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REVIEW

# Forkhead transcription factors and ageing

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**Mutations in single genes and environmental interventions can extend healthy lifespan in laboratory model organisms. Some of the mechanisms involved show evolutionary conservation, opening the way to using simpler invertebrates to understand human ageing. Forkhead transcription factors have been found to play a key role in lifespan extension by alterations in the insulin/IGF pathway and by dietary restriction. Interventions that extend lifespan have also been found to delay or ameliorate the impact of ageing-related pathology and disease, including cancer. Understanding the mode of action of forkheads in this context will illuminate the mechanisms by which ageing acts as a risk factor for ageing-related disease, and could lead to the development of a broad-spectrum, preventative medicine for the diseases of ageing.**
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## Introduction

This review deals with the role of forkhead transcription factors in lifespan, ageing and ageing-related disease. Research into ageing has been rejuvenated by the recent discovery of mutations in single genes that can extend lifespan of laboratory model organisms. Furthermore, some of the pathways involved show conservation of this effect on ageing over the large evolutionary distances between yeast, nematode worms, flies and mammals, opening up the use of the simpler and short-lived invertebrates to make discoveries about mammalian ageing (Piper *et al*., in press). Most strikingly of all, lifespan-extending mutations can also delay, ameliorate or even abolish the impact of many ageing-related diseases, including the major killers: cardiovascular disease, neurodegeneration and cancer. These findings have raised the possibility of a broad spectrum, preventative medicine for the diseases of ageing (Partridge and Gems, 2007).

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Forkhead transcription factors are turning out to play a key role in invertebrate models of extension of healthy lifespan by single-gene mutations, and evidence is mounting for their importance in mammals. Forkheads can also play a role in extension of lifespan by dietary restriction, an environmental intervention that also extends lifespan in diverse organisms (Kennedy *et al*., 2007). Here, we discuss these findings and their implications. The forkhead family of transcription factors is characterized by a type of DNA-binding domain known as the forkhead box (FOX) (Weigel and Jackle, 1990). They are also called winged helix transcription factors because of the crystal structure of the FOX, of which the forkheads contain a well-differentiated subclass. Genes encoding forkheads are present throughout the animal kingdom as well as in fungi and yeast, but not in plants (Baldauf, 1999). In humans, over 100 genes encoding forkhead proteins are present in the genome, classified into many subgroups on the basis of sequence similarity. The invertebrate genes in general show a clear association with one of these mammalian subgroups, implying that the subgroups differentiated early in animal phylogeny (Mazet *et al*., 2003). Members of the family participate in a wide range of biological functions, including development, growth, stress resistance, apoptosis, cell cycle, immunity, metabolism, reproduction and ageing (Burgering and Kops, 2002; Giannakou and Partridge, 2004; Arden, 2007; Carter and Brunet, 2007; Peng, 2007; van der Horst and Burgering, 2007; Tuteja and Kaestner, 2007a, b).

## Forkheads, ageing and lifespan

The role of forkheads in ageing and lifespan first came to light in the nematode worm *Caenorhabditis elegans*. A genetic screen for mutations that extended adult lifespan produced a collection of mutants (Klass, 1983) that were subsequently examined in further detail, revealing that a mutation the gene *age-1* robustly extended the lifespan of the adult worm (Friedman and Johnson, 1988). If *C. elegans* larvae encounter food shortage or crowding during development, instead of growing and becoming reproductive adults, they undergo developmental arrest as a dauer larva, which stores fat and is stress resistant and very long-lived. A series of temperature-sensitive mutations was isolated that caused the worms to enter

the dauer developmental pathway in the absence of food shortage or crowding, and *age-1* turned out to be one of these. A subsequent screen of these same mutations for effects on adult lifespan showed that mutations in one of them, *daf-2*, could more than double lifespan. Extension of lifespan and dauer formation by mutation of both *age-1* and *daf-2* required the presence of another gene called *daf-16*, implying that both *daf-2* and *age-1* normally function to suppress the activity of *daf-16* (Kenyon *et al*., 1993; Dorman *et al*., 1995). *Age-1* was found to encode a worm orthologue of mammalian phosphatidylinositol-3-OH kinase (Morris *et al*., 1996), while *daf-2* encodes the single worm orthologue of the mammalian insulin/IGF receptors (Kimura *et al*., 1997). *Daf-16* encodes a forkhead transcription factor that is alternatively spliced and widely expressed in the tissues of the worm (Lin *et al*., 1997; Ogg *et al*., 1997). These findings implied that the mammalian orthologue(s) of *daf-16* could play important roles in diabetes and obesity. They also showed for the first time that insulin/IGF-like signalling (IIS) could play a role in determination of the rate of ageing and lifespan, highlighting the diverse range of biological roles associated with the signalling pathway. The discoveries also raised the important issue of whether the role of IIS and its associated forkhead transcription factors in ageing and lifespan was a worm peculiarity or, instead, evolutionarily conserved.

*Daf-16* is the *C. elegans* orthologue of the FOXO subgroup of the mammalian forkhead transcription factors. *Drosophila* also has a single FOXO orthologue, dFOXO, which was initially shown to play a role in the control of growth and size and to mediate some of the effects of IIS on these traits (Junger *et al*., 2003; Kramer *et al*., 2003; Puig *et al*., 2003). A role of dFOXO in ageing and lifespan was demonstrated by the finding that overexpression of the gene in the adult fat body of flies led to extension of lifespan (Giannakou *et al*., 2004, 2007; Hwangbo *et al*., 2004). Mutations in several genes in the *Drosophila* IIS pathway have been shown to extend lifespan (Giannakou and Partridge, 2007), but it is not yet clear whether, as in *C. elegans*, dFOXO is necessary for these effects. Mutations in genes encoding components of the IIS pathway in mammals have been shown to extend lifespan: the insulin receptor (Bluher *et al*., 2003), the IGF-1 receptor (Holzenberger *et al*., 2003), klotho (Kurosu *et al*., 2005), Irs2 (Taguchi *et al*., 2007) and Irs1 (Selman *et al*., 2007). Female mice that are null for Irs1 also show clear improvement in health at older ages for all traits examined, including glucose homoeostasis, motor performance, immune system profiles, osteoporosis and ulcerative dermatitis (Selman *et al*., 2007). So far, no role in determination of mammalian lifespan has been ascribed to the FOXO family or to any other group of forkheads, nor has it yet been established if one or more FOXOs (there are three each in mouse and human) or other forkheads are required for the extension of lifespan by altered IIS. The effect of IIS on ageing and lifespan has thus been conserved over the large evolutionary distances between *C. elegans*, *Drosophila* and the mouse (Piper *et al*., In Press), and the single O class FOX transcription factor is clearly essential for extension by IIS in *C. elegans* and can itself extend lifespan when overexpressed in *Drosophila*.

Dietary restriction (DR), a reduction in food intake while avoiding malnutrition, can also extend lifespan in diverse organisms including yeast *Saccharomyces cerevisiae*, *C. elegans Drosophila* and mouse (Kennedy *et al*., 2007). It remains to be determined if the mechanisms by which DR extends lifespan are similar in these organisms or whether, instead, this is a case of evolutionary convergence. Given the role of IIS in nutrient-sensing, an obvious question is whether alterations in IIS are involved in mediating the increase in lifespan in response to DR. The evidence is mixed. In *C. elegans*, several studies have shown that *daf-16* is not required for the response of lifespan to some methods of dietary restriction (Kaeberlein *et al*., 2006; Lee *et al*., 2006; Houthoofd *et al*., 2007). However, a recent study, using a different method of DR, found that the response did require *daf-16* (Greer *et al*., 2007a) suggesting that, even within a single organism, DR can act through different pathways to extend lifespan. The *Drosophila* FOXO is not required for extension of lifespan by DR through food dilution. However, like null mutation of the single fly insulin receptor substrate *chico* (Clancy *et al*., 2001, 2002), overexpression of dFOXO in the adult fat body both extends lifespan (Giannakou *et al*., 2004) and alters the response to dietary restriction in a way that suggests that *chico* flies are mildly dietarily restricted by their genotype (Giannakou *et al*, in press). The interaction between DR and extension of lifespan by IIS in mammals awaits investigation. However, a null mutation in the growth hormone receptor, which results in lowered circulating levels of Igf-1, extends lifespan in the mouse, with no further increase occurring if the mice are subjected to DR, suggesting that these two interventions may act in the same pathway (Bonkowski *et al*., 2006). There are also some similarities in changes in genes expression, cellular biochemistry and anticancer effects of DR and reduced IIS in mice (Spindler and Dhahbi, 2007). Recent work with *C. elegans* has implicated a different forkhead in mediating the response of lifespan to DR. Systematic inactivation of the 15 forkhead-like genes by RNA interference identified just one of them, *pha-4*, as necessary for the increase in lifespan by dietary restriction (Panowski *et al*., 2007). PHA-4 is orthologous to the mammalian Foxa family, members of which play key roles in development and metabolic homoeostasis (Friedman and Kaestner, 2006; Tuteja and Kaestner, 2007a, b).

A third experimental intervention that can increase lifespan is removal of the germ line. This has been demonstrated clearly in *C. elegans*, where laser ablation of the germ line precursor cells can increase lifespan by 60%. This extension of lifespan requires *daf-16* (Hsin and Kenyon, 1999). Since *daf-16* mutants are not themselves long-lived, this finding implies that the germ line normally inhibits the activity of *daf-16*. It appears to do so independently of *daf-2*, because ablation of the germ line in *daf-2* mutant animals also produced a

substantial increase in lifespan (Hsin and Kenyon, 1999). Genetic ablation of the germ line in *Drosophila* does not extend lifespan. However since this intervention disrupts the anatomy of the gonad, this finding does not rule out the possibility of a role for the germ line in determination of lifespan in this organism (Barnes *et al*., 2006). Interestingly, transplant of the ovaries of young, but not old, mice into ovariectomised mice has been reported to extend lifespan (Cargill *et al*., 2003), but any role of IIS has not been investigated.

Olfaction is also turning out to play an important role in determination of lifespan of the two invertebrates. Genetic or laser ablation, or malfunction, of specific olfactory or gustatory neurons in the amphids of *C. elegans* extends lifespan and most of this extension requires *daf-16* (Apfeld and Kenyon, 1999; Alcedo and Kenyon, 2004). Genetic ablation of olfactory function has also been shown to produce a substantial increase in lifespan in *Drosophila* (Libert *et al*., 2007), although any role of forkheads remains to be investigated.

In mammals, the evidence for a role of forkheads in determination of lifespan is indirect and comes, for instance, from changes in expression or activity during dietary restriction or in genotypes of mice that show extension of lifespan through altered IIS. The finding that alteration of IIS can increase mammalian lifespan and health during ageing clearly implicates a mechanism involving one or more forkheads, but the details remain to be elucidated. In humans, evidence for an involvement of forkheads in determination of lifespan has come from population–genetic association studies, with genetic variants in both FOXO1a and FOXO3a implicated (Kuningas *et al*., 2007; Lunetta *et al*., 2007). It will be important to confirm these results in other study populations and to discover the causes of death that are altered.

The role of forkheads in ageing and lifespan has been known for only 10 years, and has yet to be established in mammals. This field of study is still in its infancy, with almost all of the crucial questions unanswered. In this review, we shall discuss what is known about the upstream signalling mechanisms that regulate forkhead function in the determination of lifespan, the cofactors that may be important, the tissues and life history stages in which forkheads function to determine lifespan, their downstream transcriptional targets, the biochemical processes that they regulate and finally the relationship between forkheads, ageing and disease.

## Regulation of forkhead function in determination of lifespan

Although scant, the most direct evidence for mechanisms regulating forkhead function during extension of lifespan has come from work with *C. elegans*. DAF-16 (the worm FOXO orthologue) accumulates in the nucleus of long-lived *daf-2* (insulin/Igf receptor orthologue) mutant animals, and mutations in *daf-18*, the gene encoding the worm PTEN orthologue, block this nuclear localization and restore lifespan of *daf-2* mutants to control values (Lin *et al*., 2001). Lifespan-extending ablation of sensory neurons also causes nuclear localization of DAF-16, as does ablation of the germ line, but in this case only in a subset of intestinal cells (Lin *et al*., 2001). There is thus a strong association between extension of lifespan by these various interventions that depend upon *daf-16* and nuclear localization of DAF-16.

The worm DAF-16 contains four consensus Akt phosphorylation sites and, by analogy with mammalian FOXOs (see below), phosphorylation of these sites by Akt might be expected to regulate nuclear localization and hence target gene expression. Changing of these sites from serine or threonine to alanine in *C. elegans* resulted in strong nuclear localization of DAF-16, but these animals did not live significantly longer than wild-type animals. The mutant DAF-16 functioned normally in *daf-16* null animals and, taken together, these results demonstrate that nuclear localization of DAF-16 is not sufficient to extend lifespan, and other forms of regulation must occur (Lin *et al*., 2001). Work on the upstream kinases suggested that SGK-1, the *C. elegans* homologue of the serum- and glucocorticoid-inducible kinase SGK, is the critical kinase in the extension of lifespan (Hertweck *et al*., 2004), with the two Akts playing more major roles in other phenotypes controlled by IIS. However, subsequent work showed that RNA interference with Akt-1 extended lifespan (Hamilton *et al*., 2005). The discrepancy in the two results could have occurred because the original study (Hertweck *et al*., 2004) used a null mutation for *akt-1* and found no extension of lifespan; a reduction, through RNAi, rather than complete abolition, through null mutation, of *akt-1* activity may be needed to extend lifespan (Hamilton *et al*., 2005). AMP activated protein kinase (AMPK) is also important in regulation of DAF-16 during extension of lifespan in *C. elegans*. DAF-16 is required for extension of lifespan by one method of DR, and so also is AMPK. Expression of an active version of the AMPK in worms extends their lifespan. AMPK also phosphorylates DAF-16 at novel sites and activates DAF-16-dependent transcription (Greer *et al*., 2007b). There is thus clear evidence that regulation of DAF-16 by phosphorylation can be important in extension of lifespan, but the biochemical details of the modifications that are required for lifespan extension, and their precise effects upon DAF-16 function, await elucidation.

While the strongest evidence for a regulation of FOXO function specifically in control of lifespan stems from work in *C.elegans*, biochemical analyses of FOXO regulation in the invertebrates is limited, and most evidence comes from genetic analysis of epistatic interactions between mutations in different genes. Biochemical studies on the phosphorylation/acetylation status of FOXO in long-lived mutant animals have rarely been undertaken. In contrast, work in mammalian tissue culture has revealed much about the biochemical modulation of FOXO function but little about the role of this modulation in the determination of lifespan. Nevertheless, the sirtuin family members SIRT1 and SIRT2, implied in the regulation of lifespan,

have been demonstrated to be upregulated in response to DR, thus leading to deacetylation and enhanced transcriptional activation of FOXO3, offering a potential mechanism coupling DR and the IIS pathway also in mammals (Wang *et al*., 2007).

The prototypic regulation of FOXO function was characterized in the context of activation of the insulin-signalling pathway in mammals, where FOXO proteins are targets of growth-factor-stimulated Akt phosphorylation (Brunet *et al*., 1999). Insulin/IGF-1-stimulated Akt activation leads to phosphorylation of FOXO proteins at threonine 24, serine 256 and serine 319 on FOXO1, and is PI3K-dependent (Alessi *et al*., 1997). Importantly, these phosphorylation sites are conserved across all FOXO proteins with the exception of FOXO6, and across the large distance from invertebrates to humans. However, Akt is not the only kinase that phosphorylates these residues of FOXO proteins in response to growth factor signalling in a PI3K-dependent manner. IGF-stimulated activation of the serum and glucocorticoid inducible kinase (SGK)-1 also mediates FOXO phosphorylation, although preferentially on threonine 24 and serine 319 of FOXO1 (Brunet *et al*., 2001). SGK-1 was initially identified as a glucocorticoid responsive gene, and SGK-1-mediated FOXO phosphorylation has been demonstrated to contribute to the antiapoptotic effect of glucocorticoids via SGK-1-mediated FOXO inactivation (Mikosz *et al*., 2001). Nevertheless, the exact function of differential FOXO phosphorylation by SGK-1 and Akt has not been determined for mammals although, as already indicated, phosphorylation of DAF-16 by SGK-1 is involved in extension of lifespan by altered IIS in *C. elegans* (Hertweck *et al*., 2004). The functional consequence of Akt- and SGK-mediated FOXO phosphorylation is a nuclear–cytoplasmic shuttling of FOXO, thus inhibiting transcription of FOXO target genes (Biggs *et al*., 1999). The mechanisms translating FOXO phosphorylation into nuclear export are complex and only partly understood. While phosphorylation of FOXO is considered to unfold a nuclear export sequence in the C-terminus of these proteins, another important regulation of FOXO nuclear export is the phosphorylation-dependent interaction with 14-3-3 proteins (Brunet *et al*., 2002).

Besides the well-characterized insulin/IGF-1-regulated inactivation of FOXO proteins, mammalian FOXO proteins undergo regulation via multiple additional signalling pathways, which can have opposite biological effects. For instance, kinases classically implied in the regulation of inflammation and cellular stress responses, such as the cJUN-terminal kinase (Jnk) and the inhibitor of NFκB (IκB) kinases (IKKs), phosphorylate FOXO proteins at sites distinct from those targeted by Akt (Hu *et al*., 2004). While Jnk-dependent phosphorylation of FOXO4 at serine 447 and 451 promotes cytoplasmic/nuclear translocation, this cannot be the only Jnk-mediated regulation of FOXO, since this serine residue is not conserved in other FOXO proteins, which still undergo Jnk-dependent regulation (Essers *et al*., 2004). Moreover, recently another mechanism of Jnk-dependent FOXO regulation was revealed, when Sunayama *et al*. (2005) demonstrated that Jnk also directly phosphorylates 14-3-3 proteins, resulting in dissociation from FOXO1 and allowing nuclear re-entry of FOXO. Thus, these experiments also provide the molecular basis for the notion that Jnk activation can override insulin-stimulated FOXO inactivation, as insulin-induced inactivation of FOXO1 depends on 14-3-3 binding. Moreover, Jnk-dependent FOXO regulation can directly control ageing, because overactivation of Jnk in *Drosophila* has been demonstrated to extend lifespan (Wang *et al*., 2003, 2005). Any importance of Jnk activation in control of lifespan in mammals still awaits experimental confirmation.

While IKK-ß has also been demonstrated to control FOXO3 function via phosphorylation of serine 644 in the C-terminus, leading to FOXO inactivation, the role of this pathway in control of evolutionarily conserved lifespan regulation appears questionable. First, serine 644 is only conserved in FOXO3 between mice and humans and is not present in *C. elegans* or *Drosophila*. Moreover, it is not conserved in other mammalian FOXO members. Thus, IKK-dependent FOXO3 regulation may instead represent a species-specific pathway that is not involved in determination of lifespan (Hu *et al*., 2004).

Interestingly, not only Jnk mediates stress-dependent FOXO activation. More recently Lethinen *et al*. showed that the mammalian Sterile 20 like kinase (Cheung *et al*., 2003) (MST) positively regulates FOXO-dependent expression of oxidative stress defence genes: MST is induced upon oxidative stress and phosphorylates both FOXO1 and 3 on an evolutionarily conserved serine residue in the forkhead domain of these proteins, preventing 14-3-3 binding and resulting in nuclear translocation of FOXO. Interestingly, MST-dependent FOXO regulation controls lifespan in *C. elegans* (Lehtinen *et al*., 2006), offering the promising possibility that MST-dependent FOXO regulation also controls lifespan in mammals.

Another emerging pathway in control of FOXO function is via AMPK-mediated phosphorylation. Brunet *et al*. demonstrated that AMPK phosphorylates FOXO1 and 3 on multiple serine residues, activating FOXO transcriptional activity (Greer *et al*., 2007b). Importantly, as already mentioned, AMPK-regulation of FOXO proteins was subsequently shown to contribute to the lifespan-extending effect of DR in *C. elegans* (Greer *et al*., 2007a). Interestingly, AMPK serves as a principal fuel sensor in mammalian cells, as its activation is controlled by the intracellular AMP/ATP ratio as a measure of energy depletion (Hardie *et al*., 1998). Thus, for example, lowering glucose concentration results in activation of AMPK in a variety of cell types and organs (Mu *et al*., 2001). Therefore, AMPK-dependent FOXO activation may serve to couple organismal and intracellular energy availability to control of lifespan. Given the profound effect of DR on lifespan, AMPK-mediated FOXO activation provides an interesting candidate in this pathway. Similarly, it was recently demonstrated that

reducing glucose availability in *C. elegans* extends lifespan, a phenomenon attributed to altered mitochondrial respiration (Schulz *et al*., 2007). Nevertheless, glucose-deprivation-initiated AMPK/FOXO activation may provide another explanation for this phenomenon, a hypothesis that receives some support from the finding that metformin, a well-characterized AMPK-activator, initiates gene expression patterns in mice that overlap with those induced by DR (Zahn and Kim, 2007).

Taken together, these experiments reveal regulated phosphorylation of FOXO proteins as an integrative signalling platform that coordinates cellular stress responses, fuel availability, metabolism and inflammation. Some combination of these activities must also result in control of lifespan. While the kinase networks that control FOXO regulation represent a rapidly extending research field, the identification of FOXO phosphatases remains in its infancy. Nevertheless, elucidation of regulated phosphatase activities towards the multiple, functionally divergent phosphorylation sites in FOXO-proteins is expected to yield important novel insights into the control of FOXO function and thus, ultimately, to understanding the role of these transcription factors in ageing and ageing-related disease.

## Cofactors of forkheads in the determination of lifespan

Signalling cascades, such as IIS, often converge on a single transcription factor, yet these pathways influence diverse phenotypes by differentially regulating gene expression, both by repression and activation, in different tissues and under different circumstances. The interaction of these transcription factors with cofactors is therefore likely to be of great importance in bringing about spatial and temporal regulation of forkhead activity, as well as diversification of function (Wolff *et al*., 2006). Understanding these interactions will be necessary to precisely determine what changes in regulation of the action of forkheads are required for lifespan to be extended. Several cofactors for forkheads in determination of lifespan in *C. elegans* have been identified, although the biochemical details of their action and regulation mostly await elucidation.

A novel cofactor for *daf-16* emerged from a genetic screen in *C. elegans*. RNAi for the gene *smk-1*, similar to removal of *daf-16*, completely suppresses extension of lifespan by mutation of *daf-2* but, like RNAi for *daf-16*, produces only a minor reduction of lifespan of wild-type animals, and hence does not simply make the animals sick (Wolff *et al*., 2006). RNAi for *smk-1* does not affect the lifespan of animals mutant for *daf-16*. *Smk-1* is also required for lifespan-extension by other mechanisms that require *daf-16*, such as ablation of the germ line. SMK-1 protein is exclusively nuclear, and interference with its expression does not interfere with nuclear/cytoplasmic relocation of DAF-16. However, *smk-1* is required for repression or upregulation of two genes by DAF-16 (Wolff *et al*., 2006). Interestingly, although *smk-1*, like *daf-16*, is required for the resistance to DNA damage, to bacterial infection and to some stresses of *daf-2* mutants, it is not required for the resistance to heat, for which *daf-16* is required, nor is *smk-1* required for the developmental and reproductive phenotypes of *daf-2* mutants, which do require *daf-16*. These findings imply that the resistance to heat of *daf-2* mutants may be mediated by the interaction of DAF-16 with some other cofactor, that this phenotype is not required for increased lifespan, and a nuclear interaction between DAF-16 and SMK-1 leads to specific regulation of genes that increase lifespan (Wolff *et al*., 2006).

Extension of lifespan in *C. elegans daf-2* mutants requires the heat shock factor *hsf-1*. However, in the absence of HSF-1, DAF-16 accumulates normally in the nuclei of these mutants and there is normal upregulation of expression of some DAF-16-regulated genes, indicating that DAF-16 can regulate gene expression independently of HSF-1, but not in a way that increases lifespan. Overexpression of *hsf-1* itself extends lifespan, an effect that requires *daf-16*. HSF-1 can regulate heat shock genes in the absence of DAF-16, but this does not increase lifespan either. These findings imply that DAF-16 and HSF-1 might act together to regulate a subset of genes. RNA expression-profiling showed that this was the case for four small heat shock proteins and that RNAi for these genes specifically shortened the lifespan of *daf-2* mutant animals (Hsu *et al*., 2003). These findings identify HSF-1 as a cofactor for DAF-16 in the extension of lifespan, and also suggest that misfolded proteins may be an important source of ageing-related damage.

In *C. elegans*, extension of lifespan by most methods of DR does not require *daf-16* (but see (Greer *et al*., 2007a). Surprisingly, *smk-1* is required for the increase in lifespan seen in *eat* mutants, which have reduced pharyngeal pumping of food, and are hence a model for DR. This finding suggested that SMK-1 could interact with another forkhead to mediate the response to DR, and a systematic survey of the 15 candidates in the worm genome revealed that a Foxa transcription factor, PHA-4, was also essential for the response to several forms of DR, but not for extension of lifespan by reduced IIS (Panowski *et al*., 2007). The consensus DNA-binding sequences for PHA-4 and DAF-16 overlap, suggesting that there could be competition between them, and they also differentially regulate expression of the 5 worm *sod* genes (Wolff *et al*., 2006). It will be important to determine the role of Foxa transcription factors in response to DR in mammals. So far, Foxa proteins in mammals have mainly been investigated with respect to their role in metabolic control. Here, Foxa1 regulates pancreatic ß-cell function via control of oxidative phosphorylation (Vatamaniuk *et al*., 2006), while Foxa2 controls white adipose tissue function; heterozygous Foxa2-deficient mice exhibit a predisposition for the development of high-fat-diet-induced obesity (Wolfrum *et al*., 2003). On the other hand, Foxa3 has been demonstrated to play a central role in control of

glucagon expression in pancreatic alpha cells (Liu *et al*., 2002). Given the opposing metabolic effects of glucagon and insulin, and in light of the crucial role of IIS in control of longevity, analysis of mice with altered Foxa protein function or altered glucagon signalling may provide novel important insights into the control of lifespan in mammals (Sloop *et al*., 2004).

Another important cofactor in control of FOXO function is the SIR2 deacetylase. The gene *sir-2*, which encodes an $NAD^+$-dependent protein deacetylase, extends replicative lifespan in the yeast *S. cerevisiae* when overexpressed (Kaeberlein *et al*., 1999). Overexpression of the orthologous genes also extends lifespan in *C. elegans* (Tissenbaum and Guarente, 2001) and *Drosophila* (Rogina and Helfand, 2004). In *C. elegans*, the extension of lifespan by overexpression of *sir-2.1* requires *daf*-16 (Tissenbaum and Guarente, 2001), implying that *sir-2.1* could downregulate IIS. Work in mammalian cells has shown that sirtuins can bind to and deacetylate forkhead proteins (Brunet *et al*., 2004; Motta *et al*., 2004). Recent work with *C. elegans* has shown that, as in mammals, the nuclear exclusion of DAF-16 is mediated by 14-3-3 proteins (Li *et al*., 2007). These are also SIR-2.1-binding partners and they are required for the extension of lifespan from overexpression of *sir-2.1* (Berdichevsky *et al*., 2006; Wang *et al*., 2006). In contrast, neither the 14-3-3 proteins nor *sir-2.1* are required for extension of lifespan by reduced IIS, and they appear to regulate DAF-16 activity in a parallel pathway related to stress resistance (Berdichevsky *et al*., 2006). It will be important to investigate the presence of similar mechanisms in *Drosophila* and mammals.

Although signal-controlled regulation of FOXO phosphorylation represents a major regulatory mechanism of FOXO function, the identification of SIRT1 as another FOXO-interacting cofactor has, at the same time, let to the recognition of further important post-translational modifications of FOXO proteins in control of gene expression. Further important insights into the regulatory role of FOXO acetylation came from the observation that SIRT1, the mammalian homologue of the yeast class III histone deacetylase Sir2, interacts with and promotes deacetylation of FOXO1 (Brunet *et al*., 2004). Subsequent work revealed that in most, but not all studies, SIRT1-mediated deacetylation of FOXO1 results in enhanced transcription of FOXO target genes in different cellular contexts, such as the regulation of apoptosis and hepatic gene expression (van der Horst and Burgering, 2007) (Fukuoka *et al*., 2003). Frescas *et al*. (2005) provided some mechanistic insight into how SIRT1 activates FOXO1-mediated transcription, by showing that deacetylation results in nuclear immobilization, supporting the view that acetlyation controls intranuclear activation of FOXO proteins. Thus, acetylation provides another important control mechanism, which may integrate different extracellular signals from those controlling FOXO phosphorylation. Again, the role of acetylation of mammalian FOXO proteins with respect to control of lifespan still awaits experimental analysis.

As well as sirtuin family regulation of FOXO acetylation, it has also been demonstrated that FOXO interacts with the nuclear receptor coactivators CHRP response element binding protein (CREB) and p300 (Perrot and Rechler, 2005). Via the histone acetylase activity associated with these FOXO interactors, FOXO proteins themselves serve as substrates undergoing acetylation (Perrot and Rechler, 2005). Nevertheless, the functional consequences of the interaction of FOXO with CREB binding protein p300 remains controversial. While Nasrin *et al*. (2000) demonstrated that CREB binding protein inhibits FOXO1-mediated expression of IGF binding protein (BP)1, Perrot and Rechler (2005) showed that p300 interacting with FOXO1 stimulates FOXO acetylation, resulting in enhanced FOXO-mediated transcriptional activation. Nevertheless, these experiments could not rule out an indirect stimulatory effect of p300 via histone acetylation.

Another coactivator recently implied in the regulation of FOXO-dependent gene expression is the PPARγ coactivator (PGC)-1. PGC-1 was initially identified as a coactivator for the nuclear hormone receptor PPARγ in control of brown adipose tissue differentiation. Recent experiments have demonstrated that PGC-1 is a critical regulator of mitochondrial biogenesis. Interestingly, a gene expression analysis revealed an overlap of 28% between the genes regulated by dFOXO and those regulated by the *Drosophila* homologue of PGC-1 (Gershman *et al*., 2007). Direct interaction of FOXO and PGC-1 has been revealed in mammalian cells, where PGC-1 acts as a coactivator of FOXO1 in controlling expression of genes critically important for the regulation of hepatic gluconeogenesis, such as G6-phosphatase and PEPCK (Puig and Tjian, 2005). Importantly, expression of PGC-1 itself declines with ageing, and DR reverses the age-dependent decline in expression of PGC-1 and its target genes. Thus, these experiments establish a model, that controlling PGC-1 expression may improve longevity, a hypothesis that can be directly addressed in the invertebrate model organisms.

## Tissues and life history stages in which forkheads act to influence ageing

The tissues in which altered IIS can affect lifespan have been most thoroughly investigated in *C. elegans*, with some work in *Drosophila* and the mouse. Key findings are that the pathway has both tissue-autonomous and non-autonomous effects and that it is also autoregulatory in the invertebrates (Puig *et al*., 2003; Puig and Tjian, 2005, 2006; Casas-Tinto *et al*., 2007; Marr *et al*., 2007), as in mammals. The role of this autoregulation in determination of lifespan is yet to be investigated.

Neuronal tissue has been implicated as of key importance in lifespan-extension by altered IIS in *C. elegans*. In an initial study, restoration of expression of *age-1* or *daf-2* in neurons of *age-1* or *daf-2* mutant animals, respectively, shortened lifespan to control values. In contrast, restoration of gene expression in muscle or the intestine (which also acts as the fat and liver of the worm) appeared to play little role (Wolkow *et al*., 2000). These findings implied that *daf-16*, as the

IIS effector, would show a similar pattern of tissue specificity of its effect on lifespan. However, in another study, *daf-16* expression was restored in the neurons of worms mutant for *daf-16* and *daf-2*, which have lifespan similar to wild-type controls, and only a 5–20% increase in lifespan was seen. In contrast, restoration of *daf-16* expression in the intestine produced a substantial, 50–60% increase in lifespan (Libina *et al*., 2003). Mosaic analysis confirmed these results and also suggested that tissues other than neurons and the gut can make a substantial contribution to the increase in lifespan (Libina *et al*., 2003). In addition, when *daf-2* mutants were subjected to RNAi for *daf-16*, expression of *daf-16* was reduced in all tissues except neurons (consistent with the known refractoriness of *C. elegans* neurons to RNAi), and lifespan was reduced to about 20% greater than that of *daf-2*/*daf-16* mutant animals, again suggesting only a minor role for neurons in the extension of lifespan (Libina *et al*., 2003). The somewhat inconsistent results of these two studies could stem from differences in the level of gene mis-expression or of branching of the IIS pathway below *daf-2* or *age-1*. Whatever the explanation, it seems clear that the intestine, the nervous system and other unidentified tissues play a cell autonomous role in the increase in lifespan from reduced IIS.

In addition to its cell autonomous effects on lifespan, revealed by these studies in which IIS activity was restored in specific tissues of an otherwise IIS-unresponsive animal, *daf-16* can have cell non-autonomous effects on lifespan in *C. elegans*. Production of several of the worm insulin-like peptides is regulated by *daf-16* (Murphy *et al*., 2003). Furthermore, upregulation of *daf-16* expression in the intestine of wild-type animals resulted in an increase in activity of *daf-16* not only in the intestine but also in epidermis and muscles, as did upregulation in neurons, but with a smaller non-autonomous effect (Libina *et al*., 2003). Whether or not these non-autonomous effects are mediated by insulin-like ligands awaits investigation.

A role of neuronal tissue in longevity has also been highlighted for flies and mice. In *Drosophila*, ablation of cells in the brain that produce three of the fly insulin-like ligands extends lifespan, presumably by reducing levels of circulating insulin ligands that in turn reduce peripheral insulin signalling (Broughton *et al*., 2005). In mice, brain-specific disruption of the insulin receptor substrate, Irs2, extends lifespan and has been proposed to protect the brain from age-related hyperinsulinaemia (Taguchi *et al*., 2007). The role of forkheads in these two models has not yet been explored. Fat is also implicated in *Drosophila* and mice as an important tissue in the extension of lifespan by IIS. Fat-specific overexpression of the pathway antagonist dPTEN or dFOXO itself in flies extends lifespan (Giannakou *et al*., 2004, 2007; Hwangbo *et al*., 2004). One study suggested that this extension of lifespan occurred through a cell non-autonomous effect on transcription of one of the genes encoding insulin-like ligands in the brain of the fly (Hwangbo *et al*., 2004), but this non-autonomous effect was not seen in another study (Giannakou *et al*., 2007). Understanding the action of dFOXO in fat body will be important for discovering exactly how IIS extends lifespan. Fat has also been identified as an important tissue for extension of lifespan by altered IIS in the mouse. Knockout of the insulin receptor in white adipose tissue produced a lean, long-lived mouse (Bluher *et al*., 2003), but any role of forkheads awaits elucidation.

The timing of the effect of interventions that increase lifespan is important for several reasons. Pharmacological interventions will be effective only while the system is responsive to altered inputs. In addition, DR in *Drosophila* has been shown to act acutely to lower risk of mortality, because switching flies to a new dietary regime causes them to switch their mortality rate to become equal to that of flies held permanently in the new regime (Mair *et al*., 2003). In contrast, lowered temperature reduces the rate of accumulation of irreversible, ageing-related damage, because flies with a low-temperature history have permanently lower mortality rates than flies with a hotter thermal history when they are both examined at the same temperature (Mair *et al*., 2003). The use of timed genetic manipulations allows these issues to be addressed for extension of lifespan by altered IIS. In *C. elegans*, switching in IIS status using RNAi showed that IIS acts specifically during early adulthood to determine adult survival, with no effect of altered IIS during the preadult period or after the main period of reproduction (Dillin *et al*., 2002). This study did not specifically address the issue of reversibility of the effects of IIS on survival. This was subsequently addressed in a study of timed induction or removal of overexpression of dFOXO in the adult fat body of *Drosophila*, which showed that the effect on survival was completely reversible in young adults, but gradually became less so with age (Giannakou *et al*., 2007). A complexity in these studies came from the finding that dFOXO shows an age-related increase in expression levels, so that the system may become less switchable at the molecular level (Giannakou *et al*., 2007). Nonetheless, the findings suggest that dFOXO acts in part acutely to reduce mortality rates. It would be informative to have similar information from mice about reversibility of the effects on mortality of DR and IIS.

## Transcriptional and biochemical targets of forkheads in ageing

Because forkheads transcription factors can have such a major effect on ageing and lifespan, a considerable amount of work has been devoted to measuring changes in RNA transcript profiles of long-lived mutant animals, to identify the genes and biochemical processes that are being differentially regulated. A significant amount of this work has been done with the invertebrates and has provided highly informative leads to target genes and biochemical processes, in some cases with subsequent functional validation. However, much remains to be learned. Most RNA transcript profiles for the

invertebrates have come from whole animals. Work with *Drosophila* has made it clear that pooling tissues with different transcript profiles can lead to large changes in expression in individual tissues being missed (Chintapalli *et al*., 2007). In mammals, where transcript profiles are more tissue-specific, this is likely to be less of a problem. Another issue is that transcript profiles of, for instance, altered activity of DAF-16, include changes in expression of genes that are both direct and indirect targets of the transcription factor, and the pattern of causality requires further analysis.

An initial, bioinformatic, study searched for orthologous genes in the *C. elegans* and *Drosophila* genomes that had the FOXO binding site present in the promoter region, which could be expected to identify direct targets of FOXO. Seventeen such genes were identified, and one third of these showed altered expression in IIS mutant worms. Furthermore, RNAi with two of these genes, a worm orthologue of retinoblastoma-binding protein 2 and hydroxyphenylpyruvate dioxygenase, extended lifespan (Lee *et al*., 2003). Further work identifying direct targets of DAF-16 would be valuable. Two studies used whole-genome RNA transcript profiling to identify all genes regulated by *daf-16* in long-lived mutants, both direct and indirect targets. One study found upregulation of genes involved in cellular stress response, defense against microbial infection and metabolic genes. In addition to the previously characterized FOXO target genes, *mtl-1* and *sod-3*, they found that expression of the catalase genes *ctl-1* and *ctl-2*, glutathione-*S*-transferase gene *gst-4* and the small heat-shock protein genes were all increased in animals with reduced *daf-2* activity and decreased in animals with reduced *daf-16* activity. Inhibition of these genes with RNAi resulted in shortened lifespan of *daf-2* mutants, revealing that many of these genes significantly affected lifespan, but not much, implying that DAF-16 exerts its effects on lifespan by regulating the expression of many genes with small additive effects (Murphy *et al*., 2003). A second, similar study also identified genes involved in cellular stress response and metabolism (McElwee *et al*., 2003). These studies were useful for generating hypotheses for subsequent experimental testing, but were inevitably limited in precision by the use of whole animals for the studies. A recent study used RNA transcript profiles of long-lived IIS mutant *C. elegans* and *Drosophila*, together with livers of long-lived mice mutant for the somatotopic axis and with reduced circulating Igf-1, to search for commonly regulated processes. Interestingly, in all three organisms, there was downregulation of genes involved in protein synthesis and upregulation of gene involved in cellular detoxification through metabolism of xenobiotic and endobiotic toxins (McElwee *et al*., 2007). Downregulation of protein synthesis has recently been directly demonstrated to increase lifespan in *C. elegans* (Hansen *et al*., 2007; Pan *et al*., 2007; Syntichaki *et al*., 2007). Direct manipulation of cellular detoxification pathways would also be informative.

While the initial identification of FOXO target genes in mammals has focussed on candidate pathways in the regulation of lifespan, such as metabolic control of oxidative stress, approaches aiming at the genome-wide coverage of FOXO targets such as CHIP-cloning has unravelled numerous additional targets in a whole range of pathways. Some of these could be confirmed to control lifespan. On the other hand, FOXO target-gene identification in mammals is complex. It can be expected that the four different FOXO proteins will exhibit overlapping but also distinct, most likely tissue-specific, sets of target genes. Thus, extensive work will have to be performed to identify these networks using combinatory approaches, such as ChIP-cloning, ChIP-on-chip analysis and gene expression analysis in mutants with tissue-specific gain and loss of function. Similarly, functional validation of identified target genes with respect to control of lifespan and development of ageing-associated diseases is still in its infancy. For example, lifelong reduction of superoxide dismutase (SOD2) activity in heterozygous $SOD^{+/-}$ mice increases DNA damage and cancer incidence but surprisingly has no impact on ageing of these mice, questioning the functional role of FOXO-regulated SOD expression in control of lifespan (Van Remmen *et al*., 2003). These experiments underline the importance of subsequent functional target gene validation *in vivo*.

Moreover, FOXO1 has been demonstrated to exert important functions not only by directly binding DNA but also by acting itself as a signalling control coactivator or repressor. Similarly, the role of indirect FOXO action on other transcription factors in control of lifespan has not been addressed.

## Forkheads and ageing-related disease

The world-wide increase in human life expectancy is leading to increasing impact of the diseases of ageing: for example, obesity, diabetes, atherosclerosis, cancer and neurodegenerative diseases. To date, research efforts have concentrated on investigating the pathophysiology of single diseases. However, the remarkable recent discovery that mutations in single genes can extend healthy lifespan implies that these mutations could also reduce the impact of a broad spectrum of ageing-related damage and pathology. Indeed, in both *C. elegans* (Cohen *et al*., 2006; Pinkston-Gosse and Kenyon, 2007) and in *Drosophila* (Wessells *et al*., 2004), lifespan-extending mutations have been shown to ameliorate pathology in specific models of ageing-related disease. In addition, a null mutation in Irs1 both extends lifespan and improves health in the mouse (Selman *et al*., 2007). FOXO proteins have been demonstrated to play a central role not only in the regulation of lifespan but also directly in control of the development and progression of metabolic diseases and neurodegenerative diseases such as Alzheimer's disease and cancer (Tannenbaum and Silverstone, 1949; Patel *et al*., 2005). Given the pivotal role of FOXO proteins in the regulation of lifespan, these discoveries have profound implications for ageing research and could revolutionize approaches for prevention and treatment of ageing-associated diseases.

*Metabolism, diabetes and obesity*

Given the prominent role of FOXO proteins in the insulin/IGF signalling pathway, not only in invertebrates but also in mammals, numerous studies have been directed to elucidation of the role of FOXO proteins in control of metabolism. These studies have revealed multiple effects of FOXO-regulated gene expression on adipocyte differentiation (Nakae *et al*., 2003), pancreatic ß-cell survival (Kitamura *et al*., 2005), regulation of hepatic glucose production (Naimi *et al*., 2007) and the central regulation of energy homoeostasis (Kitamura *et al*., 2006). Thus, it was shown that FOXO1 is induced in early stage of the adipocyte differentiation and premature FOXO1 activation prevents differentiation of pre-adipocytes while reducing FOXO1 activity restores adipocyte differentiation in adipocyte precursors lacking the insulin receptor (Nakae *et al*., 2003). Importantly, *in vivo*, FOXO1 haploinsufficiency protects from diet-induced diabetes in mice (Nakae *et al*., 2003). With respect to the direct role of FOXO1 regulation of hepatic glucose metabolism, FOXO1, particularly by interacting with its coactivator PGC-1α, promotes expression of key enzymes of gluconeogenesis, such as glucose-6-phosphatase and PEPCK (Puigserver *et al*., 2003). Conversely, insulin-stimulated PGC-1α phosphorylation abrogates its interaction with FOXO1 besides directly phosphorylating FOXO1 (Puigserver *et al*., 2003). Therefore, FOXO1 represents a critical signalling molecule in the regulation of gluconeogenesis, a key step whose dysregulation is responsible for the progression of insulin resistance towards overt diabetes. Besides directly regulating glucose metabolism in adipose tissue and liver, FOXO1 has more recently been demonstrated to play a central role in the expression of neuropeptides, critically implied in the maintenance of energy homoeostasis. Thus, it was shown that FOXO1 contributes to leptin- and insulin-stimulated reduction of the orexigenic (food intake-stimulating) neuropeptide agouti-related peptide in the arcuate nucleus of the hypothalamus (Kitamura *et al*., 2006). Thus, modulating FOXO activity not only provides a candidate pathway to improve glucose metabolism but also energy balance as a novel obesity target.

*Neurodegeneration*

Dietary restriction not only affects ageing-associated disease that are obviously linked to metabolism, such as diabetes and obesity, but also positively affects onset and progression of neurodegenerative diseases such as Alzheimer's disease (Patel *et al*., 2005). Alzheimer's disease is characterized by the deposition of macromolecular complexes of β-amyloid, as a consequence of which neuronal death and degeneration develop. Interestingly, several lines of evidence indicate direct involvement of FOXO regulation in control of amyloid deposition and subsequent regulation of neurodegeneration. Aberrant protein aggregation of the amyloid (A) β1-42 peptide and aggregation-mediated Aβ1-42 toxicity is reduced in *C. elegans*, when ageing is slowed by decreased insulin/IGF-1-like signalling (Cohen *et al*., 2006). Here, insulin/IGF-1 signalling controls two pathways: one leading to a heat shock factor (HSF)-1-dependent disaggregation of aberrant protein aggregates followed by rapid degradation. While this pathway appears to be FOXO/DAF-16-independent, an alternative FOXO/DAF-16-dependent pathway can lead to detoxification of protein aggregates by promoting formation of high molecular weight less toxic aggregates. These experiments have directly implied FOXO/DAF-16 in a key mechanism, affecting the development of Alzheimer's disease (Cohen *et al*., 2006). Nevertheless, conservation of these pathways in the mammalian system still has to be validated. More indirect evidence for the role of FOXO regulation in control of the development of neurodegenerative diseases stems from the recent notion that the FOXO regulator SIRT1 controls amyloid β aggregation in mice. DR upregulates SIRT1 expression in the brain, and SIRT overexpression in the central nervous system can protect from the onset of Alzheimer's disease in the transgenic mouse overexpressing human Aβ1-42 (Qin *et al*., 2006). DR-mediated SIRT1 overexpression controls α-secretase activity, which processes the amyloid precursor protein (Qin *et al*., 2006). Although these experiments have clearly placed the FOXO regulator SIRT1 in the pathway of amelioration of amyloid peptide toxicity by DR, the direct involvement of FOXO in this pathway in mammals still has to be investigated. Taken together, there is accumulating evidence that the same molecular pathways that can extend lifespan, including FOXO regulation, also play a critical role in the development of neurodegenerative diseases.

*Cancer*

Given that FOXO target genes directly control cell cycle arrest, DNA repair and apoptosis, FOXO proteins are putative candidates for tumor suppressor genes. Indeed, initial experiments have characterized FOXO1, -3 and -4 as genes at chromosomal breakpoints, in rhabdomyosarcomas for FOXO1 (Galili *et al*., 1993) (Davis *et al*., 1994) and acute myeloid leukaemias for FOXO3 and -4 (Parry *et al*., 1994) (Borkhardt *et al*., 1997). These translocations occur at a breakpoint in intron 2 of the different FOXO family members. Hence, the resulting fusion proteins exhibit constitutive nuclear localization and transcriptional activity. Nevertheless, to date, it is not clear whether tumorigenesis is primarily promoted by the resulting fusion proteins or haploinsufficiency for the respective wild-type FOXO allele. Experiments aimed at elucidation of this question have so far provided limited clarification, because expression of the human Pax3/FOXO1-fusion protein detected in human rhabdomyomas failed to induce tumorigenesis, either on the background of control or FOXO1 heterozygous knockout mice (Lagutina *et al*., 2002) (Keller *et al*., 2004). Thus, these experiments may reflect the general difficulty of mimicking human rhabdomyosarcomas in mice and underline the importance of developing different models to ultimately distinguish between these potential mechanisms.

Further evidence for an involvement of FOXO proteins in tumorigenesis stems from the observations that increased nuclear localization of FOXO1 is correlated with a poor prognosis in breast cancer (Jin *et al*., 2004), and that FOXO4 overexpression inhibits tumour growth of Her2-oncogene overexpressing cells in nude mice (Yang *et al*., 2005). More indirect evidence for a potential, critical role of mammalian FOXO proteins in control of tumorigenesis comes from their regulatory and functional similarities to the well-characterized tumour suppressor p53. Both FOXO proteins and p53 are activated upon cellular stress conditions, inducing cell cycle arrest to allow for cellular adaptation and repair. While FOXO-mediated cell cycle arrest depends on $p27^{kip1}$ (Medema *et al*., 2000), p53 induction of cell cycle arrest primarily occurs via regulation of $p21^{cip1}$ (el-Deiry *et al*., 1993). Similarly, both FOXO proteins and p53 induce apoptosis upon prolonged activation as an ultimate response of the organism to dispose of damaged and potentially harmful cells. Among many pathways activated by FOXO and p53 proteins, induction of apoptosis by both proteins depends on upregulation of Bcl-2 homology (BH)3-only proapoptotic proteins, such as BIM in the case of FOXO and PUMA in case of p53 (Yu *et al*., 2001). Besides parallel modes of action of the tumour suppressor p53 and FOXO proteins, both pathways are moreover functionally interconnected: p53 directly upregulates expression of SGK-1, one of the main kinases inhibiting transcriptional activation of FOXO proteins via phosphorylation-dependent cytoplasmatic translocation (see above) (You *et al*., 2004). On the other hand, p53 upregulates the PTEN tumour suppressor gene (Stambolic *et al*., 2001), which acts as a phosphatidylinositol-phosphatase, negatively regulating the PI3-kinase pathways, whose activation is critical for FOXO inactivation via Akt- and SGK-1-mediated phosphorylation. Although, p53-dependent FOXO regulation via both mechanisms appears to have opposing effects, inhibition in the former and activation in the latter case, the biological outcome of this regulation may differ depending upon cellular context. Specifically, a functional role for FOXO proteins in PTEN-deficient tumours is supported by the finding that overexpression of FOXO1 can inhibit their growth in nude mice (Ramaswamy *et al*., 2002). Interestingly, tumor-inhibition is also achieved by FOXO1-mutants, which lacks the ability to induce the proapoptotic BIM proteins but retain the ability to induce cyclinD2, indicating that tumor suppression by FOXO proteins primarily depends on cell cycle control, as opposed to the induction of apoptosis (Ramaswamy *et al*., 2002).

Novel mechanistic insights into how FOXO proteins control tumor growth stem from a genetic screen in *C. elegans*, investigating the functional role of DAF-16/FOXO target genes in control of longevity and tumor resistance. Twenty-nine out of 734 DAF-16 target genes tested in this screen affected germ line tumor cell proliferation or p53-dependent apoptosis (Pinkston-Gosse and Kenyon, 2007). Many of the mammalian orthologues of these genes are characterized tumor suppressor or oncogenes. Given the age-dependent onset of tumorigenesis in general, half of the genes identified as controlling tumorigenesis also affected lifespan, further supporting the direct mechanistic link between these traits (Pinkston-Gosse and Kenyon, 2007). Interestingly, two of the components identified in this screen resembled homologues of the human Tpr, a component of the nuclear core complex. In *C. elegans*, normal germ cell apoptosis is p53- and *daf-2*-independent, while germ-tumor apoptosis can be triggered by genotoxic stress and *daf-2*-mutations. The latter phenomenon can be blocked by siRNA-mediated knockdown of the Tpr homologue npp-21, indicating that nuclear pore formation is critical for *daf-2*/*daf-16*-mediated germ-tumor apoptosis (Pinkston-Gosse and Kenyon, 2007). Further work clearly has to functionally address the role of Tpr-mediated regulation of nuclear pore formation in mammalian tumorigenesis.

## Conclusions

Despite the surprising and exciting discoveries of the last few years, work to date has but scratched the surface of how the ageing process itself is affected by forkhead proteins. Future work will elucidate the precise signalling mechanisms at work and the ways in which gene expression in different tissues is altered. Almost no work has yet been done on how cellular biochemistry is altered or on identification of the types of molecular damage that are ameliorated to extend lifespan. Understanding exactly how slowing down the ageing process ameliorates the impact of ageing-related disease is also a major challenge for the future.

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