Available online at www.sciencedirect.com
ScienceDirect
Mechanisms of Ageing and Development 127 (2006) 922–936
mechanisms of ageing and development
www.elsevier.com/locate/mechagedev

Erratum to "Diapause-associated metabolic traits reiterated in long-lived daf-2 mutants in the nematode *Caenorhabditis elegans*" [Mech. Ageing Dev. 127 (5) (2006) 458–472]☆

Joshua J. McElwee [a], Eugene Schuster [b], Eric Blanc [b], Janet Thornton [b], David Gems [a,*]

[a] *Department of Biology, University College London, Gower Street, London WC1E 6BT, UK*
[b] *European Bioinformatics Institute, Hinxton, Cambridge CB10 1SD, UK*

Abstract

The longevity of the *Caenorhabditis elegans* diapausal dauer larva greatly exceeds that of the adult. Dauer formation and adult ageing are both regulated by insulin/IGF-1 signalling (IIS). Reduced IIS, e.g. by mutation of the *daf-2* insulin/IGF-1 receptor gene, increases adult lifespan. This may reflect mis-expression in the adult of dauer longevity-assurance processes. Since IIS plays a central role in the regulation of metabolism, metabolic alterations shared by dauer larvae and *daf-2* adults represent candidate mechanisms for lifespan determination. We have conducted a detailed comparison of transcript profile data from dauers and *daf-2* mutant adults, focusing on expression of metabolic pathway genes. Our results imply up-regulation in both dauers and *daf-2* mutant adults of gluconeogenesis, glyoxylate pathway activity, and trehalose biosynthesis. Down-regulation of the citric acid cycle and mitochondrial respiratory chain occurs in dauers, but not *daf-2* adults. However, the F_1 ATPase inhibitor was up-regulated in both, implying enhanced homeostasis in conditions where mitochondria are stressed. Overall, the data implies increased conversion of fat to carbohydrate, and conservation of ATP stocks in *daf-2* mutant adults, suggesting a state of increased energy availability. We postulate that this fuels increased somatic maintenance activity, as suggested by the disposable soma theory.

Keywords: Insulin/IGF-1 signalling; *Caenorhabditis elegans*; Diapause; Metabolism; Microarray

1. Introduction

The biological processes underlying longevity and ageing remain largely unknown. However, recent model organism studies have identified many genes that influence lifespan (reviewed in (Kenyon, 2005)). Reduction of insulin/IGF-1 signalling (IIS) can increase lifespan in *Caenorhabditis elegans* (Friedman and Johnson, 1988; Kenyon et al., 1993; Kimura et al., 1997), *Drosophila* (Clancy et al., 2001; Tatar et al., 2001), and mice (Bluher et al., 2003; Holzenberger et al., 2003). In *C. elegans*, IIS also regulates larval diapause (Riddle and Albert, 1997). Under conditions which are not propitious for reproductive success (e.g. high temperature, low food and high population density), developing larvae form a long-lived, non-feeding, stress resistant form, the dauer larva (Cassada and Russell, 1975). Wild-type dauers can survive for more than 3 months, during which time they may resume development if dauer-inducing conditions are reversed; by contrast, adult *C. elegans* age and die after only 2–3 weeks (Klass, 1977). Many mutations which reduce IIS result in constitutive dauer formation (the Daf-c phenotype), even under non-dauer inducing conditions. The increases in adult lifespan resulting from reduced IIS may be the result of mis-scheduled expression of dauer longevity-assurance processes in the adult (Kenyon et al., 1993).

A number of observations support this view. For example, strong similarities were observed between microarray-derived transcript profiles of dauers (compared to recovered dauers) and *daf-2* mutant adults (compared to *daf-16*, *daf-2* mutants; see below) (McElwee et al., 2004). This is consistent with several

DOI of original article: 10.1016/j.mad.2006.01.006.
☆ Please note that this paper was previously published in volume 127, issue 5 of Mechanisms of Ageing and Development, pages 458–472. It is reproduced here due to problems with figure quality in the original publication. Please use the original article for citation purposes.
* Corresponding author. Tel.: +44 20 7679 4381; fax: +44 20 7679 7096.
E-mail address: david.gems@ucl.ac.uk (D. Gems).

0047-6374/$ – see front matter
doi:10.1016/j.mad.2006.10.002

earlier observations on individual genes or enzymes. For example, dauers and *age-1* and *daf-2* mutant adults have increased SOD activity levels (Anderson, 1982; Vanfleteren, 1993; Vanfleteren and De Vreese, 1995), and the MnSOD gene *sod-3* shows increased expression in both dauers and *daf-2* mutants (Honda and Honda, 1999). Glyoxylate cycle enzyme levels are also elevated in dauers and *daf-2* mutant adults (O'Riordan and Burnell, 1990; Vanfleteren and De Vreese, 1995). In addition, severe *daf-2* mutant adults show dauer-like behaviour, including cessation of feeding, and adoption of an immobile, dauer-like posture (Gems et al., 1998).

The increase in adult lifespan resulting from reduced IIS requires the gene *daf-16* (Kenyon et al., 1993; Larsen et al., 1995). This encodes a FOXO class forkhead transcription factor (Lin et al., 1997; Ogg et al., 1997), and it is likely that the action of genes differentially regulated by *daf-16* determine the effects of IIS on ageing. *daf-16*-regulated genes have been identified using DNA microarray analysis (McElwee et al., 2003, 2004; Murphy et al., 2003). However, it is not easy to identify longevity-assurance processes by studying *daf-16*-regulated genes because there are so many of them. By a recent estimate from our laboratory, 1077 genes are up-regulated and 748 genes down-regulated in *daf-2* mutants relative to *daf-16*; *daf-2* mutants (McElwee et al., 2004).

In an attempt to gain insight from these large gene sets, we recently compared them to published sets of genes differentially expressed between dauers and recovered dauers (McElwee et al., 2004; Wang and Kim, 2003). We reasoned that at least some of the longevity-determining processes in dauers and *daf-2* adults are likely to be the same. Thus, transcriptional changes shared between dauers and *daf-2* adults are longevity-associated, and this association might be causal. A non-biased screen was conducted for gene classes over-represented in either up- or down-regulated genes in both long-lived milieus. This analysis implicated two main processes in longevity assurance. One had been previously identified (chaperonin activity; small heat shock proteins); the other had not (phase 1, phase 2 drug detoxification; cytochrome P450s, short-chain dehydrogenase/reductases and UDP-glucuronosyltransferases)(Gems and McElwee, 2005; McElwee et al., 2004).

Previous studies have suggested *daf-2* mutant longevity is a consequence of altered metabolism, for example, reduced mitochondrial respiratory chain activity (Feng et al., 2001; Van Voorhies and Ward, 1999) or increased anaerobic pathway activity (Holt and Riddle, 2003; Rea and Johnson, 2003); reviewed in (Burnell et al., 2005). In this study, we use transcript profile data to explore links between metabolism and ageing by means of detailed metabolic pathway reconstructions, comparing data from dauers and *daf-2* mutants. We have examined glycolysis, the citric acid (TCA) cycle, gluconeogenesis, the glyoxylate cycle, the PEPCK-succinate (fumarate reductase or malate dismutation) pathway, storage carbohydrate metabolism, mitochondrial and peroxisomal fat metabolism, mitochondrial respiratory chain activity and mitochondrial uncoupling. To this end, expression of some 250 key metabolic genes has been examined. In this way, we have tested metabolic theories of ageing, and identified novel aspects of energy metabolism whose activity levels are correlated with longevity.

2. Materials and methods

2.1. Microarray data analysis

Prior to analysis, we performed a quality control procedure on the *C. elegans* Affymetrix microarray to ensure the specificity of each individual probe set. The details of this procedure have been described previously (McElwee et al., 2004). Probe sets which mapped to more than one gene target (promiscuous, 1548 probe sets) or had no identifiable gene target (orphan, 894 probe sets) were removed from further analysis. Similarly, the microarray used for the dauer analysis has been subjected to an in silico quality control procedure (J. Lund, personal communication, http://elegans.uky.edu/MA/primers/new_primer_names.html), and probes predicted to be promiscuous or orphan were also removed from the dauer analysis.

Array data for dauer larvae compared to larvae 12 h after the onset of dauer recovery was from Wang and Kim (2003). Generation of array data from *daf-2* mutants has been previously described (McElwee et al., 2004). Briefly, two *daf-2* mutant alleles were tested, *m577* (class 1) and *e1370* (class 2) using whole genome oligonucleotide arrays (Affymetrix). Pooling data for the two *daf-2* alleles, comparisons were made between *daf-2* (long-lived, DAF-16 ON) and *daf-16(0)*; *daf-2* (not long-lived, DAF-16 OFF), using five biological replicates per genotype. We identified genes where transcript abundance is significantly different between *daf-2* and *daf-16*; *daf-2*.

Because methods for analyzing microarray data are rapidly evolving, we have reanalysed the data originally reported in both previous studies using more up-to-date techniques (McElwee et al., 2004; Wang and Kim, 2003). To analyse the *daf-2* experiment, we employed a normalisation routine derived from a recent methodological optimisation of Affymetrix microarrays using a defined mRNA spike-in population (Choe et al., 2005). The raw data (cel files) were treated as follows, and all manipulations were performed using R (version 2.0.1) (http://www.r-project.org/): background correction = MAS5, first normalisation = loess, summary = medianpolish, second normalisation = loess. This normalisation routine is what is recommended by Choe et al. (2005) as the best method for analysis for Affymetrix arrays, and was shown to outperform most other commonly used normalisation routines. Probe sets which could not be reliably measured above background levels (identified as those with 'absent' calls following MAS5 background correction) in all 20 microarray hybridisations were removed from further analysis prior to the second loess normalisation step. The output from this normalisation routine was restricted to only include probe sets which report genes within the metabolic pathways examined (see below), and was then analysed with SAM (version 2.1) (Tusher et al., 2001) to identify probe sets which are differentially expressed between *daf-2* and *daf-16*; *daf-2*.

To analyse the dauer dataset, the dauer-adjusted time zero comparisons (four replicates, comparing dauers at time zero to a reference mRNA control) were compared to the dauer-adjusted time 12 comparisons (four replicates, comparing animals 12 h after the initiation of dauer recovery to a reference mRNA control). As for the *daf-2* analysis, this dataset was restricted to only include metabolic probes, and was then analysed using SAM to identify probes, which are differentially expressed between dauers and recovered dauers.

We have used the q value (global false-discovery rate) from SAM (Tusher et al., 2001) as a measure for significance in our analyses, and consider probes where $q = 0$ to be differentially expressed. We also flag cases where $0 < q < 0.05$ as potentially differentially expressed. While the q value gives a measure of significance for each gene, it is not a true estimate of the probability for the gene to be a false positive (q is generally lower). Because of this, we place special emphasis on genes which show differential expression in both biological milieus, as this likely represents a true transcriptional response that might underlie the longevity of both dauers and *daf-2* mutants. Furthermore, the local FDR (Efron et al., 2001), which may be considered as the probability for a gene to be a false positive, is very low for most genes with $q < 0.05$ (on average, the local FDR for genes with $q < 0.05$ is 0.118 for the *daf-2* experiment, and 0.099 for the dauer experiment). Both the q value and local FDR from SAM for all genes in both experiments are available in supplemental materials.

2.2. Construction of metabolic pathways

Metabolic pathways were derived partly from previous pathway constructions (Holt and Riddle, 2003; Petriv et al., 2002; Tsang and Lemire, 2003; Wang and Kim, 2003), and partly from biochemical pathways described in other organisms (Nelson and Cox, 2000; Salway, 1999; Tielens et al., 2002). *C. elegans* genes for enzymes were identified using protein database tools, e.g. WormBase annotations (http://www.wormbase.org), or location of the protein sequence of the equivalent mammalian enzyme using NCBI Entrez protein searches (http://www.ncbi.nlm.nih.gov/). The BLASTP tool (Altschul et al., 1990) in WormBase (http://www.wormbase.org/db/searches/blat) was then used to identify similar *C. elegans* proteins. Where necessary, the NCBI BLASTP tool (http://www.ncbi.nlm.nih.gov/BLAST/) was then used to confirm the predicted identity of the *C. elegans* sequence(s) found. Detailed pathway figures (Figs. 2–8) were created using Canvas 8.0.5 (Deneba Systems, Inc).

3. Results

3.1. Overview of metabolism during diapause

Dauer larva metabolism differs from that of later larval stages and adults in ways that reflect the fact that they are non-feeding. Dauers subsist on storage nutrient provisions: glycogen (O'Riordan and Burnell, 1989; Popham and Webster, 1979), trehalose (Pellerone et al., 2003) and, particularly, lipid (Kimura et al., 1997). As in adults, energy generation from lipid involves conversion of fatty acids into acetyl-CoA by β-oxidation, and then combustion of acetyl-CoA via the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. In contrast to adults, precursors for biosynthesis (e.g. amino acids and glucose) must be derived from fat. Glycerol derived from breakdown of fat can be fed into the glycolytic or gluconeogenic pathways by conversion into glyceraldehyde-3-phosphate, by the action of glycerol kinase and glycerol-3-phosphate dehydrogenase (Nelson and Cox, 2000; Salway, 1999).

In mammals, it is likely that acetyl-CoA cannot be used as a substrate for gluconeogenesis. This is because the two carbons of the acetate entering the cycle are effectively lost as CO_2 (Fig. 1). However, in nematodes the two decarboxylation reactions in the citric acid cycle can be side-stepped by the glyoxylate pathway. This allows net conversion of acetyl-CoA into oxaloacetate (Fig. 1). More specifically, acetyl-CoA is converted into citrate and isocitrate as in the citric acid cycle. Isocitrate is then converted by isocitrate lyase into succinate and glyoxylate, and glyoxylate into malate by malate synthase. These two enzymes, not found in mammals, form distinct structural domains within a single polypeptide (glyoxylate enzyme), which in *C. elegans* shows regulated expression in intestine and muscle (Liu et al., 1995). Glyoxylate enzyme expression can be induced throughout larval development by fasting (Liu et al., 1997). The glyoxylate cycle is completed by conversion of succinate and malate into oxaloacetate. This may be converted into phosphoenol pyruvate (PEP) by PEP carboxykinase (PEPCK), a key gluconeogenic enzyme. A second gluconeogenic enzyme is pyruvate carboxylase, which converts pyruvate into oxaloacetate (Fig. 1). Glyoxylate cycle activity has been linked to a decline in triglycerides and an increase in carbohydrates in *C. elegans* embryos (Kahn and McFadden, 1980).

In dauer larvae, citric acid cycle activity is reduced relative to that of the glyoxylate cycle, consistent with utilisation of stored lipids (O'Riordan and Burnell, 1990; Wadsworth and Riddle, 1989). Measured metabolic rate in dauer larvae, e.g., by oxygen consumption, is also reduced (Houthoofd et al., 2002; Vanfleteren and De Vreese, 1996). Other data point to the existence of a distinct physiological state within dauer larvae, for example, reduced levels of ATP, and an increased intracellular pH (pH 7.3, as opposed to 6.3 in recovering dauers) (Wadsworth and Riddle, 1988).

A previous study has shown that in dauers there are increased mRNA levels for genes encoding key enzymes involved in glycolysis, the glyoxylate cycle, gluconeogenesis, and β-oxidation of fatty acids (Wang and Kim, 2003). To explore the extent of reiteration of dauer energy metabolism in *daf-2* adults, we have compared in dauers and *daf-2* mutants changes in expression at the transcript abundance level of genes involved in a variety of key metabolic processes (results summarised in Table 1). Changes in mRNA levels are likely to reflect changes in activity of the enzyme encoded; however, one must bear in mind that actual pathway activity is also influenced by other factors, e.g., post-transcriptional aspects of gene expression, allosteric regulation and substrate availability. We also screened for the presence near the 5′-end of the gene of the DAF-16-binding element (DBE) (McElwee et al., 2004), to identify components of energy metabolism, which might be

Table 1
Summary comparison of dauer larvae and daf-2 adults

Process	Alteration implied by mRNA profiles	
	Dauers[a]	*daf-2* adults[a]
Central metabolism		
Glycolysis	Up	Up
Gluconeogenesis	Up	Up
Glyoxylate pathway	Up	Up
Citric acid (TCA) cycle	Down	No change
PEPCK-succinate pathway	[Up]	[Up]
Mitochondrial complexes I–IV	Down	No change
Complex V (ATPase), overall	Down	No change
IF_1 (ATPase inhibitor)	Up	Up
Uncoupling protein (UCP-4)	[Down]	No change
Storage carbohydrate metabolism		
Glycogen synthesis	Down	[Up]
Glycogen breakdown	No change	No change
Trehalose synthesis	Up	Up
Trehalose catabolism	Up	Up/down
Lipid metabolism		
Lipolysis (lipase)	Up	No change
Glycerol catabolism	Up	[Up]
Carnitine shuttle (fatty acid uptake)	Up	Down
Mitochondrial β-oxidation of fatty acids	Up	[Up]
Peroxisomal β-oxidation of fatty acids	Up	No change
Peroxisomal biogenesis, division (peroxins)	Up	No change
Peroxisomal bile acid formation	[Up]	Down
Fatty acid synthesis (steroyl CoA desaturases)	Up	Up

[a][Up] and [Down] indicate weak evidence suggesting increased or decreased activity.

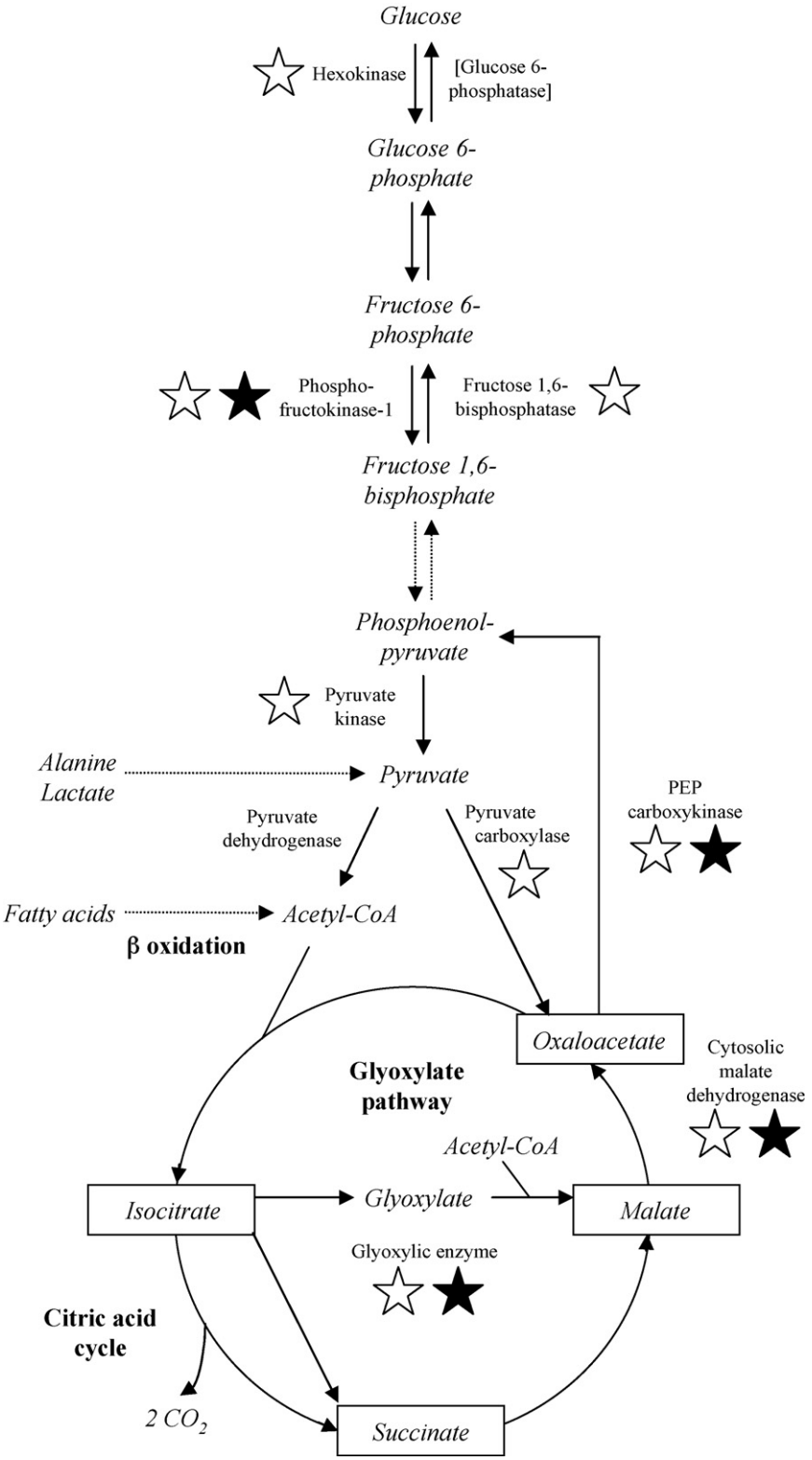

Fig. 1. Glycolysis, TCA cycle, gluconeogenesis and glyoxylate pathways: alterations of gene expression in dauers and *daf-2* mutant adults. Stars indicate that at least one gene encoding this enzyme is significantly up-regulated ($p < 0.01$). Open stars, dauers; black stars, *daf-2* adults.

directly regulated by DAF-16. Genes containing this sequence are likely to be direct transcriptional targets of DAF-16, rather than being further downstream in a DAF-16-regulated cascade.

3.2. Glycolysis and gluconeogenesis

In glycolysis, glucose is converted into pyruvate. Gluconeogenesis proceeds in the opposite direction, creating glucose

(and other biosynthetic precursors) from pyruvate or other substrates such as amino acids and lactate (Nelson and Cox, 2000; Salway, 1999) (Fig. 1). Most of the enzymes in glycolysis and gluconeogenesis catalyse reversible reactions. Whether glycolysis or gluconeogenesis occurs is influenced by the activity levels of a few non-reversible reactions (Fig. 1). The irreversible steps in glycolysis are catalysed by hexokinase, phosphofructokinase-1 (PFK) and pyruvate kinase (PK). Those of gluconeogenesis are catalysed by phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase, fructose 1,6-bisphosphatase (F-1,6-bisPase) and glucose 6-phosphatase.

In dauers there is increased expression of hexokinase, PFK and PK ($q = 0$) relative to recovered dauers. Of these, PFK (a different isozyme) and perhaps also PK ($q = 0.013$) show increased expression in *daf-2* mutants relative to *daf-16*; *daf-2* mutants (Figs. 1 and 2). The genes encoding gluconeogenic enzymes pyruvate carboxylase *(pyc-1)*, PEPCK (R11A5.4, W05G11.6), and F-1,6-bisPase (*fbp-1*) show increased expression in dauers. Strikingly, all of these gluconeogenic genes contain DBE elements (Fig. 2), implying direct regulation by DAF-16. Of these DBE-containing genes, both PEPCKs are up-regulated in *daf-2* mutants, and perhaps PK also ($q = 0.013$), while F-1,6-bisPase is not. In the case of the

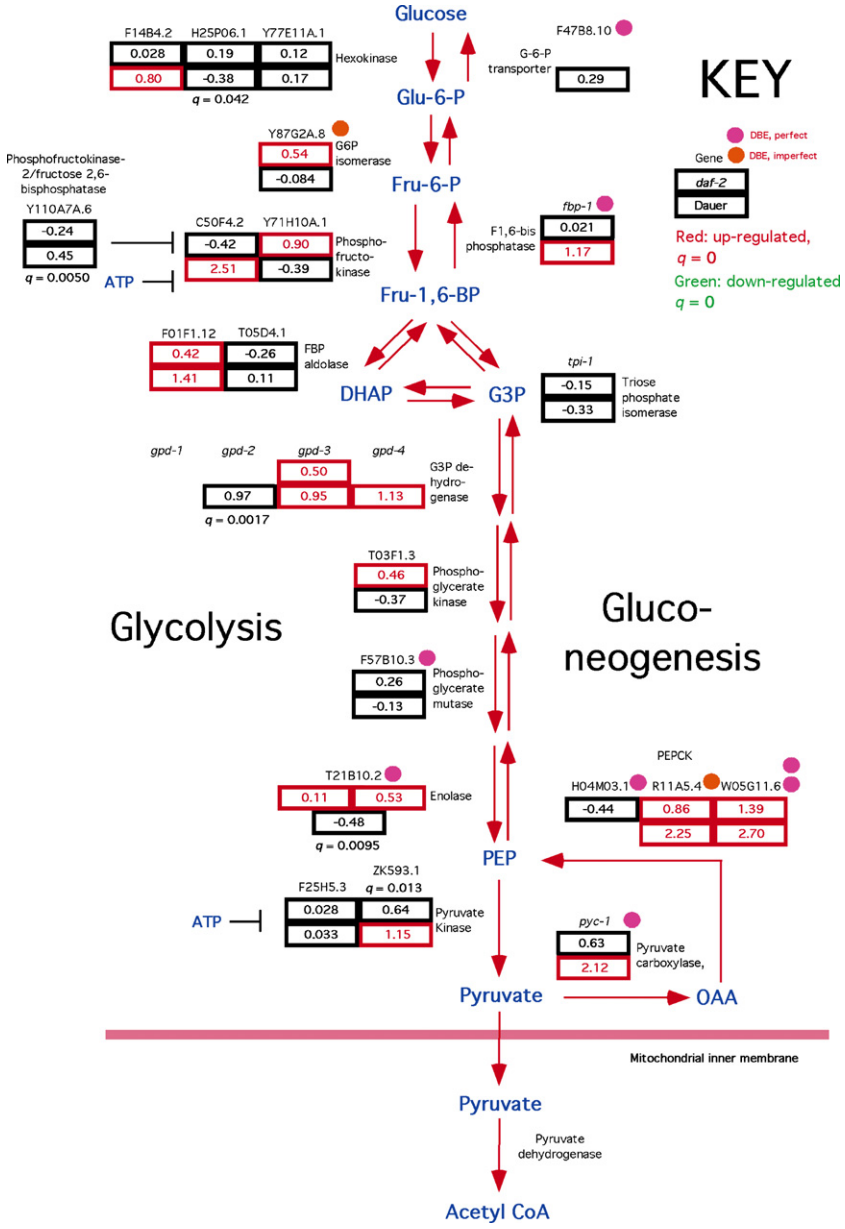

Fig. 2. Glycolysis and gluconeogenesis. Where data for two probe sets was available, one value was selected at random where no significant alteration was seen. If a significant alteration was seen in both cases, the most significant change is represented here. Where data from the two probe sets did not concur with respect to significance, both are shown. Genes where $q = 0$, i.e., with significant alteration in expression, are shown in colour: red where up-regulated in dauers or *daf-2* adults, or green where down-regulated. Where $0 < q < 5$, suggesting an alteration of expression, the value for q is given. If no q value is given, this indicates $q > 5$, i.e., that the gene shows no detectable change in expression.

fourth major regulatory gluconeogenic enzyme, glucose-6-phosphatase (G-6-Pase), the *C. elegans* genome contains no homologue. Perhaps, trehalose rather than glucose is used as a transport sugar in *C. elegans*—hence, the absence of G-6-Pase (see below).

Consistent with increased gluconeogenesis, one of the two genes encoding glyoxylate enzyme (*gei-7*) is up-regulated in dauers and *daf-2* adults (Fig. 1). This tallies with elevated levels of isocitrate lyase and malate synthase activity previously observed in dauers and *daf-2*(*e1370*) mutant adults (Vanfleteren and De Vreese, 1995). Cytosolic malate dehydrogenase (MDH) can link the glyoxylate cycle to gluconeogenesis by converting malate from the former to oxaloacetate for consumption by the latter. One MDH isozyme, encoded by F46E10.10, is up-regulated in dauers and *daf-2* adults. Based on sequence homology, it has been proposed that this MDH is cytosolic (Holt and Riddle, 2003).

Overall, these data imply that glycolysis, and especially gluconeogenesis, are up-regulated in both dauers and *daf-2* mutant adults. Given the increased glyoxylic enzyme expression, and the elevated fat levels in dauers and *daf-2* mutants (Kimura et al., 1997), it seems likely that the main gluconeogenic substrate is acetyl-CoA generated by β-oxidation of fatty acids (see below).

Amino acids are another major gluconeogenic substrate. In mammals, insulin inhibits transcription of PEPCK, and also tyrosine aminotransferase (TAT) (O'Brien et al., 2001), which is involved in tyrosine catabolism. *C. elegans* TAT (F42D1.2) is also up-regulated in *daf-2* mutants (two-fold increase, $q = 0$), but unchanged in dauers. Thus, PEPCK and TAT regulation by IIS is evolutionarily conserved and ancient.

3.3. Citric acid cycle

Pyruvate dehydrogenase (PDH) links glycolysis to the citric acid (TCA) cycle via conversion of pyruvate to acetyl-CoA. mRNA levels for most PDH subunits are reduced in dauers (Wang and Kim, 2003) (Fig. 3). Key rate-limiting enzymes within the citric acid cycle are citrate synthase and isocitrate dehydrogenase, neither of which show altered mRNA levels in dauers or *daf-2* adults. However, in dauers, mRNA levels for a number of citric acid cycle enzymes are reduced, consistent with lowered citric acid cycle activity. By contrast, expression of citric acid cycle enzymes is unchanged in *daf-2* adults (apart from MDH isozyme F46E10.10, which may be cytosolic; see above). Thus, the reduction in citric acid cycle activity in dauers appears not to be reiterated in *daf-2* adults.

3.4. PEPCK-succinate pathway

A possible alternative function of PEPCK in *C. elegans* is to drive the opposite reaction to that in gluconeogenesis,

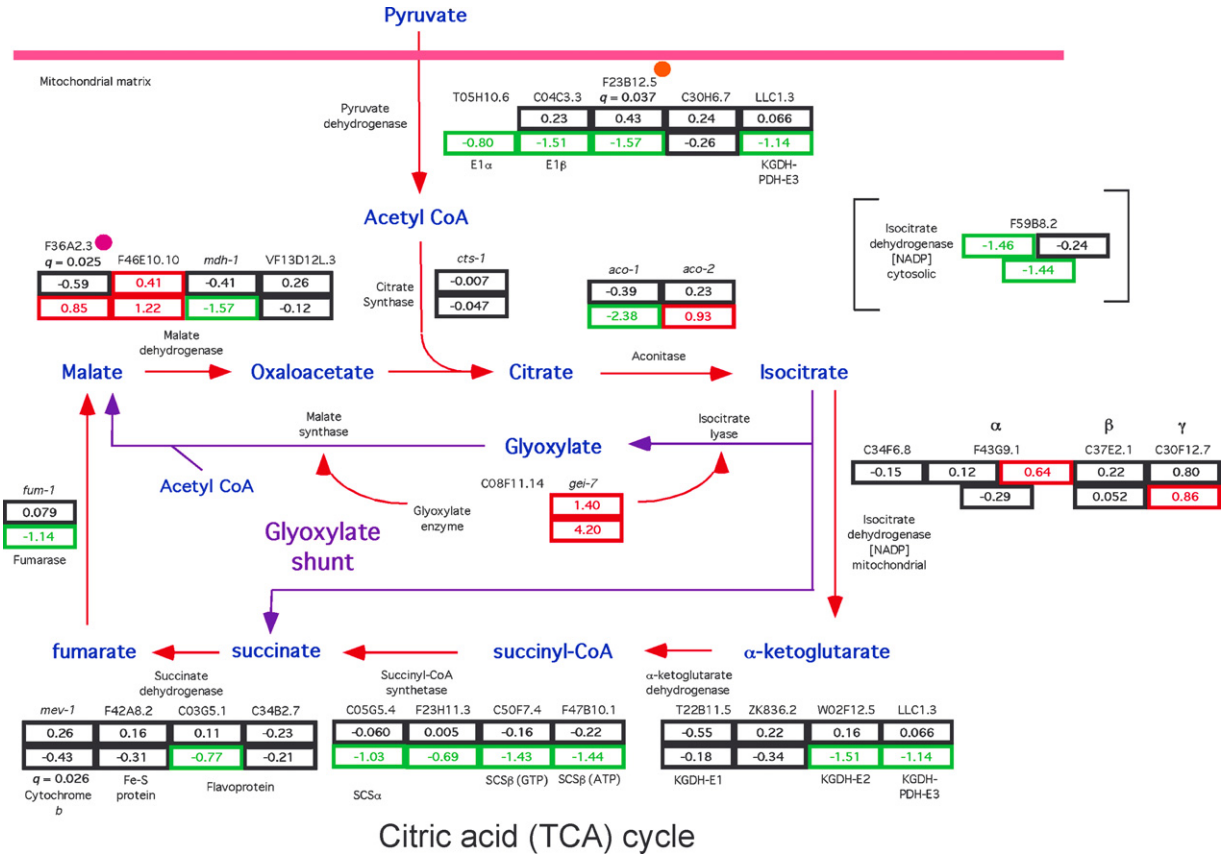

Fig. 3. Citric acid (tricarboxylic acid, TCA) cycle. Malate dehydrogenase: *mdh-1* (F20H11.3) is predicted to be mitochondrial, while F46E10.10 may be cytosolic (Holt and Riddle, 2003). Cellular localisation of F36A2.3 and VF13D12L.3 is unclear.

converting PEP into oxaloacetate. This generates fumarate (via malate) to act as an electron acceptor in anaerobic respiration (Tielens et al., 2002) (Fig. 4). Reduction of fumarate involves electron transfer from mitochondrial complex II to fumarate, via the electron carrier rhodoquinone and fumarate reductase (i.e. succinate dehydrogenase operating in the opposite direction) (Tielens et al., 2002; Tielens and van Hellemond, 1998). As part of this pathway, malate is also converted into pyruvate to maintain cellular redox balance.

Reduction of fumarate in this way, generating succinate, is well-documented in the parasitic nematode *Ascaris suum*, and has also been observed in *C. elegans*, even (slightly) under aerobic conditions (Takamiya et al., 1999). Moreover, under anoxic conditions *C. elegans* excrete more succinate, consistent with increased PEPCK-succinate pathway activity (Foll et al., 1999). Based on an analysis of data on gene expression in dauers derived from SAGE analysis (Jones et al., 2001), it was proposed that PEPCK-succinate pathway activity is increased in dauers (Holt and Riddle, 2003). It has also been postulated that anaerobic respiration involving fumarate might lower production of damaging reactive oxygen species, thereby contributing to the longevity of dauers and IIS mutants (Rea and Johnson, 2003).

Whether complex II acts as a succinate dehydrogenase or a fumarate reductase is determined by the flavoprotein (F_p) subunit, which contains the fumarate/succinate catalytic site. *C. elegans* has two genes for this F_p subunit (C03G5.1 and C34B2.7). Potentially, these represent succinate dehydrogenase and fumarate reductase subunits. However, neither subunit is up-regulated in dauers or *daf-2* mutants (Fig. 3), nor is malic enzyme, also important in the PEPCK-succinate pathway.

F48E8.3 encodes a protein equally similar to baker's yeast soluble fumarate reductases FRDS1 and FRDS2, which are cytosolic and mitochondrial, respectively (Muratsubaki and Enomoto, 1998). FRDS catalyses the irreversible conversion of fumarate to succinate, oxidising reduced flavin nucleotides in the process. FRDS activity is required for yeast growth under anaerobic conditions, apparently to maintain cellular levels of NAD^+ to support glycolytic generation of ATP during anaerobic respiration (Enomoto et al., 2002). This gene is up-regulated in dauers (Wang and Kim, 2003), consistent with earlier analysis of SAGE data (Holt and Riddle, 2003), and also in *daf-2* mutants (Fig. 4).

Succinyl-CoA synthetase and acetate:succinate-CoA transferase are involved in anaerobic generation of acetate (Fig. 4). mRNA profiles provide no evidence of up-regulation of the

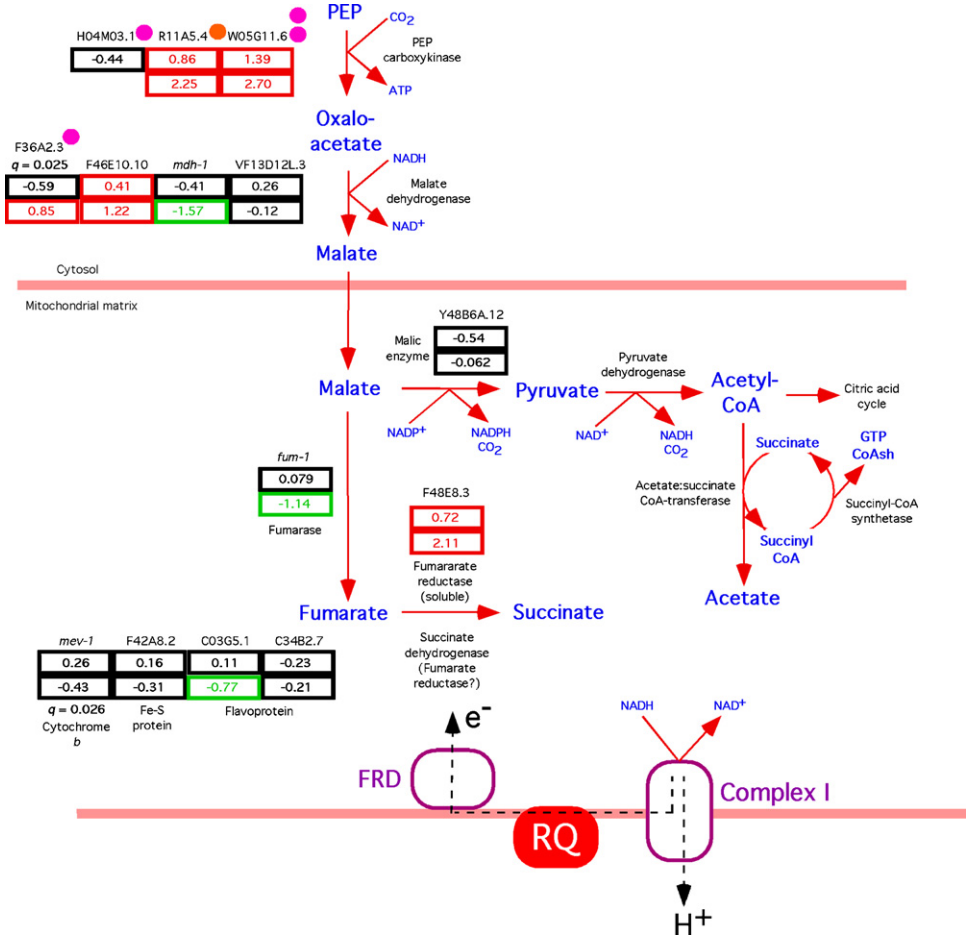

Fig. 4. PEPCK-succinate pathway. Derived from (Holt and Riddle, 2003; Rea and Johnson, 2003; Tielens et al., 2002). FDR, fumarate reductase; e^-, electron. Translocation of proteins by complex I drives ATP production by ATP synthase. In *S. cerevisiae*, soluble fumarate reductase oxidises FADH, $FMNH_2$ or reduced riboflavin.

former enzyme in dauers or *daf-2* adults (Fig. 3); the sequence of the latter enzyme remains unknown. Analysis of SAGE data also suggested that other forms of fermentation might be increased in dauers, with lactate dehydrogenase (LDH) generating lactate, and alcohol dehydrogenase (AD) generating acetaldehyde (for conversion into acetate). Expression of *ldh-1* (F13D 12.2) is reduced in dauers ($q = 0$), but unchanged in *daf-2* adults. Of three AD genes, two (*sodh-1* and D2063.1), showed increased expression in dauers ($q = 0$), and a third (*sodh-2*), showed decreased expression ($q = 0.0017$). However, none of these genes showed altered expression in *daf-2* adults. In summary, among transcript profile data, only the up-regulation of the yeast fumarate reductase homologue (F48E8.3) suggests that fermentative metabolism might be increased in dauers or *daf-2* adults.

3.5. Mitochondrial respiratory chain

Transcript profile analysis is consistent with reduced mitochondrial TCA cycle activity in dauers but not *daf-2* adults. We next examined expression levels of mitochondrial respiratory chain (MRC) and ATP synthase genes, which generate ATP via oxidative phosphorylation. In this process, oxidation of NADH and succinate is coupled to formation of an electrochemical (proton) gradient across the inner mitochondrial membrane via the action of four electron transport complexes. These are I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase), III (ubiquinol-cytochrome *c* oxidoreductase) and IV (cytochrome *c* oxidase)(-Nelson and Cox, 2000). The proton gradient generated drives ATP synthesis via complex V (ATP synthase, or F_0F_1-ATPase).

Genes encoding most of the MRC genes have been identified in the *C. elegans* genome (Tsang and Lemire, 2003). In the main, these are encoded by the nuclear genome, but 12 are encoded by the mitochondrial genome. We compared the effects of the dauer state and reduced IIS on expression of 80 MRC genes (Fig. 5). In the case of dauers, of 64 genes for which data was available, 41 were clearly down-regulated ($q = 0$), and a further 9 with less certainty ($0 < q < 0.05$). This is consistent with lowered MRC activity in dauers. By contrast, in *daf-2* mutants, no genes were down-regulated, even given a permissive statistical cut-off ($q < 0.05$).

Three genes showed up-regulation ($q = 0$). One was fumarate reductase (F48E8.3), discussed above. Interestingly, the remaining two, B0546.1 and *mai-1* (K10B3.9), encode the two *C. elegans* members of the F_1 ATPase inhibitor (IF_1) family. *mai-1* was up-regulated in both dauers and *daf-2* adults. Anoxia can result in failure of oxidative phosphorylation, and

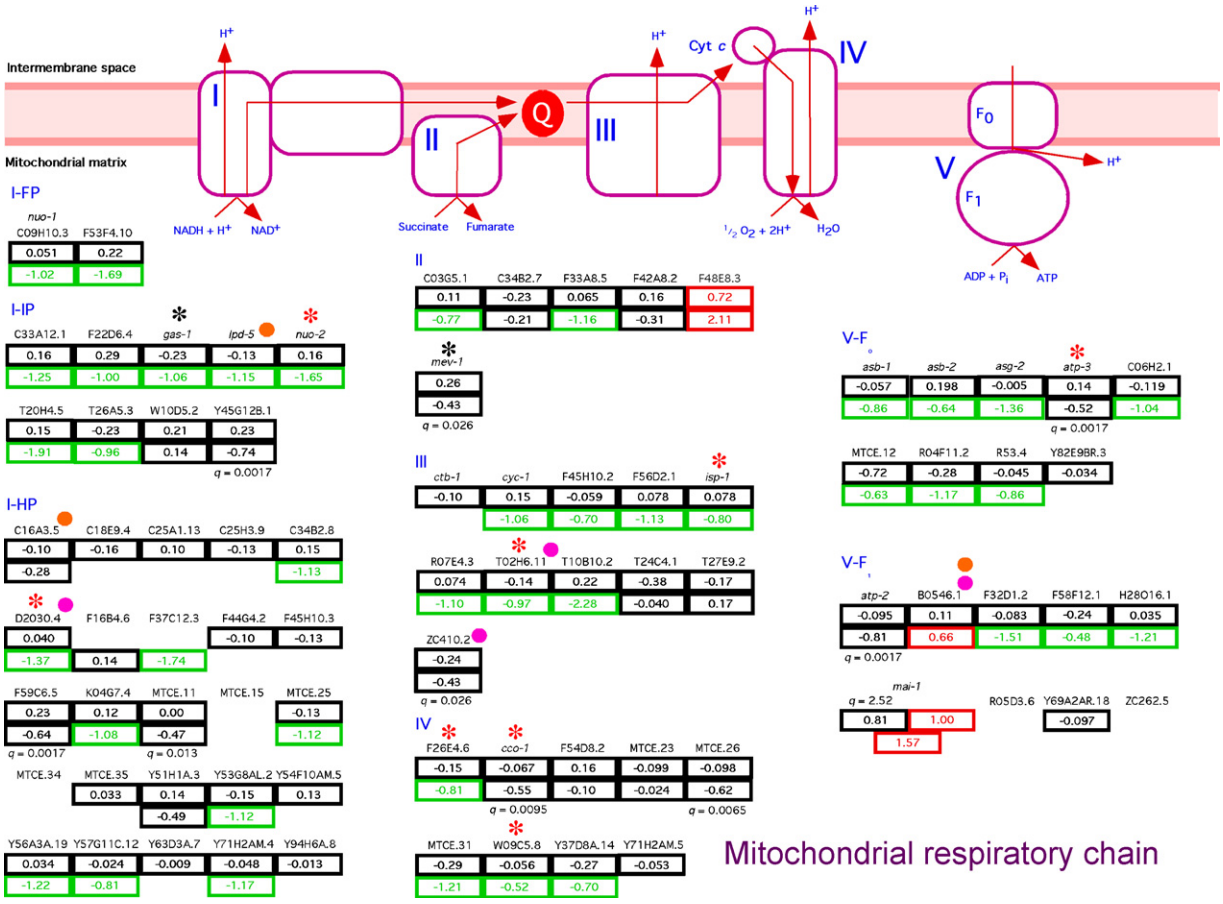

Fig. 5. Mitochondrial respiratory chain. Bovine complex I can be dissociated into soluble flavoprotein (I-FP) and iron–sulphur (I-IP) subunits, plus an insoluble (I-HP) fragment. Genes marked with a red or black asterisk are those where reduced activity (mutation or RNAi knockdown) increases or decreases adult lifespan, respectively (Dillin et al., 2002; Feng et al., 2001; Hartman et al., 2001; Ishii et al., 1990; Lee et al., 2003). In addition, mutations in *nuo-1* and *atp-2* extend lifespan of arrested L3 larvae (Tsang et al., 2001).

conversion of complex V ATP synthase into an ATPase. This destroys the ATP that remains, with deleterious consequences. IF_1 blocks this ATPase activity, thereby protecting mitochondria (Green and Grover, 2000), and thus, represents an important somatic maintenance function (see Section 4).

Partial reduction of oxygen during oxidative phosphorylation results in production of superoxide free radicals, and it has been proposed that this causes ageing (Beckman and Ames, 1998). Uncoupling proteins (UCPs) allow protons to cross the inner mitochondrial membrane, thus, uncoupling proton-motive force generation from ATP synthesis. This results in lower levels of reduced ubiquinone and therefore of superoxide production. One possibility is that uncoupling promotes longevity (Brand, 2000; Skulachev, 1996). *ucp-4*, a *C. elegans* gene encoding a possible UCP (Hanak and Jezek, 2001), did not show increased expression in either dauers or *daf-2* mutant adults; in fact, in dauers there was a potential decrease in expression ($q = 0.026$).

In conclusion, a number of studies have shown that interference with MRC activity (e.g. by mutation or RNAi) can increase lifespan (Dillin et al., 2002; Hekimi and Guarente, 2003; Larsen and Clarke, 2002; Lee et al., 2003). Our transcript analysis suggests that while lowered MRC activity might contribute to dauer larva longevity, this is not the case for *daf-2* mutant longevity. However, IF_1 activity represents a novel candidate mechanism for longevity assurance.

3.6. Metabolism of storage carbohydrates (glycogen and trehalose)

Dauers contain elevated levels of the storage polysaccharide glycogen (Popham and Webster, 1979). Glycogen synthase (GS) appears to be down-regulated in dauers ($q = 0$ in one of two probes), consistent with glycogen utilisation as an energy source (Fig. 6).

The disaccharide trehalose is an invertebrate sugar transport and storage material (Behm, 1997). Trehalose is present at all life stages in *C. elegans* at up to 2.3% of dry weight, with the highest concentrations found in eggs and dauer larvae (Pellerone et al., 2003). Long-lived IIS mutant *age-1*(*hx546*) adults show a two-fold increase in trehalose levels relative to wild-type (Lamitina and Strange, 2005).

The *C. elegans* genome contains two putative trehalose-6-phosphate synthase (*tps*) genes, which catalyse trehalose biosynthesis, and five trehalase (*tre*) genes, which catalyse hydrolysis of trehalose into glucose. Both *tps-1* and *tps-2* are strongly up-regulated in dauer larvae and *daf-2* mutants (Fig. 6), consistent with increased trehalose synthesis in both milieus. Of the *tre* genes, only one shows altered expression in dauers (*tre-5*, up-regulated); *tre-2* and *tre-3* show possible down-regulation ($q < 0.01$ in each case). In *daf-2* mutants *tre-4* and *tre-5* are up-regulated, and *tre-3* down-regulated. The simultaneous up-regulation of *tps* and *tre* genes suggests a futile cycle of trehalose synthesis and breakdown. However, this could reflect biosynthesis of trehalose in one tissue, and its breakdown in another. In mammals, glucose generated by hepatic gluconeogenesis, is consumed in peripheral tissues (e.g. the brain).

3.7. Triglyceride catabolism

Wang and Kim (2003) report that certain β-oxidation enzymes are up-regulated in dauers, consistent with increased catabolism of provisioned fat. We compared expression of genes encoding enzymes involved in catabolism of triglycerides (Fig. 7). The first step in this process is hydrolysis of triglycerides by hormone-sensitive lipase to glycerol and fatty acids. Expression of hormone-sensitive lipase (C46C11.1) is up-regulated in dauers, but not *daf-2* adults. Glycerol kinase and

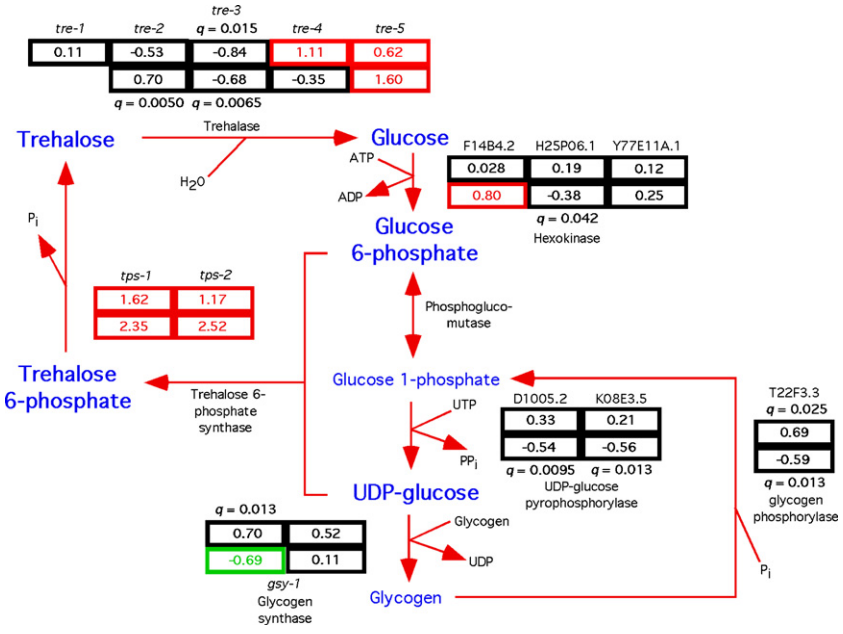

Fig. 6. Metabolism of storage carbohydrates.

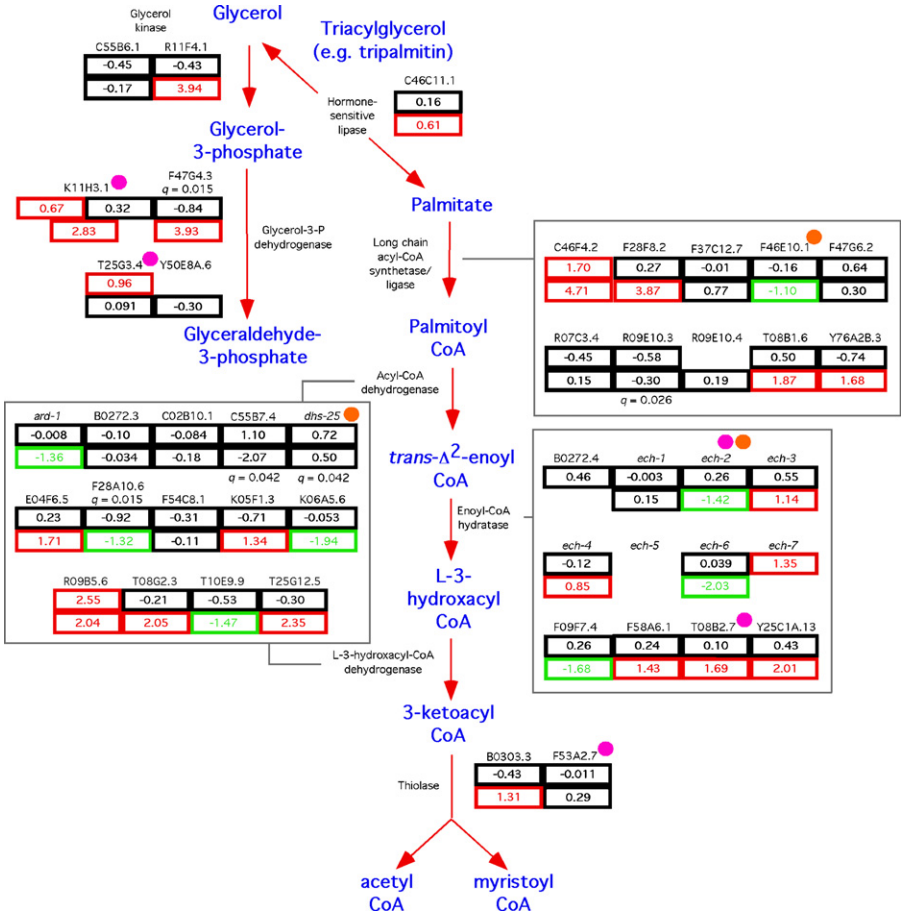

Fig. 7. Lipid catabolism pathway.

glycerol-3-phosphate dehydrogenase convert glycerol into glyceraldehyde-3-phosphate, which can then enter the glycolytic pathway. In dauers, genes for both enzymes are up-regulated ($q = 0$). In *daf-2* adults two G3PD genes are up-regulated ($q = 0$), but a third may be down-regulated ($q = 0.015$).

The rate-limiting step in β-oxidation is the formation of fatty acyl-CoA, catalysed by long-chain fatty acyl-CoA synthetases. *C. elegans* has numerous potential fatty acyl-CoA synthase/ligase genes. Of these examined, four were clearly up-regulated in dauers (Wang and Kim, 2003) (Fig. 7). Only C46F4.2 was up-regulated in *daf-2* mutants. Of genes encoding other β-oxidation enzymes, only one, an L-3-hydroxyacyl-CoA dehydrogenase (R09B5.6) was up-regulated in both milieus.

The carnitine palmitoyltransferase (CPT) shuttle system is a rate-limiting step in the import and oxidation of fatty acids into mitochondria. Of eight CPT homologues (B0395.3, F09F3.9, K11D12.4, R07H5.2, W01A11.5, W03F9.4, Y46G5A.17, Y48G9A.10), five were up-regulated in dauers (B0395.3, R07H5.2, W01A11.5, W03F9.4, Y48G9A.10, $q = 0$; data available for seven). This suggests increased transport of fatty acids into mitochondria. By contrast, in *daf-2* adults no CPT genes were up-regulated, and three (F09F3.9, K1 1D12.4, W01A11.5) were down-regulated ($q = 0$). In conclusion, while *daf-2* mutants have elevated lipid levels (Kimura et al., 1997), transcript profiles provide little evidence of altered fat catabolism.

3.8. Peroxisomal fatty acid metabolism

While some 90% of oxidation of short- and medium-chain length fatty acids takes place in mitochondria, peroxisomes are the main site for oxidation of very-long-chain fatty acids (C_{22} and longer). Peroxisomes also catabolise most of the less common fatty acids, including dicarboxylic acids, prostaglandins, leukotrienes and xenobiotic fatty acids. Protein effectors of peroxisomal lipid catabolism and biogenesis in *C. elegans* have been identified previously (Petriv et al., 2002; Thieringer et al., 2003). Those involved in fat catabolism include very-long-chain acyl-CoA synthases, ABC transporters (homologues of the adrenoleukodystrophy protein, ADLP), and peroxisomal thiolases. In mammals, peroxisome assembly, division and inheritance is regulated by a set of at least 23 proteins called peroxins; homologues of 10 have been identified in *C. elegans* (Petriv et al., 2002; Thieringer et al., 2003).

In dauers, for fatty acid catabolism enzymes and transporters, of 10 genes with altered expression ($q = 0$), 8 showed up-regulation (Fig. 8). Moreover, 7 of the 10 peroxins show increased expression. This implies that peroxisomal activity is elevated in dauers. By contrast, in *daf-2* mutants none of these genes examined were up-regulated, and two were down-regulated.

In peroxisomes, oxidation of some fatty acids occurs slowly, and this can lead to sequestration of coenzyme A (CoASH)

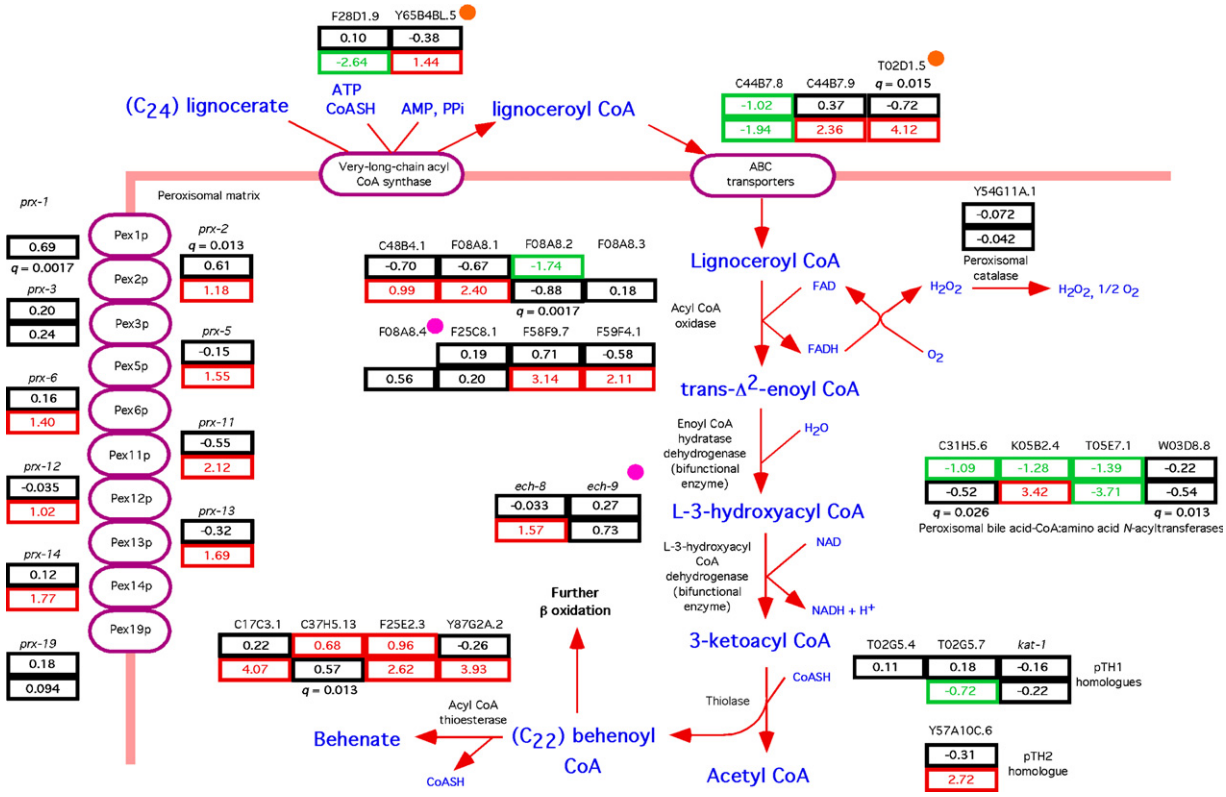

Fig. 8. Peroxisomal fatty acid oxidation. Behenic (docosanoic) acid, shown here, is the shortest of the very-long-chain fatty acids.

within peroxisomes. Acyl-CoA thioesterases hydrolyse fatty acyl-CoAs to release this CoASH. In mammals, the broad substrate range acyl-CoA thioesterase PTE-2 is a key regulator of peroxisomal lipid metabolism (Hunt et al., 2002). *C. elegans* has four PTE-2 homologues, of which three are up-regulated in dauers, and two in *daf-2* adults ($q = 0$; Fig. 8). Increased PTE activity in dauers and *daf-2* adults suggests an elevated requirement for CoASH for other metabolic processes (possibly mitochondrial β-oxidation), and elevated excretion of fatty acids.

A further role of peroxisomes in mammalian liver is formation of bile acids, such as cholic acid and chenodeoxycholic acid, which are sterol derivatives destined for excretion. Bile acid synthesis involves modification of the cholesterol backbone by cytochrome P450 enzymes, and production of choloyl-CoA and chenodeoxycholoyl-CoA. The final step in bile acid generation is the conjugation of these latter compounds with glycine or taurine, catalysed by peroxisomal bile acid-CoA:amino acid *N*-acetyltransferase (BAAT). There are four BAAT homologues in the *C. elegans* genome. In dauers one is up-regulated, and one down-regulated ($q = 0$), and the remaining two may also be down-regulated ($q < 0.05$); similarly, in *daf-2* adults, three are down-regulated (Fig. 8). This suggests that the increased expression of cytochrome P450 enzymes in *daf-2* mutants (McElwee et al., 2004) is not due to increased excretion of sterols. Overall, these results imply increased peroxisomal activity in dauers but not *daf-2* adults.

3.9. Triglyceride synthesis

Both dauers and *daf-2* adults contain large quantities of stored fat in the form of lipid droplets, mainly in the intestine and hypodermis. We therefore examined the following key genes in fatty acid synthesis: two acetyl-CoA carboxylases (T28F3.5, W09B6.1), a malonyl-CoA transacylase (C50D2.9, no data for dauers), two fatty acid synthases (F32H2.6, F32H2.5), and three enoyl-ACP reductases (F53C11.3, T05C12.3, W01C9.4). Of these, the only significant change was seen in dauers in the F53C11.3 2,4-dienoyl-CoA reductase (6.4-fold increase, $q = 0$). With respect to triglyceride synthesis, one glycerol kinase (R11F4.1) was strongly up-regulated in dauers (15.3-fold increase, $q = 0$). None were up-regulated in *daf-2* adults.

In mammals, steroyl-CoA desaturase (SCD) is an important determinant of adiposity (Dobrzyn and Ntambi, 2004). This enzyme of the smooth endoplasmic reticulum is rate limiting in the biosynthesis of monounsaturated fatty acids, the most abundant form of fatty acid in triglycerides. High levels of activity of SCDs leads to increased fat levels, and deletion of murine *scd1* leads to reduced body adiposity. The *C. elegans* genome contains three genes similar to *scd*: *fat-5*, *fat-6* and *fat-7*. FAT-6 and FAT-7 proteins have steroyl-CoA desaturase activity, while FAT-5 is a palmitoyl-CoA desaturase (Watts and Browse, 2000). Interestingly, all three are strongly up-regulated in dauers, and *fat-5* and *fat-6* are up-regulated in *daf-2* adults too ($q = 0$ in all cases). *fat-1–fat-4* encode other fatty acyl-CoA

desaturases, some of which are up-regulated in dauers, but none in *daf-2* adults. This suggests the possibility that fatty acyl-CoA desaturases contribute to the elevated lipid levels in dauers and *daf-2* adults.

4. Discussion

The primary aim of this study was to gain insight into mechanisms regulated by insulin/IGF-1 signalling which control ageing and longevity. To this end, we performed metabolic pathway reconstructions, to allow careful comparison of transcript profile data derived from microarray studies of dauer larvae versus recovered dauers (Wang and Kim, 2003), and long-lived *daf-2* mutant adults versus non-long-lived *daf-16*; *daf-2* mutants (McElwee et al., 2004). Our analysis implies reiteration of some but not other aspects of dauer larva metabolism in *daf-2* adults, thereby identifying candidate IIS-regulated determinants of ageing (conclusions summarised in Table 1).

4.1. Up-regulation of gluconeogenesis in dauers and daf-2 adults

Transcript profiles of dauer larvae and *daf-2* adults imply that glycolysis and gluconeogenesis are up-regulated in both milieus. Generally, these two processes are regulated co-ordinately and reciprocally, with one or the other predominating. However, our analysis implies that phosphofructokinase-1 and pyruvate kinase (glycolytic) are up-regulated simultaneously with pyruvate kinase and PEPCK (gluconeogenic)(Fig. 1). This would be predicted to result in a futile cycle of catabolism and anabolism. However, up-regulation may be occurring in different tissues, as previously suggested (Wang and Kim, 2003). One possibility is that sugars are generated in the gut from stored lipid, and transported to muscle and neurons; in mammals, hepatic gluconeogenesis supplies glucose to muscle and the nervous system.

Some *daf-2* adults show reduced capacity to feed (Gems et al., 1998), yet increased PEPCK expression was seen in *daf-2* mutants both with and without this feeding defect (J.J. McElwee, D. Gems, unpublished). Given that (unlike dauers) *daf-2* mutants are able to feed, increased gluconeogenesis would seem to be surplus to requirement. How might increased gluconeogenesis affect the biology of *daf-2* mutants? The transcript profiles lend themselves to the following interpretation. Increased glyoxylate cycle and gluconeogenic activity uses acetyl-CoA derived from stored lipid. The resulting sugars may be transported, perhaps in the form of trehalose, to other tissues where glycolysis predominates. Such IIS-controlled alterations resemble those in *D. melanogaster*, where ablation of median neurosecretory cells (which secrete insulin-like peptides) results in increased lifespan, and increased levels of lipid, glycogen and trehalose (Broughton et al., 2005).

Could increased gluconeogenesis contribute to longevity? One possibility is that it renders energy from storage lipids available for somatic maintenance processes. Theory predicts that a major determining mechanism in the evolution of ageing is selection for pleiotropic alleles, which enhance fitness through effects early in the life history, but cause ageing later (Williams, 1957). A candidate mechanism by which such pleiotropy may act is resource (especially energy) allocation away from somatic maintenance processes that protect against ageing, and into processes that increase reproductive success, resulting in a short-lived (or disposable) soma (Kirkwood, 1977). Our earlier study implies over-expression of small heat shock proteins and drug detoxification enzymes in dauers and *daf-2* adults, both of which consume energy (McElwee et al., 2004). For example, each glucuronidation reaction catalysed by UDP-glucuronosyltransferase consumes an entire hexose sugar molecule (glucuronic acid). This interpretation is consistent with the disposable soma theory, which predicts that lifespan is limited by energy consuming somatic maintenance processes (Gems and McElwee, 2005; Kirkwood, 1977).

4.2. Reduced mitochondrial respiration in dauers but not daf-2 adults

A number of prior observations indicate that respiration is lowered in dauer larvae, such as lowered oxygen consumption (Houthoofd et al., 2002; Vanfleteren and De Vreese, 1996), reduced numbers of mitochondria (Popham and Webster, 1979), reduced expression of citric acid cycle enzymes (O'Riordan and Burnell, 1989; Wang and Kim, 2003), and of mitochondrial respiratory chain genes (this study). Several studies have suggested that lowered respiration in *daf-2* mutants might contribute to their longevity (Feng et al., 2001; Van Voorhies and Ward, 1999). However, transcript analysis shows no evidence of lowered expression of genes for mitochondrial components in *daf-2* mutants. Transcript profiles are consistent with an overall reduction in mitochondrial number or volume in dauer larvae, but not *daf-2* adults. This suggests that mitochondrial function in *daf-2* adults is relatively unaltered, and that mutant longevity is not attributable to lowered mitochondrial energy metabolism. Consistent with this, oxygen consumption is not reduced in *daf-2* mutant adults relative to wild-type, and may even be slightly increased (Houthoofd et al., 2005).

4.3. F_1 ATPase inhibition: a candidate longevity-assurance mechanism

Under anoxic conditions (e.g. myocardial ischemia in mammals), the F_1 component of mitochondrial complex V changes from an ATP synthase into an ATPase. This ATPase activity hydrolyses much of remaining ATP, which could otherwise be used for cellular recovery, and is therefore highly deleterious. IF_1 inhibits this ATPase activity, thus preventing wasteful destruction of ATP, and providing protection against anoxia (Green and Grover, 2000). We observed increased expression of two *C. elegans* genes encoding IF_1 proteins: one in both dauers and *daf-2* adults, and one in dauers alone (Fig. 5). This suggests the hypothesis that during ageing, uncontrolled F_1 ATPase activity depletes ATP levels, contributing to senescent decline.

F_1 ATP synthase activity is pH-dependent, and requires a proton gradient across the inner mitochondrial membrane to function (Nelson and Cox, 2000). Hypoxia reverses the ATP synthase into an ATPase because it causes collapse of the proton gradient (Green and Grover, 2000). We suggest that a similar collapse happens in senescent mitochondria. This might contribute to the decline in ATP levels during ageing in *C. elegans* (Braeckman et al., 1999).

Increased IF_1 activity could account for a number of characteristics of *daf-2* mutants. Firstly, they are resistant to hypoxic death (Scott et al., 2002). Secondly, they have elevated levels of ATP (Braeckman et al., 1999; Dillin et al., 2002). Thirdly, they have a lower level of heat production than wild-type adults (Houthoofd et al., 2005): blocking F_1 ATPase activity could prevent a futile cycle of ATP synthesis and hydrolysis, which might otherwise generate more heat.

4.4. Trehalose: a longevity-assurance sugar?

The increased expression of both trehalose phosphate synthase genes seen here is consistent with elevated trehalose levels previously seen in dauer larvae and *age-1*(*hx546*) mutants (Lamitina and Strange, 2005; Pellerone et al., 2003). Besides its role as a sugar transport and storage material, this non-reducing disaccharide of glucose also confers protection against environmental stresses, acting as a cryoprotectant and an anhydrobiotic preservative (Behm, 1997; Crowe and Crowe, 2000; Pellerone et al., 2003; Singer and Lindquist, 1998). Possibly, elevated trehalose levels contribute to the increased survival of *daf-2* mutant adults, relative to wild-type, after freezing (−80 °C; R. Wong, D. Gems, unpublished observation). However, long-lived *D. melanogaster* mutants with increased trehalose levels are cold sensitive (Broughton et al., 2005).

In baker's yeast and *Escherichia coli* increased trehalose is protective against heatshock. Trehalose is thought to block aggregation of unfolded, denatured proteins, allowing them to retain their native conformation (Singer and Lindquist, 1998). Chaperonin activity has previously been shown to be a potent longevity-assurance mechanism in *C. elegans* (Hsu et al., 2003; Lithgow et al., 1995; Walker and Lithgow, 2003). Possibly, the elevated trehalose levels in dauer larvae and IIS mutants contribute to their heatshock resistance (Anderson, 1978; Lithgow et al., 1994) and their longevity.

An important point here is that in mammals there is little evidence of glyoxylate pathway activity or trehalose biosynthesis. Thus, if elevated levels of either process does promote longevity, this would represent private (non-evolutionarily conserved) mechanisms of lifespan determination.

One possibility is that in *C. elegans* trehalose is the only circulating sugar. This could explain the absence of a predicted glucose-6-phosphatase, and also the high levels of trehalose phosphate synthase and trehalase activity. By this view, glucose made via gluconeogenesis (perhaps in the gut) is converted into trehalose and secreted internally, then taken up by peripheral tissues, and broken down into free glucose via trehalase. Consistent with this, measured glucose levels in *C. elegans* are very low indeed compared to trehalose (Hanover et al., 2005).

4.5. Representation of the DAF-16-binding element (DBE) among metabolic genes

The DBE is found in the promoter element of some DAF-16-regulated genes, and is over-represented among gene that are up-regulated (but not down-regulated) in *daf-2* mutants and dauers (McElwee et al., 2004). To identify metabolic processes that may be directly regulated by DAF-16, we surveyed metabolic pathway genes for the presence of the DBE in the promoter region. The one process where genes showed striking over-representation of DBEs was gluconeogenesis (Fig. 2). All three PEPCK genes contain one or two DBE or DBE-like sequences, as do *pyc-1* and *fbp-1*. This suggests that gluconeogenesis is directly controlled by DAF-16 in *C. elegans*. No other metabolic process showed such over-representation of DBEs. Notably, apart from one PEPCK-encoding gene, the only other gene with two DBEs was B0546.1, which encodes a predicted IF_1, and is up-regulated in dauer larvae.

Acknowledgements

This research was supported by a grant from the Wellcome Trust. We thank Richard Wong for performing cryoresistance tests, and Ann Burnell, Matthew Piper and Colin Selman for critical reading of the manuscript.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Anderson, G.L., 1978. Responses of dauerlarvae of *Caenorhabditis elegans* (Nematoda: Rhabditidae) to thermal stress and oxygen deprivation. Can. J. Zool. 56, 1786–1791.

Anderson, G.L., 1982. Superoxide dismutase activity in dauerlarvae of *Caenorhabditis elegans* (Nematoda: Rhabditidae). Can. J. Zool. 60, 288–291.

Beckman, K.B., Ames, B.N., 1998. The free radical theory of aging matures. Physiol. Rev. 78, 547–581.

Behm, C.A., 1997. The role of trehalose in the physiology of nematodes. Int. J. Parasitol. 27, 215–229.

Bluher, M., Kahn, B.B., Kahn, C.R., 2003. Extended longevity in mice lacking the insulin receptor in adipose tissue. Science 299, 572–574.

Braeckman, B.P., Houthoofd, K., De Vreese, A., Vanfleteren, J.R., 1999. Apparent uncoupling of energy production and consumption in long-lived Clk mutants of *Caenorhabditis elegans*. Curr. Biol. 9, 493–496.

Brand, M.D., 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Exp. Gerontol. 35, 811–820.

Broughton, S.J., Piper, M.D., Ikeya, T., Bass, T.M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D.J., Leevers, S.J., Partridge, L., 2005. Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. Proc. Natl. Acad. Sci. U.S.A. 102, 3105–3110.

Burnell, A.M., Houthoofd, K., O'Hanlon, K., Vanfleteren, J.R., 2005. Alternate metabolism during the dauer stage of the nematode *Caenorhabditis elegans*. Exp. Gerontol. 40, 850–886.

Cassada, R.C., Russell, R.L., 1975. The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev. Biol. 46, 326–342.

Choe, S.E., Boutros, M., Michelson, A.M., Church, G.M., Halfon, M.S., 2005. Preferred analysis methods for Affymetrix GeneChips revealed by a wholly defined control dataset. Genome Biol. 6, R16.

Clancy, D., Gems, D., Harshman, L.G., Oldham, S., Hafen, E., Leevers, S.J., Partridge, L., 2001. Extension of lifespan by loss of *chico*, a *Drosophila* insulin receptor substrate protein. Science 292, 104–106.

Crowe, J.H., Crowe, L.M., 2000. Preservation of mammalian cells-learning nature's tricks. Nat. Biotechnol. 18, 145–146.

Dillin, A., Hsu, A.L., Arantes-Oliveira, N., Lehrer-Graiwer, J., Hsin, H., Fraser, A.G., Kamath, R.S., Ahringer, J., Kenyon, C., 2002. Rates of behavior and aging specified by mitochondrial function during development. Science 298, 2398–2401.

Dobrzyn, A., Ntambi, J.M., 2004. The role of stearoyl-CoA desaturase in body weight regulation. Trends Cardiovasc. Med. 14, 77–81.

Efron, B., Tibshirani, R., Storey, J., Tusher, V., 2001. Empirical Bayes analysis of a microarray experiment. J. Am. Stat. Assoc. 96, 1151–1160.

Enomoto, K., Arikawa, Y., Muratsubaki, H., 2002. Physiological role of soluble fumarate reductase in redox balancing during anaerobiosis in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 215, 103–108.

Feng, J., Bussiere, F., Hekimi, S., 2001. Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*. Dev. Cell. 1, 633–644.

Foll, R.L., Pleyers, A., Lewandovski, G.J., Wermter, C., Hegemann, V., Paul, R.J., 1999. Anaerobiosis in the nematode *Caenorhabditis elegans*. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 124, 269–280.

Friedman, D.B., Johnson, T.E., 1988. A mutation in the *age*-1 gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. Genetics 118, 75–86.

Gems, D., McElwee, J.J., 2005. Broad spectrum detoxification: the major longevity-assurance process regulated by insulin/IGF-1 signaling? Mech. Ageing Dev. 126, 381–387.

Gems, D., Sutton, A.J., Sundermeyer, M.L., Larson, P.L., Albert, P.S., King, K.V., Edgley, M., Riddle, D.L., 1998. Two pleiotropic classes of *daf-2* mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. Genetics 150, 129–155.

Green, D.W., Grover, G.J., 2000. The IF(1) inhibitor protein of the mitochondrial F(1)F(0)-ATPase. Biochim. Biophys. Acta 1458, 343–355.

Hanak, P., Jezek, P., 2001. Mitochondrial uncoupling proteins and phylogenesis-UCP4 as the ancestral uncoupling protein. FEBS Lett. 495, 137–141.

Hanover, J.A., Forsythe, M.E., Hennessey, P.T., Brodigan, T.M., Love, D.C., Ashwell, G., Krause, M., 2005. A *Caenorhabditis elegans* model of insulin resistance: altered macronutrient storage and dauer formation in an OGT-1 knockout. Proc. Natl. Acad. Sci. U.S.A. 102, 11266–11271.

Hartman, P.S., Ishii, N., Kayser, E.B., Morgan, P.G., Sedensky, M.M., 2001. Mitochondrial mutations differentially affect aging, mutability and anesthetic sensitivity in *Caenorhabditis elegans*. Mech. Ageing Dev. 122, 1187–1201.

Hekimi, S., Guarente, L., 2003. Genetics and the specificity of the aging process. Science 299, 1351–1354.

Holt, S.J., Riddle, D.L., 2003. SAGE surveys *C. elegans* carbohydrate metabolism: evidence for an anaerobic shift in the long-lived dauer larva. Mech. Ageing Dev. 124, 779–800.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P.C., Cervera, P., Le Bouc, Y., 2003. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 421, 182–187.

Honda, Y., Honda, S., 1999. The *daf*-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. FASEB J. 13, 1385–1393.

Houthoofd, K., Braeckman, B.P., Lenaerts, I., Brys, K., De Vreese, A., Van Eygen, S., Vanfleteren, J.R., 2002. Ageing is reversed, and metabolism is reset to young levels in recovering dauer larvae of *C. elegans*. Exp. Gerontol. 37, 1015–1021.

Houthoofd, K., Fidalgo, M.A., Hoogewijs, D., Braeckman, B.P., Lenaerts, I., Brys, K., Matthijssens, F., De Vreese, A., Van Eygen, S., Munoz, M.J., Vanfleteren, J.R., 2005. Metabolism, physiology and stress defense in three aging Ins/IGF-1 mutants of the nematode *Caenorhabditis elegans*. Aging Cell 4, 87–95.

Hsu, A.L., Murphy, C.T., Kenyon, C., 2003. Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science 300, 1142–1145.

Hunt, M.C., Solaas, K., Kase, B.F., Alexson, S.E., 2002. Characterization of an acyl-coA thioesterase that functions as a major regulator of peroxisomal lipid metabolism. J. Biol. Chem. 277, 1128–1138.

Ishii, N., Takahashi, K., Tomita, S., Keino, T., Honda, S., Yoshino, K., Suzuki, K., 1990. A methyl viologen-sensitive mutant of the nematode *Caenorhabditis elegans*. Mutat. Res. 237, 165–171.

Jones, S.J., Riddle, D.L., Pouzyrev, A.T., Velculescu, V.E., Hillier, L., Eddy, S.R., Stricklin, S.L., Baillie, D.L., Waterston, R., Marra, M.A., 2001. Changes in gene expression associated with developmental arrest and longevity in *Caenorhabditis elegans*. Genome Res. 11, 1346–1352.

Kahn, F.R., McFadden, B.A., 1980. Embryogenesis and the glyoxylate cycle. FEBS Lett. 115, 312–314.

Kenyon, C., 2005. The plasticity of aging: insights from long-lived mutants. Cell 120, 449–460.

Kenyon, C., Chang, J., Gensch, E., Rudener, A., Tabtiang, R., 1993. A *C. elegans* mutant that lives twice as long as wild type. Nature 366, 461–464.

Kimura, K.D., Tissenbaum, H.A., Liu, Y., Ruvkun, G., 1997. *daf*-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science 277, 942–946.

Kirkwood, T.B.L., 1977. Evolution of ageing. Nature 270, 301–304.

Klass, M.R., 1977. Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. Mech. Ageing Dev. 6, 413–429.

Lamitina, S.T., Strange, K., 2005. Transcriptional targets of DAF-16 insulin signaling pathway protect *C. elegans* from extreme hypertonic stress. Am. J. Physiol. Cell. Physiol. 288, C467–C474.

Larsen, P., Clarke, C.F., 2002. Extension of life-span in *Caenorhabditis elegans* by a diet lacking coenzyme Q. Science 295, 120–123.

Larsen, P.L., Albert, P.S., Riddle, D.L., 1995. Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics 139, 1567–1583.

Lee, S.S., Lee, R.Y., Fraser, A.G., Kamath, R.S., Ahringer, J., Ruvkun, G., 2003. A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. Nat. Genet. 33, 40–48.

Lin, K., Dorman, J.B., Rodan, A., Kenyon, C., 1997. *daf*-16: an HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. Science 278, 1319–1322.

Lithgow, G.J., White, T.M., Hinerfield, D.A., Johnson, T.E., 1994. Thermotolerance of a long-lived mutant *of Caenorhabditis elegans*. J. Gerontol. 49, B270–B276.

Lithgow, G.J., White, T.M., Melov, S., Johnson, T.E., 1995. Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc. Natl. Acad. Sci. U.S.A. 92, 7540–7544.

Liu, F., Thatcher, J.D., Barral, J.M., Epstein, H.F., 1995. Bifunctional glyoxylate cycle protein of *Caenorhabditis elegans*: a developmentally regulated protein of intestine and muscle. Dev. Biol. 169, 399–414.

Liu, F., Thatcher, J.D., Epstein, H.F., 1997. Induction of glyoxylate cycle expression in *Caenorhabditis elegans*: a fasting response throughout larval development. Biochemistry 36, 255–260.

McElwee, J., Bubb, K., Thomas, J.H., 2003. Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16. Aging Cell 2, 111–121.

McElwee, J.J., Schuster, E., Blanc, E., Thomas, J.H., Gems, D., 2004. Shared transcriptional signature in *C. elegans* dauer larvae and long-lived *daf-2* mutants implicates detoxification system in longevity assurance. J. Biol. Chem. 279, 44533–44543.

Muratsubaki, H., Enomoto, K., 1998. One of the fumarate reductase isoenzymes from *Saccharomyces cerevisiae* is encoded by the OSM1 gene. Arch. Biochem. Biophys. 352, 175–181.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., Kenyon, C.J., 2003. Genes that act downstream of DAF-16 to influence the lifespan of *C. elegans*. Nature 424, 277–284.

Nelson, D.L., Cox, M.M., 2000. Lehninger Principles of Biochemistry. Worth, New York.

O'Brien, R.M., Streeper, R.S., Ayala, J.E., Stadelmaier, B.T., Hornbuckle, L.A., 2001. Insulin-regulated gene expression. Biochem. Soc. Trans. 29, 552–558.

O'Riordan, V.B., Burnell, A.M., 1989. Intermediary metabolism in the dauer larva of the nematode *Caenorhabditis elegans*—1. Glycolysis, gluconeogenesis, oxidative phosphorylation and the tricarboxylic acid cycle. Comp. Biochem. Phys. 92B, 233–238.

O'Riordan, V.B., Burnell, A.M., 1990. Intermediary metabolism in the dauer larva of the nematode *Caenorhabditis elegans*—II. The glyoxylate cycle and fatty-acid oxidation. Comp. Biochem. Phys. 95B, 125–130.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., Ruvkun, G., 1997. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature 389, 994–999.

Pellerone, F.I., Archer, S.K., Behm, C.A., Grant, W.N., Lacey, M.J., Somerville, A.C., 2003. Trehalose metabolism genes in *Caenorhabditis elegans* and filarial nematodes. Int. J. Parasitol. 33, 1195–1206.

Petriv, O.I., Pilgrim, D.B., Rachubinski, R.A., Titorenko, V.I., 2002. RNA interference of peroxisome-related genes in *C. elegans*: a new model for human peroxisomal disorders. Physiol. Genom. 10, 79–91.

Popham, J., Webster, J., 1979. Aspects of the fine structure of the dauer larva of the nematode *C. elegans*. Can. J. Zool. 57, 794–800.

Rea, S., Johnson, T.E., 2003. A metabolic model for lifespan determination in *Caenorhabditis elegans*. Dev. Cell 5, 197–203.

Riddle, D.L., Albert, P.S., 1997. Genetic and environmental regulation of dauer larva development. In: Riddle, D.L., et al. (Eds.), *C. elegans* II. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 739–768.

Salway, J.G., 1999. Metabolism at a Glance. Blackwell Science, Oxford.

Scott, B.A., Avidan, M.S., Crowder, C.M., 2002. Regulation of hypoxic death in C. *elegans* by the insulin/IGF receptor homolog DAF-2. Science 296, 2388–2391.

Singer, M.A., Lindquist, S., 1998. Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. Trends Biotechnol. 16, 460–468.

Skulachev, V.P., 1996. Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. Q. Rev. Biophys. 29, 169–202.

Takamiya, S., Matsui, T., Taka, H., Murayama, K., Matsuda, M., Aoki, T., 1999. Free-living nematodes *Caenorhabditis elegans* possess in their mitochondria an additional rhodoquinone, an essential component of the eukaryotic fumarate reductase system. Arch. Biochem. Biophys. 371, 284–289.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.-P., Yin, C.-M., Garofalo, R.S., 2001. Mutations in the *Drosophila* insulin receptor homologue retard senescence and impair neuroendocrine function. Science 292, 107–110.

Thieringer, H., Moellers, B., Dodt, G., Kunau, W.H., Driscoll, M., 2003. Modeling human peroxisome biogenesis disorders in the nematode *Caenorhabditis elegans*. J. Cell Sci. 116, 1797–1804.

Tielens, A.G., Rotte, C., van Hellemond, J.J., Martin, W., 2002. Mitochondria as we don't know them. Trends Biochem. Sci. 27, 564–572.

Tielens, A.G.M., van Hellemond, J.J., 1998. The electron transport chain in anaerobically functioning eukaryotes. Bba – Bioenergetics 1365, 71–78.

Tsang, W.Y., Lemire, B.D., 2003. The role of mitochondria in the life of the nematode, *Caenorhabditis elegans*. Biochim. Biophys. Acta 1638, 91–105.

Tsang, W.Y., Sayles, L.C., Grad, L.I., Pilgrim, D.B., Lemire, B.D., 2001. Mitochondrial respiratory chain deficiency in *Caenorhabditis elegans* results in developmental arrest and increased life span. J. Biol. Chem. 276, 32240–32246.

Tusher, V.G., Tibshirani, R., Chu, G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U.S.A. 98, 5116–5121.

Van Voorhies, W.A., Ward, S., 1999. Genetic and environmental conditions that increase longevity in *Caenorhabditis elegans* decrease metabolic rate. Proc. Natl. Acad. Sci. U.S.A. 96, 11399–11403.

Vanfleteren, J.R., 1993. Oxidative stress and ageing in *Caenorhabditis elegans*. Biochem. J. 292, 605–608.

Vanfleteren, J.R., De Vreese, A., 1995. The gerontogenes *age-1* and *daf-2* determine metabolic rate potential in aging *Caenorhabditis elegans*. FASEB J. 9, 1355–1361.

Vanfleteren, J.R., De Vreese, A., 1996. Rate of aerobic metabolism and superoxide production rate potential in the nematode *Caenorhabditis elegans*. J. Exp. Zool. 274, 93–100.

Wadsworth, W., Riddle, D., 1988. Acidic intracellular pH shift during *Caenorhabditis elegans* larval development. Proc. Natl. Acad. Sci. U.S.A. 85, 8435–8438.

Wadsworth, W.G., Riddle, D.L., 1989. Developmental regulation of energy metabolism in *Caenorhabditis elegans*. Dev. Biol. 132, 167–173.

Walker, G.A., Lithgow, G.J., 2003. Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals. Aging Cell 2, 131–139.

Wang, J., Kim, S., 2003. Global analysis of dauer gene expression in *Caenorhabditis elegans*. Development 130, 1621–1634.

Watts, J.L., Browse, J., 2000. A palmitoyl-CoA-specific delta9 fatty acid desaturase from *Caenorhabditis elegans*. Biochem. Biophys. Res. Commun. 272, 263–269.

Williams, G.C., 1957. Pleiotropy, natural selection and the evolution of senescence. Evolution 11, 398–411.