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Progress paper

Broad spectrum detoxification: the major longevity assurance process regulated by insulin/IGF-1 signaling?

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Abstract

Our recent survey of genes regulated by insulin/IGF-1 signaling (IIS) in *Caenorhabditis elegans* suggests a role for a number of gene classes in longevity assurance. Based on these findings, we propose a model for the biochemistry of longevity assurance and ageing, which is as follows. Ageing results from molecular damage from highly diverse endobiotic toxins. These are stochastic by-products of diverse metabolic processes, of which reactive oxygen species (ROS) are likely to be only one component. Our microarray analysis suggests a major role in longevity assurance of the phase 1, phase 2 detoxification system involving cytochrome P450 (CYP), short-chain dehydrogenase/reductase (SDR) and UDP-glucuronosyltransferase (UGT) enzymes. Unlike superoxide and hydrogen peroxide detoxification, this system is energetically costly, and requires the excretion from the cell of its products. Given such costs, its activity may be selected against, as predicted by the disposable soma theory. CYP and UGT enzymes target lipophilic molecular species; insufficient activity of this system is consistent with age-pigment (lipofuscin) accumulation during ageing. We suggest that IIS-regulated longevity assurance involves: (a) energetically costly detoxification and excretion of molecular rubbish, and (b) conservation of existing proteins via molecular chaperones. Given the emphasis in this theory on investment in cellular waste disposal, and on protein conservation, we have dubbed it the green theory.
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1. Introduction

Many advances in the understanding of the biological determinants of ageing have been made recently by means of mutant analysis using model organisms. A particular virtue of this approach is that its only premise is that genes influence ageing and longevity, and this premise has to be true (for example, given the large differences in lifespan between different animal species). Thus, it is unaffected by bias due to pre-conceptions derived from pre-existing theories (Kenyon, 1997). This circumvents one of the problems with earlier work on ageing: a surfeit of different theories of ageing (over 300 by one estimate)(Medvedev, 1990), many conceived with little reference to empirical evidence.

Studies utilizing mutants with altered rates of ageing in the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila*, and the baker's yeast *Saccharomyces cerevisiae* have implicated a range of processes in lifespan determination; reviewed in (Hekimi and Guarente, 2003; Tatar et al., 2003). These include nutritional alteration of chromatin structure, mediated by the Sir2 NAD-dependent histone deacetylase (Imai et al., 2000; Motta et al., 2004), and mitochondrial electron transport (Branicky et al., 2000; Dillin et al., 2002; Lee et al., 2003). The most powerful determinant of animal ageing identified to date is an endocrine system resembling the mammalian insulin and insulin-like growth factor 1 (IGF-1) systems. The role of this system in ageing was originally identified in *C. elegans* (Friedman and Johnson, 1988; Kenyon et al., 1993; Kimura et al., 1997), but was subsequently found to play a similar role in ageing in *Drosophila* and mice; reviewed in (Liang et al., 2003; Longo and Finch, 2003). However, the role of this endocrine system

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in ageing gives little insight into the proximal biochemical determinants of ageing which it presumably regulates. The effects of insulin/IGF-1 signaling (IIS) on ageing in *C. elegans* are entirely dependent upon DAF-16, a FOXO class transcription factor which it regulates (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). Thus, the direct determinants of ageing are likely to exist among transcriptional targets of DAF-16 and perhaps genes acting further downstream in a DAF-16-regulated transcriptional cascade (Kenyon et al., 1993).

Thanks to the sequencing of the *C. elegans* genome, and the development of DNA microarray technology for genome-wide surveys of transcriptional changes, it has been possible to systematically identify genes that are regulated by DAF-16 (McElwee et al., 2003, 2004; Murphy et al., 2003). Among DAF-16-regulated genes are some that are up-regulated and others that are down-regulated. Using RNA-mediated interference (RNAi), it was demonstrated that many DAF-16 up-regulated genes are determinants of mutant IIS longevity (i.e. knock-down of gene expression shortens lifespan). Likewise, many DAF-16 down-regulated genes are determinants of ageing (i.e. knock-down increases lifespan) (Murphy et al., 2003).

These studies imply that some of the transcriptional targets of DAF-16 specify biochemical processes, which are primary determinants of longevity and ageing. But what are these processes? As often occurs in microarray experiments, large lists of regulated genes have been identified associated with a wide range of processes. Moreover, the genes range from those with known and well-characterised function, to others that remain entirely uncharacterised.

There is a potential error that can arise in analysis of large gene lists of this type. Faced with a list of largely unfamiliar genes, an investigator may pick out the small proportion of genes which fit pre-existing ideas about ageing. This biased approach is sometimes referred to *as fishing* (as in fishing for a desired answer when asking a question). The main reason to avoid fishing is that it cannot discover new processes linked to ageing by studying microarray data. To avoid it, what is required is a means of performing unbiased analysis of gene lists to identify previously unrecognised processes linked to lifespan determination.

2. A non-biased and comparative analysis of *daf-2*-regulated genes

One standard way to perform an unbiased analysis of a list of differentially-expressed genes defined by microarray analysis is as follows. One may ask whether the number of genes of a particular gene class (say, the transthyretin-related proteins, or TRPs) that are present in a gene list is more than what one would expect in a random list of genes of that size. In our analysis, we estimated (using a conservative version of Fisher's exact test)(Hosack et al., 2003) that the probability that the overlap between TRP genes and genes up-regulated in *daf-2* mutants had occurred by chance was only $p = 0.001$ (McElwee et al., 2004). This correlation suggests (but of course does not demonstrate) a role for TRPs in longevity assurance.

When *C. elegans* develop under conditions of crowding and poor nutrition, they form a diapausal alternative third stage larva, the dauer larva (Riddle and Albert, 1997). Dauer larvae can live for several months (Klass and Hirsh, 1976) in contrast to *C. elegans* adults, which live only 2–3 weeks. Dauer larva formation is regulated by IIS, and reduced IIS can cause both constitutive dauer larva formation and increased adult lifespan. It has been suggested that in IIS mutant adults (e.g. *daf-2*), increased longevity results from expression of dauer larva longevity (Kenyon et al., 1993). On the assumption that similar processes may assure the longevity of dauer larvae and *daf-2* mutant adults, in our recent study we screened for gene classes which were enriched for genes up- or down-regulated in both dauer larvae and *daf-2* mutant adults.

The efficacy of this approach is implied by three observations (McElwee et al., 2004). Firstly, the lists of genes with altered transcript abundance in dauer larvae were enriched among *daf-2*-regulated genes, supporting the view that dauer longevity processes are expressed in *daf-2* mutant adults. Secondly, this analysis identified only two dozen non-redundant gene classes over-represented in genes up-regulated in *daf-2* mutants and dauers, and a similar number in down-regulated genes. This is a feasible number of entities to analyse, in contrast to the ∼2000 individual genes showing altered expression in *daf-2* mutants. Thirdly, one of the gene groups enriched in *daf-2* and dauer up-regulated genes was the HSP-20/α-crystallins, or small heatshock proteins (smHSPs). This is important because this gene class is one where a causal role in longevity assurance in *C. elegans* is securely demonstrated (Hsu et al., 2003; Walker and Lithgow, 2003). This suggests that the list of *daf-2*/dauer-associated gene groups will include others that determine lifespan.

The next step in our analysis of *daf*-2/dauer-associated gene groups was unavoidably subjected to bias. We studied these gene groups, and wondered how they might be linked to ageing. This, of course, involves fishing of a sort: something that we initially were at pains to avoid. In our defense, what we hoped at least to achieve was for the data to lead us to novel hypotheses about ageing, and this it has done. This was possible given the small number of gene classes defined by the analytical approach used.

3. Up-regulation of detoxification systems in dauer larvae and *daf-2* mutants

We studied the *daf-2*/dauer-regulated gene groups to identify potential IIS-regulated processes that might be determinants of ageing and longevity. Among up-regulated gene groups were several encoding genes which share a role

in drug detoxification: cytochrome P450s (CYPs), short-chain dehydrogenase/reductases (SDRs), UDP-glucuronosyltransferases (UGTs) and (in *daf-2* adults only) glutathione S-transferases (GSTs). In mammals these four enzyme classes act in concert to dispose of toxic endobiotic or xenobiotic compounds (e.g. toxins, drugs, carcinogens), mainly in the liver. The detoxification system involves two stages, phase 1 and phase 2 (Gibson and Skett, 2001). Phase 1 reactions (functionalisation reactions) result in addition of chemically reactive functional groups, which allow further metabolism of otherwise unreactive, typically lipophilic compounds. Phase 1 reactions are often (but not always) required for phase 2 (conjugative reactions): the addition of side groups which increase solubility, aiding excretion.

Cytochrome P450s (CYPs) and short-chain dehydrogenase/reductases (SDRs) are major agents of phase 1 metabolism in mammalian cells. CYPs act most often as mixed function oxidases, bioactivating lipophilic toxins usually by hydroxylation. SDRs typically catalyse the reduction of carbonyl groups in aldehydes and ketones. UGTs are the major effectors of phase 2 metabolism, with assistance from GSTs, sulphotransferases and acetyltransferases (Gibson and Skett, 2001). In their detoxifying capacity, CYPs and UGTs act in the smooth endoplasmic reticulum (ER), GSTs in the cytosol, and SDRs in both locations.

Cytochrome P450s (CYPs) function not only in drug metabolism but also in a range of biosynthetic processes (e.g. of steroids, fatty acids, prostaglandins, vitamin D). Yet the coincidental up-regulation of UGTs, SDRs and GSTs in *daf-2* mutants/dauers argues that CYP action in phase 1 metabolism is activated. Moreover, some of the most strongly up-regulated CYP genes have previously been shown to be activated by xenobiotics (Menzel et al., 2001).

The up-regulation of these gene groups implies that the capacity for phase 1 and phase 2 metabolism is increased in dauer larvae and *daf-2* mutants. This in turn suggests that phase 1, phase 2 detoxification and excretion is a possible longevity assurance mechanism. If correct, this would mean that the toxic compounds that this system targets are a cause of ageing, presumably due to the molecular damage that they cause.

4. The green theory of ageing

Among IIS-regulated gene groups where increased expression is correlated with longevity, we identified gene groups linked to detoxification, and chaperonins. Based on this, we suggest that ageing entails accumulation of damage to macromolecules due to a range of toxic by-products of metabolism. We propose that longevity assurance mechanisms involve either removal of diverse molecular species that cause damage (e.g. by CYPs, SDRs, UGTs, GSTs), or repair of damaged proteins (e.g. by smHSPs). According to this view, the cell is under constant threat from metabolic waste products and xenobiotics. We suggest that the smooth ER works as a cellular filter, deploying phase 1 and phase 2 metabolism to mobilise and excrete these mainly lipophilic toxins. This clears the cell of molecular rubbish, thereby preventing molecular damage, and ageing.

The problems for the cell in terms of the theory proposed here have certain parallels with environmental problems arising from human industry. The principals of longevity assurance that it suggests—increased expenditure of energy on waste disposal to prevent damage to the cell, and the conservation of cellular constituents by the action of molecular chaperones resemble the recommendations of green activists: to increase investment in clean waste disposal, to reduce pollution and to conserve resources (e.g. by recycling). For this reason we have nick-named the hypothesis presented here the green theory. We are currently pursuing several approaches to test whether this theory is true. These include testing the xenobiotic resistance of long-lived IIS mutants, employing drugs which induce expression of xenobiotic detoxification genes and testing effects on xenobiotic resistance and ageing, and studying effects of over-expression of xenobiotic detoxification using transgenes.

5. Relationship to other theories: the oxidative damage theory

A number of pre-existing theories of ageing may be viewed in a new light in the context of the green theory. Perhaps the most influential theory of the biological mechanism of ageing is the oxidative damage theory. According to this, ageing results from molecular damage caused by reactive oxygen species (ROS), particularly superoxide and its derivatives, which are produced mainly as a by-product of mitochondrial oxidative phosphorylation (Beckman and Ames, 1998; Sohal and Weindruch, 1996). Our new model can potentially incorporate both the oxidative damage theory of ageing, and the diverse observations of correlation between increased longevity and increased stress resistance (Lithgow and Walker, 2002).

We suggest that the oxidative damage theory is only partially true. It is likely that molecular damage caused by ROS contributes to ageing, and antioxidant defences to longevity. The long-term survival of biological systems in an atmosphere containing oxygen, a highly reactive gas, is a challenge to homeostasis. Yet our results draw attention to the fact that oxygen and its by-products are not by any means the only challenge. The exquisitely ordered molecular machines that living systems are must also somehow deal with an enormous diversity of other unwanted molecular species, some of them highly toxic. This is presumably a particular problem with organic (typically lipophilic) compounds, where molecular diversity is almost infinite. This is reflected by the large number and diversity of genes

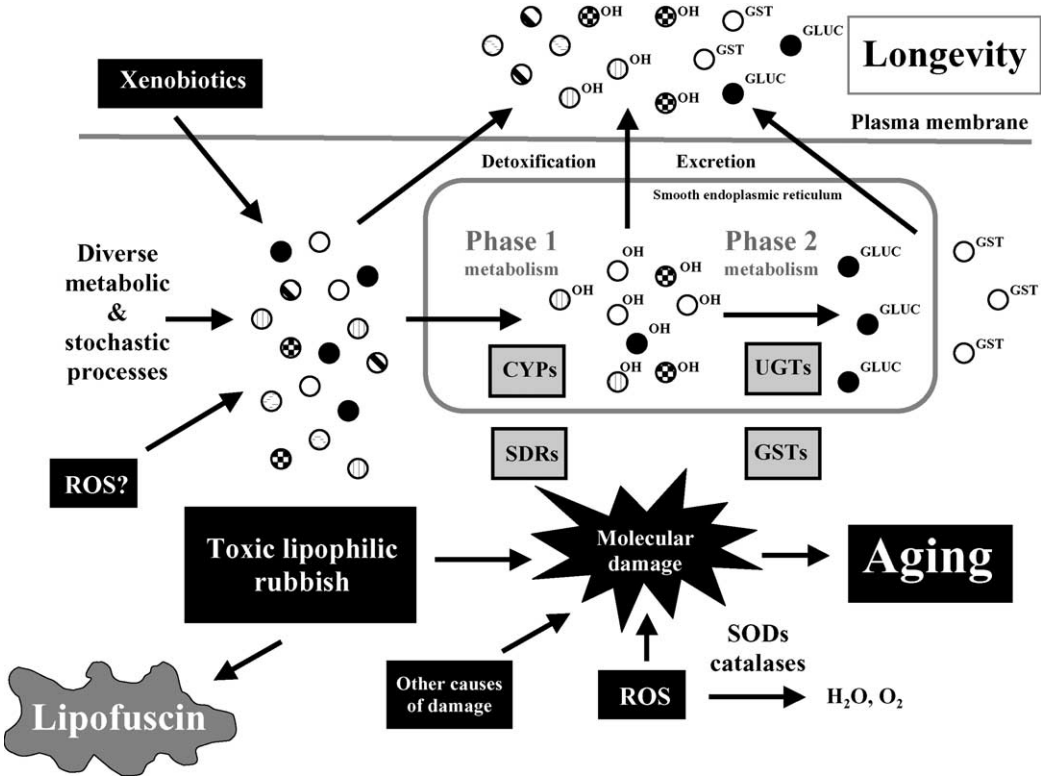

Fig. 1. Broad spectrum detoxification and longevity assurance. We propose that a major contributor to the ageing process is molecular damage resulting from toxins (mainly endobiotic and lipophilic) which are targets of the phase 1, phase 2 detoxification system. In this model, the smooth endoplasmic reticulum functions as a longevity organelle, derivatising and excreting lipophilic toxins. However, this process is energetically costly, and most cells take the energy saving step of down-regulating the detoxification system, and dumping toxins within lipofuscin depots. The consequence of this is accelerated ageing. CYP, cytochrome P450 (mainly oxidases); GST, glutathionyl; Gluc, glucuronosyl/glucosyl; SDR, short-chain dehydrogenase/reductase; UGT, UDP-glucuronosyl-(or glucosyl-) transferases.

in the CYP, SDR, UGT and GST families and, typically, the broad substrate range of the individual enzymes.

This interpretation is consistent with the finding that treatment with antioxidants does not result in extended lifespan; reviewed in Sohal et al. (2000). Some *Drosophila* studies have shown increases in lifespan resulting from over-expression of antioxidant genes, mainly when using heterologous promoters (Parkes et al., 1998; Sun et al., 2002; Sun and Tower, 1999). Yet it now appears likely that much of this effect was due to rescue of sub-viability and short lifespan in the fly stocks used (Orr and Sohal, 2003; Spencer et al., 2003). In *C. elegans* one study reported increases in lifespan from treatment with synthetic superoxide dismutase (SOD) mimetics (Melov et al., 2000); however, this effect appears unreproducible (Bayne and Sohal, 2002; Keaney and Gems, 2003; Keaney et al., 2004). Elevated SOD levels do not extend lifespan in mice (Huang et al., 2000). We suggest that the failure of antioxidants to extend lifespan is because given such a treatment, lifespan is still limited by damage from diverse other agents of molecular damage, particularly lipophilic molecular rubbish.

It should be pointed out that our microarray study (McElwee et al., 2004) did not exclude a role for antioxidant defences in longevity assurance. The statistical analysis used was suitable for identifying large gene classes correlated with lifespan, but less so for small gene groups, and not at all for single genes. For example, our microarray analysis showed significant up-regulation of a number of antioxidant defense genes in *daf-2*(−) mutants, including *sod-1* and ZK430.3 (Cu/Zn SOD), *sod-3* (Mn SOD) and *ctl-1* (catalase) (McElwee et al., 2004). Consistent with earlier studies, this suggests an association between longevity assurance and increased defences against a broad range of sources of molecular damage (Fig. 1).

6. The evolutionary theory of ageing

It is unlikely that ageing per se enhances evolutionary fitness in any way. Rather it is believed to evolve as a fitness-neutral secondary consequence of selection for other fitness traits which maximise reproductive success (Partridge and Barton, 1993; Williams, 1957). One interpretation is that biological processes that assure longevity are energetically costly, and that fitness may be optimised by investing into somatic maintenance processes only the minimum resources required to assure survival through the reproductive period (Kirkwood, 1977). This disposable soma theory predicts that longevity assurance genes whose activity levels limit

lifespan are likely to specify energetically costly processes of somatic maintenance.

Interestingly, both phase 1 and phase 2 metabolism of toxins require a lot of energy. The mixed function oxidase (MFO) reactions of phase 1 metabolism, catalysed by CYPs, consume NADPH.

$$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH$$

Similarly, SDR reactions consume energy from NADH or NADPH (Kallberg et al., 2002). More strikingly, each UGT glucuronidation reaction consumes a molecule of glucose (~30 ATP equivalents), and glutathionylation by GSTs a molecule of the tripeptide glutathione. Potentially, increased phase 1 and 2 metabolism in *daf-2* mutant adults consumes energy that could otherwise be expended on reproduction, thus reducing fitness.

An assumption of the green theory is that the enormous structural diversity of unwanted organic molecular forms must represent an informatic challenge to the cell. By contrast, detoxifying superoxide should not, arguably, represent a major challenge to the cell, since the SOD and catalase enzymes achieve this efficiently, and without requiring energy input. Moreover, there is no requirement for disposal of the products of neutralisation of superoxide (water and oxygen). By contrast, lipophilic, organic junk will be difficult to dispose of because of its complexity, the energy required, and also the need to excrete it from the body. In terms of specificity and energy efficiency, catalase and the UGTs are quite antithetical. But it is easy to see why; the problem to the genome of disposing of lipophilic molecular rubbish is, in evolutionary terms, insurmountable. While lipophilic garbage disposal enzymes with the specificity and energy efficiency of catalase might be possible in principle, evolution would not be expected to generate them. This is because of the predicted molecular diversity of the rubbish targets. The phase 1, phase 2 detoxification system represents the best of a bad evolutionary job.

7. Lipofuscin accumulation and targets of detoxification

Our model implies the existence of a wide range of lipophilic compounds causing molecular damage. But where would such compounds come from, and what direct evidence is there for their existence? The CYP/UGT detoxification system has both endobiotic and xenobiotic substrates. The fact that the detoxification system is up-regulated in dauer larvae, which are non-feeding and whose buccal cavity and anus are sealed, suggests that xenobiotics are not the major cause of ageing. Where might endobiotic lipophilic toxins come from?

One possibility is that stochastic errors in a range of metabolic pathways generate molecular intermediates which are unrecognisable to the cell, and which therefore accumulate. Throughout the animal kingdom ageing is accompanied by progressive accumulation of lipofuscin (Yin, 1996). This is poorly characterised, fluorescent molecular waste, which in *C. elegans* accumulates in secondary lysosomes (Clokey and Jacobsen, 1986). Lipofuscin accumulation is consistent with a link between ageing and failure to detoxify molecular waste. Notably, the age-dependant accumulation of autofluorescent material is retarded in long-lived *daf-2* mutant *C. elegans* (Garigan et al., 2002).

Lipofuscin accumulation may be merely an indicator of an overall impaired disposal of molecular waste, including that which causes the molecular damage underlying ageing; alternatively lipofuscin itself may cause such damage. *C. elegans* is a very short-lived organism (lifespan, 2–3 weeks), and autofluorescent material begins to accumulate even before the end of larval development. Potentially, this species may have evolved to cut costs on excretion of molecular rubbish, instead dumping it in the cell within secondary lysosomes. Ultrastructural studies of ageing nematodes suggest a catastrophic accumulation of electron dense inclusions (lipofuscin) in the cytoplasm of intestinal cells. This has been particularly well-described in studies of *C. briggsae* (Epstein et al., 1972). Possibly this accumulation limits lifespan.

8. Evolutionary conservation of dauer and *daf-2*-regulated gene groups

The role of IIS in the regulation of ageing has proved to be evolutionarily conserved, representing a public rather than a private mechanism of ageing (Martin et al., 1996; Partridge and Gems, 2002). An important question is whether the biochemistry of lifespan regulated by IIS is public or private. The genes regulated by IIS in *C. elegans* include some for which there are clear mammalian orthologues (e.g. SODs, smHSPs), and which could correspond to public mechanisms. For other gene groups it is less clear whether a public mechanism is a possibility.

The CYP genes are a good example of this. This large gene family falls phylogenetically into several distinct groups, or clans, some of which show little variation in family or subfamily size (e.g. CYP1, CYP5 and higher) and others which show great variation (e.g. CYP2, 3 and 4) (Nelson, 1999). Phylogenetic analysis indicates that a high degree of lineage-specific expansions occurred in the CYP2, 3, 4 and mitochondrial CYP families after the divergence of major vertebrate and invertebrate groups some 600 million years ago (Nelson, 1999). The majority of *C. elegans* CYPs (44/77) belong to a clan not found in insects or vertebrates that is derived from a common ancestor of the CYP2 clan. Vertebrates also largely expanded the CYP2 clan; by contrast, insects expanded the CYP4 and CYP3 clans (Nelson, 1999).

Lineage-specific expansions are common among gene families linked to stress resistance (Lespinet et al., 2002).

This applies to the UGTs, the TRPs and the GSTs. The latter group include seven taxon-independent classes (Alpha, Mu, Pi, Sigma, Theta and Omega) (Board et al., 2000). While some *C. elegans* GSTs belong to the Alpha, Pi and Sigma types, most appear unique to the Nematoda (Campbell et al., 2001). One such nematode-specific GST is K08F4.7, which has a confirmed role in oxidative stress resistance (Leiers et al., 2003; Tawe et al., 1998), and is up-regulated in *daf-2* mutants (McElwee et al., 2004). Thus, in many cases it is not possible to identify orthologues between *C. elegans* stress-resistance genes and those of other model organisms. Yet it seems likely that although these genes are not orthologous, many perform the same biological function in nematodes and mammals. This is implied, e.g. by the induction of expression of members of the *C. elegans* specific CYP2 clan by xenobiotics (Menzel et al., 2001). Thus, it is likely that genetic orthology is not a pre-requisite for public mechanisms of longevity assurance.

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