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Nematology 19 (2017) 499-508

Nematology
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Forum article

Lifespan extension in *Caenorhabditis elegans* insulin/IGF-1 signalling mutants is supported by non-vertebrate physiological traits

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Received: 7 December 2016; revised: 6 February 2017
Accepted for publication: 6 February 2017; available online: 1 March 2017

Summary – The insulin/IGF-1 signalling (IIS) pathway connects nutrient levels to metabolism, growth and lifespan in eukaryotes ranging from yeasts to humans, including nematodes such as the genetic model organism *Caenorhabditis elegans*. The link between ageing and the IIS pathway has been thoroughly studied in *C. elegans*; upon reduced IIS signalling, a genetic survival program is activated resulting in a drastic lifespan extension. One of the components of this program is the upregulation of antioxidant activity but experiments failed to show a clear causal relation to longevity. However, oxidative damage, such as protein carbonyls, accumulates at a slower pace in long-lived *C. elegans* mutants with reduced IIS. This is probably not achieved by increased macroautophagy, a process that sequesters cellular components to be eliminated as protein turnover rates are slowed down in IIS mutants. The IIS mutant *daf-2*, bearing a mutation in the insulin/IGF-1 receptor, recapitulates the dauer survival program, including accumulation of fat and glycogen. Fat can be converted into glucose and glycogen *via* the glyoxylate shunt, a pathway absent in vertebrates. These carbohydrates can be used as substrates for trehalose synthesis, also absent in mammals. Trehalose, a non-reducing homodimer of glucose, stabilises intracellular components and is responsible for almost half of the lifespan extension in IIS mutants. Hence, the molecular mechanisms by which lifespan is extended under reduced IIS may differ substantially between phyla that have an active glyoxylate cycle and trehalose synthesis, such as ecdysozoans and fungi, and vertebrate species such as mammals.

Keywords – antioxidants, fat, glycogen, glyoxylate shunt, proteostasis, trehalose.

The ageing process of free-living nematodes was first explored in 1970 by David Gershon using the vinegar eel *Turbatrix aceti*. By applying synchronisation and chemical sterilisation techniques, he established the first survival curves of worm cohorts and monitored age-specific loss of enzyme activities (Erlanger & Gershon, 1970; Gershon, 1970; Gershon & Gershon, 1970). A few years later, ageing in *Caenorhabditis elegans* was first described in studies covering survival at varying environmental conditions (Klass, 1977) and the non-ageing characteristics of the dauer diapause stage (Klass & Hirsh, 1976). Two studies in the early 1980s nucleated the field of the genetics of ageing in *C. elegans*. First, Tom Johnson showed that in *C. elegans* the genetic components of recombinant inbred lines dictate lifespan (Johnson & Wood, 1982), and, second, Michael Klass discovered the first *C. elegans* mutants with extended lifespan (Klass, 1983). The latter strains were not outcrossed and showed pleiotropic phenotypes and, therefore, the author assumed the worms lived longer due to a dietary restriction effect rather than by mutation in one specific gene that regulates ageing rate. Subsequent genetic analysis of these strains by Tom Johnson resulted in the discovery of *age-1* (*age*ing alteration), the first mutated gene ever described that causes lifespan extension in a metazoan (Friedman & Johnson, 1988). With the discovery of a second longevity mutant, *daf-2* (abnormal *da*uer *f*ormation), a few years later (Kenyon *et al*., 1993), the genetics of ageing field gained substantial momentum. The

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DOI 10.1163/15685411-00003060

daf-2 mutant was not recovered from a genetic longevity screen but was isolated in an effort to analyse genetically dauer formation (Kenyon, 2011). In the Kenyon study, it was shown that lifespan extension in *daf-2* mutants is fully dependent on *daf-16* gene activity. A few years later, these genes were cloned, characterised and found to be part of an Insulin/Insulin Growth Factor 1-mediated signalling (IIS) pathway, with *daf-2* coding for the Ins/IGF-1 receptor (Kimura *et al*., 1997), *age-1* being a homologue of a phosphoinositide 3-kinase subunit (Morris *et al*., 1996) and *daf-16* representing the *C. elegans* forkhead box O (FOXO) transcription factor (Lin *et al*., 1997; Ogg *et al*., 1997).

The IIS pathway

After the seminal discoveries of *age-1*, *daf-2* and *daf-16*, other genes of the IIS pathway (Fig. 1) were identified and characterised (Ogg & Ruvkun, 1998; Paradis & Ruvkun, 1998; Paradis *et al*., 1999; Wolkow *et al*., 2002; Hertweck *et al*., 2004). Most IIS genes have homologues in species ranging from yeast to humans, indicating the strong evolutionary conservation of this pathway. Moreover, the IIS pathway seems to influence lifespan in many of these species, including humans (Kenyon, 2010). These discoveries elicited quite some optimism and many research groups have since focused on the IIS pathway and the mechanisms by which it extends

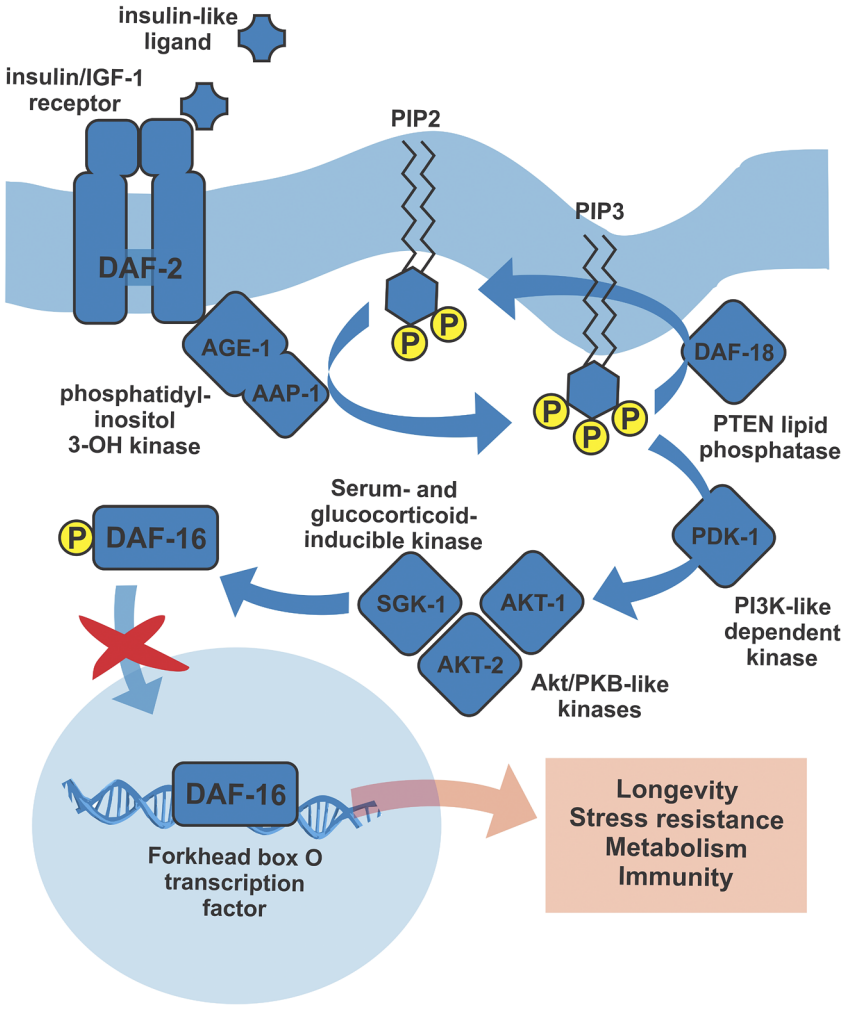

Fig. 1. Overview of the most important components of the IIS pathway in *Caenorhabditis elegans*.

C. elegans lifespan. The gene index in the abstract book of the 2015 International *C. elegans* Meeting (Los Angeles, CA, USA) leaves no doubt that *daf-2* and *daf-16* are, currently, by far the most studied *C. elegans* genes.

Although the IIS pathway generally regulates growth and metabolism in all eukaryotes, its constituents have diverged into networks that differ among phylogenetic groups (Papatheodorou *et al*., 2014; McGaugh *et al*., 2015). As a result, there is no one-to-one gene homology between the IIS components of *C. elegans* and humans. The worm genome encodes for 40 insulins, most of which have unidentified functions (Pierce *et al*., 2001; Cornils *et al*., 2011), whilst in humans only three related insulin-like peptides occur: insulin, IGF-1 and IGF-2. On the other hand, *C. elegans* has only one known IIS receptor, *daf-2*, whilst humans express several receptors, each controlling downstream pathways that trigger specific physiological functions (Clayton *et al*., 2011). In *C. elegans*, the FOXO transcription factor DAF-16 is the endpoint of the IIS cascade. A forkhead box (FOX) is an 80-100 amino-acid DNA-binding motif that is present in a wide variety of FOX proteins. These proteins are clustered in subfamilies ranging from A to S based on sequence homology and DAF-16 belongs to the O subfamily. A single *daf-16* locus in *C. elegans* encodes five isoforms (Kwon *et al*., 2010; Chen *et al*., 2015), two of which influence lifespan, whilst in humans four separate genes make up the FOXO family. Also here, two FOXO forms have been linked to lifespan determination (Martins *et al*., 2016). Hence, despite their diversity, FOXO transcription factors seem to regulate lifespan in a wide range of eukaryotes, but likely *via* different downstream programs.

The FOXO/DAF-16 lifespan program

Being identified as a master switch in lifespan regulation, *daf-16* has received much attention over the last two decades. The identification of genes under control of this transcription factor could reveal the molecular mechanism(s) of lifespan extension.

With the dawn of the omics era, transcriptomic (McElwee *et al*., 2003; Murphy *et al*., 2003; McElwee *et al*., 2004; Halaschek-Wiener *et al*., 2005), proteomic (Dong *et al*., 2007; Jones *et al*., 2010; Depuydt *et al*., 2013, 2014; Stout *et al*., 2013; Walther *et al*., 2015) and metabolomic (Fuchs *et al*., 2010) studies showed that IIS mutants undergo massive changes in gene expression and shifts in metabolic networks. DAF-16 targets were also determined by analysing DNA binding sites of this transcription factor with DamID and ChIP (Oh *et al*., 2006; Schuster *et al*., 2010). In an early microarray study, many of the differentially expressed genes of IIS mutants were tested for their influence on lifespan. Most of these genes only had a partial effect, suggesting that the total IIS lifespan extension is caused by the upregulation of a wide variety of genes, including genes involved in the cellular stress and antimicrobial response, as well as metabolic genes (Murphy *et al*., 2003). These differentially expressed genes largely overlap with the transcriptional pattern observed in *C. elegans* dauers (McElwee *et al*., 2004, 2006), which is not surprising as in the long-lived IIS mutants, the dauer program is probably activated heterochronically during adulthood.

The DAF-16/dauer longevity program: a struggle against free radicals?

In an early effort to characterise the physiology of the long-lived IIS mutant *age-1*, two researchers independently found that the antioxidant activity is increased in this strain (Larsen, 1993; Vanfleteren, 1993). This fits well with the free radical theory of ageing that states that free radicals, produced as a by-product of oxidative metabolism in the mitochondria, cause molecular damage that accumulates over time and underlies the functional decline that characterises ageing (Harman, 1956, 1972). In this reasoning, the high antioxidant levels in long-lived *age-1* mutants would scavenge free radicals, preventing damage accumulation and lead to lifespan extension. High antioxidant activity was later also confirmed for the long-lived IIS mutant *daf-2* (Houthoofd *et al*., 2003, 2005). Also in the transcriptomic studies mentioned above, it was confirmed that a broad antioxidant response is activated in IIS mutants (Murphy *et al*., 2003; Halaschek-Wiener *et al*., 2005). The expression and activity of the primary ROS scavengers superoxide dismutase (SOD) and catalase (CTL), in particular, are strongly upregulated. However, are antioxidant upregulation and lifespan extension causally related? An early test using RNAi knockdown of *sod-3*, *ctl-1* and *ctl-2* genes suggested that these genes are only marginally involved in IIS lifespan extension as knockdown resulted in a slight (10-15%) decrease of *daf-2* lifespan (Murphy *et al*., 2003). Later studies using knockout mutants did not find any effect. Knocking down the two mitochondrial SODs as well as two cytosolic SODs did not shorten *daf-2* lifespan, whilst knockdown of the extracellular SOD extended *daf-2* lifespan even more (Doonan *et al*., 2008). Later it was shown that

quintuple mutants, lacking all five *C. elegans* SODs, had a normal lifespan but were extremely sensitive to oxidative stress. This suggests that, under normal conditions, oxidative stress is not a lifespan determinant (Van Raamsdonk & Hekimi, 2012). It is still to be tested whether the lack of all five *sod* genes reduces the lifespan of long-lived IIS mutants.

Damage accumulation in IIS mutants

If antioxidant enzymes play no or only a minor role in IIS longevity, does oxidative damage accumulate in these worms at the same rate as in wild type controls? Several studies suggest this is not the case; IIS mutants seem to have low rates of oxidative damage accumulation. Protein carbonylation, a standard measure of oxidative protein damage, increases at slower rates in the *age-1* mutant (Ishii *et al*., 2002) and in *daf-2* mitochondria (Brys *et al*., 2007) compared to the wild type control. This decrease in the accrual of oxidative protein modifications in IIS mutants was confirmed in studies using mass spectrometry (Knoefler *et al*., 2012; Dhondt *et al*., 2016). This phenotype may be linked to increased antioxidant activity in IIS mutants but, again, a direct causal link to lifespan has not been shown.

Damage clearance and protein turnover

Oxidative damage may just be one type of damage that accumulates over time and its contribution to ageing may vary from species to species (Gladyshev, 2014). This can explain the limited effect of antioxidants to lifespan in the very short-lived *C. elegans* species. Here, more general processes that prevent damage accumulation may be of greater importance. Protein turnover has been put forward as a potential key player in ageing as it allows the maintenance of proteome quality over time (Gafni, 1990; Ryazanov & Nefsky, 2002; Tavernarakis & Driscoll, 2002). As protein turnover rates have been shown to decline strongly with age, it is comprehensible that ageing could be caused by a gradual self-reinforcing collapse of protein homeostasis (proteostasis).

The finding that autophagy is increased and genetically required for lifespan extension of IIS mutants supports this view (Melendez *et al*., 2003; Lapierre *et al*., 2013). Increased autophagy rates in IIS mutants may clear protein damage efficiently thereby postponing damage accumulation and ageing. However, not all studies are in accordance with this view. RNAi knockdown of 14 autophagy-related genes in *daf-2* mutants showed that RNAi of only two genes caused lifespan shortening while the other 12 genes caused further extension of lifespan or had no effect (Hashimoto *et al*., 2009). Hence, the role of autophagy in IIS longevity is still unclear.

Recent findings cast further doubt on the importance of high bulk protein turnover rates in IIS longevity. Quantitative proteomics data showed a clear decrease in ribosomal subunits and translation factors in *daf-2* mutants suggesting a decrease in protein synthesis rates in these worms (Depuydt *et al*., 2013; Stout *et al*., 2013). Active insulin and IGF pathways are known to support anabolic growth. Hence, a reduction-of-function mutation in these pathways, such as *daf-2*(*e1370*), is indeed expected to decrease anabolic processes such as protein synthesis. This was recently confirmed using classical ^{35}S pulse chase labelling (Depuydt *et al*., 2016) and stable isotope labelling combined with mass spectrometry (Dhondt *et al*., 2016; Visscher *et al*., 2016). About half of the protein species show decreased turnover rates in the long-lived *daf-2* mutants, while turnover of the other proteins remains unchanged (Dhondt *et al*., 2016). The IIS mutants do not seem to invest in the energy-consuming process of protein turnover to maintain proteostasis. Nevertheless, proteostasis is well maintained during adulthood in these mutants (Walther *et al*., 2015).

Investment in fat and glycogen synthesis

If antioxidants and protein turnover are not the (main) strategies to postpone molecular damage and ageing in IIS mutants, which processes do matter? The morphology of IIS mutants may provide a first clue: *daf-2* mutants tend to accumulate large amounts of fat (Ogg *et al*., 1997; Depuydt *et al*., 2014) and glycogen (Frazier & Roth, 2009; Depuydt *et al*., 2014) in the intestine, hypodermis and to some extent in the body wall muscles, thereby phenocopying dauers (Fig. 2). Hence, IIS mutants seem to invest heavily in carbon storage in the form of carbohydrates and triglycerides. In dauers, accumulated fat serves as an energy source for long-term survival in the absence of feeding and its use is tightly controlled by intracellular energy sensors capable of switching lipases on and off (Narbonne & Roy, 2009). Glycogen accumulation in IIS mutants may seem linked to energy storage as well but it also relates to osmotic and anoxic stress resistance (Frazier & Roth, 2009; LaMacchia *et al*., 2015; Possik *et al*., 2015), which are important features in dauer survival.

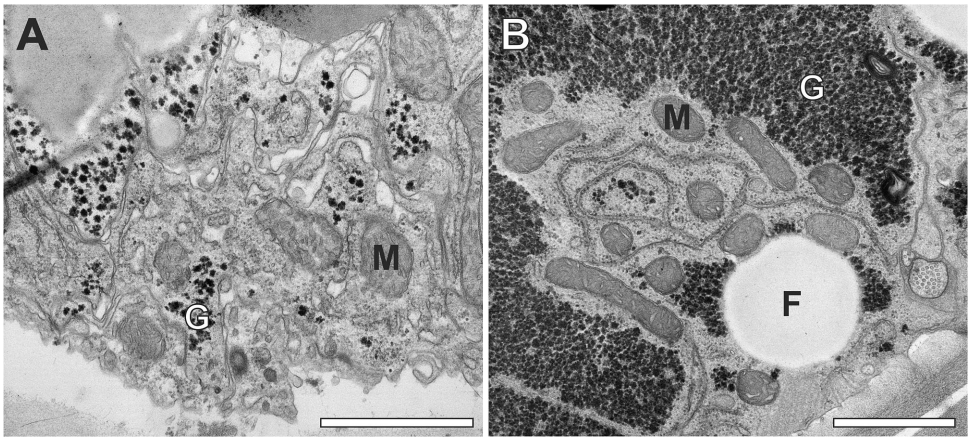

Fig. 2. Transmission electron micrographs of *Caenorhabditis elegans*. A: A reference strain with normal lifespan, *glp-4*(*bn2*) *daf-16*(*mgDf*50);*daf-2*(*e1370*); B: The long-lived IIS mutant *glp-4*(*bn2*);*daf-2*(*e1370*). Abbreviations: M: mitochondrium; G: glycogen accumulation; F: fat droplet. Sample preparation and electron microscopy were carried out as described in Depuydt *et al*. (2014). (Scale bar = 1 μm.)

The fat and glycogen storage phenotype is supported in proteomic studies. Many enzymes involved in fatty acid β-oxidation are upregulated in adult *daf-2*(*e1370*) mutants, while food uptake is drastically lowered, hinting at the use of stored fat as an energy source during adulthood in IIS mutants (Depuydt *et al*., 2014). In these mutants, most enzymes involved in carbohydrate metabolism are also strongly upregulated with glycogen synthase (GSY-1) belonging to the top most upregulated proteins. This agrees with the copious amounts of glycogen observed in the *daf-2* intestine and hypodermis (Fig. 2).

Unlike in vertebrates, fat can be easily converted into glycogen in *C. elegans* (Fig. 3): fatty acids are degraded to acetyl-CoA by β-oxidation, which in turn can be fed to the glyoxylate cycle. The glyoxylate cycle is a shortcut of the tricarboxylic acid (TCA) cycle bypassing two decarboxylation steps. This is managed by a single polypeptide with two enzymatic functions: isocitrate lyase and malate synthase activity (Liu *et al*., 1995). In *C. elegans*, two isoforms of this multifunctional protein exist: the mitochondrial ICL-1 (Erkut *et al*., 2016) and the less characterised C08F11.14 (Frazier & Roth, 2009). When the glyoxylate cycle is activated, carbon is not completely oxidised and lost as CO_2 but instead malate and succinate are synthesised that can be converted into oxaloacetate. The latter substrate can be fed into the gluconeogenesis pathway and the resulting glucose phosphate can finally be incorporated into glycogen. In short, fat can be converted into glycogen by glyoxylate cycle activity. It comes as no surprise that *icl-1* is drastically upregulated in dauers (Holt & Riddle, 2003; Wang & Kim, 2003; Erkut *et al*., 2016) and IIS mutants (Murphy *et al*., 2003; Depuydt *et al*., 2014; Shen *et al*., 2014). Moreover, *icl-1* activity is responsible for up to 45% of the lifespan extension observed in *daf-2* mutants (Murphy *et al*., 2003; Shen *et al*., 2014). But how can the fat-to-glycogen conversion or glycogen itself support the longevity phenotype of IIS mutants?

Trehalose, a chemical chaperone that stabilises proteins and membranes

It is very likely that glycogen provides a fast and easily accessible source of glucose units for the synthesis of trehalose, a disaccharide with well known cytoprotective properties (Elbein *et al*., 2003; Perry & Wharton, 2011). In *C. elegans* dauers, trehalose is required for successful anhydrobiosis, an ametabolic desiccated state that allows individuals to survive sustained periods of drought stress (Erkut *et al*., 2011). Moreover, the glyoxylate shunt is required for anhydrobiotic survival as well in *C. elegans*, clearly linking fat-to-sugar conversion and trehalose metabolism (Erkut *et al*., 2016). Again this metabolic pattern is mirrored in IIS mutants; endogenous trehalose synthesis explains a considerable portion of the extended lifespan of *daf-2* mutants. Exogenously added trehalose extends lifespan of wild-type worms while in *daf-2* mutants lifespan is not further extended, which also indicates

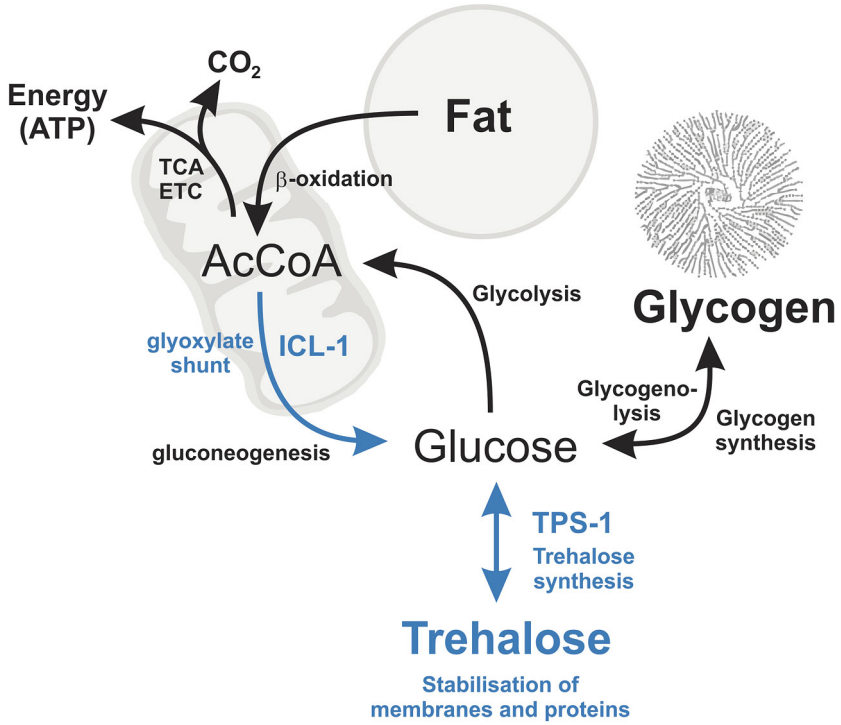

Fig. 3. Simplified overview of *Caenorhabditis elegans* dauer and IIS mutant metabolism with emphasis on the interrelation between fat, glycogen and trehalose. Non-mammalian pathways, enzymes and metabolites are indicated in blue.

that longevity in *daf-2* is (at least in part) caused by endogenous trehalose (Honda *et al*., 2010).

Using a recent *C. elegans* metabolic network model, trehalose production was predicted to be strongly supported by glyoxylate cycle activity under micro-aerobic conditions (Yilmaz & Walhout, 2016). However, in the absence of glyoxylate shunt activity, trehalose can still be produced in lower quantities, probably explaining the partial rescue of *icl-1* mutation on *daf-2* lifespan extension (Shen *et al*., 2014).

Finally, exogenously added trehalose increases protein stability, measured as the trichloroacetic acid-soluble protein fraction, in *C. elegans*. This fraction also correlates well with endogenous trehalose levels in *C. elegans*, indicating that intracellular trehalose acts as a potent protein stabiliser in this worm (Depuydt *et al*., 2016).

Hence, the protein and membrane stabilising properties of trehalose are likely key to IIS mutant longevity. The field of trehalose biology in *C. elegans* in underexplored and future work should reveal in which tissues it is synthesised, how it is transported between tissues, and whether certain tissue types benefit more from trehalose protection than others (with lifespan as a readout). *Caenorhabditis elegans* would also allow for easy genetic manipulation to increase artificially intracellular trehalose levels by overexpressing trehalose synthesis genes and/or knockdown of trehalases and test whether there is a beneficial effect on lifespan. Trehalose can act in concert with LEA proteins for its protective function (Chakrabortee *et al*., 2007). The *C. elegans* genome encodes a single *lea* gene homologue, known to be upregulated in *daf-2* mutants (Dong *et al*., 2007; Depuydt *et al*., 2016) and awaiting functional characterisation.

Relevance to human biology

There is no doubt that *C. elegans* fuelled genetic ageing research in other models including vertebrate species. One of the most successful discoveries so far was the role of IIS signalling in lifespan determination, first revealed in *C. elegans* and later confirmed in many other species (Kenyon, 2010). Nevertheless, one should always bear in mind that, despite evolutionary conservation of many genes and pathways, *C. elegans* and humans differ widely in their molecular physiology as a result

of differences in size, thermobiology and environmental challenges. Although reduced IIS pathway signalling may lead to broadly comparable phenotypes in worms and mammals, the means by which these characteristics are achieved may differ widely. IIS longevity in *C. elegans* is, at least in part, supported by metabolic pathways that are absent in mammals, such as glyoxylate shunting and trehalose synthesis (Fig. 3). Also the glutamate synthase homologue W07E11.1, which, like ICL-1, only occurs in bacteria, plants and nematodes, is highly upregulated in *daf-2* mutants hinting at an important function in the IIS longevity phenotype (Depuydt *et al*., 2014). These metabolic patterns seem all part of an ancient genetic program that allows small organisms with limited mobility to undergo anhydrobiosis and survive repetitive drought/rehydration cycles (Alpert, 2006; Erkut *et al*., 2016). In *C. elegans* IIS mutants, this non-mammalian genetic program is switched on heterochronously in adults, making these worms stress resistant and long-lived in a way that cannot be directly achieved in humans. Nevertheless, detailed functional knowledge of this elaborate program may lead to future medical or pharmaceutical applications that may support human longevity or reduce frailty at advanced age.

Acknowledgements

We are grateful to Myriam Claeys for preparation of the electron microscopy samples. We are indebted to the Special Research Fund at Ghent University (BOF15/24j/013) and the Fund for Scientific Research – Flanders (G.04371.0N and G.0D64.14N) for financial support.

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