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FOXO3 and Exceptional Longevity: Insights From Hydra to Humans

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Abstract

Aging is a complex, multifactorial process with significant plasticity. While several biological pathways appear to influence aging, few genes have been identified that are both evolutionarily conserved and have a strong impact on aging and age-related phenotypes. The FoxO3 gene (*FOXO3*), and its homologs in model organisms, appears especially important, forming a key gene in the insulin/insulin-like growth factor-signaling pathway, and influencing life span across diverse species. We highlight some of the key findings that are associated with FoxO3 protein, its gene and homologs in relation to lifespan in different species, and the insights these findings might provide about the molecular, cellular, and physiological processes that modulate aging and longevity in humans.

1. FoxO GENES AS DETERMINANTS OF LONGEVITY

Biological aging is a process that can be difficult to define outside of a specific context. However, for individuals, the process of aging over time consistently incurs the loss of homeostasis that eventually results in death. The lifespan of an individual in relation to other members of their species can be expressed as longevity or “exceptional longevity” if it exceeds some specified threshold (Willcox, Willcox, & Ferrucci, 2008). Many extrinsic factors interact with intrinsic genetic factors to influence longevity, resulting in significant plasticity of the life span of multicellular organisms from invertebrates to humans (Kenyon, 2005). Both entropic loss of order and genetic programs are deterministic factors in the rate of aging and overall longevity of living organisms (Hayflick, 2007). Although we have limited tools for influencing the second law of thermodynamics in cells, we have many tools

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for studying the impact of genetic variations on longevity (Deelen, Beekman, Capri, Franceschi, & Slagboom, 2013). The exact contribution of the lottery of genetics to longevity varies between individuals, but has been consistently shown to exceed 20% in average-lived populations (Budovsky et al., 2013; Shadyab & LaCroix, 2015) and approaches 50% in select long-lived populations, such as centenarians (Willcox, Willcox, Hsueh, & Suzuki, 2006; Yashin, Iachine, & Harris, 1999).

The first candidate genes to be associated with exceptional longevity (100+ years of age) in humans were *HLA-DRW9* (P =0.0001) and *HLA-DR1* (P =0.037), which were found in human leukocyte antigen (HLA) complex region genes in long-lived Okinawans in lower and higher frequency, respectively, by the Okinawa Centenarian Study (Takata, Suzuki, Ishii, Sekiguchi, & Iri, 1987). The first widely replicated longevity-associated gene was *APOE*, a lipid-binding protein involved in important transport functions in the lymphatic and vascular systems (Schächter et al., 1994). The frequency of the *APOE* ε4 allele, considered a risk allele for cardiovascular disease (CVD) and Alzheimer's disease (AD), was originally observed to be lower in centenarians than in adult controls (age 20–70) by 6% (P <0.001), whereas the frequency of the protective ε2 allele was 6% higher (P <0.01), leading to an apparent association with longevity (Schächter et al., 1994). This initial finding in Caucasians (French) has since been confirmed in multiple Caucasian and Asian populations (Garatachea et al., 2015). Carriers of the ε2 allele have an approximately 20% lower prevalence of coronary heart disease (Bennet et al., 2007) and significantly lower cognitive decline in extreme old age (97+, odds ratio 0.58) (Lindahl-Jacobsen et al., 2013). Attrition of ε4 allele carriers from mortality at earlier ages may explain the association of the ε2 allele with longevity (Sebastiani et al., 2017).

Unlike ApoE (Davignon, Gregg, & Sing, 1988), interest in the role of the forkhead box O (FoxO) family of transcription factors with lifespan began not in humans—but in nematodes. Kenyon and colleagues reported that mutations in the *daf-2* gene of *Caenorhabditis elegans* resulted in a 2.3-fold increase in their lifespan, with the majority of mutants also retaining their health after their wild-type counterparts had already died (Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993). The morbidity compression (i.e., healthspan) and longevity-conferring (i.e., lifespan) action of the *daf-2* mutant were dependent on a related gene, *daf-16* (Kenyon et al., 1993), which proved to be the *C. elegans* ortholog of human FoxO genes (Ogg et al., 1997).

We carried out the first investigation into the possible association of variants in the 125 kb FoxO gene (FOXO) with human longevity in a nested case–control candidate gene study targeting insulin signaling and glucose homeostasis genes, which are associated with the longevity benefits of caloric restriction (Willcox, Donlon, et al., 2008). Utilizing a large, longitudinal cohort of Japanese-American men well characterized for aging phenotypes—the Honolulu Heart Program—we found the first association of several single nucleotide polymorphisms (SNPs) in human *FOXO3* (and to a lesser extent *FOXO1*) with longevity. Heterozygotes and homozygotes of the protective variant of the *rs2802292* SNP in particular had a 1.9-fold (P <0.0003), or 2.8-fold (P <0.0007) increased chance, respectively, of living to 95 years compared with homozygote carriers of the nonprotective variant (Willcox, Donlon, et al., 2008). This significant association between *FOXO3* SNPs and longevity in

humans has been independently replicated in multiple populations (Morris et al., 2014), including a genome-wide association study (GWAS) (Broer et al., 2015).

A GWAS follows the same broad design concept as a candidate gene study in that each use a defined outcome within the sample population to investigate the association of genetic variation with a specific phenotype. GWAS are considered to produce more rigorous associations because they assess polymorphisms across the entire genome, and because they employ a Bonferroni correction for multiple testing that is at least three orders of magnitude greater than those used to assess the statistical significance of candidate gene hypotheses. The drawback of requiring a strength of association such that the likelihood of the null hypothesis being disproved is less than 1 in 20 million ($P < 5\ 10^{-8}$) is that a large cohort must be recruited for a GWAS. Studies from the CHARGE consortium exemplify this. A meta-analysis of potential longevity associations performed in 2010 involved 1836 longevity cases (90 + years of age) and 1955 controls (aged 55–80 years), but *FOXO3* SNP(s) did not appear in the 24 most statistically significant candidates despite consistent replication with longevity in independent studies of Japanese-American (Willcox, Donlon, et al., 2008), Italian (Anselmi et al., 2009), German (Flachsbart et al., 2009), Han Chinese (Li et al., 2009), and Danish (Soerensen et al., 2010), among other populations. In fact, none of those 24 candidates from the original CHARGE meta-analysis achieved genome-wide significance (Newman et al., 2010). Just 5 years later, however, the CHARGE consortium had expanded its meta-analysis to 6036 longevity cases and 3757 controls and confirmed the candidacy of SNPs in *APOE* (odds ratio=1.20; corrected *P* value=4.8×10^{-4}) and *FOXO3* (odds ratio=1.17; uncorrected *P* value=1.85×10^{-10}) as potential longevity genes (Broer et al., 2015). Although the Japanese-American and Italian cohorts were exclusively male, female participants were included in the studies of German, Danish, Han-Chinese, and various Caucasian populations included in the CHARGE consortium of cohorts. This is important since women have a greater average lifespan than men, and because gender-specific effects are observed in studies of association of polymorphisms and can affect the power of genetic association studies (Magi, Lindgren, & Morris, 2010). Both males and females display an association between different *FOXO3* SNPs and longevity, with some variability between each when studied separately (Li et al., 2009; Soerensen et al., 2010). Given the high linkage disequilibrium between *FOXO3* SNPs (Soerensen et al., 2010), we can reasonably assume that effects observed for one SNP apply to another, and therefore that the “protective” alleles of *FOXO3* may confer longevity to both men and women.

The exact physiological mechanism(s) by which *FOXO3* SNPs confer longevity were not immediately clear from the genetic association studies. The effect does not appear to be mediated by a coding variant in an exon but rather is associated with a ~100 kb region in or near intron 2, which contains noncoding SNPs in high LD (Donlon et al., 2012). To quantify the effect size of the protective *FOXO3* allele(s) on risk of mortality, we studied the association of *rs2802292* SNP alleles with major age-associated clinical causes of death—i.e., coronary heart disease (CHD), cancer, hypertension, diabetes, and stroke, among others. We investigated the effect size on all-cause mortality and cause-specific mortality for the *FOXO3* protective allele across 17 years of prospective data in two cohorts that represented three distinct ethnic groups. The risk for all-cause mortality was 10% lower in carriers of the protective allele (hazard ratio =0.90; 95% CI 0.84–0.95; *P* =0.001), with CHD being the

only cause of death significantly associated with lack of the protective allele. A meta-analysis of results from three ethnically different populations (Asian, Black, and Caucasian) revealed a 26% reduction in mortality from CHD mortality (combined hazard ratio =0.74; 95% CI 0.64–0.86; P =0.00004) for carriers of the protective allele (Willcox et al., 2016). Thus, the protective alleles of both *APOE* and *FOXO3* each reduce the risk of death from CHD (Bennet et al., 2007; Willcox et al., 2016), albeit likely via different principal mechanisms.

2. THE INSULIN SIGNALING PATHWAY AND LIFE SPAN

The link between the insulin/IGF-1 signaling (IIS) pathway and longevity was first apparent from research involving *C. elegans* in the 1990s when *daf-2* was identified as the homolog of the insulin receptor, and its downstream effector, *daf-16*, was found to be the homolog of *FOXO* (Dorman, Albinder, Shroyer, & Kenyon, 1995; Kenyon et al., 1993; Ogg et al., 1997). The IIS pathway begins with the transmembrane DAF-2/insulin receptor (invertebrates/mammals). Binding of insulin or growth hormones initiates a phosphorylation cascade that involves AGE-1/PI3K and results in the AKT (protein kinase B)-mediated phosphorylation of DAF-16/FoxO3, causing the repression of FoxO3 translocation to the nucleus (Fig. 1). In this manner, the energy-sensing pathway regulates the FoxO3 protein by blocking its activity as a transcription factor in the presence of the appropriate stimulus. In *C. elegans*, the extension of lifespan associated with repression of the IIS pathway, for example, as a result of genetic mutation(*s*) (Kenyon et al., 1993), is entirely dependent on a functional copy of *DAF-16*.

The functions of FoxO3 and its homologs in the IIS pathway are highly conserved (O'Neill, Kiely, Coakley, Manning, & Long-Smith, 2012). The link between the IIS pathway and longevity in mice (Blüher, Kahn, & Kahn, 2003; Shimokawa et al., 2015), *Drosophila melanogaster* (Clancy et al., 2001; Giannakou et al., 2004; Tatar et al., 2001), and *Saccharomyces cerevisiae* (Fabrizio, Pozza, Pletcher, Gendron, & Longo, 2001; Postnikoff, Malo, Wong, & Harkness, 2012), formed the basis for our studies of insulin signaling genes in candidate gene studies of human longevity (Willcox, Donlon, et al., 2008). Interestingly, although we observed a strong association of *FOXO3* variants with longevity, SNPs in other genes in the IIS pathway (specifically, *ATF4*, *CBL*, *CDKN2*, *EXO1*, and *JUN*) did not exhibit an association (Morris et al., 2014). Nevertheless, a previous study found an association between an *AKT* SNP and longevity (Pawlikowska et al., 2009). The range of species across which the IIS pathway affects longevity supports the hypothesis that a highly conserved longevity mechanism is centered around *FOXO3*. Given the key role of FoxO3 in translating IIS pathway signals into changes in the pattern of gene expression, and a significant difference in longevity between carriers of *FOXO3* alleles, it seems reasonable to conclude that FoxO3 forms part of an evolutionarily conserved control node with considerable influence on longevity.

Caloric restriction (CR) is the single strongest intervention for extension of lifespan, with a 30% CR resulting in approximately 30% increased life span in rodents and more across the spectrum of other model organisms (Bonkowski, Rocha, Masternak, Regaiey, & Bartke, 2006; Harrison, Archer, & Astle, 1984; Lakowski & Hekimi, 1998; McCay, Crowell, &

Maynard, 1935). The effects of CR are strongly connected to the IIS pathway. Mutations in the insulin receptor of various species lead to longer life spans (Bartke et al., 2001; Blüher et al., 2003; Clancy et al., 2001; Kimura, Tissenbaum, Liu, & Ruvkun, 1997; Lakowski & Hekimi, 1998). Adipose tissue seems to be key to the effects of FoxO and IIS on lifespan. In *D. melanogaster*, induced expression of the native FoxO gene (*dFOXO*) in the fat body of adults produced a sex-specific increase in lifespan of 20%–50% favoring female flies (Giannakou et al., 2004). In mice, fat-specific knockout of the insulin receptor results in an 18% increase in lifespan, accompanied by reduced adiposity (50%–70% less fat mass) (Blüher et al., 2003). Additionally, without functional FoxO3, mice cannot benefit from the lifespan extension effects of CR (Shimokawa et al., 2015). Studies of nonhuman primates (Rhesus monkeys) have shown that CR improves healthspan and may increase lifespan (Mattison et al., 2017). The possibility of an effect in humans is supported by improvements in various biomarkers of healthy aging, cardiovascular and metabolic health in particular (Das, Balasubramanian, & Weerasekara, 2017; Fontana, Meyer, Klein, & Holloszy, 2004; Most, Tosti, Redman, & Fontana, 2017; Ravussin et al., 2015; Walford, Mock, Verdery, & MacCallum, 2002). The traditional Okinawan diet, which results in a 10%–15% CR vs the average diet of other Japanese, may contribute to these people having the world's longest lifespan and a remarkable healthspan (Bernstein et al., 2004; Willcox & Willcox, 2014; Willcox et al., 2007). There is no question that most people in Western society consume an excess of calories and that this poses a significant hazard to healthspan and lifespan. On the other hand, diets that push the limits of caloric restriction are associated with negative effects on lifespan and healthspan (Walford et al., 2002; Willcox et al., 2004).

3. TRANSCRIPTIONAL IMPACTS OF FoxO GENES ACROSS SPECIES

FoxOs are transcriptional regulators that sit at the interface between multiple signaling pathways and gene expression. FoxO3 influences expression of a wide variety of genes and plays a key role in numerous cellular processes (Morris, Willcox, Donlon, & Willcox, 2015). These include redox regulation, autophagy, energy homeostasis, DNA repair, cell cycle arrest, and stem cell homeostasis. The role of FoxO3 can be accurately summarized as being a central component of cellular stress resistance, be it oxidative, metabolic, or replicative (Morris et al., 2015). FoxO3 binds a 5'-TTGTTTAC-3' sequence found at relatively high frequency throughout the genome. This sequence is recognized by a highly conserved DNA-binding domain (DBD) that is shared across the forkhead box family (Furuyama, Nakazawa, Nakano, & Mori, 2000). There is thus a need for additional levels of variation in the mode of action between FoxO transcription factors besides DNA–protein interactions; e.g., posttranslational modifications (PTM) and interaction with coregulators, including microRNAs (Eijkelenboom & Burgering, 2013). FoxO proteins range in size from ~400 to 700 amino acids in length. Besides sharing a similar DBD, they also contain two nuclear localization sequences and a single nuclear export sequence. FoxO proteins across species share highly conserved PTM sites of phosphorylation, acetylation, and ubiquitination (Smith & Shanley, 2010).

Following activation, the IIS pathway represses FoxO3 activity by increasing the levels of the lipid phosphatidylinositol-3-phosphate, causing phosphoinositide-3-kinase (PI3K) to activate the serine/threonine kinase AKT, which then phosphorylates FoxO3 (Brunet et al.,

1999). These phosphorylation events create binding sites for the regulator protein 14–3-3, which disrupts FoxO3/DNA interactions and promotes exclusion of FoxO3 from the nucleus, thus effectively blocking its transcriptional activity and permitting degradation of FoxO3 in the cytoplasm (Dobson et al., 2011). However, in the presence of reactive oxygen species (ROS), phosphorylation of FoxO3 by MST1 or JNK at alternative PTM sites dissociates 14–3-3, leading to increased expression of oxidative stress response genes such as catalase, thioredoxin-dependent peroxide reductase, sentrin-specific peptidase 1, and superoxide dismutase (SOD) 2 (Eijkelenboom & Burgering, 2013; Morris et al., 2015). In response to nutrient deprivation, FoxO3 is upregulated by the energy sensor AMP-activated protein kinase (AMPK), which phosphorylates FoxO3 at several serine residues, leading to activation of FoxO3, which in turn activates specific target genes involved in stress resistance and energy metabolism. In addition to affecting nuclear translocation, activation of FoxO3 by AMPK increases its binding affinity to CBP–p300 (Wang, Chan, et al., 2012; Wang, Marshall, et al., 2012), a transcription coactivation complex that functions as a histone acetyltransferase to relax chromatin. In an apparent contradiction, the CBP–p300 complex mediates the acetylation of FoxO proteins, which appears to inhibit their DNA binding (van der Heide & Smidt, 2005). Sirtuins 1 and 2—which have been linked to longevity in mice and nematodes—deacetylate FoxO3, inhibiting its transcriptional activity and promoting ubiquitin-mediated degradation (Wang, Chan, et al., 2012; Wang, Marshall, et al., 2012). The extension of lifespan in *C. elegans* in response to oxidative stress requires the FoxO3 gene homolog *daf-16* (Heidler, Hartwig, Daniel, & Wenzel, 2010). The *daf-16* protein upregulates ROS-neutralizing SOD (Honda & Honda, 2002) via an oxidative stress response conserved in *Drosophila* (Curtis et al., 2007). Thus, multiple interdependent mechanisms continuously adjust the subcellular location and transcriptional regulatory capacity of FoxO3 to influence its role in energy homeostasis and redox regulation.

Autophagy is a critical cell process that maintains cellular homeostasis by regulating the turnover and degradation of damaged cellular components by packaging and delivering them to the lysosome for degradation. In skeletal myocytes, FoxO3 transcriptionally activates autophagy via the LC3 and BNIP3 genes (Mammucari et al., 2007). In human and mouse embryonic cell lines the prelude to this is the upregulation of PI3K, causing AKT1 to exclude FoxO1 from the nucleus (Zhou et al., 2012). This process impacts skeletal muscle atrophy (Mammucari et al., 2007) and cardiomyocyte size in rodents (Sengupta, Molkentin, & Yutzey, 2009), hinting at a possible influence of FoxO3-mediated autophagy on longevity. FoxO3-mediated activation of autophagy during osteoblast differentiation in mesenchymal stem cells to help maintain redox homeostasis (Gómez-Puerto et al., 2016) represents another cellular and physiological mechanism by which FoxO3 may contribute to longevity, namely, stem cell homeostasis.

The stem cell theory of aging postulates that gradual loss of the ability of adult stem cell populations to contribute to tissue homeostasis is a prime driver of organismal aging (Schultz & Sinclair, 2016). This theory is supported by evidence of reductions in the number and/or function of a variety of stem cell populations across the range of organismal complexity from *C. elegans* to humans (Schultz & Sinclair, 2016). Therefore, mechanisms by which stem cell homeostasis is maintained are of great relevance to the longevity of different organisms, and the essential role of FoxO3 in this process has been demonstrated

repeatedly. The Cnidarian, *Hydra vulgaris*, is considered to be immortal because it shows no evidence of senescence (Martínez, 1998). The constant renewal of its tissues is achieved by highly efficient maintenance of its stem cells (Bosch, Anton-Erxleben, Hemmrich, & Khalturin, 2010) in a FoxO-dependent manner (Boehm et al., 2012). *C. elegans* has only one stem cell population, the germline stem cells. The regulatory mechanism controlling proliferation of these stem cells has also been shown to be dependent on the activity of *daf-16* (Michaelson, Korta, Capua, & Hubbard, 2010). Similarly, dFoxO regulates germline stem cells in *Drosophila* by upregulating the Notch-suppressing gene, *fng*, in the niche cells supporting this stem cell population (Yang et al., 2013).

Mice present the best evidence that FoxO3-mediated regulation of stem cell homeostasis could have an impact on human longevity. In stem cells (satellite cells) of mouse skeletal muscle, FoxO3-mediated Notch signaling maintains their quiescence and thereby the long-term self-renewal capacity of the tissue (Gopinath, Webb, Brunet, & Rando, 2014). Hematopoietic stem cells (HSC) in *Foxo3* knockout mice have an impaired oxidative stress response and there is a significantly greater decline in their frequency in bone marrow with age (Miyamoto et al., 2007). Independent of ROS management, *Foxo3* knockout mice have defects in HSC mitochondrial metabolism that affect their long-term homeostasis, suggesting a broader role for FoxO3 in stem cell maintenance (Rimmelé et al., 2015). Indeed, the role of FoxO3 in autophagy is important to HSC maintenance, particularly for old HSC (Warr et al., 2013). In the absence of functional FoxO3, murine HSC demonstrated significant downregulation of proautophagic genes in response to external stress. This FoxO3-dependent mechanism is essential for the continued survival of old HSC (Warr et al., 2013). FoxO3 knockout mice also exhibit fewer neural stem cells (NSC), with impaired self-renewal and differentiation capacity (Renault et al., 2009). This increases significantly with age compared to wild-type controls and may be attributable to the loss FoxO3-mediated redox balance (Yeo et al., 2013). FoxO3 also plays a role in another important regulator of NSC self-renewal and differentiation control, Wnt. A mouse knockout model in which *Foxo1*, *Foxo3*, and *Foxo4* were deleted demonstrated that negative regulation of Wnt played a role in the early loss of NSC (Paik et al., 2009). In humans, both circulating HSC counts and differentiation capacity (Moresi et al., 2005), as well as NSC frequency (Maslov, Barone, Plunkett, & Pruitt, 2004) decline with age. HSC aging in the human thymus results in loss of T cell diversity (Naylor et al., 2005) which may partly explain the decline in adaptive immune response in the elderly (Ginaldi et al., 1999).

DNA repair and cell cycle control are essential to the maintenance of genome stability in replicating cells, and the increase in genome instability with age (Burhans & Weinberger, 2007) suggests a relation between these processes and longevity. ROS directly and indirectly cause DNA damage, which induces FoxO3-dependent cell cycle arrest via the mitogen-activated protein kinase signaling pathway, leading to downregulation of the proliferation regulatory gene, *Myc* (Kress et al., 2011). The main target of the DNA damage-sensing factor ataxia telangiectasia mutated is H2AX. H2AX is a specialized histone whose mouse knockout model exhibits decreased *FoxO3* expression and changes in expression of FoxO3-regulated genes in response to irradiative damage (Tarrade et al., 2015). The changes to FoxO3-regulated gene expression in *H2ax* knockout mice result in decreased DNA repair response (involving genes *Ddb2* and *Hspa1b*), weaker assertion of cell cycle control (via

Cdkn1a), and increased apoptosis signaling (via Bcl2l11) (Tarrade et al., 2015). Together, these results indicate a significant role for FoxO3 in the DNA damage response and repair process independent of redox homeostasis.

4. EXTRINSIC INFLUENCES AND THE FUTURE OF FoxO3-MEDIATED LONGEVITY

All of the earlier evidence supporting the many mechanisms by which FoxO3 influences longevity is good news for those lucky enough to receive a protective genotype of *FOXO3* from the genetic lottery that is reproduction. Identification of SNPs that affect phenotype, particularly one as complex as longevity, is vital to the investigation of genetic contributions to aging. The knowledge we have gained regarding the role of FoxO3 in human longevity can be leveraged to benefit healthspan and perhaps lifespan for everyone, irrespective of whether they carry the protective haplotype of *FOXO3* or the common haplotype. An important part of this process will be the identification of extrinsic modifiers of *FOXO3* expression—diet, life-style, and small molecules—and assessment of their effect, both short-term and long-term, on age-related diseases and longevity. The highly conserved nature of the *FOXO*/longevity relationship should yield a rich translation of beneficial effects from model organisms to humans.

Energy restricted diets improve biomarkers and other phenotypes of healthy aging in humans (Civitarese et al., 2007; Fontana et al., 2004; Ravussin et al., 2015; Walford et al., 2002; Willcox & Willcox, 2014; Willcox et al., 2007; Witte, Fobker, Gellner, Knecht, & Flöel, 2009). In the general population, however, adherence to diet-management plans is generally poor. In contrast, nutraceutical and dietary supplement usage, which is on the rise, is more likely to be effective. Identification of FoxO3 activators is in its infancy. We know from *C. elegans* that under conditions of stress, the AGE-1 hypertonic stress resistance mechanism includes the DAF-16-mediated upregulation of trehalose synthesis enzymes TSP-1 and TPS-2 (Lamitina & Strange, 2005). Trehalose is a glucose disaccharide linked to cytoprotection across multiple species. When fed to young worms, trehalose extends their life span by 30%; even when the feeding started in late adulthood, trehalose compressed morbidity and extended remaining lifespan in *C. elegans* (Honda, Tanaka, & Honda, 2010). Trehalose also stimulates autophagy—a FoxO3-mediated process—in multiple human cells (Belzile et al., 2016; Chen et al., 2016). When fed to older mice, trehalose improved arterial endothelium-dependent dilation (LaRocca et al., 2012). Endothelium-dependent dilation of arteries occurs when the endothelial cells produce nitric oxide (NO), which signals relaxation in the underlying vascular smooth muscle cells. Trehalose treatment of aged mice reduces vascular oxidative stress and normalizes cytokine expression (LaRocca et al., 2012). In a similar manner, oral supplementation with trehalose in humans results in improvement in endothelium-dependent microvascular function, which is considered a major indicator of CVD risk (Kaplon et al., 2016). While it remains to be demonstrated directly that trehalose activates FoxO protein(s) in humans, given these associations, trehalose may offer an approach to nutraceutical activation of FoxO3. It also suggests that a FoxO3-dependent mechanism in endothelial cells may play a role in the impact that protective variant(s) in linkage disequilibrium with *FOXO3* SNP *rs2802292* has on CHD mortality.

Supplementation with the potent polyphenol curcumin improves endothelial function of resistance arteries by 37% (P =0.03) via the same kind of increase in NO bioavailability and sensitivity as trehalose supplementation (Kaplon et al., 2016; Santos-Parker et al., 2017). However, curcumin may also have a more direct link to FoxO3 regulatory pathways. Curcumin can reduce phosphorylation of FoxO3, leading to its nuclear translocation and resultant upregulation of expression of stress resistance and lipid transport genes (Zingg et al., 2012). This may explain the dFOXO-dependent 21% increase in *Drosophila* lifespan following treatment with the curcumin metabolite tetrahydrocurcumin (Xiang et al., 2011). Green tea also contains potent polyphenolic compounds, one of which—epigallocatechin-3-gal-late—has been shown to activate FoxO3 (Belguise, Guo, & Sonenshein, 2007). In breast cancer cells, this natural compound, via activation of FoxO3, had the added benefit of inhibiting their invasive phenotype by upregulation of estrogen receptor alpha (Belguise et al., 2007). The microalgae *Haematococcus pluvialis* produces astaxanthin (a xanthophyll carotenoid), under conditions of stress. This compound is a potent activator of FoxO3 and increases *C. elegans* life span by up to 30% (Yazaki, Yoshikoshi, Oshiro, & Yanase, 2011). The effect is abrogated in a *daf-16* null mutant.

Although these natural compounds are promising options for nutraceutical-mediated activation of FoxO3, pharmaceutical compounds approved for other uses have been shown to activate FoxO3. Notably, bepridil (now withdrawn in the United States) and trifluoperazine (an anti-psychotic) promote FoxO3 translocation to the nucleus by inhibiting AKT phosphorylation (Park et al., 2016). The drug metformin induces AMPK-mediated phosphorylation and nuclear translocation of FoxO3 (Sato et al., 2012). It should be noted that metformin is being used in the first clinical trial approved to study the effect of a drug on longevity (Barzilai, Crandall, Kritchevsky, & Espeland, 2016). The screening of novel and existing libraries of small molecular compounds for FoxO nuclear translocation capabilities utilizing cells modified with green fluorescent protein-tagged FoxO has produced a wealth of potential activators (Cautain et al., 2016; Zanella, Rosado, García, Carnero, & Link, 2008). When measuring nuclear translocation of FoxO as the endpoint, compounds may act by influencing the activity of other components of the IIS pathway (Zanella et al., 2008), or the exclusion of active FoxO proteins from the nucleus (Cautain et al., 2016). These successful small-scale investigations of pharmaceutical activation of FoxO(s) suggest that high-throughput screening may reveal a veritable library of old drugs that can be put to new uses for individuals with age-related morbidities modifiable by FoxO3. Those seeking a better chance of achieving exceptional longevity may soon have the option of utilizing methods that take advantage of the positive effect of FoxO3 on lifespan and healthspan, regardless of their luck in the genetic lottery.

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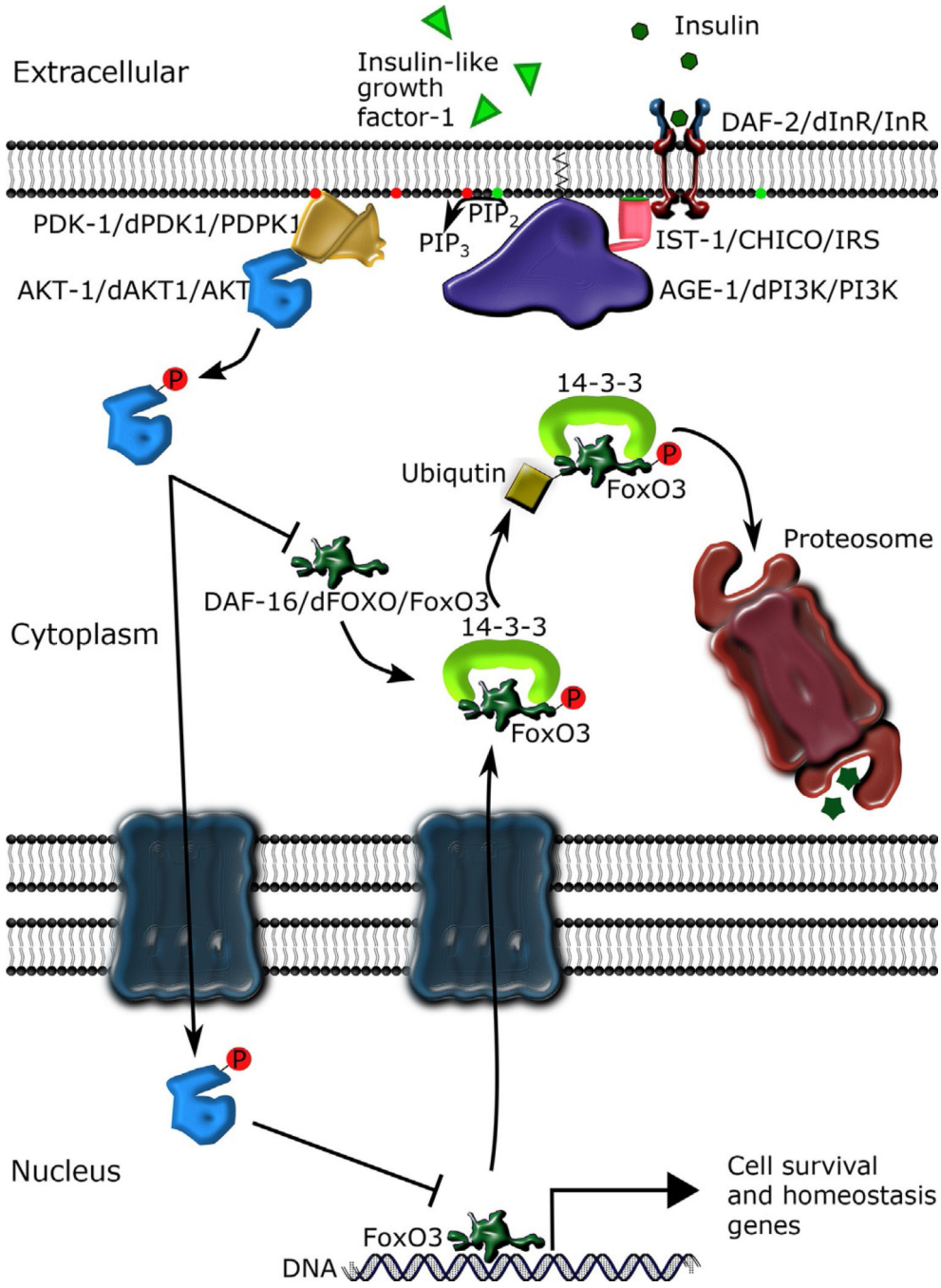

Fig. 1.

The role of the insulin/IGF-1 signaling pathway in regulating FoxO3 activity is conserved between *C. elegans*/*D. melanogaster*/humans. Following extracellular binding of the insulin receptor (InR) by a substrate (e.g., insulin or insulin-like growth factor-1), the insulin receptor substrate (IRS) associates with InR on the cytoplasmic side of the cellular membrane. IRS in turn recruits PI3K which phosphorylates the membrane lipid phosphatidylinositol-2-phosphate (PIP_2), creating phosphatidylinositol-3-phosphate (PIP_3). PIP_3 is bound by phosphoinositide-dependent protein kinase-1 (PDPK1) which activates

AKT via phosphorylation. Active AKT inhibits the nuclear translocation of FoxO3 and promotes its exclusion from the nucleus via a phosphorylation event. Phosphorylated FoxO3 is bound by the 14–3-3 regulator protein, leading to ubiquitination and proteasomal degradation.