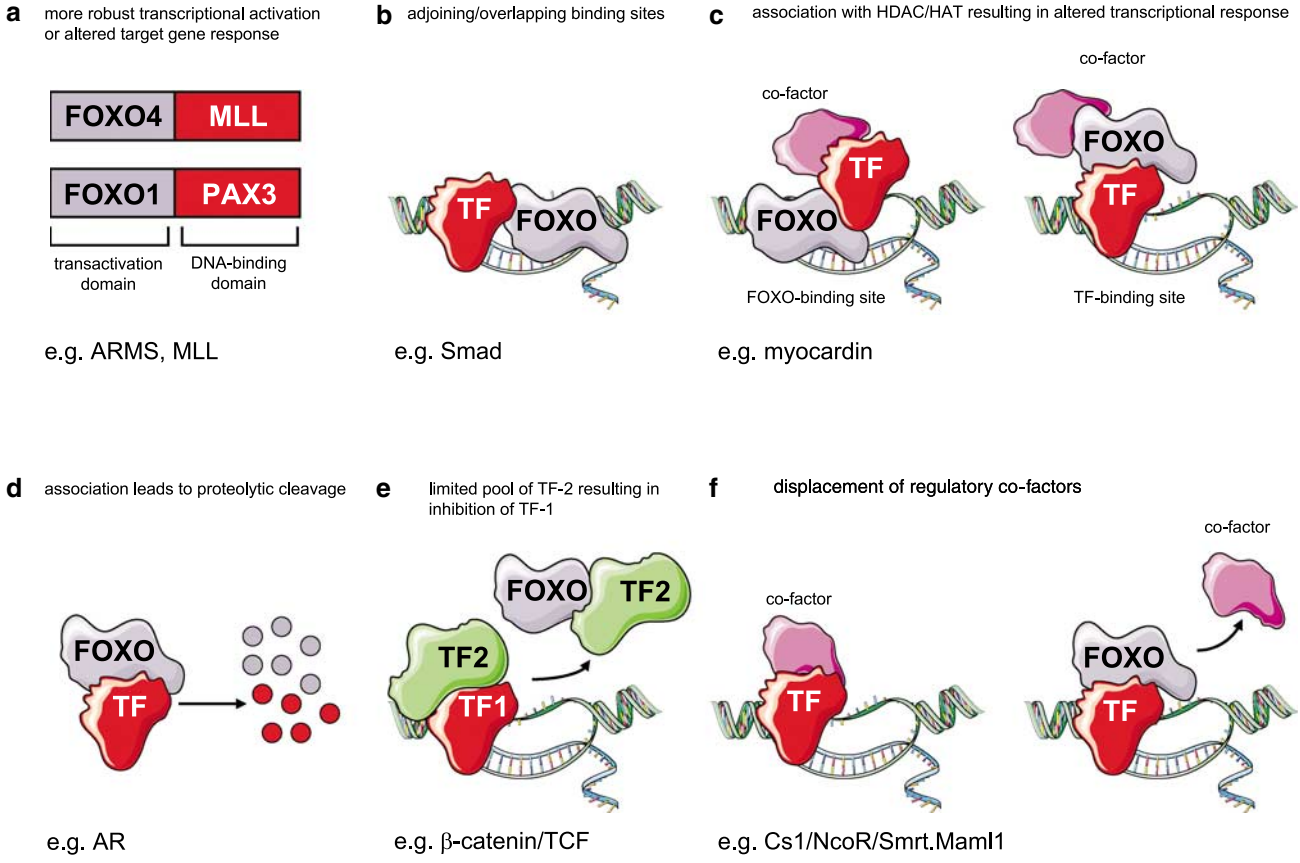

Figure 2 Mechanisms of altered transcriptional regulation through FOXO-interactions. Interaction of FOXO proteins with diverse transcription factor families or cofactors can lead to altered transcriptional responses through a variety of mechanisms. (**a**) Fusion proteins. chromosomal translocations in mixed lineage leukaemia or alveolar rhabdomyosarcoma result in the generation of FOXO fusion proteins. These are thought to have both more robust and altered transcriptional responses resulting in oncogenesis. (**b**) Transcriptional synergy. often FOXO-binding elements are found adjoining or overlapping with other transcription factors. Association between these proteins can often result in enhanced transcriptional responses. (**c**) Recruitment of conventional cofactors. the recruitment of histone acetylase transferases (HATs) or histone deacetylases (HDACs) to promoters through association with transcription factors can lead to activation or suppression of transcription. FOXO-transcription factor associations can result in altered cofactor distribution to target promoters. (**d**) Proteolytic degradation. association of FOXO proteins may lead to increased proteolytic degradation of FOXOs or associating transcription factors. (**e**) Transcription factor sequestration. transcription factors often form heterodimeric complexes when binding DNA. When one of these components is a limiting factor and also binds FOXOs, it may result in inhibition of transcription. (**f**) Displacement of regulatory cofactors. association of transcription factors with coactivators or corepressors modulates transcription. Displacement of these complexes by FOXO binding will result in altered transcriptional responses.

programmes. For example, the ability of Foxo4 to repress myocardin-mediated transcription is not recapitulated by Foxo1 or Foxo3 (Liu *et al*., 2005). Adding complexity to this, cell context-specific effects have also been observed. For example HOXA5 represses FOXO-induced IGFBP-1 transcription in liver cells but cooperatively activates transcription in fibroblasts (Foucher *et al*., 2002). Since FOXO proteins are exquisitely regulated by a variety of post-translational modifications, modulation of these events also allows a further level of control modulating FOXO transcriptional targets.

It is likely that we have only just started to uncover the full complement of FOXO transcriptional targets and the possibilities of therapeutically modulating FOXO function in disease has only recently been investigated. Total ablation of FOXO activity might have detrimental consequences since FOXOs can be considered to be tumour suppressors (Paik *et al*., 2007). The ability to design pharmacological compounds that subtly manipulate FOXO-interactions with other transcription factors might prove to have beneficial therapeutic effects for treatment of a wide variety of diseases.

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