MISC-1/OGC IS A NEW STRESS RESPONSE GENE AND REGULATOR OF APOPTOSIS, GERMLINE STEM CELL PROLIFERATION AND INSULIN SECRETION

by

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ABSTRACT

The present work produced new insights on the function of an ascaroside molecule and a gene that affect formation of dauer larvae, a diapause stage in *Caenorhabditis elegans*.

Our results indicate that the ascaroside daumone, although a component of the dauer pheromone, does not act through signalling pathways active in the cilia. Furthermore, daumone has toxic effects if the animals are exposed to dauer-inducing concentrations of the compound. This ascaroside is not able, by itself, to recapitulate the full spectrum of events that occur during dauer formation.

We identified *misc-1* (MItochondrial Solute Carrier) as a novel suppressor of the dauer phenotype of *daf-2/IGF1R* mutants. We provide evidence that MISC-1 is the putative orthologue of mammalian OGC (2-OxoGlutarate Carrier), a metabolic carrier of the inner mitochondrial membrane. We show that a *misc-1* null allele suppresses the dauer phenotype of *daf-2* mutants by increasing insulin secretion. Consistent with this result, *misc-1* mutants have increased proliferation of germline stem cells. Furthermore, we show that MISC-1 and OGC are involved in a phylogenetically conserved apoptosis pathway. Reduced levels of MISC-1 in *C. elegans* and OGC in human cells result in mitochondrial fragmentation. MISC-1 and human OGC physically interact with the anti-apoptotic Bcl-2-family members CED-9 and Bcl-x_L, respectively, and are novel components of the mitochondrial permeability transition pore. Decreased levels of MISC-1 and OGC induce apoptosis in *C. elegans* and mouse cells. Finally, our experiments confirm that MISC-1/OGC has Reactive Oxygen Species (ROS)-detoxifying functions *in vivo*. We use *misc-1* mutants to show that increased levels of ROS do not have negative effects on life span, contrary to current theories of aging. Using *misc-1* mutants as a model, we show that extended life span of some *C. elegans* mitochondrial mutants is dependent on upregulation of extra-mitochondrial pathways of energy production. In conclusion, we show that the metabolic protein MISC-1/OGC affects insulin secretion, mitochondrial dynamics, apoptosis and ROS detoxification. We propose that MISC-1/OGC integrates metabolic and cell survival decisions by its physical interaction with the apoptotic machinery.

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LIST OF ABBREVIATIONS

AGE: ageing alteration

AIF: apoptosis inducing factor

AKT: AKT kinase family

ANT: adenine nucleotide translocase

Apaf1: apoptotic protease activating factor

Bcl: B-cell lymphoma

BH: Bcl-2-homology domain

BMP: Bone Morphogenetic Protein

Ca^{2+}: calcium

cAMP: cyclic adenosine monophosphate

CARD: caspase recruitment domain

CED: cell death abnormality

CEP-1: *C. elegans* p53 orthologue

cGMP: cyclic guanosine monophosphate

CK: creatine kinase

Cyp-D: cyclophilin-D

DAF: abnormal dauer formation

DAPI: 4',6-diamidino-2-phenylindole

Drp: dynamin-related protein

DT: diphtheria toxin

EGL: egg-laying defective

ETC: electron transport chain

FOXO: forkhead box O

G_{α}: G-protein, α subunit

GPA: G-protein, α subunit (*C. elegans* protein class)

GPCR: G-protein coupled receptor

GSH: glutathione

HEK293: human embryonic kidney cell lines

HK: hexokinase

IGF1R: insulin growth factor 1 receptor

IIS: insulin/insulin-like signalling

IP3: inositol-3 phosphate

L1-L4: the four larval stages during *C. elegans* development

LET-60: *C. elegans* K-Ras

LIN-35: *C. elegans* homologue of human Retinoblastoma protein

MAD: mothers against Decapentaplegic

mBzR: mitochondrial benzodiazepine receptor

MIN6: mouse insulinoma cell lines

MISC-1: mitochondrial solute carrier

Mit: mitochondrial mutants

Mfn: mitofusins

MOMP: mitochondrial outer membrane permeabilization

MPTP: mitochondrial permeability transition pore

N2: wild-type *C. elegans* strain

NaN_3: sodium azide

NHR: nuclear hormone receptor

OGC: 2-oxoglutarate carrier

OPA1: optic atrophy 1

PDK: 3-phosphoinositide-dependent kinase

PI3K: phosphatidylinositol 3-kinase

Rb: retinoblastoma

RNAi: RNA interference

ROS: reactive oxygen species

SAGE: serial analysis of gene expression

sCPX: sterol carrier protein X

SGK: serum- and glucocorticoid-inducible kinase homologue

shRNA: short hairpin RNA

siRNA: small interfering RNA

SOD: superoxide dismutase

SolCar: solute carrier domain

TAX: abnormal chemotaxis

TEM: transmission electron microscopy

TM-GC: transmembrane guanylate cyclase

TGF-β: transforming growth factor-β

VDAC: voltage-dependent anion channel

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DEDICATION

To my Grandfathers,

Who would have loved to see this day

CO-AUTHORSHIP STATEMENT

I wrote all the chapters contained in this thesis. My supervisor, Dr. Riddle, and the collaborators listed in the authors' line in each chapter, provided important comments and editorial changes.

I performed all the work described in this thesis, with a few exceptions.

(i) Katarzyna Kida and Oliver E. Blacque performed the electron transmission microscopy experiments described in Chapter 2 (Figure 2.3).

(ii) Dan S. Luciani and James D. Johnson performed the shRNA experiments described in Chapter 2 (Figure 2.4C). However, I performed the Western blot represented in Figure 2.4C.

(iii) Ferdinando Palmieri developed the antibody against human OGC. I used this antibody for the Western blot represented in Figure 2.5.

(iv) Donha Park did the work that resulted in Figure 2.7A-B.

Furthermore, I wrote half of the review article presented in Appendix 4, and the full review article in Appendix 5. Dr. Riddle made important editorial changes to both reviews.

1 INTRODUCTION

1.1 The dauer diapause stage in *Caenorhabditis elegans*

The life cycle of the nematode *C. elegans* includes embryogenesis, four larval stages (L1 to L4) and the adult stage. Transition between larval stages is marked by molting of the cuticle (Singh and Sulston, 1978). It was noted that adverse environmental conditions lead to the formation of an alternative L3 stage, called dauer (from the German word for 'enduring') (Cassada and Russell, 1975).

The dauer larva is in a diapause state optimized for dispersal to environments with better conditions. These animals can live for months without feeding (Klass and Hirsh, 1976) and move only if subjected to stimulus. Upon entry into the dauer stage, several morphological changes occur (reviewed in Riddle and Albert, 1997). The composition of the cuticle changes and becomes less permeable, probably to confer resistance to desiccation or environmental insult. Second, the larvae become radially constricted with the typical 'thin' appearance, compared to other larvae. Third, the buccal cavity is closed. This blockage prevents food from entering the mouth. The dauer larva is effectively isolated from the external environment, if it were not for its amphid sensilla. The *C. elegans* amphids, which comprise six pairs of bilaterally symmetrical ciliated neurons (Ward et al., 1975; Ware et al., 1975), are the only chemosensory organs that are still exposed to the external environment through pores in the cuticle. The ciliated neurons detect signals from the environment and are pivotal players in the decision to enter and exit the dauer stage.

Some of these ciliated neurons have been shown in cell ablation studies and in cilia-defective mutants to be essential for dauer entry, while others are required for dauer exit (Albert et al., 1981; Bargmann and Horvitz, 1991). It has been proposed that some of these neurons detect a constitutively produced pheromone, which is used by the animals as a measure of population density (Golden and Riddle, 1984). When population density is too high (high pheromone concentration), these dauer-inducing ciliated neurons activate gene networks that

lead to dauer formation. On the contrary, other ciliated neurons function by detecting environmental signals that are conducive to reproductive development.

1.2 The dauer pheromone

C. elegans constitutively secretes a pheromone, which is sensed by the worms as a measure of population density (Golden and Riddle, 1984). When population density becomes too high, and consequently pheromone concentration increases beyond a certain threshold, L1 worms make a decision that will lead to dauer formation. This pheromone is called dauer pheromone.

The dauer pheromone was initially identified as a mixture of hydroxylated fatty acids reminiscent of bile acids (Golden and Riddle, 1984). Golden and Riddle determined by thin layer chromatography that the dauer pheromone contains at least three different chemical species. It was also shown that the dauer pheromone competes with a 'food signal' produced by the bacteria, on which *C. elegans* feeds, to induce dauer entry (Golden and Riddle, 1982).

However, the chemical components of the dauer pheromone have not been identified until recently (Jeong et al., 2005; Butcher et al., 2007; Butcher et al., 2008; Srinivasan et al., 2008). These components were purified and their chemical structures determined. All the pheromone components are glycosides of the dideoxysugar ascarilose and have been named dauer ascarosides.

Mutants with defective sensory cilia or defective amphidial pores are unable to respond to the pheromone (Golden and Riddle, 1984; Vowels and Thomas, 1994). It was therefore concluded that the dauer pheromone is sensed by the ciliated neurons. The signal transduction pathway initiated by the pheromone in the ciliated neurons was partially elucidated, mainly at the genetic level.

In different species, from insects to mammals, pheromones act by binding to G-Protein Coupled Receptors (GPCRs) located on sensory neurons (Buck and Axel, 1991). GPCRs are localized to the plasma membrane and are characterized by seven transmembrane domains, with an extracellular N-terminus and a cytoplasmic C-terminus [reviewed in (Buck, 2000)]. They

are the most important class of molecules mediating the effects of neurotransmission, including odorant and visual cues. It is known that binding of a pheromone ligand to its cognate GPCR elicits conformational changes that lead to recruitment of G-protein subunits. Activated G-protein subunits then relay the GPCR signal by activating downstream neuronal effectors. Rhodopsin is one of the best established examples of GPCR-G-protein interaction in mammals. In this case, photons activate Rhodopsin, which then recruits and activates a G-protein α subunit. This G_α protein then activates cAMP signalling. Similarly, it was suggested that two G-protein α subunits, GPA-2 and GPA-3, relay the signal from the dauer pheromone receptor to the Trans-Membrane Guanylate Cyclase (TM-GC) DAF-11 in *C. elegans* (Zwaal et al., 1997). DAF-11 ultimately becomes inhibited and the consequent drop in cGMP levels induces dauer formation. GPA-2 and GPA-3 are partially redundant in their function. Over-expression of GPA-2 and GPA-3 makes worms more sensitive to dauer pheromone extracts, while knock-out mutants in each gene or in both genes have severely reduced abilities to respond to pheromone and enter the dauer stage.

Although the backbones of the signal transduction pathway downstream of the dauer pheromone receptor have been partially determined, the molecular identity of the pheromone receptor itself is currently not known. Attempts at identifying this molecule have been hampered by the fact that the *C. elegans* genome encodes over 700 GPCRs (Bargmann, 1998) and that the pure components of the dauer pheromone have not been isolated until recently.

Since 2005, nine components of the dauer pheromone have been identified and synthesized by several groups (Butcher et al., 2007; Butcher et al., 2008; Srinivasan et al., 2008). These molecules are ascarosides that differ in the length and chemical properties of their side chains. It was shown that these ascarosides differ in their efficiency at inducing dauer formation and that some of them act in a synergistic or additive fashion. It was recently determined that some of these ascarosides have other roles beside dauer induction. Some of them, for instance, also act as male attractants (Srinivasan et al., 2008) and another one is produced under specific conditions of temperature (Butcher et al., 2008).

1.3 Signal transduction pathways involved in dauer formation

Several mutagenesis screens were performed to isolate animals that could be classified into two classes of abnormal dauer entry phenotypes. Mutants in one class enter the dauer stage constitutively, irrespective of favourable environmental conditions, a phenotype called Daf-c (Dauer formation constitutive). Mutants in the other class are unable to enter the dauer stage, a phenotype called Daf-d (Dauer formation defective). Daf-c and Daf-d mutants were ordered in genetic pathways through epistasis analyses involving crosses between a temperature sensitive *daf-c* and a non-conditional *daf-d* mutant.

These genetic studies placed the Daf mutants into three distinct signal transduction pathways: Insulin/Insulin-Like signalling (IIS), Transforming Growth Factor-β (TGF-β) signalling and cyclic nucleotide signalling (Figure 1.1; [Riddle et al., 1981;Vowels and Thomas, 1992; Thomas et al., 1993; Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Grenache et al., 1996]).

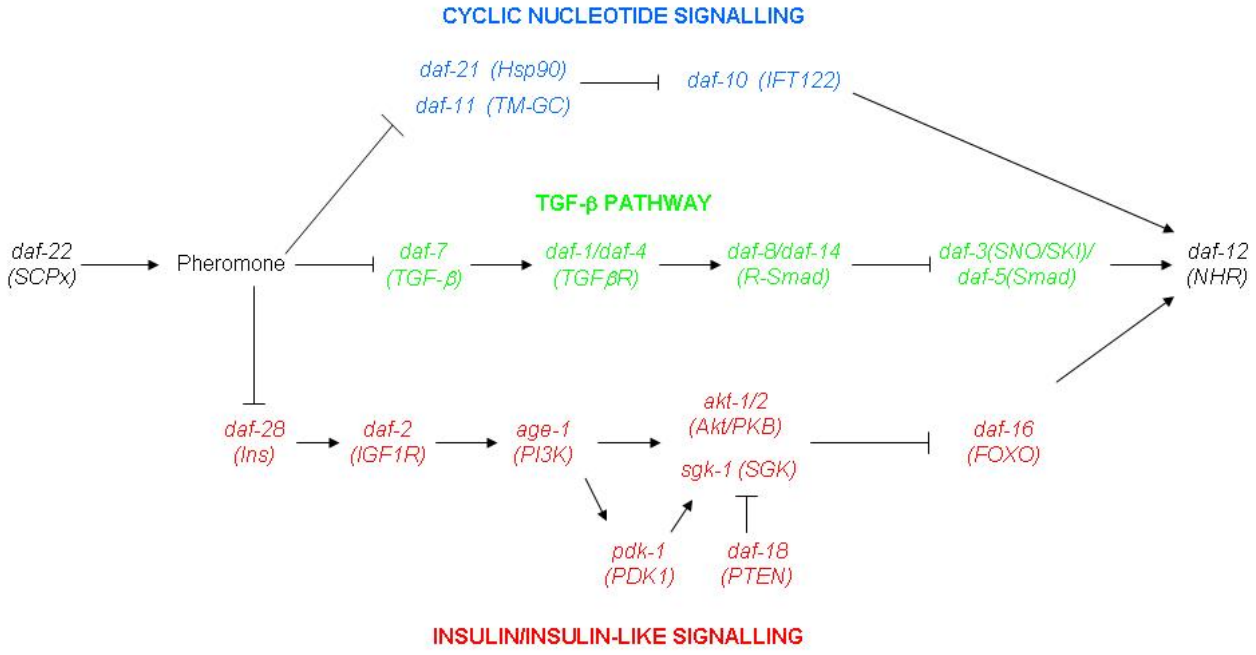

Figure 1.1 Signalling pathways involved in dauer formation. Three major signalling pathways control dauer formation: cyclic nucleotide signalling (in blue), the TGF-β pathway (in green) and the Insulin/Insulin-like Signalling (IIS) pathway (in red). Most genes involved in dauer formation have mammalian orthologues (in brackets). Genetically, these three pathways are downstream of *daf-22*, which is involved in pheromone biosynthesis, and upstream of *daf-12*, a gene encoding a Nuclear Hormone Receptor (NHR).

1.3.1 The Insulin/Insulin-like Signalling pathway

The IIS pathway is remarkably conserved with the mammalian insulin pathway [reviewed in (Christensen et al., 2006)]. *daf-2* encodes the *C. elegans* Insulin Receptor/Insulin Growth Factor 1 Receptor [IR/IGF1R; (Kimura et al., 1997)]. Several hypomorphic alleles exist for this gene and all result in Daf-c phenotypes. Complete loss-of-function alleles for this gene have a lethal phenotype, similarly to loss-of-function mutations of mammalian *IGF1R* (Liu et al., 1993; Gems et al., 1998). DAF-2 is a plasma membrane receptor with tyrosine kinase function. Upon activation by insulin-like ligands, DAF-2 acts by phosphorylating and activating its downstream target AGE-1, which is a Phosphatidylinositol 3-Kinase [PI3K; (Morris et al., 1996)]. Activation of AGE-1 leads to the production of Phosphatidylinositol-3,4,5-triphosphate (IP3), which acts as an activator of PDK-1 [3-Phosphoinositide-Dependent Kinase; (Paradis et al., 1999)]. However, IP3 also activates the PTEN protein DAF-18 (Ogg and Ruvkun, 1998), which inhibits AGE-1 in a negative feedback loop. PDK-1 phosphorylates and activates the downstream Serine/Threonine kinases AKT-1, AKT-2 (Paradis and Ruvkun, 1998) and SGK-1 (Hertweck et al., 2004). These kinases are able to phosphorylate and inhibit the ultimate effector of IIS signalling, the FOXO (FOrkhead boX O) transcription factor DAF-16 (Ogg et al., 1997). Phosphorylated DAF-16 is retained in the cytosol and cannot therefore perform its activating and repressive functions with respect to its target genes.

In summary, binding of insulin-like ligands to DAF-2 induces IIS, which results in DAF-16 inhibition and reproductive growth. Environmental conditions or mutations that abrogate IIS upstream of *daf-16* result in constitutively active DAF-16 and a Daf-c phenotype.

1.3.2 The TGF-β pathway

The first gene in the TGF-β pathway to be cloned was *daf-1*, which encodes a type I receptor (Georgi et al., 1990;Kingsley, 1994; Wrana et al., 1994), based on the presence of a GS domain similar to the phosphorylation site of the mammalian type I TGF-β receptor. Later it was found that *daf-4* encodes a type II TGF-β receptor (Estevez et al., 1993). DAF-1 and DAF-4 have

protein serine/threonine domains and by homology with their mammalian counterparts are thought to bind to each other to form functional heterodimers. The type II TGF-β receptor binds its cognate ligand, recruits the type I receptor and activates it through phosphorylation. The activated type I receptor then activates the downstream signalling cascade by phosphorylating its targets.

Activation of the TGF-β receptor complex in *C. elegans* results in the phosphorylation of the Receptor Smads (R-Smads) DAF-8 (Park et al., 2010) and DAF-14 (Inoue and Thomas, 2000). R-Smads were originally discovered in *C. elegans* and *Drosophila melanogaster* and named Sma (for the small phenotype caused by mutations in some of these proteins) and MAD (Mothers Against Decapentaplegic), respectively. The name Smad was obtained from the fusion of the names Sma and MAD. Activated R-Smads form oligomers and migrate to the nucleus, where they participate in the transcriptional activation or repression of target genes. It is believed that DAF-8 is required by DAF-14 for proper transcriptional control of target genes, because DAF-14 lacks a DNA binding domain. Mutations in *daf-1*, *daf-4*, *daf-8* and *daf-14* result in abrogation of TGF-β signalling and Daf-c phenotypes.

On the contrary, mutations in *daf-3*, which encodes a co-Smad-like protein (Patterson et al., 1997), and *daf-5*, which encodes a co-factor of DAF-3 with SKI/SNO domains (da Graca et al., 2004), result in Daf-d phenotypes. DAF-5/DAF-3 function is inhibited by DAF-8 and DAF-14.

Another important gene in the TGF-β pathway is *daf-7*, which encodes a TGF-β ligand (Ren et al., 1996). Mutations in this gene result in a Daf-c phenotype. DAF-7 has sequence similarity to Bone Morphogenetic Protein (BMP) and TGF-β. It was argued that a protein like DAF-7 could represent the common ancestor to both BMP and TGF-β ligands (Riddle and Albert, 1997).

daf-7 has a very restricted expression pattern. The gene is only expressed in the ASI ciliated neurons, which control dauer entry. It was shown that expression of a *gfp* reporter under control of the *daf-7* promoter declined in L2d animals that are destined to enter the dauer stage. *daf-7* is not expressed in dauer larvae. It was also observed that *daf-7* expression was

abrogated when animals were exposed to dauer pheromone extracts (Ren et al., 1996). The TGF-β pathway is therefore extremely interesting in relation to the study of the molecular pathways exploited by the dauer pheromone to induce dauer formation.

1.3.3 Cyclic nucleotide signalling

Several results implicate cyclic nucleotide signalling in dauer formation. Birnby et al., (2000) showed that mutations in the TM-GC *daf-11*, confer a Daf-c phenotype. This dauer constitutive phenotype was rescued by exogenous addition of Br-cGMP. Furthermore, mutations in *tax-2* and *tax-4* - which code for the α and β subunits, respectively, of a cyclic nucleotide gated ion channel (Coburn and Bargmann, 1996; Komatsu et al., 1996) – have weak Daf-c phenotypes.

The finding that cGMP signalling has a role in dauer formation led to the speculation that the dauer pheromone acts through this pathway. This hypothesis stemmed from the observation of a similar mechanism involved in mammalian visual transduction [reviewed in (Hurley, 1987)], in which the G-protein transducin mediates activation of a cGMP phosphodiesterase in response to activation of the GPCR Rhodopsin by light (Baehr et al., 1982) to cause membrane depolarization. Similarly, it was hypothesized that the putative dauer pheromone receptor GPCR might act via the G-proteins GPA-2 and GPA-3 to modulate the function of the TM-GC DAF-11, and consequently the function of the TAX-2/TAX-4 ion channel to cause membrane depolarization. Epistasis analyses indicate that the TGF-β and the cGMP signalling pathways are partially parallel (reviewed in Riddle and Albert, 1997).

1.3.4 Genes upstream and downstream of the major dauer pathways

Some genes have been identified that are either upstream or downstream of IIS, TGF-β and cyclic nucleotide signalling pathways. The gene upstream of all these pathways is *daf-22*, which was originally identified for its putative involvement in the synthesis of the dauer pheromone (Golden and Riddle, 1985). It was, in fact, observed that *daf-22* mutants have a Daf-d phenotype, but that this phenotype could be corrected by exogenous addition of dauer

pheromone extracts. It was also observed that although *daf-22* worms do not secrete dauer pheromone, a solution containing lysed mutants had dauer-inducing abilities (Riddle, personal communication). It was therefore speculated that *daf-22* encodes an enzyme involved in some step of dauer pheromone biosynthesis.

The molecular identity of *daf-22* was recently determined (Butcher et al., 2009). DAF-22 is the homologue of the human sterol carrier protein SCPx, which catalyzes the final step in peroxisomal fatty acid β-oxidation. *daf-22* mutants accumulate long-chain fatty acid derivatives with weak dauer-inducing function.

The three major dauer pathways seem to converge genetically on *daf-12*, which encodes a nuclear hormone receptor (Antebi et al., 1998; Antebi et al., 2000). Mutations in *daf-12* result in a Daf-d phenotype and are epistatic to all IIS and TGF-β Daf-c mutants, suggesting that the activity of this gene is absolutely required for entry into the dauer stage.

1.4 Some dauer-formation genes control life span

Dauer larvae can live for months without feeding. This basic observation led to studies that tried to link known genes involved in dauer formation and longevity. One of the first studies that were able to find such an association focused on *age-1* (Morris et al., 1996). *age-1* encodes a phosphatidylinositol-3-OH kinase and its mutations confer a Daf-c phenotype. Mutants in this gene have an adult life span more than twice as long as wild type.

Other groups showed that hypomorphic alleles of *daf-2* confer longevity as well, with some alleles inducing a two-fold increase in maximum adult life span (Kenyon et al., 1993; Gems et al., 1998).

These studies suggested that regulation of insulin signalling might be important for the regulation of longevity not only in dauer larvae, but also in adults. Studies in other model organisms seem to corroborate these findings. Mutations in the *Drosophila* gene *chico*, which encodes an insulin receptor substrate, increase life span (Clancy et al., 2001). Homozygous mutants have a 48% extension of median life span, while heterozygotes have a 36% increase in

median life span. Similarly, studies in mouse showed that female animals heterozygous for mutations in *IGF1R*, the homologue of *daf-2*, had a 33% increase in life span (Holzenberger et al., 2003). However, male heterozygotes did not have a significantly increased life span, compared to wild type litter mates.

The apparent importance of the IIS pathway in life span control sparked a flurry of studies that focused on whole genome gene expression changes in mutants with compromised insulin signalling. Some of these genomic studies focused on *C. elegans* mutants carrying *daf-2* alleles and analyzed global gene expression changes by either micro array (Murphy et al., 2003) or SAGE [Serial Analysis of Gene Expression; (Halaschek-Wiener et al., 2005; Ruzanov et al., 2007; Ruzanov and Riddle, 2010)]. These studies highlighted the importance of IIS in regulating a wide range of metabolic functions. Specifically, mutations in *daf-2* seem to decrease overall expression of metabolic genes, although pathways involved in fat homeostasis were up-regulated. These studies also revealed the wide range of cellular functions regulated by the main effector of the IIS pathway, the transcription factor DAF-16. These genomic studies, together with other genetic analyses, suggested that IIS might regulate life span through the repression of general metabolic processes, including protein translation, and of germline proliferation.

Although the role of IIS in modulation of longevity is becoming well established and seems to be phylogenetically conserved, a novel role for the TGF-β pathway in life span regulation is also emerging. Shaw et al., (2007) found that mutations in some genes in the TGF-β pathway, notably *daf-1*, *daf-4*, daf-7, *daf-8* and *daf-14*, extend adult life span. However, these data seem to contradict results from other laboratories (Larsen et al., 1995; Donha Park, personal communication).

Some evidence also exists regarding a possible role of cyclic nucleotide signalling in longevity (Hahm et al., 2009). It was shown that mutations in the TM-GC gene *daf-11* or constitutively active forms of the G-protein α-subunit GPA-3 have a positive effect on life span. Interestingly, the proposed positive effects of mutations in the TGF-β and cGMP pathways on

life span require DAF-16 (Shaw et al., 2007; Hahm et al., 2009), the IIS effector. Once again, these data suggest that insulin signalling may play a pivotal role in the determination of organismal life span and that the other dauer pathways regulate longevity by repressing the IIS.

1.5 Longevity in mitochondrial mutants

Aging studies in *C. elegans* have shown that several nuclear genes encoding mitochondrial proteins can modulate life span. An RNAi (RNA interference) screen targeting over 5900 genes indicated that mitochondria may play an important role in determining life span (Lee et al., 2003). Specifically Lee *et al.* found that RNAi against genes encoding components of the Electron Transport Chain (ETC) complexes I and II, two mitochondrial carriers and other mitochondrial enzymes resulted in extended life spans. Other groups employed RNAi knock-down techniques and mutants to show that reducing the function of genes encoding components of the ETC (*nuo-2*, complex I; *cyc-1*, complex III; *cco-1*, complex IV) or the ATP synthase (*atp-3*) can increase life span (Dillin et al., 2002; Rea et al., 2007). Interestingly, these groups found that knock-down of gene expression was effective at increasing life span only if it occurred during larval development and they identified the critical window of gene inactivation to be between the L3 and L4 stages. Using an RNAi dilution strategy, Rea et al. (2007) showed there exists an optimal level of gene knock-down conducive to longevity, following a bell-shaped curve. Any stronger or weaker knock-down led to either deleterious or no effect on life span. These *C. elegans* studies suggested that careful manipulation of mitochondrial function could have beneficial effects on aging. However, disruption of the ETC in *Drosophila* did not increase life span (Rera et al., 2010). RNAi treatments targeting ETC genes were able to further increase the life span of *daf-2* mutants (Dillin et al., 2002), leading to speculations that DAF-16 may be mediating this effect. However, the mechanism(s) by which mitochondrial function controls life span is not understood.

One possible explanation for the longevity of mitochondrial mutants is that decreased mitochondrial function may also decrease the levels of Reactive Oxygen Species (ROS). It has

been postulated that a reduction in ETC function would lead to a consequent reduction in ROS production and cellular damage and hence favour longevity. ROS modify proteins and DNA with generally deleterious effects. This hypothesis is called the free radical theory of aging (Harman, 1956). Another possible explanation for the longevity of ETC mutants is that they may cause a decrease in metabolic rate (Branicky et al., 2000), according to the rate-of-living theory. As seen in *daf-2* mutants, reductions in metabolism are associated with longevity. Furthermore, it was shown with different dietary regimens and several feeding (*eat*) mutants that dietary restriction induces longevity in different organisms, from *C. elegans* to mammals [reviewed in (Koubova and Guarente, 2003)]. It seems therefore plausible that a reduction in mitochondrial function may increase life span by reducing oxidative phosphorylation and thus metabolic rate. It has been proposed that the oxidative stress and the rate-of-living theories of aging might be linked, because it was noticed that caloric restriction reduced oxidative damage (Sohal and Weindruch, 1996)

However, the oxidative stress and the rate-of-living theories of aging do not seem to explain the longevity phenotypes of all mitochondrial mutants. First of all, not all ETC mitochondrial mutants have decreased ROS levels. This conclusion was reached by indirectly assessing ROS production in mutant worms by exposing animals to a chemical (paraquat or juglone) that generates ROS. The assumption is that animals with higher basal levels of ROS are more sensitive to exogenously induced ROS production and die faster than controls in a survival experiment. The oxidative stress theory of aging would predict that ETC mutants have lower ROS levels and/or better ROS detoxification mechanisms and therefore would be more resistant to paraquat. Although some mitochondrial mutants are indeed more resistant to ROS production, this is not true for some of them. Also, no increase in protein oxidation was detected in some ETC mutants (Rea et al., 2007). Finally, it was shown that blocking ETC by inhibiting one of its complexes actually results in increased ROS production (Brand, 2000).

Also against the oxidative stress theory of aging, some laboratories generated knock-out strains carrying deletions in one or more of the five *C. elegans sod* (Superoxide Dismutase)

genes (Yang and Hekimi, 2010; Honda et al., 2008; Doonan et al., 2008; Van Raamsdonk et al., 2010). *sod* genes encode proteins that are responsible for the dismutation reaction of the reactive oxygen species O_2^- to H_2O_2 and are therefore involved in an important detoxification reaction [reviewed in (Fridovich, 1995)]. It is assumed that *sod* gene knock-outs have increased levels of ROS. Surprisingly, these single or multiple knock-out animals have normal life spans. These experiments provide further indirect evidence that ROS levels might not be negatively associated with longevity, as postulated by the oxidative stress theory of aging.

The only direct measurement of ROS levels in long-lived mitochondrial mutants was performed by Artal-Sanz and Tavernarakis, (2009). These authors showed that down-regulation of two genes encoding the two subunits of *C. elegans* prohibitin (*phb-1* and *phb-2*) decrease life span in wild type, but increase life span of *daf-2* animals and some TGF-β mutants. Prohibitin is a large protein complex that assembles in the inner mitochondrial membrane and has been implicated in several cellular processes, from mitochondrial biogenesis to cell death and replicative senescence. This group showed that longevity conferred by *phb-1* and *phb-2* knock-down was accompanied by lower ROS levels than in control animals. More direct measurements of ROS levels in long-lived mitochondrial mutants are required to better understand the association between longevity and oxidative stress.

In addition to ETC genes and prohibitin, other genes involved in mitochondrial function have been linked to longevity in *C. elegans*. One of them is the frataxin homologue *frh-1*. Mutations in frataxin lead to the autosomal recessive neurological disorder Friedreich ataxia. The effect of *frh-1* on life span is controversial. Two separate groups generated *frh-1* knock-down systems. While one group found that *frh-1* RNAi by feeding increased life span (Ventura et al., 2005), the other found that injecting dsRNA targeting *frh-1* decreased life span (Vazquez-Manrique et al., 2005). These discrepancies might be due to the different levels of gene knock-down achieved by the two groups, since the life span effects of *frh-1* knock-down seem to be dependent on the degree of knock-down (Rea et al., 2007), as is the case for other mitochondrial genes.

Although there is a plethora of data pointing to positive effects of mitochondrial mutations on life span in *C. elegans*, the scenario is quite different in other organisms. For instance, no association between ROS levels and longevity was found in *Drosophila* (Miwa et al., 2004). Knock-down of orthologues of ETC genes that confer longevity in *C. elegans* did not extend life span in *Drosophila* (Rera et al., 2010). Interestingly, mutations in ETC genes, or frataxin, cause severe disease phenotypes in mammals (Wallace, 1999). These findings suggest that the life-prolonging effects of mitochondrial dysfunction might be specific to *C. elegans*.

The reasons for the apparent difference between *C. elegans* and other organisms are not clear. It was proposed that *C. elegans* can utilize extra-mitochondrial pathways of energy production and could therefore survive with partially non-functional mitochondria (Rea and Johnson, 2003), but data to uphold this hypothesis are lacking. It has been observed, however, that *gei-7*, the only *C. elegans* gene involved in the glyoxylate shunt (Tsuboi et al., 2002), is up-regulated in dauer larvae and some long-lived mutants, like *daf-2* (Wadsworth and Riddle, 1989; Ruzanov et al., 2007). The glyoxylate shunt is used to synthesize carbohydrates from fat stores that are mobilized by β-oxidation, and provides a mechanism to bypass the Krebs cycle in the mitochondria. *gei-7* encodes a malate synthase/isocitrate lyase enzyme, which cleaves isocitrate to form glyoxylate and succinate. Succinate can then be used as a substrate for gluconeogenesis. Once carbohydrates are made, glycolysis yields 2 ATP molecules that can be expended by the cell. The glyoxylate shunt therefore allows modest energy production even in the absence of functional mitochondria. The shunt is known to be functional only in bacteria, plants and *C. elegans*, but not in mammals. Further studies are needed to elucidate the role of the glyoxylate shunt in *C. elegans* longevity.

1.6 Mitochondria and apoptosis in *C. elegans*

Mitochondria are not only implicated in life span regulation, but they also play a role in other major cellular processes such as apoptosis.

C. elegans is an established model for the study of apoptosis, also known as programmed cell death, a term that was coined to differentiate this process of regulated cell death from necrosis (Kerr et al., 1972). Studies in this model organism revealed the stereotyped patterns of cell death that occur during nematode development. In fact, of the 1090 cells generated during development of the hermaphrodite, 131 undergo apoptosis (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983), and their cell lineage has been carefully mapped. Most of the dying cells are of neuronal and ectodermal origin and apoptose during embryogenesis, but some endodermal cells (muscle and pharyngeal gland cells, 18 cells out of 131 in total) undergo apoptosis by the L2 stage (Hengartner, 1997).

While the post-mitotic soma does not undergo apoptosis in adults, the actively proliferating germline cells of hermaphrodites undergo extensive programmed cell death. The reason for this is not clear, but some hypothesized that the dying cells act like nurse cells do in *Drosophila*, by way of donating their cytoplasm to other proliferating cells (Gumienny et al., 1999). Germline apoptosis offers the tools to assess the effects of specific mutations on adult rates of apoptosis.

In wild-type germlines, oogenic cells can undergo apoptosis only in the gonadal loop region, where cells exit the pachytene stage of meiotic prophase and enter diplotene. These germline “cells” are not fully enclosed by membranes, but they share the cytoplasm of the germline syncytium by way of cytoplasmic bridges. The study by Gumienny et al. (1999) showed that apoptosis occurs normally in wild-type germlines, usually in the number of two corpses per gonadal arm. This is similar to what happens during human oocyte production.

Apoptosis is under genetic control. Genetic screens identified over one hundred genes that participate in some branch of the apoptosis pathway (Sulston, 1976; Hedgecock, 1983; Ellis and Horvitz, 1986; Ellis et al., 1991; Hengartner and Horvitz, 1994). Genes participate in apoptosis at four specific stages: (a) the decision to apoptose, (b) the execution of apoptosis, (c) the engulfment of the cell corpse by a neighbouring cell and (d) degradation of the cell corpse. Mutants affecting all four stages have been recovered and placed in genetic pathways by means of epistatic analyses.

Epistatic analyses placed *C. elegans* apoptosis genes in three major pathways: (i) the physiological pathway, mediated by the Retinoblastoma (Rb)-like protein LIN-35 (Schertel and Conradt, 2007); (ii) the DNA damage pathway, mediated by the p53 protein CEP-1 (Schumacher et al., 2005a); and (iii) the physiological cytoplasmic stress pathway, mediated by Ras (Gumienny et al., 1999).

These three pathways all converge on the core apoptotic machinery, composed of the Bcl-2-family protein CED-9, the Apaf1 protein CED-4 and the effector caspase CED-3. It was actually thanks to the discovery of the linear apoptotic pathway composed of CED-9, CED-4 and CED-3 (Hengartner et al., 1992; Hengartner and Horvitz, 1994) that the anti-apoptotic role of mammalian Bcl-2 (Vaux et al., 1988) was found (Vaux et al., 1992). It was also shown that Bcl-2 is able to partially correct for the deficit of CED-9 in *C. elegans* mutants (Vaux et al., 1992). However, the role of mitochondria in apoptosis was not clear yet at this point. All that was known was that Bcl-2 localized to the mitochondrial membrane in mammalian cells [reviewed in (Wang, 2001)]. A clearer picture started to emerge when it was noticed that *Xenopus laevis* oocyte extracts could trigger apoptosis only when the membrane fraction was enriched in mitochondria (Hockenbery et al., 1990; Newmeyer et al., 1994). Several groups then showed that proteins that normally reside in the intermembrane space of mitochondria can induce apoptosis in mammalian cells (Liu et al., 1996; Susin et al., 1999; Du, 2000;Verhagen, 2000; Li et al., 2001). In the presence of apoptotic stimuli, these proteins translocate to the cytoplasm or the nucleus, where they induce activation of caspases or DNA fragmentation.

1.7 Mitochondrial proteins control apoptosis

Cytochrome c, which normally participates in the ETC by shuttling electrons between complex III and IV, can induce apoptosis in mammalian systems. Cytochrome c localizes to the inner mitochondrial membrane, where it interacts with the mitochondrion-specific membrane lipid cardiolipin (Ott et al., 2002; Kagan et al., 2005). Upon apoptotic stimuli, cytochrome c exits the mitochondria and enters the cytosol, where it interacts with its target protein Apaf1. Apaf1 is a

cytosolic protein with a Walker's domain for nucleotide binding, multiple WD-40 domains (Zou et al., 1997) and a Caspase Recruitment Domain (CARD). Interaction of cytochrome c with Apaf1 increases its affinity for ATP 10-fold. Binding of nucleotides induces conformational changes in Apaf1 that facilitate its oligomerization. Heptameric Apaf1 (Acehan et al., 2002; Yu et al., 2005) and cytochrome c form the apoptosome (Zou et al., 1999). The configuration of the apoptosome exposes the Apaf1 CARD domains, which recruit pro-Caspase-9 and induce its autoactivation by proteolytic cleavage. Apoptosome-bound Caspase-9 then activates downstream effector caspases like Caspase-3 (Rodriguez and Lazebnik, 1999). Effector caspases then cleave their cellular targets to reproduce the hallmarks of apoptosis. Although the role of cytochrome c in mammalian apoptosis is well established, its involvement in *C. elegans* apoptosis has not been shown. This is probably because the *C. elegans* orthologue of Apaf1, CED-4, does not possess a cytochrome c binding domain (Zou et al., 1997). Instead, in its inactive form, CED-4 dimers are bound to CED-9 and localize to the outer mitochondrial membrane. It is only upon apoptotic insults that the BH3-only Bcl-2-like protein EGL-1 interacts with the CED-9/CED-4_2 complex, freeing CED-4 of its inhibition (Figure 1.2). The role of cytochrome c in apoptosis might therefore be a relatively recent adaptation that occurred in higher eukaryotes. Active dimeric CED-4 forms an octameric apoptosome, whose funnel-shaped structure can accommodate two pro-CED-3 molecules (Qi et al., 2010). As in mammals, the *C. elegans* apoptosome induces the autocatalytic processing of downstream caspases (in this case CED-3). It has recently been shown that physical interaction of CED-3 with the apoptosome greatly enhances CED-3 function (Qin et al., 2010).

On the contrary, the role of the Apoptosis Inducing Factor [AIF; (Susin et al., 1999)] in apoptosis is fully conserved from nematodes to mammals. AIF and its *C. elegans* homologue WAH-1 [Worm AIF Homologue; (Wang et al., 2002)] localize to the intermembrane space, where they are anchored by means of the insertion of their N-terminal transmembrane domain to the inner mitochondrial membrane. Apoptotic stimuli induce the proteolytic cleavage by calpain and activation of AIF/WAH-1, which can then escape from the inner mitochondrial space

via the pores opened in the outer mitochondrial membrane by Mitochondrial Outer Membrane Permeabilization (MOMP). AIF/WAH-1 then migrates to the nucleus, where it is required for DNA fragmentation (hence the DNA laddering characteristic of programmed cell death). In *C. elegans*, it was shown that WAH-1 can stimulate the activity of the lipid translocase scramblase SCRM-1, which is responsible for the translocation of phosphatidyl serine molecules – potent signals for eliciting the engulfment and the removal of cell corpses - to the extracellular domain of the plasma membrane (Wang et al., 2007).

Another mitochondrial protein that has a role in apoptosis is the endonuclease EndoG. EndoG is encoded by a nuclear gene and imported into mitochondria, where its physiological function is to remove the RNA primers required for mitochondrial DNA replication (Cote and Ruiz-Carrillo, 1993; Tiranti et al., 1995). However, there is evidence that not all EndoG proteins participate in this function. Some EndoG is not found in the mitochondrial matrix, but in the intermembrane space, and probably provides a readily available source of EndoG for apoptosis (Li et al., 2001; Wang, 2001). Upon mitochondrial damage, EndoG migrates to the nucleus, where it causes nucleosomal DNA fragmentation. The pro-apoptotic activities of both EndoG and AIF are independent of caspase activation. The *C. elegans* orthologue of EndoG was identified in a screen for suppressors of *ced-3* and was named CPS-6 [CED-3 Protease Suppressor; (Parrish et al., 2001)]. CPS-6 is also localized to mitochondria and *cps-6* mutants can be rescued by mouse EndoG.

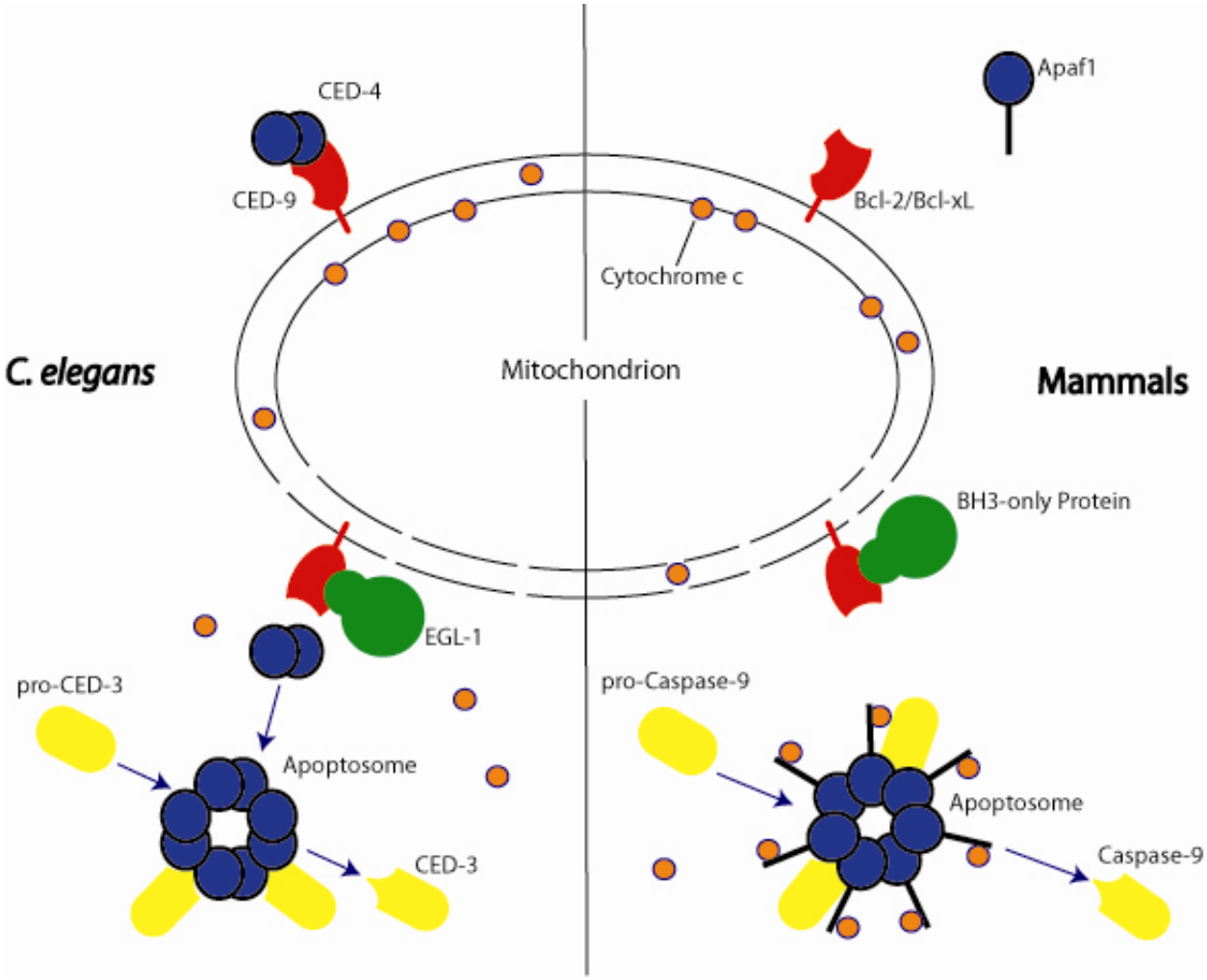

Figure 1.2 Comparison of mitochondria-mediated apoptosis in *C. elegans* and mammals. This diagram shows the mechanism of apoptosis initiation by the CED-9/Bcl-2-family protein, CED-4/Apaf1 and CED-3/Caspase-9. In *C. elegans*, dimeric CED-4 is kept in a quiescent state by its physical interaction with CED-9. Upon apoptotic stimuli, the BH3-only protein EGL-1 binds to CED-9 and causes release of CED-4, which is then free to homo-oligomerize and form the octameric apoptosome. Apoptosome formation is independent of cytochrome c, because CED-4 lacks the WD40 domains required for this interaction. The apoptosome converts pro-CED-3 into its active caspase form CED-3. Similarly, in mammals Bcl-2 and Bcl-x_L have anti-apoptotic functions. Upon apoptotic stimuli, BH3-only proteins inhibit Bcl-2/Bcl-x_L, mitochondrial fragmentation occurs and cytochrome c exits the mitochondria. Cytochrome c binds to monomeric Apaf1 and activates it, allowing it to form the heptameric mammalian apoptosome. The apoptosome activates Caspase-9. Orthologous proteins are drawn in the same colour.

1.8 Mitochondrial fusion and fission and their relevance to apoptosis

The involvement of cytochrome c, AIF and EndoG in apoptosis clearly demonstrates that mitochondria play a pivotal role in the induction of programmed cell death. However, the role of mitochondrial fragmentation in apoptosis is not clear. It is generally accepted that mitochondrial fragmentation is associated with apoptosis, but there is a lack of consensus on whether fragmentation precedes or follows initiation of the apoptotic program. Some studies showed that mitochondrial fragmentation is a prerequisite for MOMP and release of apoptogenic proteins from the mitochondria, but others disagree and even suggest that mitochondrial fragmentation inhibits MOMP [reviewed in (Arnoult, 2007)].

Mitochondria are dynamic organelles, continuously undergoing rounds of fusion and fission events. It has been hypothesized that these morphological changes are needed to regulate metabolites, Ca^{2+} and proton flux throughout the mitochondrial network and are therefore dependent upon the metabolic needs of the cell [reviewed in (Arnoult, 2007; Orrenius et al., 2003)]. In mammals, the equilibrium between fusion and fission events is maintained by dynamin-like GTPases, notably the dynamin-related protein Drp1, mitofusins (Mfn1 and Mfn2) and OPA1. Whereas Mfn1,2 and OPA1 mediate mitochondrial fusion, Drp1 is responsible for mitochondrial fission. It is usually localized to the cytosol, but it can be seen transiently associated with the mitochondrial outer membrane during fission events (Otsuga et al., 1998; Smirnova et al., 1998). Expression of a dominant-negative form of Drp1 inhibits mitochondrial fragmentation and cytochrome c release (Frank et al., 2001). At the same time, dominant-negative Drp1 or reduction of Drp1 by RNAi results in the production of long, inter-connected mitochondria (Smirnova et al., 2001; Lee et al., 2004). By contrast, Drp1 over-expression induces mitochondrial fragmentation in HeLa cells (Szabadkai et al., 2004). Although Drp1 can mediate mitochondrial fragmentation in HeLa cells, this is not sufficient to induce apoptosis. Drp1 over-expression was reported to result in increased cytochrome c release and caspase activation (Szabadkai et al., 2004), but another group using the same system did not observe an

increase in apoptotic rate (Karbowski et al., 2002). Its *C. elegans* orthologue, DRP-1 (Labrousse et al., 1999), is also involved in mitochondrial fission. So far, *C. elegans* is the only organism where it has been conclusively shown that DRP-1-induced mitochondrial fragmentation is sufficient to induce apoptosis (Jagasia et al., 2005).

Morphological changes in the outer mitochondrial membrane might not be all that is required for the release of apoptogenic factors. It was shown that profound changes in the inner mitochondrial membrane also occur prior to cytochrome c release (Scorrano et al., 2002). The inner membrane folds extensively into cristae. Cristae folds are *bona fide* compartments within mitochondria, being delimited by cristae junctions. Cristae junctions are very narrow, with a diameter of only ~18 nm, and effectively isolate the cristae from the intermembrane space (Scorrano et al., 2002). Cristae are the place where the ETC complexes are located and contain 80-85% of total cytochrome c. Scorrano and coworkers (2002) employed Transmission Electron Microscopy (TEM) and tomographic reconstructions to determine that apoptotic signals elicit profound changes in the inner mitochondrial membrane, widening the cristae junction to a mean diameter of ~57 nm and thus completely releasing cytochrome c into the intermembrane space. Here, cytochrome c can exit the mitochondria upon induction of MOMP. It was later shown that OPA1 is not only involved in mitochondrial fusion, but it is also associated with the inner mitochondrial membrane. Here, it regulates cristae junctions and is required for the complete release of cytochrome c (Arnoult et al., 2005; Frezza et al., 2006).

In conclusion, while it is still unclear what the role of mitochondrial fragmentation is in the chain of apoptotic events, it has been shown in different organisms that the mitochondrial fusion/fission machinery is involved in the induction of the apoptotic program.

1.9 The role of the Bcl-2 protein family in mitochondrial fragmentation and apoptosis

The members of the Bcl-2 family of proteins are involved in the regulation of programmed cell death. Proteins in this family can be grouped into three subfamilies based on the number of BH (Bcl-2 Homology) domains they contain [reviewed in (Lomonosova and Chinnadurai, 2009)].

The first subfamily includes the anti-apoptotic proteins Bcl-2, Bcl-x_L, Bcl-w, MCL-1 and A1/BFL-1, which contain four BH domains (*i.e.* BH1 to BH4). The second subfamily is comprised of the pro-apoptotic proteins Bax, Bak and Bok, which contain only three BH domains (BH1-3). Lastly, there are proteins possessing only the BH3 domain. The BH3-only subfamily comprises important cell death proteins like Bik, EGL-1, Bim, BMF, NOXA, Bid, Bad, BNIP3 and Beclin-1.

Some studies have provided evidence that members of the fusion/fission machinery interact with anti-apoptotic Bcl-2-family proteins. This is also the case in *C. elegans*. Conradt's group (Jagasia et al., 2005) showed that the BH3-only protein EGL-1 is able to induce mitochondrial fragmentation. However, mitochondrial fragmentation could be blocked by a gain-of-function mutation in *ced-9*, encoding the *C. elegans* homologue of Bcl-2. A gain-of-function allele of *ced-9* was also able to suppress the mitochondrial fragmentation phenotype conferred by *drp-1* over-expression. The authors therefore suggested that the Bcl-2 family of proteins might be directly involved in the regulation of mitochondrial fusion/fission events. These findings were later reinforced by the finding that CED-9 interacts with FZO-1 and EAT-3, the orthologues of the mitochondrial fusion machinery components Mfn1,2 and OPA1, respectively (Rolland et al., 2009). Furthermore, the authors also showed that CED-9 can mediate the fusion of both the inner and outer mitochondrial membranes.

The pro-apoptotic Bcl-2 family proteins do not seem to control mitochondrial fragmentation, at least directly, but some of them trigger MOMP in the outer mitochondrial membrane. The best example of MOMP induction is offered by the pro-apoptotic proteins Bax and Bak [reviewed in (Antignani and Youle, 2006)]. It has been proposed that upon pro-apoptotic stimuli, Bax and Bak change their conformation and oligomerize. These oligomers are predicted to form pores in the outer mitochondrial membranes, thereby inducing MOMP and allowing cytochrome c and other apoptogenic mitochondrial factors to be released in the cytosol. The formation of pores in the outer mitochondrial membrane by Bcl-2 proteins is still controversial, because the proposed pore has never been isolated and no biochemical information is available about it. However, two pieces of evidence seem to give weight to this

hypothesis. First, it was shown that Bax and Bak can form 2-10 large puncta on mitochondria upon apoptotic stimuli (Karbowski et al., 2002). Interestingly, these puncta co-localize with Drp1 and Mfn2, thereby linking MOMP with the fusion/fission machinery. Second, Bax, Bak and other Bcl-2 family members have protein structures reminiscent of Diphteria Toxin (DT). DT is a polypeptide secreted by *Corynebacterium diphtheriae* necessary for its pathogenesis. This polypeptide chain contains three domains: (i) an enzymatic killing domain, (ii) a cell surface receptor binding domain and (iii) a translocation (T) domain [reviewed in (Antignani and Youle, 2006)]. The T domain is inserted in membranes, where it forms aqueous pores permeable to molecules in the 3 to 10 kDa range (Sharpe and London, 1999).

The T domain of DT displays similar topology to α helices in Bcl-x_L and Bax (Muchmore et al., 1996; Suzuki et al., 2000). The fact that anti-apoptotic Bcl-2 and Bcl-x_L can form ion channels [reviewed in (Schendel et al., 1998)] suggests that these channels are not used for cytochrome c exit from mitochondria. The difference between pro- and anti-apoptotic activities of Bcl-2 family proteins might therefore lie in the specific differences in physico-chemical properties between the different channels/pores. Furthermore, it was reported that the membrane-inserted state of Bcl-2 is the preferred configuration for its inhibition of pro-apoptotic Bax (Dlugosz et al., 2006), which suggests that Bcl-2 and Bcl-x_L inhibit pore formation by Bax only when they are associated with membranes (Antignani and Youle, 2006). It has been proposed that release of apoptogenic factors from mitochondria may depend on the relative amounts of anti- and pro-apoptotic Bcl-2-family proteins in the outer mitochondrial membrane (Crompton, 1999).

Whereas the pro- and anti-apoptotic Bcl-2-family proteins are mostly membrane-bound, members of the BH3-only subfamily are not. These proteins have been placed upstream of the other two subfamilies with respect to initiation of the apoptotic program, but their exact mechanism of action is still not fully understood. Experimental results provide two possible models to explain the mechanism of action of BH3-only proteins. One model argues that BH3-only proteins directly bind and activate the Bax/Bak complex, while the other proposes that they

inhibit anti-apoptotic Bcl-2 family members and cause the displacement of Bax/Bak complexes (Gillissen et al., 2007). While in mammalian systems the debate is still ongoing, the mechanisms of action of the *C. elegans* BH3-only protein EGL-1 has been determined. EGL-1 binds to the Bcl-2 homologue CED-9 and causes its inhibition. CED-9 inhibition in turn releases the Apaf1 homologue CED-4 into the cytosol, thereby triggering a caspase cascade. Therefore, at least in the worm, it appears that BH3-only proteins function by inhibiting anti-apoptotic Bcl-2-family proteins. A similar mechanism has also been invoked for the other *C. elegans* BH3-only protein, CED-13 (Schumacher et al., 2005b). Interestingly, no BH3-only proteins have been identified in *Drosophila* (Clavería and Torres, 2003).

1.10 Involvement of Bcl-2-family proteins in the regulation of the mitochondrial permeability transition pore

Electron microscopy studies of mitochondria showed that there are contact sites between the outer and inner mitochondrial membranes, and these sites were called intermembrane junctions (Hackenbrock, 1968). It was later realized that these junctions are formed by protein complexes, including the Voltage-Dependent Anion Channel (VDAC) in the outer mitochondrial membrane (Ono and Tuboi, 1987; Brdiczka, 1991) and the Adenine Nucleotide Translocase (ANT) in the inner mitochondrial membrane (McEnery et al., 1992). These two proteins are involved in metabolism. VDAC (also known as mitochondrial Porin) forms large aqueous pores in the outer mitochondrial membrane to allow low molecular weight molecules to enter the intermembrane space. ANT catalyzes the exchange of cytosolic ADP for mitochondrial ATP [reviewed in (Crompton et al., 2002). VDAC and ANT can also interact with cyclophilin-D (CyP-D), which is the mitochondrial isoform of the peptidylprolyl *cis-trans* isomerase cyclophilin chaperone family (Halestrap et al., 1997; Woodfield et al., 1997; Crompton et al., 1998). The complex formed by VDAC, ANT and Cyp-D at membrane junctions is called the Mitochondrial Permeability Transition Pore (MPTP; Figure 1.3).

The MPTP has been implicated in cell death. It was originally identified as a mediator of necrosis following hypoxia-related injuries (Nazareth et al., 1991). It was later shown that the MPTP also has a role in apoptosis (Zamzami et al., 1995). The major functions of the MPTP during apoptosis are to regulate the concentration of matrix Ca^{2+}, pH, mitochondrial membrane potential and matrix volume [reviewed in (Bernardi et al., 1994; Crompton, 1999)]. It has been suggested that apoptotic signals, especially the increase in mitochondrial matrix Ca^{2+} concentration and ROS, can open the MPTP (Zoratti and Szabò, 1995). MPTP opening results in efflux of apoptogenic factors – including mitochondrial cytochrome c (Kantrow and Piantadosi, 1997) and AIF (Susin et al., 1996) – and changes in mitochondrial membrane potential. MPTP opening also results in influx of solvent into the mitochondria, with the consequent increase in mitochondrial volume and eventual membrane rupture (Crompton, 1999). It has been determined that the MPTP plays an important role in the modulation of programmed cell death, partially contributing to MOMP (Wolff et al., 2008).

The exact mechanism by which Cyp-D regulates the complex is still to be fully elucidated, but it was shown that this component of the MPTP mediates cell death (Schinzel et al., 2005). It was suggested that Cyp-D prevents apoptosis by repressing the activity of ANT (Schubert and Grimm, 2004). It was also hypothesized that Cyp-D provides a signal to the MPTP about the folding status of mitochondrial proteins. Creatine Kinase (CK), Hexokinase (HK) (Beutner et al., 1998) and the mitochondrial benzodiazepine receptor (McEnery et al., 1992; Hirsch et al., 1998) also interact with the MPTP. These three proteins contribute to the cell death-promoting functions of the MPTP. Identifying the other members or regulators of the MPTP is the subject of ongoing research.

Several members of the Bcl-2 family interact and/or regulate the activities of the MPTP (Marzo et al., 1998b). Bcl-2 was found to directly interact with Cyp-D (Eliseev et al., 2009), thereby suggesting that Bcl-2 can flip from the outer to the inner mitochondrial membrane to exert its anti-apoptotic function. Bcl-2 and Bcl-x_L were able to inhibit the opening of the MPTP in reconstituted liposomes. In the same system, fractionation experiments showed an

enrichment of the pro-apoptotic Bcl-2-protein Bax with the MPTP (Marzo et al., 1998b; Marzo et al., 1998a). This observation was interesting because Bax is known to disrupt mitochondrial membrane potential (Xiang et al., 1996), an activity mediated by the MPTP. However, a role for Bax in MPTP-dependent cytochrome c release is still a question for debate (Eskes et al., 1998).

On the other hand, the anti-apoptotic protein Bcl-x_L was shown to physically interact with members of the MPTP in co-immunoprecipitation experiments (Shimizu et al., 1999). It is therefore possible that the interaction of anti- or pro-apoptotic Bcl-2-family members with VDAC or other components of the MPTP may change the activation state of the channel and affect cell survival decisions.

The role of the MPTP in programmed cell death is conserved from mammals to *C. elegans*. Shen et al., (2009) recently showed that the MPTP is present in *C. elegans* and its components are highly conserved. WAN-1/ANT, CED-9/Bcl-2 and CED-4/Apaf1 physically bind to each other and reduction of *wan-1* expression by RNAi reduced the number of apoptotic corpses in the *C. elegans* germline. This group therefore showed that WAN-1, the *C. elegans* orthologue of ANT, directly participates in the execution of apoptosis and is a component of the phylogenetically conserved MPTP. Importantly, as in mammalian systems, the anti-apoptotic Bcl-2-like protein CED-9 controls the function of the MPTP. *C. elegans* is therefore an appropriate model system for the study of MPTP function.

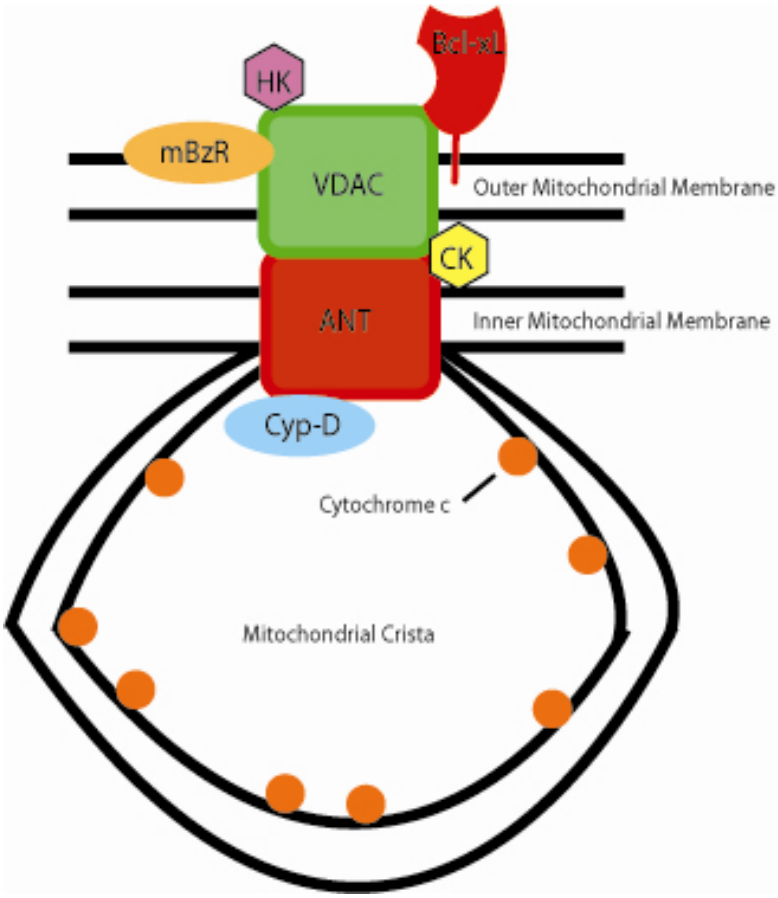

Figure 1.3 The mitochondrial permeability transition pore. The Mitochondrial Permeability Transition Pore (MPTP) is localized to the mitochondrial intermembrane junctions. VDAC in the outer membrane and ANT in the inner membrane are core components of the MPTP. However, some proteins have been found to have regulatory effects on the MPTP. Some of them are Bcl-x_L, Bcl-2, Cyclophilin-D (Cyp-D), Creatine Kinase (CK), Hexokinase (HK) and the mitochondrial Benzodiazepine Receptor (mBzR). In the presence of apoptotic stimuli, the MPTP channel opens and allows pro-apoptotic factors, like cytochrome c, to exit the cristae into the intermembrane space and eventually to the cytoplasm.

1.11 The 2-oxoglutarate carrier

This thesis describes the role of the *C. elegans* 2-oxoglutarate carrier in dauer formation, insulin signalling, mitochondrial function and apoptosis. The mammalian 2-oxoglutarate carrier (OGC, OMIM 604165) was originally purified from pig heart on the basis of its ability to exchange 2-oxoglutarate (also known as α-ketoglutarate) upon reconstitution in liposomes (Bisaccia et al., 1985). OGC is a 31.5 kDa mitochondrial protein that localizes to the inner mitochondrial membrane. As for most mitochondrial solute carriers, the OGC amino acid sequence reveals a tripartite structure produced by the repetition of three tandemly repeated, 100-amino-acid Solute Carrier (SolCar) domains (Palmieri, 1994). Each SolCar domain in turn is composed of two hydrophobic regions that represent transmembrane domains. OGC is encoded by a nuclear gene (*OGC* or *Slc25a11*) and imported into mitochondria. Unlike most other mitochondrial proteins, OGC does not have a mitochondrial import signal, but exploits a complex cascade of events to ensure its mitochondrial localization. Since OGC is a predominantly hydrophobic protein and is therefore not easily soluble in the cytosol, it is bound by the chaperones Hsc70 (Heat Shock Cognate 70) and Hsp90 (Heat Shock Protein 90) immediately following translation (Zara et al., 2009). Hsc70 and Hsp90 then transport OGC to the mitochondria. Here the chaperones interact with the specialized TRP (tetratricopeptide) domain of Tom70 (Translocase Outer Mitochondrial membrane, 70 kDa subunit) at the outer mitochondrial membrane (Palmisano et al., 1998). Tom70 and the cognate inner mitochondrial membrane translocases Tim10-Tim12-Tim22 then mediate the ATP- and membrane potential-dependent transport of mitochondrial carriers to the inner mitochondrial membrane (Sirrenberg et al., 1996; Koehler et al., 1998). Here, OGC was shown to form homodimers (Palmisano et al., 1998). However, based on recent evidence relative to the functional conformation of ANT, it is now believed that OGC is functional as a monomer (Robinson et al., 2008).

The main role of OGC is to allow the electroneutral exchange of α-ketoglutarate across the inner mitochondrial membrane (Bisaccia et al., 1985). Although OGC is primarily involved in the control of mitochondrial metabolism, it has been implicated by recent reports in other

important pathways. Yu et al., (2001) reported that over-expression of human *OGC* in HEK293 cells caused a loss of mitochondrial membrane potential, reminiscent of the effect of uncoupling proteins. Data from another group suggest that OGC is responsible for the accumulation of porphyrins in the inner mitochondrial membrane (Kabe et al., 2006). Porphyrins are essential in the mitochondria as precursors in the synthesis of heme groups in the matrix. Heme is then incorporated in proteins involved in important mitochondrial functions, like cytochromes [reviewed in (Moore, 1998)].

Several reports suggested that OGC plays an important role in the accumulation of glutathione (GSH) in mitochondria (Chen and Lash, 1998; Chen et al., 2000). Xu et al., (2006) found that wild-type rat OGC, but not its mutant form, was able to induce accumulation of GSH in proteoliposomes. Furthermore, the authors reported that over-expression of OGC in NRK-52E cells had a protective effect against chemically induced apoptosis. These data point to the involvement of OGC in stress response pathways. However, all these studies were performed either *in vitro* or in cell culture systems, not in whole organisms. Further studies are therefore required to establish *OGC* as a novel stress response gene.

1.12 Specific hypotheses

Hypothesis 1: A purified dauer pheromone ascaroside may have physiological effects other than dauer induction.

Hypothesis 2: OGC may be involved in the control of apoptosis and insulin signalling *in vivo*.

Hypothesis 3: OGC may be part of oxidative stress response pathways *in vivo* and affect life span.

1.13 Specific objectives

Objective 1: To identify the signal transduction pathways utilized by a dauer pheromone ascaroside to induce dauer formation and assess its phenotypic effects.

Objective 2: To identify the *C. elegans* orthologue of OGC and determine its involvement in germline apoptosis and in the dauer pathway, specifically the *daf-2*/insulin-like signalling pathway.

Objective 3: To determine if *C. elegans* lacking OGC have defective responses to oxidative stress and have life span phenotypes in several genetic backgrounds.

1.14 References

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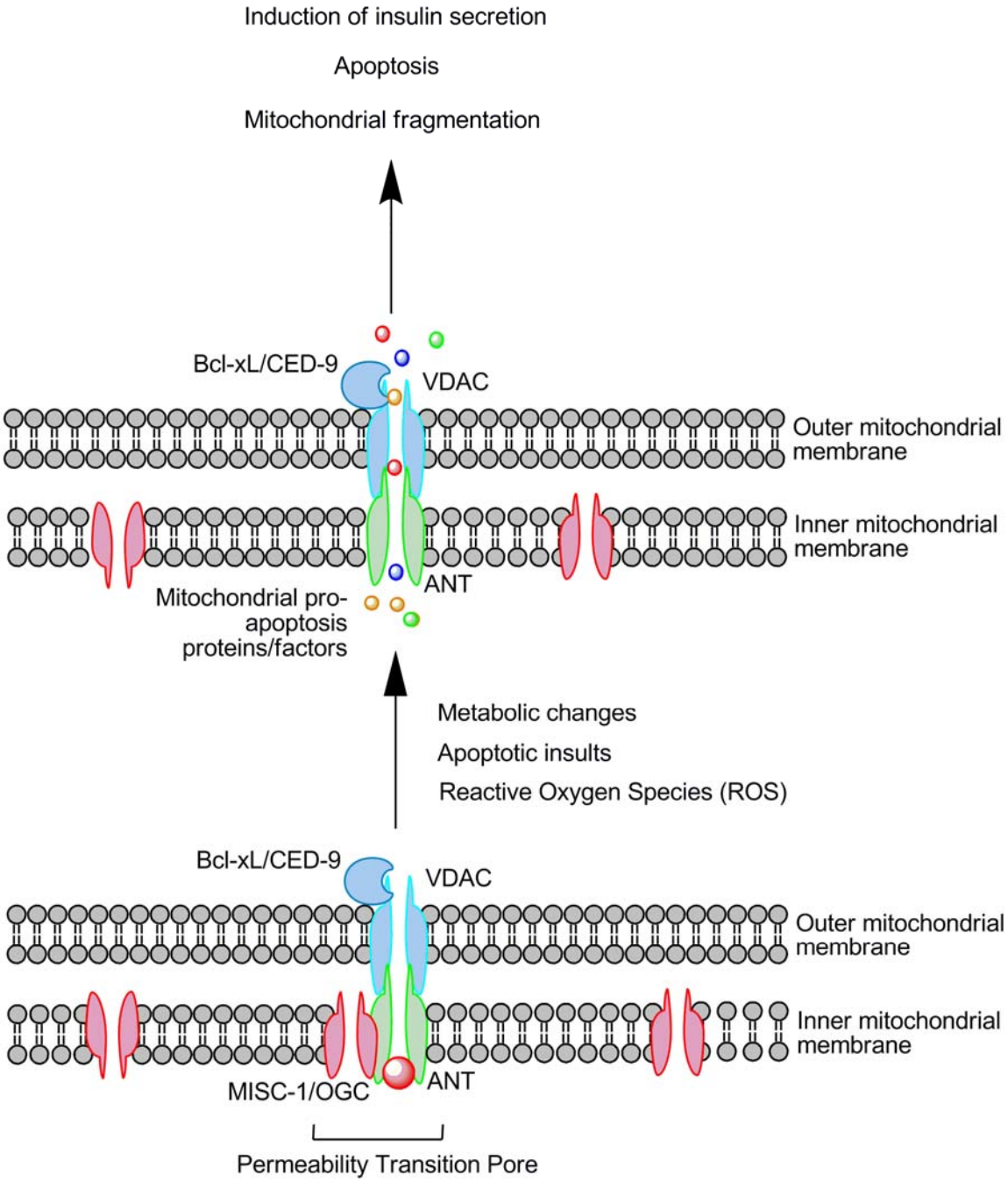
2 MISC-1/OGC LINKS MITOCHONDRIAL METABOLISM, APOPTOSIS AND INSULIN SECRETION [1]

2.1 Graphical Summary



[1] A version of this chapter was submitted for publication. Marco Gallo, Donha Park, Dan S. Luciani, Katarzyna Kida, Ferdinando Palmieri, Oliver E. Blacque, James D. Johnson, Donald L. Riddle (2010) MISC-1/OGC links mitochondrial metabolism, apoptosis and insulin secretion.

2.2 Highlights

- Lack of MISC-1 and OGC causes mitochondrial fragmentation and reduced cristae.
- MISC-1 regulates apoptosis through the physiological/LIN-35 pathway.
- MISC-1 and OGC are components of the MPTP.
- MISC-1 regulates insulin secretion and germline stem cell proliferation in *C. elegans*.

2.3 Introduction

Mitochondria are the sites of enzymatic reactions fundamental for life, producing ~95% of cellular ATP in eukaryotic cells. Mitochondria also play a dominant role in the control of apoptosis [reviewed in (Wang, 2001)]. Several mitochondrial proteins are required for the intrinsic pathway of apoptosis. The succession of events from mitochondrial fragmentation to cell death has been well characterized and shown to be highly conserved from *C. elegans* to mammals (Horvitz, 1999; Hengartner, 2000).

Mitochondria undergo constant rounds of fusion and fission to form or break their tubular structure within the cell. In addition to mitochondrial fragmentation during apoptosis (Jagasia et al., 2005), extensive mitochondrial remodelling happens in response to metabolic changes [reviewed in (McBride et al., 2006)]. The exact mechanisms that control the interdependence of metabolic rate and mitochondrial structure are currently unknown. We investigated the possibility that mitochondrial metabolic proteins interact directly with members of the apoptotic machinery to control mitochondrial morphology and cell survival decisions. We focused on the mitochondrial 2-oxoglutarate carrier (OGC) to test our hypotheses.

The nuclear gene *OGC* encodes a monomeric carrier protein that resides in the inner mitochondrial membrane and is responsible for the electroneutral transport of α-ketoglutarate (Bisaccia et al., 1985; Robinson et al., 2008). This gene has been studied mostly for its effects on cellular metabolism. However, it has recently been shown that mammalian OGC imports about 40% of mitochondrial glutathione (GSH), a potent anti-oxidant peptide involved in oxidative stress response in mammals and *C. elegans*. Also, its over-expression in NRK-52E cells prevents chemically induced apoptosis (Chen and Lash, 1998; Chen et al., 2000b; Xu et al., 2006**;** Gallo, submitted). These findings suggest that OGC might be involved in stress response as well as control of mitochondrial metabolism. In this report, we characterize the *C. elegans* homologue of *OGC*, which we called *misc-1* (Mitochondrial Solute Carrier), and find a novel role for MISC-1/OGC in apoptosis.

C. elegans has been used extensively to dissect the apoptotic pathway at the genetic level. Programmed cell death in *C. elegans* follows a linear genetic pathway involving *ced-9*/Bcl-2-like (Hengartner and Horvitz, 1994), *ced-4*/Apaf1 (Yuan and Horvitz, 1992; Zou et al., 1997) and *ced-3*/Caspase-9 (Yuan et al., 1993). Under non-apoptogenic conditions, CED-9 binds to CED-4 at the outer mitochondrial membrane to maintain it in its quiescent state (Spector et al., 1997). Upon apoptotic stimuli, the BH3-only Bcl-2-family protein EGL-1 binds to CED-9 (Conradt and Horvitz, 1998), which liberates CED-4 (del Peso et al., 1998). The now active CED-4 interacts with and activates the effector caspase CED-3 in the cytoplasm, thereby initiating a cascade of caspase activations that causes the hallmark phenotypes of apoptosis.

Mitochondria-induced apoptosis can result from the opening of channels called Mitochondrial Permeability Transition Pores (MPTPs), which are located at the junctions of inner and outer mitochondrial membranes. MPTP contributes to Mitochondrial Outer Membrane Permeabilization (MOMP) during apoptosis and allows exit of cytochrome c (Shimizu et al., 1999; Scorrano et al., 2002) and other mitochondrial pro-apoptotic factors to the cytoplasm. MPTPs are multi-protein complexes, composed of the Voltage-Dependent Anion Channel (VDAC), the Adenine Nucleotide Translocase (ANT) and Cyclophilin D [Cyp-D; (Halestrap et al., 1997; Woodfield et al., 1997; Crompton et al., 1998)]. Multiple regulators have been identified, including the anti-apoptotic Bcl-2-family proteins Bcl-2 and Bcl-x_L (Marzo et al., 1998; Shimizu et al., 1999). The identity of all MPTP components and regulators is not yet known. ANT is an established component of the MPTP in both mammals (Crompton et al., 2002) and *C. elegans* (Shen et al., 2009). We tested the possible interaction of OGC and MISC-1 with components of the MPTP.

Here we show that OGC and its *C. elegans* orthologue MISC-1 maintain the balance of fusion and fission events in human cells and in *C. elegans*. The absence of MISC-1/OGC results in increased apoptosis rates through the physiological pathway. Furthermore, we provide genetic and molecular evidence of the involvement of MISC-1/OGC in control of insulin secretion and germline stem cell proliferation. Our work points to a novel, conserved and direct

interaction between mitochondrial metabolic carriers and the apoptotic machinery to determine cell survival outcomes.

2.4 Results

2.4.1 *misc-1* expression

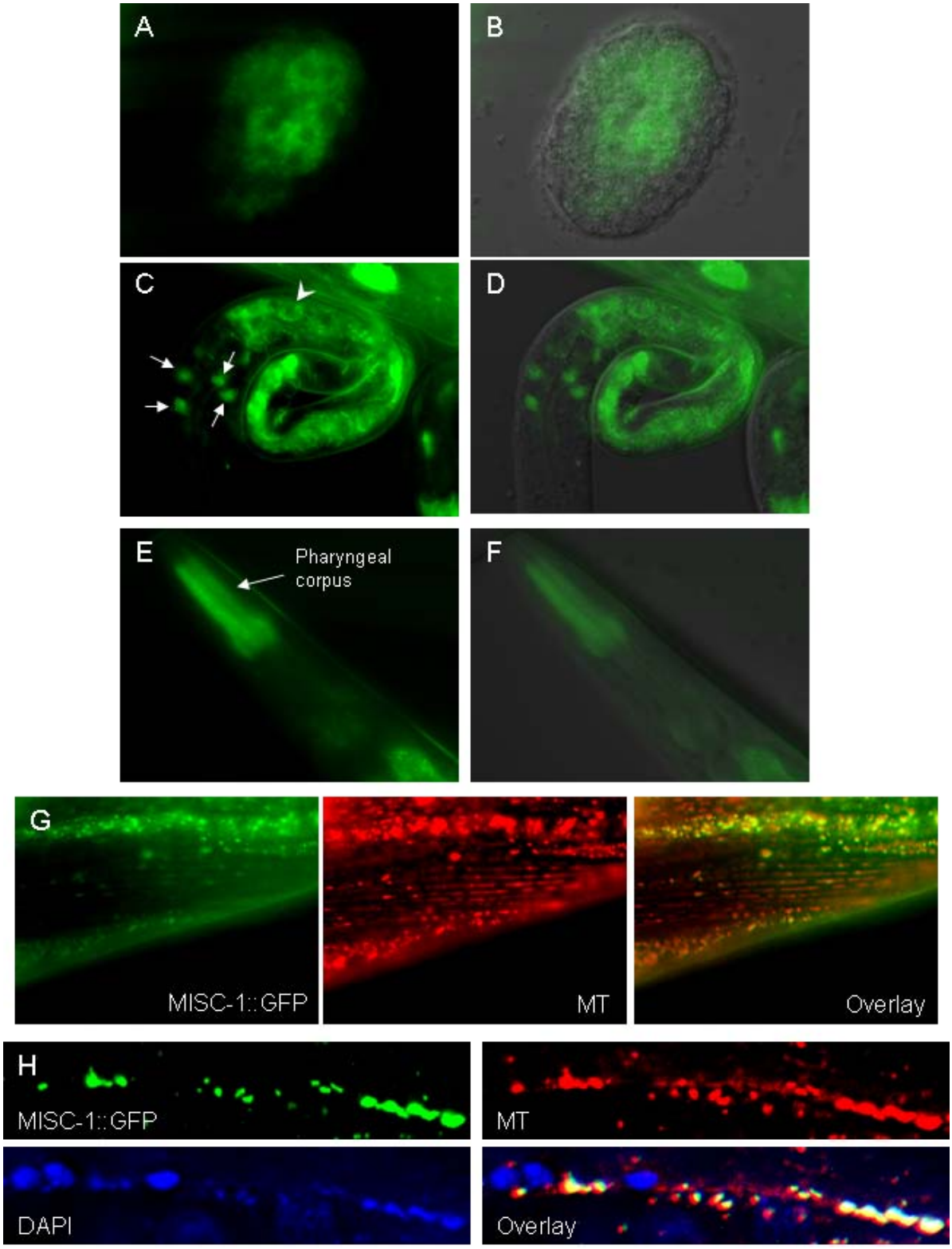
We investigated the expression profile of the 2-oxoglutarate carrier in *C. elegans*. Based on sequence similarity between MISC-1 and human OGC (72% amino acid identity; Appendix 1, Figure S1), their localization to mitochondria, and the shared roles of the two proteins (see below), we conclude that MISC-1 is the *C. elegans* orthologue of OGC.

We generated an extrachromosomal transgenic line carrying the full-length *misc-1* gene fused to *gfp* and under control of ~1100 bp of the region 5' of the ATG codon. Although low levels of reporter expression were observed ubiquitously (as expected), a subset of organs showed higher expression of MISC-1::GFP. GFP expression began early in embryogenesis in the intestinal precursor cells (Figure 2.1A-B). The protein exhibited intestinal and neuronal localization in newly hatched L1s (Figure 2.1C-D), while it was mainly present in the anterior pharynx in adults (Figure 2.1E-F). A similar expression pattern was observed in a separate extrachromosomal transgenic line carrying a *misc-1* promoter GFP fusion construct (data not shown). Overall, this expression pattern suggests that MISC-1 is required in tissues with high energy demands, *i.e.* the intestine in developing animals and the pharynx in adults. This is consistent with the increased expression of our MISC-1::GFP reporter in calorie-restricted animals (Appendix 1, Figure S2). It is interesting to note that in humans, *OGC* is most highly expressed in the heart [UCSC Genome Browser, (Kent et al., 2002)], another organ with high energy demands.

Although MISC-1/OGC is expected to be a mitochondrial carrier, we could find no published *in vivo* localization data for any organism. *C. elegans* embryonic and larval cells exhibited a typical mitochondrial pattern (Chen et al., 2000a) for the localization of MISC-1::GFP (Figure 2.1A-D). Co-localization of MISC-1::GFP and the mitochondria-specific marker MitoTracker Red CMXRos in muscle and seam cells indicates that MISC-1 is a mitochondrial protein (Figure 2.1G-H).

Figure 2.1. MISC-1::GFP reporter expression and its subcellular localization during *C. elegans* development. (A-B) GFP image and GFP/DIC overlay of MISC-1::GFP expression in an early embryo. Expression is in intestinal precursor cells and has a typically mitochondrial sub-cellular localization. (C-D) GFP image and GFP/DIC overlay of reporter expression in a newly hatched L1 animal. Expression can be observed in the intestine (arrowhead points to one typical cell) and in four head neurons (arrows). As in (A,B), the GFP reporter also has a string-like appearance typical of mitochondria. (E-F) GFP image and GFP/DIC overlay of reporter expression in an adult. Expression is strongest in the pharyngeal corpus. (G) Muscles of a MISC-1::GFP adult. MISC-1::GFP co-localizes with muscle mitochondria stained with the dye Mitotracker CMXRos (MT), as seen in the overlay. (H) Seam cell syncytium of a MISC-1::GFP adult. Mitochondria were stained with Mitotracker CMXRos and nuclei were counterstained with DAPI. The overlay shows the alternating pattern of mitochondria and nuclei in the seam cell syncytium, and illustrates how MISC-1::GFP co-localizes with the mitochondria.

Figure 2.1


A
B
C
D
E
Pharyngeal corpus
F
G
MISC-1::GFP
MT
Overlay
H
MISC-1::GFP
MT
DAPI
Overlay

2.4.2 MISC-1 and OGC regulate mitochondrial morphology

Under normal cellular conditions, mitochondria are mostly found fused in branched structures. To assess the effects of *misc-1* knock-down on mitochondrial morphology, we used a transgenic line with GFP targeted to mitochondria and under control of the muscle-specific *myo-3* promoter [*myo-3*p::mito*::gfp*; (Labrousse et al., 1999)]. *misc-1* RNAi resulted in robust (84%) down-regulation of MISC-1 protein levels (Appendix 1, Figure S3) and was therefore deemed an appropriate system for the study of *misc-1* function. *misc-1* RNAi treatment resulted in mitochondrial fragmentation in the mito::*gfp* strain (Figure 2.2A,B). In order to understand if this phenotype was specific to the mitochondria and not the result of *misc-1*-mediated defects in muscle fibres, we also tested the effect of *misc-1* RNAi on a strain carrying *myo-3*p::*myo-3*::*gfp* (Campagnola et al., 2002), a marker for the structure and organization of body wall muscle. Our results indicate that *misc-1* RNAi does not affect muscle structure (Appendix 1, Figure S4; see also Figure 2.3C-D). MISC-1 seems therefore to specifically control mitochondrial morphology.

To determine whether the effect of the 2-oxoglutarate carrier on mitochondrial morphology is conserved in mammals, we knocked down the *misc-1* human orthologue, *OGC*, in Human Embryonic Kidney 293 (HEK293) cells (Appendix 1, Figure S3). Staining with MitoTracker showed that siRNA against *OGC* resulted in a mitochondrial fragmentation phenotype reminiscent of *misc-1* knock-down worms (Figure 2.2C,D). MISC-1 and OGC have the same effect on mitochondrial morphology in *C. elegans* and human cells, respectively.

We then analyzed the mitochondria from our knock-out *misc-1* mutant strain with transmission electron microscopy. On average, the mitochondria of knock-out animals were reduced in size and more spherical in shape compared to N2 controls (Figure 2.3A,B). Moreover, they displayed a reduced number of disorganized and abnormal mitochondrial cristae (Fig. 2.3C,D). These features suggest that absence of MISC-1 shifts the equilibrium of mitochondrial dynamics in the direction of fission and are in agreement with the mitochondrial fragmentation phenotype we observed with the mito::*gfp* strain. Furthermore, the absence of a key inner membrane protein disrupts normal formation or maintenance of cristae.

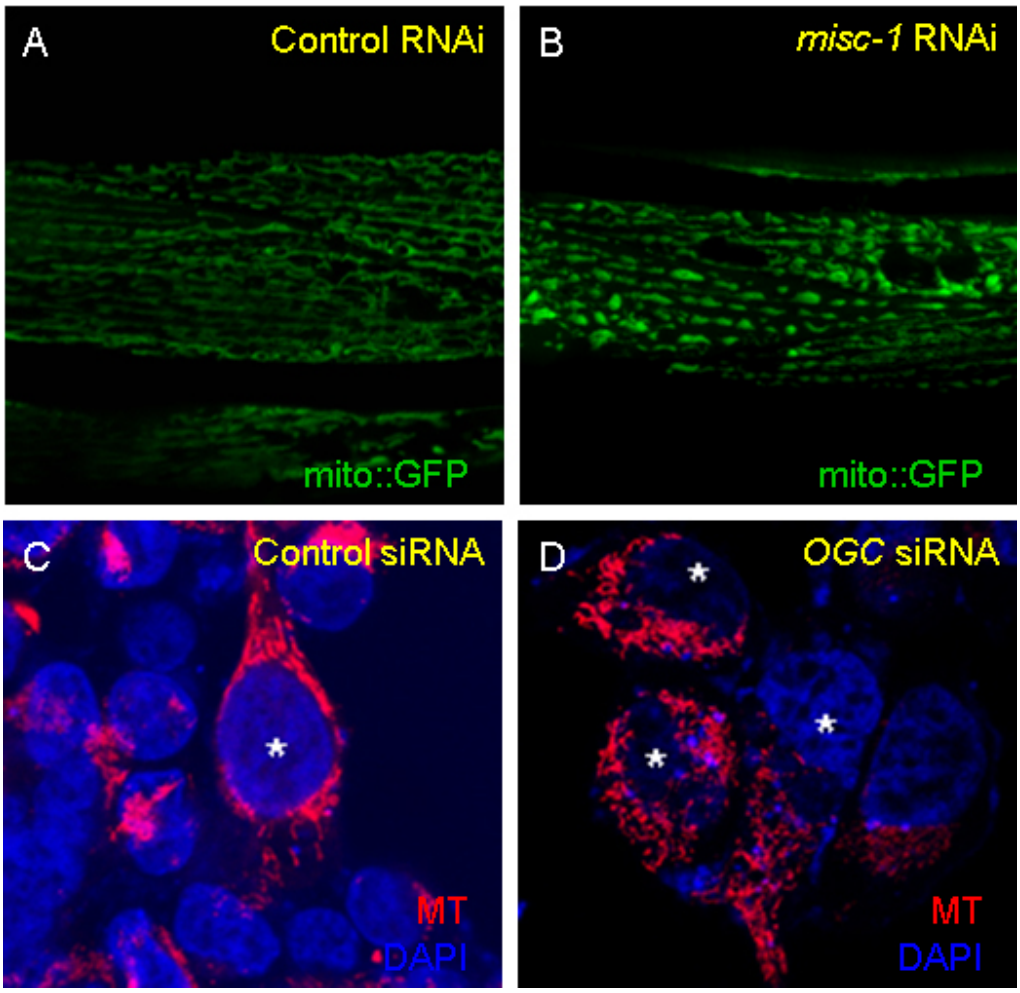


Figure 2.2. MISC-1/OGC is required for normal mitochondrial morphology in *C. elegans* and human cells. (A) Control RNAi and (B) *misc-1* RNAi on a transgenic strain carrying a muscle-specific, mitochondrially tagged form of GFP (mitoGFP). These confocal images show that *misc-1* knock-down causes mitochondrial fragmentation: the mitochondrial network breaks down because fission is more prevalent than fusion and total mitochondrial length is shorter than wild type. (C) Mitotracker staining and DAPI counterstaining of HEK293 cells transfected with the mammalian scrambled siRNA control vector. Mitochondria of transfected cells form organized networks, as expected. (D) Mitotracker staining and DAPI counterstaining of cells transfected with the *OGC* siRNA vector. These images show that downregulation of *OGC* in HEK293 cells results in mitochondrial fragmentation. *Transfected cells, as assessed by GFP fluorescence conferred by the transfected siRNA vector. MT: staining with MitoTracker CMXRos.

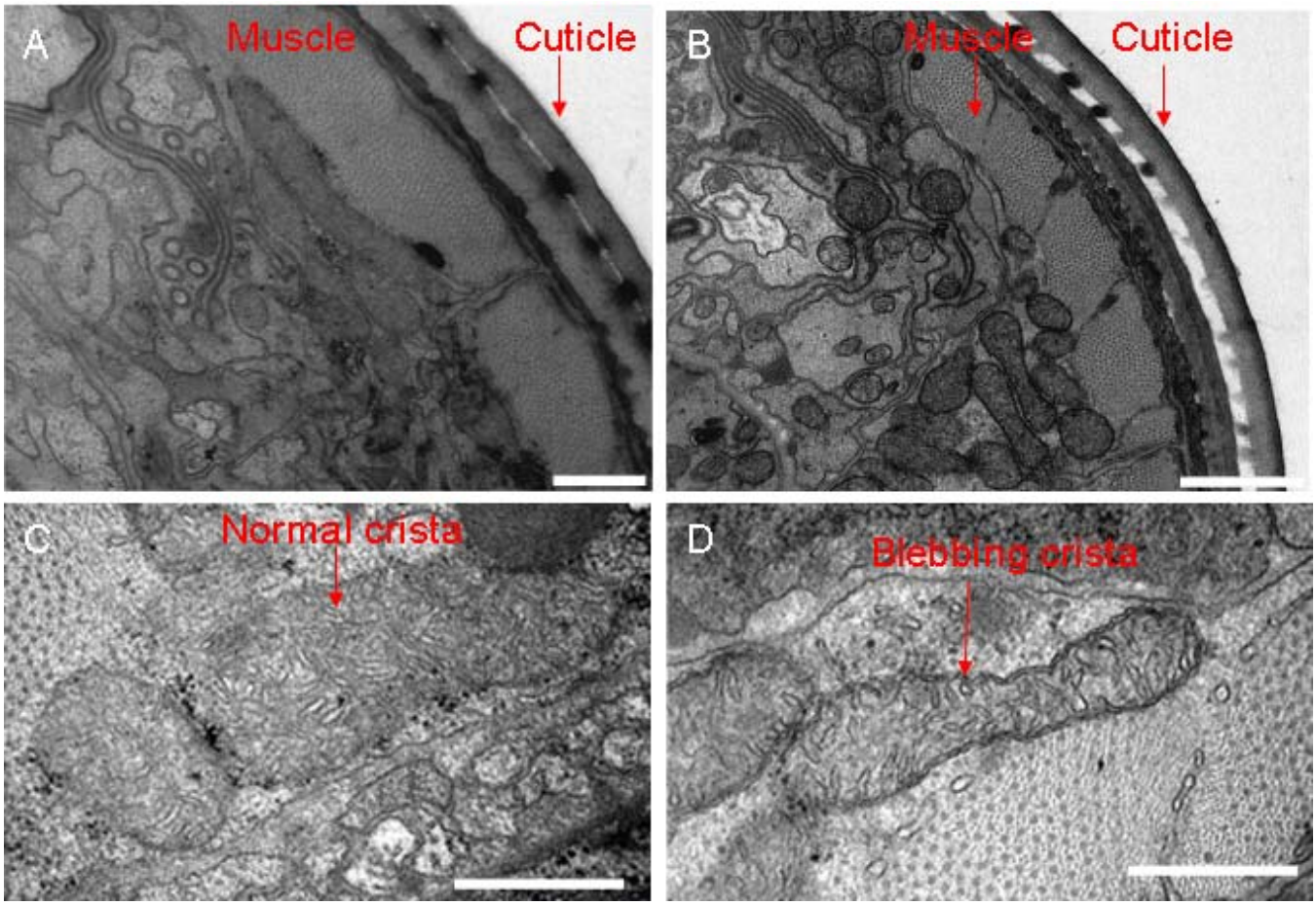


Figure 2.3. Mitochondrial ultrastructure is abnormal when MISC-1 is absent. Shown are low (A,B) and high (C,D) magnification images of TEM cross sections taken from the head region of N2 (A,C) and *misc-1* mutant (B,D) day 1 adults. Low magnification images show that compared with N2, the mitochondria of *misc-1* worms are smaller and more spherical. High magnification images indicate that compared to N2 controls, the mitochondrial cristae of the *misc-1* mutant are more disorganized, possess increased blebbing characteristics and adopt more irregular orientations. Furthermore, the cristae of *misc-1* animals are fewer in number. These images point to a function of MISC-1 in the stabilization of the inner mitochondrial membrane. The lower number of cristae in the absence of MISC-1 suggests that mutants have lower mitochondrial metabolic rates. Bars correspond to 500 nm in A, C and D, 1 μm in B.

2.4.3 Lack of MISC-1 results in increased germline apoptosis

Mitochondrial fragmentation is a hallmark of apoptosis in mammals and in *C. elegans* (Jagasia et al., 2005). We used SYTO12 to stain apoptotic bodies in the adult germline in N2 and in the *misc-1* knock-out. We observed a two-fold increase in the number of apoptotic corpses per gonadal arm in *misc-1* knock-outs (Figure 2.4A,B).

Since an increase in the number of apoptotic corpses could be caused either by increased apoptosis or by a defect in corpse removal, we used a transgenic line carrying a CED-1::GFP reporter (Zhou et al., 2001) to monitor corpse engulfment. CED-1 is a receptor localized to the membrane of the sheath cells in the somatic gonad and is responsible for the recognition of cell corpses and the initiation of their engulfment and removal. We observed a two-fold increase in the number of corpses engulfed by CED-1::GFP(+) cells in animals treated with *misc-1* RNAi, compared to adults treated with control RNAi (Appendix 1, Figure S5). These results indicate that *misc-1* RNAi does not impair the mechanism of cell corpse removal. We conclude that absence or decreased levels of MISC-1 increase the frequency of germline apoptosis.

In order to assess if reduced levels of OGC cause apoptosis in mammalian cells, we knocked-down mouse *OGC* in the MIN6 mouse insulinoma cell line with an shRNA strategy. *OGC* knock-down caused robust cleavage and activation of Caspase-3, an established marker of activation of apoptosis (Figure 2.4C). We conclude that lower levels of the 2-oxoglutarate carrier induce a phylogenetically conserved apoptosis program that is conserved from nematodes to mammals.

2.4.4 *misc-1* acts through the physiological pathway of apoptosis

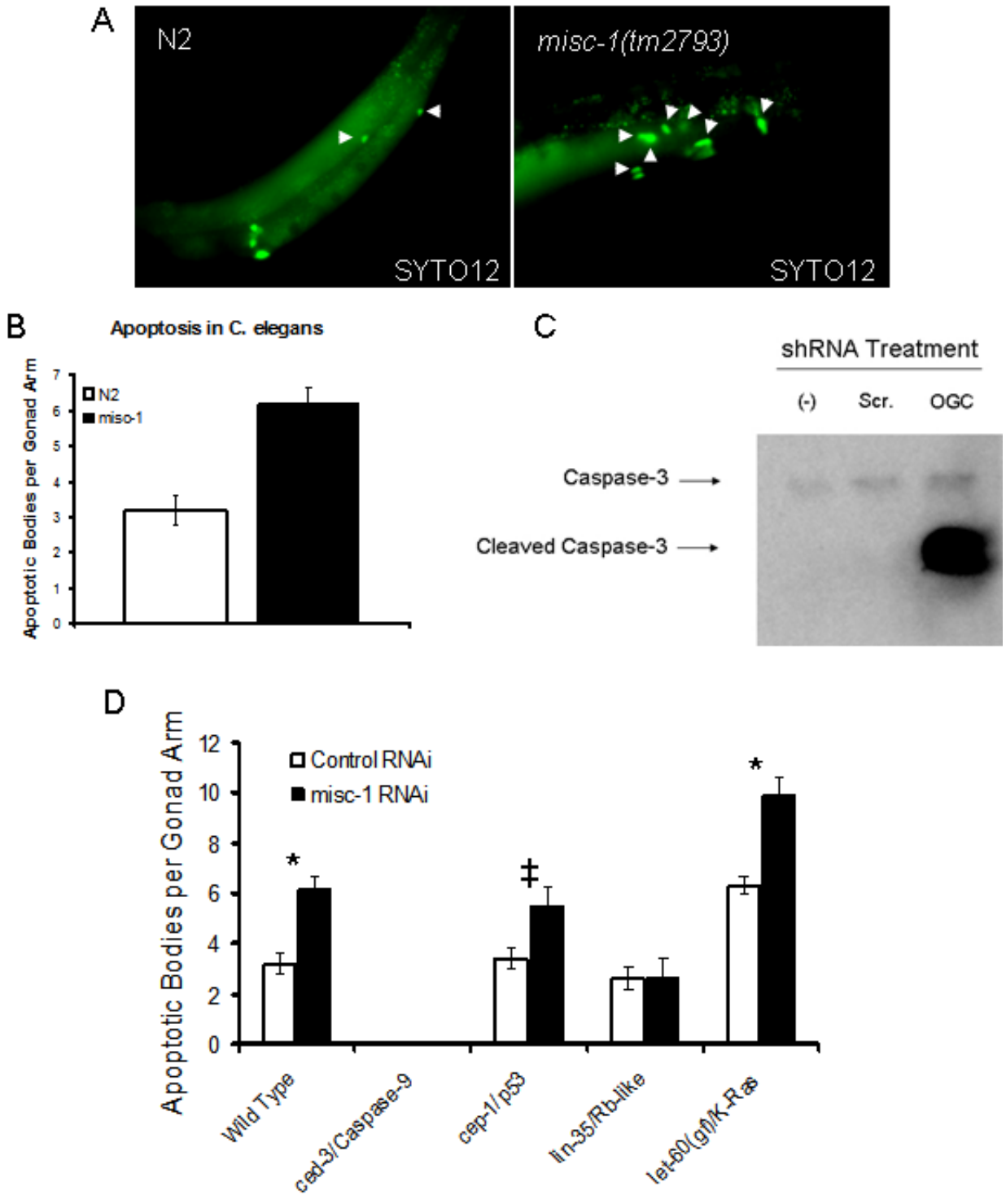
The increased levels of apoptosis observed upon knocking down *misc-1* were controlled at the genetic level, since a *ced-3(lf)/Caspase-9* genetic background obliterated apoptosis caused by *misc-1* RNAi (Figure 2.4D). In *C. elegans*, apoptosis can be triggered by three main pathways: (a) the DNA damage pathway, the main mediator being the *C. elegans* p53 orthologue CEP-1 (Schumacher et al., 2005); (b) the cytoplasmic stress pathway, mediated by LET-60/K-Ras

(Gumienny et al., 1999), and (c) the physiological pathway, mediated by the Retinoblastoma (Rb)-like protein LIN-35 (Schertel and Conradt, 2007). All three pathways ultimately converge on the CED-9/CED-4/CED-3 core apoptotic machinery.

misc-1 RNAi administered to wild-type N2 (Figure 2.4D) recapitulated the germline apoptosis phenotype observed in *misc-1* knock-out worms (see Figure 2.4B). This result, and the assessed efficiency of *misc-1* RNAi in down-regulating MISC-1 protein levels (Appendix 1, Figure S3), further confirms that RNAi is an appropriate tool to study *misc-1* function. We treated mutants in each of the three major apoptosis pathways with *misc-1* RNAi and assessed apoptosis levels by SYTO12 staining (Figure 2.4D). The *cep-1(lf)/p53* background did not affect the ability of *misc-1* RNAi to increase the number of apoptotic corpses. Using the *let-60(n1046)/K-Ras* gain of function allele, which increases the basal rate of apoptosis through the cytoplasmic stress pathway, *misc-1* RNAi increased the apoptotic rate in an additive fashion (not synergistic), indicating that *misc-1* does not function through this pathway. By contrast, a *lin-35(lf)* mutant abrogated the apoptosis phenotype induced by *misc-1* RNAi. These results suggest that *misc-1* acts through the LIN-35/Rb-mediated physiological pathway of apoptosis.

Figure 2.4. Absence of MISC-1 increases apoptosis in the *C. elegans* germline. (A) SYTO12 staining of apoptotic cell corpses (arrowheads) in the germline of N2 and *misc-1* knock-out. *misc-1* knock-out day 1 adults have an increased number of apoptotic events per gonadal arm. (B) Scoring of SYTO12-positive apoptotic corpses in N2 and *misc-1* knock-out. N2 had on average 3.2 apoptotic corpses per gonad arm, whereas *misc-1* had 6.2 per gonad arm. Absence of MISC-1 therefore results in a two-fold increase in apoptotic events in the germline. *t-test $P < 0.0001$. N = 27 for N2 and N = 30 for *misc-1*. Error bars: ±SEM. (C) *OGC* knock-down induces apoptosis in MIN6 cells. MIN6 cells were either untransfected [(-) lane], transfected with a scrambled control shRNA vector (Scr. lane) or with an shRNA vector targeting mouse *OGC* (OGC lane). Cell lysates from the three treatments were used for a Western blot employing an antibody that recognizes both total Caspase-3 and cleaved Caspase-3. The latter is the active, pro-apoptotic form of the caspase and is therefore a marker of apoptosis. The Western blot shows that knock-down of *OGC* results in Caspase-3 activation and therefore apoptosis in MIN6 cells. Similar results were obtained with MIN6 cells at two different passages. (D) *misc-1* mediates apoptosis through the physiological pathway of apoptosis. Synchronized animals with different genetic backgrounds were fed either control or *misc-1* RNAi from the L1 stage and were stained with SYTO12 on day 1 of adulthood. SYTO12-positive apoptotic bodies per gonad arm were scored. *ced-3* mutants were used as a control, since mutations in this gene encoding an effector caspase obliterates germline apoptosis. *cep-1/p53* mutants suppress the DNA-damage pathway of apoptosis. *lin-35* mutants lack the physiological pathway of apoptosis. Worms carrying a *let-60/K-Ras* gain-of-function allele have increased apoptotic rates through the cytoplasmic stress pathway. The data indicate that mutations in *lin-35* suppress the apoptotic phenotype of *misc-1* RNAi, indicating that *misc-1* controls apoptosis through the physiological pathway of apoptosis. *t-test $P < 0.001$. ‡t-test $P < 0.05$.

Figure 2.4


A
N2
SYTO12
misc-1(tm2793)
SYTO12
B
Apoptosis in C. elegans
Apoptotic Bodies per Gonad Arm
N2
miso-1
C
shRNA Treatment
(-)
Scr.
OGC
Caspase-3
Cleaved Caspase-3
D
Apoptotic Bodies per Gonad Arm
Control RNAi
misc-1 RNAi
Wild Type
ced-3/Caspase-9
cep-1/p53
lin-35/Rb-like
let-60(gf)/K-Ras

2.4.5 MISC-1 and OGC are novel components of the mitochondrial permeability transition pore

Since we found that *misc-1* modulates mitochondrial fragmentation, cristae morphology and apoptosis, which are features also regulated by the MPTP, we asked if MISC-1 might be a novel component or regulator of the MPTP. We performed a co-immunoprecipitation experiment using an antibody against ANT in HEK293 cells. ANT pulled down Bcl-x_L as expected, and it also precipitated OGC (Figure 2.5). Likewise, Bcl-x_L co-precipitated ANT and OGC (data not shown). As a control, we tested a subunit of complex IV of the electron transport chain, which is also a mitochondrial inner membrane protein. This protein was not detected in the co-immunoprecipitated fraction; it was only present in the input/supernatant.

MISC-1 also participates in formation of the MPTP in *C. elegans*. We did pull-down experiments in wild type and in our MISC-1::GFP over-expressing transgenic line with several combinations of antibodies and were able to confirm the interaction of MISC-1, CED-9/Bcl-2 and ANT (Appendix 1, Figure S6). These pull-down experiments indicate that MISC-1 and OGC are novel components of the phylogenetically conserved MPTP.

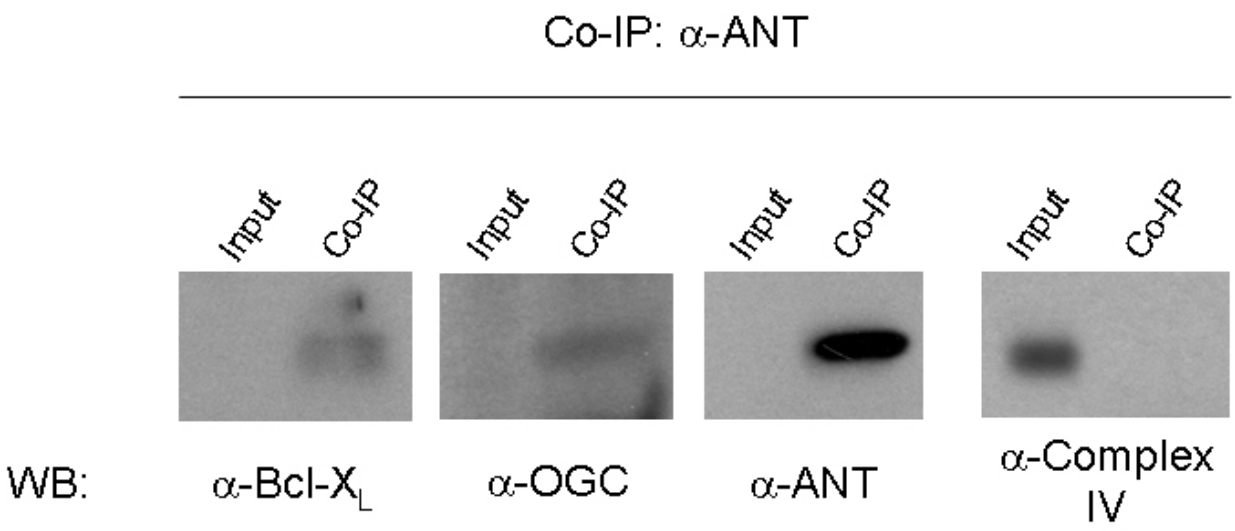


Figure 2.5. OGC interacts with Bcl-x_L and ANT, and is a component of the MPTP. We performed a co-immunoprecipitation experiment in HEK293 cells using an antibody against ANT, which is an integral component of the MPTP. We were able to detect Bcl-x_L, OGC and ANT in the co-immuno precipitant. As a control protein, we chose subunit 1 of complex IV of the electron transport chain, which is also localized to the inner mitochondrial membrane. This control protein did not co-immunoprecipitate with Bcl-x_L, OGC and ANT, but was present in the supernatant (input). We obtained similar results by immunoprecipitation with an antibody against Bcl-x_L (data not shown). Co-IP: co-immunoprecipitation. WB: western blot.

2.4.6 *misc-1* mutations suppress the dauer constitutive phenotype of *daf-2*

MISC-1 is a metabolic protein, controls mitochondrial function and interacts with CED-9, whose orthologue is Bcl-x_L. Interestingly, transgenic mice with strong over-expression of Bcl-x_L in their pancreatic beta-cells were previously shown to have altered glucose-sensing at the mitochondria and impaired glucose-stimulated insulin release (Zhou et al., 2000). This observation suggests the possibility that Bcl-x_L, like the related pro-apoptotic protein Bad (Danial et al., 2008), may play a role in insulin release independent of its role in apoptosis. We took advantage of the genetic tools afforded by *C. elegans* to investigate a possible role for *misc-1* in insulin signalling *in vivo*. The Insulin/Insulin-like Signalling (IIS) pathway is well conserved between *C. elegans* and mammals [reviewed in (Christensen et al., 2006)]. The IIS pathway was actually first assembled at the genetic level in this model organism (Riddle, 1977). In *C. elegans*, the Insulin-like Growth Factor 1 Receptor (IGF1R) is encoded by *daf-2* (DAuer Formation) (Kenyon et al., 1993; Kimura et al., 1997; Gems et al., 1998). Mutations in this gene and reduced IIS result in dauer formation, which is a larval diapause state (Klass and Hirsh, 1976; Golden and Riddle, 1982; Golden and Riddle, 1984).

To test the possibility that MISC-1 might play a role in insulin secretion in *C. elegans,* we first tested a possible genetic interaction between *misc-1* and *daf-2/IGF1R.* We created a double mutant carrying the hypomorphic allele *daf-2(m41)* and the knock-out allele *misc-1(tm2793)* to ask whether *misc-1* modified the *daf-2* dauer-constitutive phenotype (Figure 2.6A). *m41* animals constitutively enter the dauer diapause stage at non-permissive temperatures (>22°C). All *daf-2* animals entered the dauer stage at both 22.5°C and 25°C, but no *daf-2;misc-1* animals entered the dauer stage at 22.5°C and only 22% formed dauer larvae at 25°C. Therefore *misc-1* substantially suppresses the dauer phenotype of *daf-2*. The suppressive effects of the mutation are stronger at lower temperatures, probably because *m41* is a temperature-sensitive allele with a stronger phenotype at higher temperatures.

2.4.7 MISC-1 modulates insulin secretion in *C. elegans*

The suppression of the *daf-2* dauer-constitutive phenotype by *misc-1(tm2793)* could be interpreted in at least two ways: (a) *misc-1* could be genetically downstream of *daf-2* or part of a pathway downstream of the insulin-like pathway; (b) mutations in *misc-1* may positively affect insulin secretion, which in turn would compensate for the partial loss of DAF-2 function due to the hypomorphic mutation *m41*. To address this issue, we employed a strain carrying a translational fusion of the *C. elegans* insulin DAF-28 and GFP [DAF-28::GFP; (Li et al., 2003; Kao et al., 2007)]. We subjected the DAF-28::GFP strain to *misc-1* RNAi by feeding. DAF-28::GFP is secreted by the head neurons and the posterior intestine into the pseudo-coelomic fluid, where it is picked up by scavenging cells called coelomocytes. Among a total of six coelomocytes, the number containing DAF-28::GFP (GFP(+)) is proportional to the amount of secreted insulin (Li et al., 2003). When we compared the number of GFP(+) coelomocytes in worms treated with control and *misc-1* RNAi, we observed that knock-down of *misc-1* induced a ~44% increase in GFP(+) coelomocytes (average of two independent experiments; Figure 2.6B).

The DAF-28 protein acts as a DAF-2 receptor agonist, but the *C. elegans* genome encodes over 40 insulin-like proteins, some being agonists and some antagonists of DAF-2 (Kawano et al., 2000; Pierce et al., 2001; Li et al., 2003). To corroborate the DAF-28::GFP reporter results as a general quantitative marker of insulin secretion, we measured the amount of glucose in *misc-1* and wild type N2 mixed-stage populations. We reasoned that if indeed insulin secretion is increased in *misc-1* mutants, then their glucose utilization should increase and the total glucose level should drop, compared to normal animals. In fact, we observed a 6% decrease (t-test $P = 0.0054$) in glucose levels in the *misc-1* mutant (Figure 2.6C), a result consistent with an increase in insulin secretion when MISC-1 levels are reduced. Organismal levels of ATP are not affected by absence of MISC-1 (Figure 2.6D), indicating that ATP may not be responsible for the induction of insulin secretion in the case of *misc-1* insufficiency.

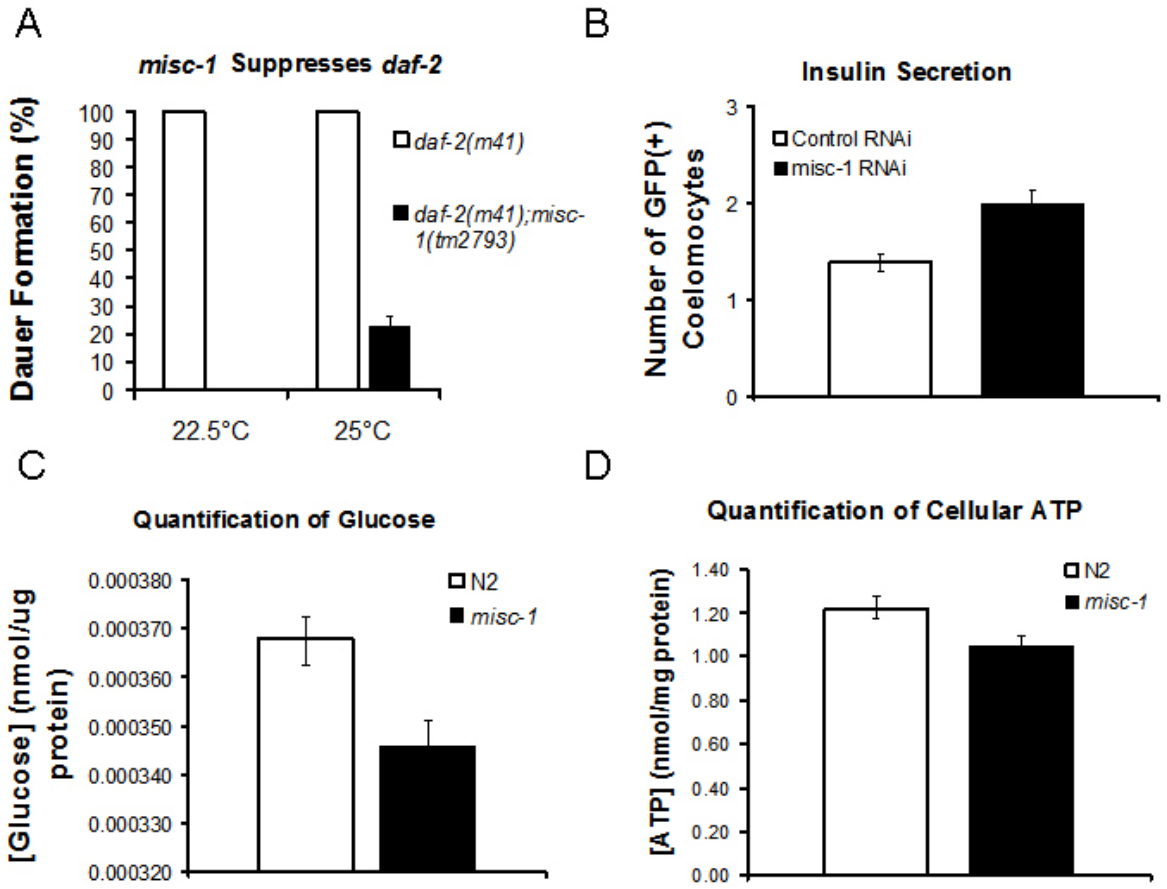


Figure 2.6. Absence of MISC-1 has positive effects on insulin secretion. (A) We assessed genetic interactions between *misc-1* and the hypomorphic allele *daf-2(m41)*. Mutations in *daf-2* result in constitutive dauer formation at 22.5°C and 25°C. However, the double mutant *daf-2;misc-1* worms did not arrest at the dauer stage at 22.5°C, and only 22.5% of the double mutants formed dauer larvae at 25°C. These data show that absence of *misc-1* expression suppresses the dauer phenotype conferred by *daf-2* mutations. T-test $P = 0.0021$. (B) We tested the possibility that the *daf-2* suppression by *misc-1* was due to an increase in insulin secretion, which could over-ride the hypomorphic allele *m41*. We measured insulin secretion by scoring GFP(+) (*i.e.* DAF-28::GFP-positive) coelomocytes in a transgenic strain upon *misc-1* knock-down. *misc-1* RNAi animals (N = 44) had on average 2 GFP(+) coelomocytes, while control worms (N = 33) had on average 1.4 (t-test $P = 0.00079$). These data suggest that *misc-1* knock-down increases insulin secretion. (C) We quantified glucose concentration in N2 wild type or *misc-1* knock-out animals, and observed a 6% reduction in glucose concentration in *misc-1* mutants, compared to control (t-test $P = 0.0054$). The amount of glucose was normalized to protein content. (D) We quantified the amount of cellular ATP in N2 and *misc-1* mutants, and observed no significant difference in the concentration of ATP between the two strains (t-test $P = 0.09$).

2.4.8 *misc-1* activity negatively modulates germline proliferation

The *C. elegans* germline is a syncytium of nuclei that mature as they migrate from a distal to a proximal position and eventually become oocytes. Notch signaling initiated from the Distal Tip Cell (DTC) regulates the balance between proliferation (mitosis) and differentiation (meiosis) of these germline nuclei. The DTC produces the Notch ligand LAG-2 (Henderson et al., 1994; Henderson et al., 1997; Nadarajan et al., 2009), which activates the GLP-1/Notch receptor in nearby germ cells and maintains these cells in the undifferentiated (mitotic) state (Austin and Kimble, 1987; Berry et al., 1997). A recent report showed that increased insulin signalling can increase germ cell proliferation independently of LAG-2 activation (Michaelson et al., 2010). A similar effect of insulin signalling on female reproduction was observed in mouse (Burks et al., 2000).

We investigated the possibility that the increased insulin secretion in *misc-1* mutants could have an effect on germline proliferation. Proliferative germ cells in late prophase and early mitotic M-phase are positive for histone 3 (H3) phosphorylation [pH3; (Gurley et al., 1978; Ajiro et al., 1996)]. We performed immunostaining of dissected germlines from wild type and *misc-1* knock-out mutants with a α-pH3 antibody. The results show that absence of MISC-1 greatly increases the number of pH3-positive, mitotic germline stem cells (Figure 2.7A,B) and an extended mitotic zone (data not shown). pH3 immunostaining strongly suggests that absence of MISC-1 favours mitosis (self-renewal) over meiosis (differentiation) in germline stem cells.

To assess the involvement of LAG-2, we employed a transgenic line expressing a GFP reporter under the control of the *lag-2* promoter [*lag-2*p::*gfp*; (Narbonne and Roy, 2006)]. This reporter is expressed in the DTCs and its processes, called cytonemes (Figure 2.7C,D). We found that the cytonemes of animals treated with *misc-1* RNAi were longer than the ones in control animals (Figure 2.7C-E). Knock-down of *misc-1* resulted in a two-fold increase in the length of the cytonemes (mean ± SEM: control RNAi = 74.9 ± 10.80 μm, *misc-1* RNAi = 150.9 ± 29.14 μm; t-test P = 0.0080). We assessed *lag-2*p::*gfp* expression by microscopy in animals treated with control or *misc-1* RNAi, but were unable to detect statistically significant differences

between the two treatments (Figure 2.7F). We concluded that upregulation of *lag-2* was not responsible for the extended cytonemes and mitotic zones of *misc-1* mutants. Although the length of the cytonemes may be positively correlated with the number of germline cells in a mitotic state, this is independent of *lag-2* expression. The germline stem cell proliferation phenotype obtained by lack of MISC-1 is therefore consistent with an increase in insulin signalling.

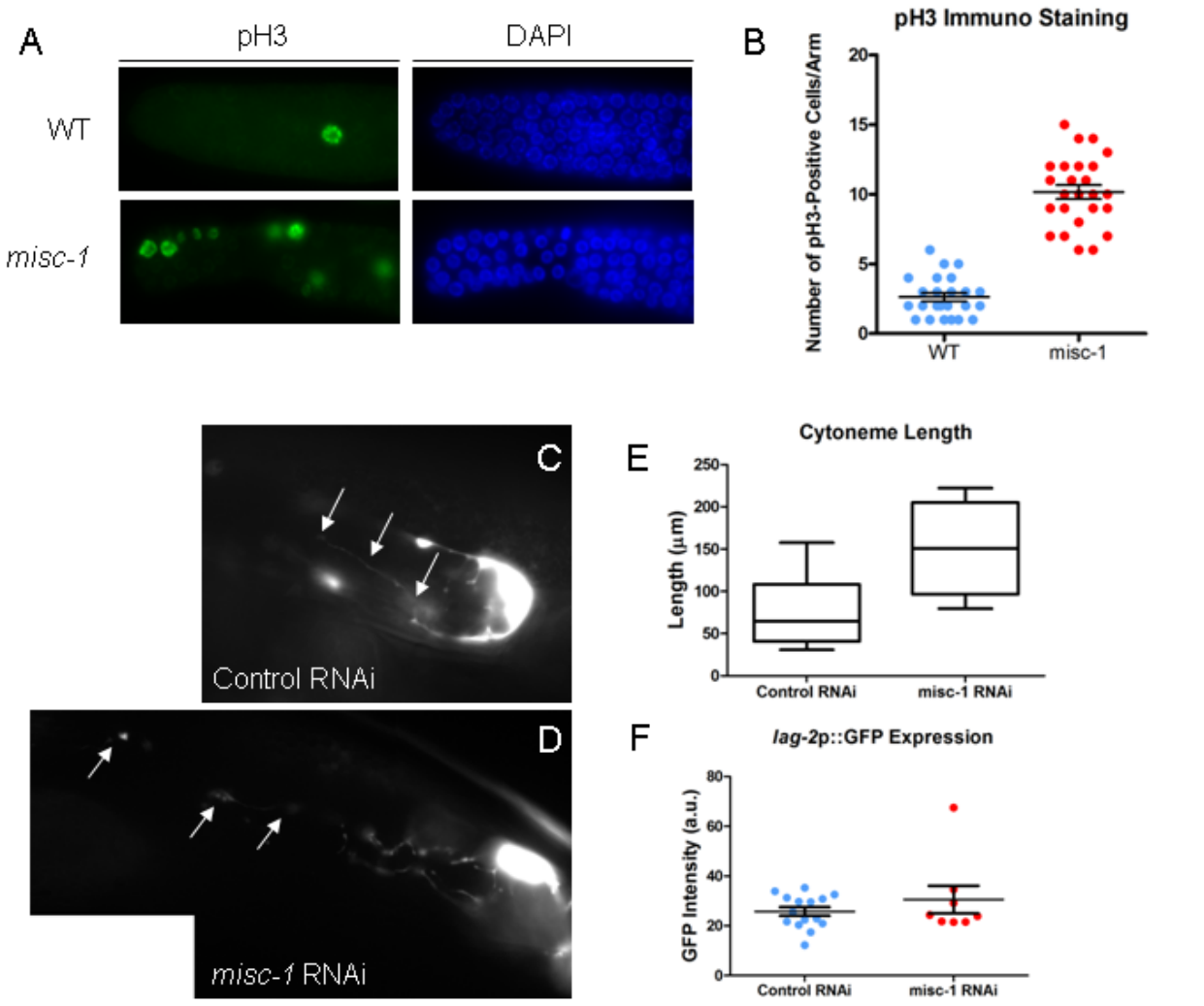


Figure 2.7. Absence of MISC-1 positively affects germline proliferation. (A) Immunostaining with α-phospho-H3 (pH3, left panel) and DAPI staining (right panel) of dissected germlines. Nuclei positive for phospho-H3 are actively undergoing mitosis. We observed an increase in mitotic cells in the germline of *misc-1* knock-out animals, compared to wild type (WT) control. (B) Quantification of phospho-H3-positive nuclei in dissected germlines of wild type (WT) and *misc-1* knock-out animals. While wild type had on average 2.63 pH3-positive nuclei, *misc-1* worms had 10.2 pH3 positive cells per gonadal arm (t-test $P < 0.0001$). (C,D) Transgenic animals expressing a *lag-2*p*::gfp* reporter were exposed to either control (empty) RNAi vector (C) or *misc-1* RNAi (D). *misc-1* knock-down results in longer DTC cytonemes than control (arrows delineate the length of the cytonemes). (E) Measurement of cytoneme length in control animals and animals exposed to *misc-1* RNAi. The mean cytoneme length was 74.9 μm in control, while it was 150.9 μm in *misc-1* knock-down worms. Cytonemes in *misc-1* knock-down worms were therefore twice as long as in control (unpaired t-test $P = 0.0080$). (F) Absence of MISC-1 does not affect *lag-2* expression. We quantified reporter expression with ImageJ. We observed no difference in GFP expression between control worms and *misc-1* RNAi-treated worms (t-test $P = 0.3137$). All error bars represent ±SEM.

2.5 Discussion

We identified *misc-1*, the *C. elegans* orthologue of human *OGC*. By studying *misc-1* in nematodes, we uncovered new functions for this gene that are conserved in higher eukaryotes. We showed that MISC-1/OGC is not merely involved in regulation of mitochondrial metabolism by participating in the malate-aspartate shunt. The protein seems to participate in a higher order of metabolic regulation by interacting with Bcl-2-family members and by regulating insulin secretion. Specifically, our experiments in *C. elegans* and mammalian cells show that MISC-1 and OGC regulate mitochondrial fusion/fission, cell death and are novel components of the MPTP.

First, we showed that reducing or eliminating expression of MISC-1/OGC resulted in mitochondrial fragmentation in *C. elegans* and in human cells. The mitochondrial fragmentation phenotype and the concomitant reduction in cristae number in *misc-1* knock-out animals suggest a reduction in mitochondrial metabolism [reviewed in (McBride et al., 2006)]. Mitochondrial metabolism also decreases and cristae condense in the absence of the fusion machinery (Bach et al., 2003). For instance, loss of function of Mitofusin-2 (*Mfn2*) – a key protein involved in mitochondrial fusion - in cell cultures inhibits pyruvate, glucose and fatty acid oxidation and reduces mitochondrial potential (Pich et al., 2005). *Mfn2* expression is down-regulated in the Zucker rat model of obesity (Bach et al., 2003; Pich et al., 2005) and in human subjects with obesity or diabetes (Bach et al., 2005). Hence, mutations affecting mitochondrial structure also affect metabolism. Here we provide an example that the opposite is also true: a metabolic protein affects mitochondrial structure.

The mitochondrial fragmentation conferred by *misc-1* knock-out or knock-down could be mediated by its physical interaction with CED-9/Bcl-2, which is a component of the core apoptotic machinery. The fact that knocking-down *misc-1* in *C. elegans* and *OGC* in human cells resulted in the same phenotype – mitochondrial fragmentation – suggests that MISC-1/OGC might be part of an ancient, phylogenetically conserved mechanism to induce

mitochondrial fragmentation. This hypothesis is strengthened by the observation that CED-9 physically interacts with FZO-1/Mfn1,2 to promote mitochondrial fusion (Rolland et al., 2009).

The interaction of MISC-1/OGC with CED-9/Bcl-2 may not be unique, as other mitochondrial proteins localized to the inner mitochondrial membrane might similarly interact with Bcl-2-family members in order to regulate the mitochondrial fusion/fission equilibrium according to metabolic needs. An example of such a protein is ANT, the primary role of which is to mediate the exchange of ADP and ATP across the inner mitochondrial membrane. MISC-1/OGC and ANT are of similar size, share protein domains (three solute carrier or SolCar domains), and are both localized to the inner mitochondrial membrane (Palmieri, 1994). Interestingly, we found that MISC-1/OGC is a novel component of the MPTP. ANT was previously characterized as a structural component of the MPTP, together with Bcl-2 proteins (Crompton et al., 2002; Shen et al., 2009). Finding other members of the MPTP could elucidate the role of this channel as a hub that integrates cues regarding metabolic status and cell survival.

Our data suggest that the MPTP links the function of MISC-1/OGC in cell death and insulin secretion. MPTP opening triggers apoptosis through the release of pro-apoptotic factors, like cytochrome c (Shimizu et al., 1999), AIF (Susin et al., 1996) and Ca^{2+} [reviewed in (Orrenius et al., 2003)]. However, cytochrome c does not act as a pro-apoptotic factor in *C. elegans* [reviewed in (Huang and Strasser, 2000)]. In mammals, cytoplasmic cytochrome c binds to its target Apaf1, which then activates the effector caspase Caspase-9 (Zou et al., 1999). In contrast, the *C. elegans* orthologue of Apaf1, CED-4, does not have WD40 cytochrome c binding domains. Instead, in its repressed state, CED-4 is bound by CED-9/Bcl-2 on the outer mitochondrial membrane. Following an apoptotic signal, the BH3-only protein EGL-1 binds to CED-9, which releases CED-4. CED-4/Apaf1 is then free to activate the effector caspase CED-3 (Spector et al., 1997; Conradt and Horvitz, 1998; del Peso et al., 1998). Efflux of pro-apoptotic factors other than cytochrome c from the mitochondria, mediated by MISC-1 and the MPTP, may play a pivotal role in this process. Our data suggest that MISC-1/OGC and the

MPTP participate in an evolutionarily conserved apoptosis program. Furthermore, we showed that *misc-1* modulates apoptosis through the physiological (LIN-35/Rb) pathway. This pathway is not activated by stress, but functions in the *C. elegans* germline during oocyte production (Gumienny et al., 1999; Schertel and Conradt, 2007), similarly to apoptosis during oocyte production in humans. It has been shown that the physiological pathway activates the core apoptotic machinery through an unknown mechanism, independent of the BH3-only protein EGL-1 (Gumienny et al., 1999). We propose that the physiological pathway of apoptosis is triggered by the efflux of mitochondrial pro-apoptotic factors controlled by the function of MISC-1/OGC and the MPTP.

It has been suggested that the MPTP might function as a low-affinity Ca^{2+} channel (Crompton, 1999). It is interesting to speculate that Ca^{2+} may mediate the apoptosis and insulin secretion phenotypes observed in the absence of MISC-1/OGC, since we were unable to detect an increase in ATP levels in whole-organism extracts from *misc-1* mutants that exhibited higher insulin secretion. However, it would be interesting to measure ATP levels specifically in the *C. elegans* insulin-secreting cells. However, because of the lack of techniques to dissect single tissues in the adult, we measured total ATP levels, thereby possibly diluting any effect *misc-1* mutations might have on ATP production in insulin-producing cells. Whether through release of mitochondrial Ca^{2+} or not, we offer genetic and molecular evidence that absence of MISC-1 results in increased insulin secretion in *C. elegans*. α-ketoglutarate, the main substrate of MISC-1/OGC, is an insulin secretagogue [reviewed in (MacDonald et al., 2005)]. Furthermore, over-expression of Bcl-x_L in mouse β-cells impairs glucose- and KCl-stimulated insulin secretion and intracellular Ca^{2+} levels (Zhou et al., 2000). Bcl-2-proteins could modulate insulin secretion via release of mitochondrial and ER Ca^{2+} (Lam et al., 1994). It is therefore possible that MISC-1/OGC affects insulin secretion by modulating the function of the MPTP and Bcl-2 proteins, probably through regulation of Ca^{2+} release from mitochondrial stores. Interestingly, an expression microarray study (Patti et al., 2003) showed that the gene encoding OGC is also

down-regulated in muscles of individuals affected by type 2 diabetes or with a family history of the disease, compared to controls.

Absence of MISC-1 favours mitotic proliferation of germline stem cells. This result seems to further corroborate the increase in insulin secretion in *misc-1* knock-down and knock-out animals. In fact, increased insulin secretion and insulin signalling induces germline proliferation in *C. elegans*, independently of the Notch ligand LAG-2 (Michaelson et al., 2010). Here we show that absence of MISC-1 induces germline stem cell proliferation independently of LAG-2. We considered the possibility that increased germline stem cell proliferation may represent a compensatory mechanism for the increased apoptotic rate in the germline of *misc-1* animals. However, several reports suggest that germline stem cell proliferation and apoptotic rates are not interdependent. For example, absence of the germline RNA helicase CGH-1 results in increased apoptosis and decreased germline proliferation (Navarro et al., 2001), whereas absence of the RNA binding protein GLD-1 results in reduced apoptosis and increased germline proliferation (Pinkston et al., 2006). We speculate that increased insulin secretion stimulates the growth of DTC cytonemes and that longer cytonemes are responsible for maintaining the extended mitotic zone observed in *misc-1* germlines. To the best of our knowledge, such an association between cytoneme length and germline stem cell proliferation has not been reported before.

In conclusion, we identified MISC-1/OGC as a novel component of the MPTP and a regulator of mitochondrial fusion/fission, apoptosis, insulin secretion and germline proliferation. The functions of this protein seem to be phylogenetically conserved from nematodes to mammals. Our data indicate that MISC-1/OGC and the MPTP have a role in assessing the metabolic status of a cell and subsequently in making cell survival decisions. We find it intriguing that a primarily metabolic protein like MISC-1/OGC plays roles in such a wide array of functions. The study of other metabolic mitochondrial carriers in the context of their wider cellular functions might elucidate the complex mechanisms underlying the integration of cell survival signals with cell metabolism.

2.6 Methods

Strains

The following strains were used: N2, *daf-2(m41)* (DR41), *misc-1(tm2793)* (DR2520), *daf-2(m41);misc-1(tm2793)* (DR2540), *daf-28*p::*daf-28::gfp*, *ced-1::gfp* (MD701), *ced-3(n717)*, *cep-1(gk139)*, *lin-35(n745)*, *let-60(n1046)*, *mgIs[rol-6(su1006);myo-3*::mito::*gfp*] (DM8006), *myo-3*p::*myo-3*::*gfp* (RW1596), *qIs56[lag-2p::gfp; unc-119(+)]*. All strains were maintained on agar plates spread with *E. coli OP50* using standard techniques (Brenner, 1974). DR2520 was obtained by out-crossing the original knock-out strain received from the Knock-out Consortium three times with wild-type N2.

Cell culture

HEK293 and MIN6 cells were cultured in Dulbecco/Vogt modified Eagle's Minimal Essential Medium (DMEM) containing 25 mM glucose, 10% heat-inactivated Fetal Calf Serum (FCS) and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Transgenic lines

For the transgenic line carrying the MISC-1::GFP reporter, we amplified 1165 bp upstream of the *misc-1* ATG codon and the full *misc-1* gene from N2 genomic DNA. We then fused *misc-1* to *gfp* using the stitching PCR method (Hobert, 2002). The transgenes were injected with the *rol-6(su1006)* co-injection marker in the gonads of N2 hermaphrodites according to established protocols (Mello et al., 1991). Although the transgene was not integrated in the genome, ~90% of animals in each generation carried the transgene.

RNAi, siRNA and shRNA

RNAi by feeding was performed as previously described (Timmons and Fire, 1998; Kamath et al., 2003). The clone used for *misc-1* RNAi was B0432.4. *misc-1* RNAi and the empty RNAi

vector [L4440; Timmons and Fire, 1998) were propagated in *Escherichia coli* HT115 and administered to worms according to established feeding RNAi protocols (Kamath et al., 2001). For siRNA in HEK293 cells, we designed oligos targeting *OGC* with the software OligoEngine Workstation. The oligos were annealed in the siRNA vector pSuper.gfp/neo (OligoEngine) to make pSuperOGC. 1 μg of pSuperOGC or a control vector containing a scrambled mammalian sequence (X-Scramble) were transfected in HEK293 cells using Lipofectamine (Invitrogen). HEK293 transfection with Lipofectamine was done for 6-8 h at 37°C. Cells were grown for 24-48 h before being used for microscopy or RNA extraction. For *OGC* knock-down in MIN6 β-cells, an shRNA vector targeting mouse *OGC* was employed (TG504014, OriGene). The cells were grown in 24 well plates and transfected for 6-8 hours using 500 ng of either *OGC* shRNA vector or a vector containing a scrambled shRNA cassette as control and 1 μl Lipofectamine 2000 (Invitrogen) in 500 μL Opti-MEM, according to the manufacturer's instructions. Following transfection, MIN6 cells were cultured in DMEM with no penicillin/streptomycin for 72 hours before the assessment of apoptosis activation.

Microscopy

Worms were mounted on a 3% agarose pad and immobilized with 10 mM Levamisol (Sigma-Aldrich). Fixed HEK293 cells were mounted on microscope slides using the Vectashield mounting solution (Vector Laboratories). Images were taken using a Zeiss Axioskop with a QImaging Retiga 2000R camera. The imaging software used was Openlab 5.5.0. For some images (Figure 1G-H, Figure 2), we used a Zeiss Axiovert 200 M confocal microscope with the LSM 5 Pascal laser system. Quantification of GFP reporter expression was performed with ImageJ (Rasband WS, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.).

qRT-PCR

L1 larvae synchronized by sodium hypochlorite treatment and overnight starvation in M9 buffer were spotted on plates and grown until day 1 of adulthood. RNA was extracted with TRIzol (Invitrogen) and chloroform and purified with RNeasy Mini Kit (Quiagen). Equimolar concentrations of control and experimental RNA were used to make cDNA with SuperScript II Reverse Transcriptase (Invitrogen). Amplification of target genes was done with iTaq SYBR Green Supermix with ROX (Bio-Rad) using an AB 7500 Fast Real-Time PCR System with standard settings. Target gene expression was normalized against endogenous levels of *act-2* expression. The same protocol was followed for qRT-PCR experiments with HEK293 cells and normalization was done with respect to *Act*β expression.

Co-immunoprecipitation and Western blots

Cells were harvested in co-immuno precipitation buffer (25 mM TRIS pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1% sodium deoxycholate) with protease inhibitor cocktail (#539134, Calbiochem). Following sonication, the solution was pre-cleared with protein G-sepharose at slow rotation for 1.5 h at 4°C. The antibodies targeting the protein to be co-immunoprecipitated were then added at a concentration of 1:50 and allowed to bind at slow rotation at 4°C overnight. The next morning protein G-sepharose beads were added and the immuno-precipitation was allowed to run at slow rotation for 4 h at 4°C. Beads were washed four times, Laemmli buffer was added to the beads and the protein samples were heated at 65°C for 10 min before being loaded on an SDS-PAGE gel. Western blots were performed using standard techniques. We generated an α-MISC-1 antibody with GenScript Corporation. Other antibodies used: OGC (developed by FP); Bcl-xL (#2762, Cell Signaling Technology); α-ANT (# MSA02) and α-Complex IV subunit 1 (# MS404, MitoSciences). Western blots for activation of apoptosis employed a Caspase-3 antibody (#9662, Cell Signaling).

Staining *C. elegans* and HEK293 mitochondria

Mitochondria were stained with MitoTracker CMXRos (Invitrogen). To stain mitochondria in worms, we resuspended MitoTracker in molten (55°C) NG agar to a final concentration of 2 μg/mL. We poured 3-4 mL of the solution in 50 mm plates. The plates were then spotted with 100 μL of OP50. Plates were stored in the dark before and during the experiment. Synchronized L1 larvae were spotted on the plates and allowed to grow until used for microscopy on day 1 of adulthood. To stain mitochondria in HEK293 cells, we added MitoTracker CMXRos to fresh growth medium at a final concentration of 100 nM. The cells were incubated at 37°C for 45 min, fixed and counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma) according to suppliers' protocols.

Germline apoptosis and DAPI staining and phospho-H3 immunostaining

To stain apoptotic bodies in the germline, we used the dye SYTO12 green fluorescent nucleic acid stain (Invitrogen) according to established protocols (Gumienny et al., 1999).
DAPI staining was done following standard techniques. Phospho-H3 immunostaining was performed as previously described (Park et al., 2010).

Electron microscopy

Adults worms were washed directly into a primary fixative of 2.5% glutaraldehyde in 0.1M Sorensen phosphate buffer. Worms were transferred to microcentrifuge tubes and fixed for one hour at room temperature. Samples were then centrifuged at 3,000 rpm for one minute, supernatant removed and washed for ten minutes in 0.1M Sorensen phosphate buffer. The worms were then post-fixed in 1% osmium tetroxide in dH2O for one hour at room temperature. Following washing in buffer, specimens were processed for electron microscopy by standard methods; briefly, they were dehydrated in ascending grades of alcohol to 100%, infiltrated with Epon and placed in aluminum planchetes orientated in a longitudinal aspect and polymerized at 60^{o}C for 24 hours. Using a Leica UC6 ultramicrotome, individual worms were sectioned in cross

section at 1μm intervals, starting outside the worm, until the anterior tip of the animal was located as judged by examining the sections stained with toluidine blue by light microscopy. Thereafter, serial ultra-thin sections of 90nm were taken. Sections were picked onto 200 mesh copper grids, stained with uranyl acetate and lead citrate and examined under a Tecnai Twin (FEI) electron microscope. All mitochondrial images were taken from muscle cells observable in anterior sections of the worm (*i.e.,* typically 5-15 μm from the nose tip). Where required, sections were tilted using the Compustage of the Tecnai to ensure an exact geometrical normalcy to the imaging system. All images were recorded at an accelerating voltage (120 kV) and objective aperture of 10 μm, using a MegaView 3 digital recording system.

Metabolic assays

Glucose concentration in worms was measured with a Glucose Assay Kit (#K606-100, BioVision) according to manufacturer's instructions. ATP contents were assayed with the Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (#FL-AA, Sigma) according to manufacturer's instructions.

Statistical analyses

Results are expressed as mean ± S.E.M. A *P*-value of less than 0.05 was considered significant. Statistical analyses were performed with the statistical package GraphPad Prism 5.02.

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3 ACTIVATION OF AN ALTERNATIVE ENERGY-PRODUCING PATHWAY EXPLAINS THE LONGEVITY OF *C. ELEGANS* MITOCHONDRIAL MUTANTS[1]

Caenorhabditis elegans is one of the favourite models for aging studies (Guarente and Kenyon, 2000), including the role of Reactive Oxygen Species (ROS) and mitochondria in the determination of life span. The literature supports the notions that ROS accumulation (Harman, 1956) and mitochondrial function (Dillin et al., 2002; Lee et al., 2003; Rea et al., 2007) are both negatively correlated with life span. Here we show that ROS levels do not correlate with life span in _C. elegans_ and provide evidence that the negative correlation between mitochondrial function and life span might be nematode-specific. We identified a new role in ROS detoxification and oxidative stress response for the metabolic gene _misc-1/OGC_ (MItochondrial Solute Carrier/2-Oxoglutarate Carrier). Absence of this gene results in a two-fold increase in basal ROS levels in _C. elegans_, while not affecting organismal life span in several genetic backgrounds. We show the direct dependence of mitochondrial mutants' longevity on the glyoxylate shunt, an extra-mitochondrial pathway of energy production. Elevation of glyoxylate shunt enzyme transcription levels is a predictor of life span extension in mitochondrial mutants. Since the glyoxylate shunt is not active in mammals, _C. elegans_ is not a straightforward model for the role of mitochondria in mammalian aging.

Although originally identified for its ability to transport 2-oxoglutarate (a.k.a. α-ketoglutarate) across the inner mitochondrial membrane (Bisaccia et al., 1985), OGC was later shown to (a) have mitochondrial uncoupling functions in human cell lines (Yu et al., 2001), (b) import at least some of the porphyrins required for synthesis of heme groups in mitochondria (Kabe et al., 2006), and (c) to mediate the transport into mitochondria of ~40% of total mitochondrial glutathione (GSH) (Xu et al., 2006). The antioxidant GSH is a low-molecular-weight thiol that decreases oxidative stress by conjugating with reactive molecules. We therefore decided to

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study the *in vivo* function of MISC-1/OGC, with respect to its possible role as a regulator of cellular damage produced by ROS.

We identified OGC's best reciprocal BLAST match in *C. elegans* (72% amino acid identity; Appendix 2, Fig. S1) and called it MISC-1 (MItochondrial Solute Carrier). Our hypothesis that *misc-1* might be involved in oxidative stress response was strengthened by the presence of two putative DAF-16/FOXO (Forkhead box O) (Ogg et al., 1997) and two putative SKN-1/bZip (Bowerman et al., 1992; An and Blackwell, 2003) binding sites in the ~1100 bp upstream of the predicted *misc-1* translation start. Both of these transcription factors have been shown to have pivotal roles in activating stress response genes (Henderson and Johnson, 2001; Oliveira et al., 2009). To test whether MISC-1 is involved in mitochondrial ROS detoxification *in vivo*, we assayed the effects of lack of MISC-1 using the *misc-1(tm2793)* knock-out allele. *tm2793* harbours a 203-bp deletion that removes the first intron and most of the second exon (Appendix 2, Fig. S2). Consistent with a knock-out mutation, we were unable to detect the *misc-1* transcript by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR, data not shown) or the MISC-1 protein by Western Blot (Appendix 2, Fig. S2) in *misc-1(tm2793)* worms. Loss of MISC-1 function affects stress response. We induced ROS generation by exposing synchronized one-day old adults to Juglone (5-hydroxy-1,4-naphtoquinone) (de Castro et al., 2004). Animals lacking MISC-1 were more sensitive to Juglone than wild-type N2 (Fig. 3.1a), confirming that MISC-1 is involved in oxidative stress response *in vivo*. This finding corroborates *in vitro* data suggesting that human OGC is involved in ROS detoxification (Xu et al., 2006). Next, we exposed N2 and *misc-1* animals to a mitochondrial poison, sodium azide (NaN_3), which binds and irreversibly inhibits cytochrome c oxidase (Hasinoff, 1990). Surprisingly, the *misc-1* mutant was more resistant to NaN_3 than wild type (Fig. 3.1b), suggesting that the mutant is less dependent on mitochondrial energy production. Finally, we tested N2 and *misc-1* strains for thermal resistance at 35°C (Fig. 3.1c) and 37°C (data not shown), and were unable to observe a significant difference between the two strains ($P > 0.05$). Our assays indicate that MISC-1 is specifically involved in preventing oxidative damage.

We measured physiological ROS levels in unstressed animals to determine whether *misc-1* mutants are sensitive to Juglone-induced ROS production because of inherent high basal levels of ROS or because of problems in clearing exogenously-induced ROS. *misc-1* mutants had an almost two-fold increase in the levels of physiological ROS, compared to N2 control (Fig. 3.1d), indicating that MISC-1 is normally required for ROS detoxification. Absence of the protein results in high ROS levels that make the mutants extremely sensitive to exogenously-induced ROS production.

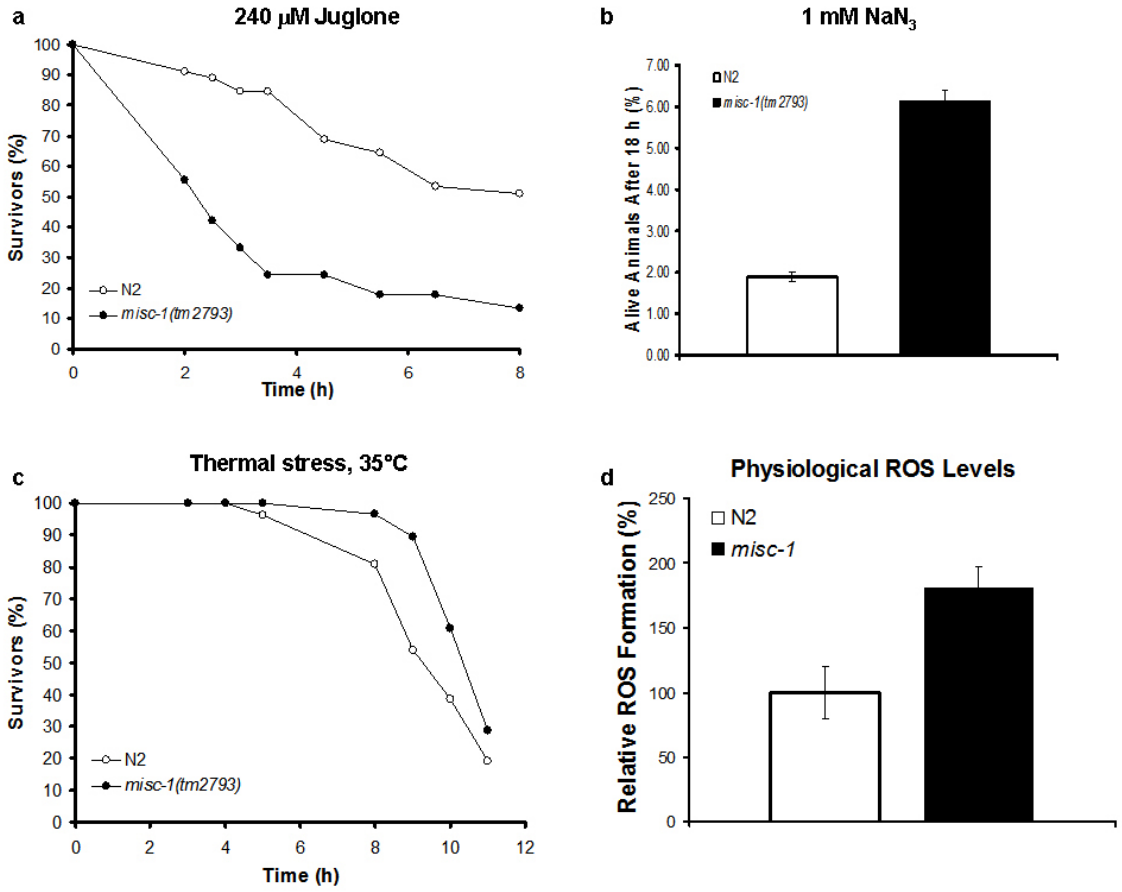

Figure 3.1 | MISC-1 is required for oxidative stress response.
a, *misc-1* mutants were more sensitive to Juglone than N2. T-test $P = 0.0026$. N = 45 for each strain used. Using established protocols (de Castro et al., 2004) *misc-1* knock-out mutants and wild-type N2 one day-old adults were exposed to 240 μM Juglone (Aldrich), a compound known to induce ROS formation. **b,** *misc-1* mutants are resistant to mitochondrial poisoning by sodium azide. One day-old adults were incubated in 24-well plates for 18 h in a solution of 1 mM sodium azide (Sigma), with OP50 added as a food source to prevent starvation. Live animals were scored based on their ability to swim upon perturbation of the medium. *misc-1* knock-out mutants were three-fold more resistant to NaN_3 than N2 (t-test $P = 0.0061$). N = 208 for N2; N = 214 for *misc-1(tm2793)*. Error bars: ± SEM. **c,** The N2 and *misc-1* strains showed similar sensitivity to thermal stress (t-test $P = 0.4736$). Assays were performed as described (Lithgow et al., 1995). N2 (N = 26) and *misc-1* (N = 28) animals were stressed at 35°C and scored for survival at the times indicated. **d,** *misc-1* day one adult mutants showed an almost two-fold increase in the level of basal cellular ROS compared to N2 (t-test $P = 0.0025$). ROS levels were assayed with 2',7'-Dichlorofluorescein Diacetate (Sigma) and normalized to protein concentration, as described (Artal-Sanz and Tavernarakis, 2009).

In *C. elegans*, decreased expression of some mitochondrial proteins results in a Mit phenotype, consisting of ~20-40% increase in mean adult life span, slowed embryonic and larval development, decreased pharyngeal pumping, reduced defecation rates and small body size (Rea et al., 2007). However, loss of *misc-1* function allowed wild-type developmental timing, body size (data not shown) and pharyngeal pumping (Fig. 3.2a). Although our assays showed that *misc-1* knock-out animals had high levels of ROS (Fig. 3.1d), their life span was indistinguishable from wild type (Fig. 3.2b; Appendix 2, Table 1). However, a two-fold increase in ROS levels is enough to affect life span in other genetic backgrounds (Artal-Sanz and Tavernarakis, 2009). We next tested *misc-1* for subtle additive effects on the life span of long-lived mutants in the insulin/IGF1-like pathway (i.e., *daf-2*, dauer formation) or the caloric restriction pathway (i.e., *eat-2*, eating abnormal). *misc-1(tm2793)* did not affect *daf-2* longevity (Fig. 3.2c), nor did *misc-1* RNAi, which reduced MISC-1 protein levels by ~87% (Appendix 2, Figure S4), affect *eat-2* life span (Fig. 3.2d; Appendix 2, Table 1). All together, our data are in agreement with recent reports that showed that genetic abrogation of ROS-detoxifying pathways does not have negative effects on life span (Yang and Hekimi, 2010; Honda et al., 2008; Doonan et al., 2008; Van Raamsdonk et al., 2010). Hence, oxidative stress (Harman, 1956) does not seem to play an important role in *C. elegans* life span, at least within a roughly two-fold range of physiological ROS (Fig. 3.1d).

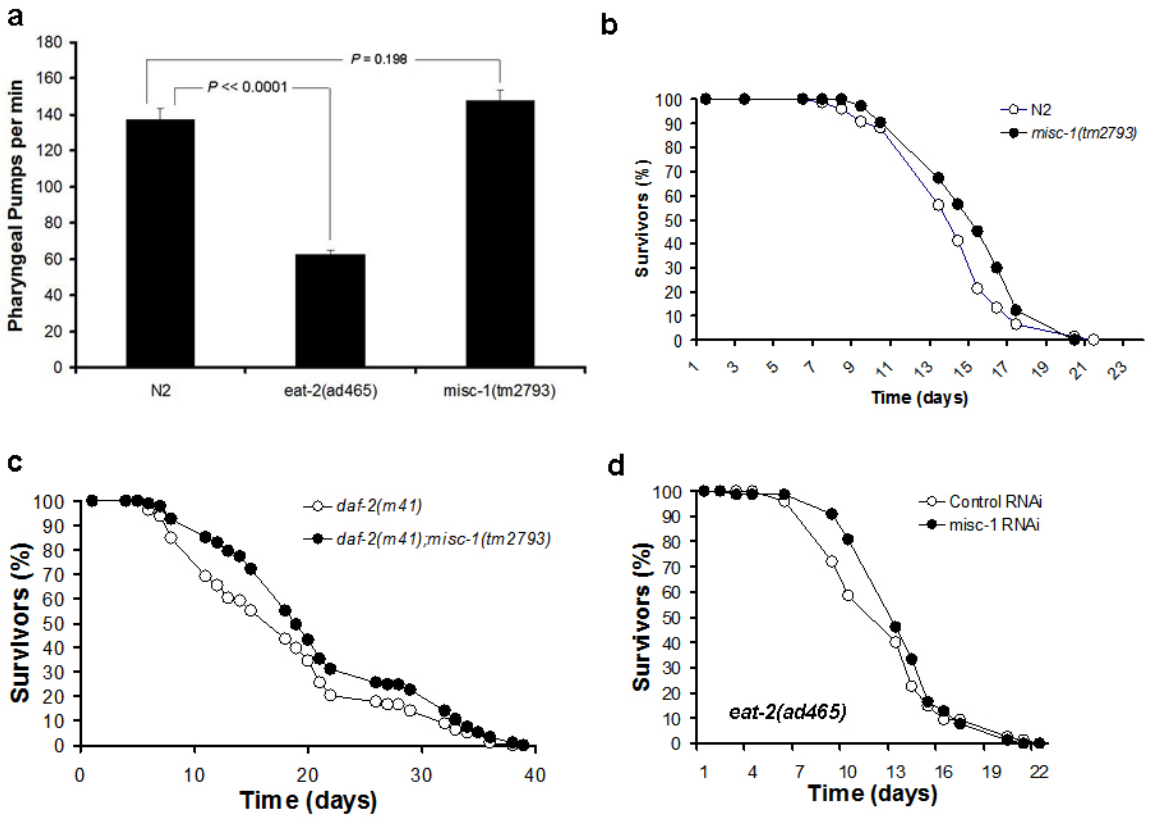

Figure 3.2 | *misc-1* knock-out and knock-down do not confer longevity.
a, *misc-1* knock-out day-one adults do not have the slow pharyngeal pumping typical of Mit mutants. The *eat-2(ad465)* mutant was used as a control, because its pharyngeal pumping rate was previously shown to be slower than wild-type (Raizen et al., 1995). N = 21 for each strain. Error bars: ± SEM. *P* values were obtained by t-test. An independent trial was performed with similar results. **b,** Life spans of N2 and *misc-1(tm2793)* knock-out adults do not differ significantly. 0.1 mg/mL FUDR (2'-Deoxy-5-fluorouridine; Sigma) was dissolved in the medium to prevent production of progeny (Hosono et al., 1982). See supplementary table 1 (Appendix 2) for full life span statistics. **c,** Life spans of long-lived *daf-2(m41)* and *daf-2(m41);misc-1(tm2793)* double mutants show no significant difference. FUDR was used as in **b**. **d,** *eat-2* mutants were treated with either control RNAi or *misc-1* RNAi. All survival assays were performed at 25°C; none showed a significant effect of *misc-1* on longevity. We conclude that the increased ROS levels do not significantly affect life span.

We hypothesized that the lack of a Mit phenotype was owing to upregulation of genes that could compensate for the lack of MISC-1-mediated detoxification. The natural candidates for *misc-1* compensation were the *sod* (superoxide dismutase) genes, which encode enzymes that catalyze the dismutation reaction that converts superoxide radicals O_2^- to H_2O_2 (reviewed by Fridovich, 1995). While we observed ~50% up-regulation of cytoplasmic Cu/Zn *sod-5* transcription in *misc-1* mutants, all the other *sod* genes were expressed at wild-type levels, as determined by qRT-PCR (Appendix 2, Fig. S3). These data raise the possibility that absence of MISC-1 affects ROS levels in the entire cell, not just in mitochondria.

MISC-1/OGC affects a wide array of mitochondrial functions predicted to affect life span (metabolism and energy production, uncoupling and ROS detoxification), yet animals lacking this protein have virtually no gross morphological or life span phenotype, compared to the Mit mutants. *misc-1* and Mit mutants show that *C. elegans* is not fully dependent on mitochondrial function for energy production. Many Mit mutants are long-lived, whereas humans with mutations in corresponding Electron Transport Chain (ETC) genes develop severe metabolic syndromes (reviewed by Wallace, 1999). Furthermore, RNAi-mediated down-regulation of the *Drosophila* orthologues of *C. elegans* Mit genes results in all the pleiotropic phenotypes of Mit mutants, except for life span extension (Rera et al., 2010). Knocking-down these genes in *Drosophila* was actually neutral or decreased life span, in both males and females. Furthermore, *C. elegans* mitochondrial polymerase γ (*polg-1*) mutants, possessing only ~4% of wild type mitochondrial genome copy number, are viable (Bratic et al., 2009). This is consistent with the increased resistance of *misc-1* mutants to NaN_3, which also suggests that *misc-1* worms are less dependent on mitochondrial energy production than wild type under normal conditions. These observations suggest that *C. elegans* is less dependent on overall mitochondrial function than other animals and the longevity of Mit mutants might be *C. elegans*-specific.

It has been proposed that Mit mutant longevity might result from their ability to produce energy in a mitochondrion-independent way, perhaps through the glyoxylate shunt (Braeckman et al., 2002; Rea and Johnson, 2003; Lee et al., 2003), which is upregulated in the dauer diapause state and in *daf-2/IGF1R* mutants (Murphy et al., 2003; Ruzanov and Riddle, 2010). Both dauer larvae and *daf-2* mutant adults are long-lived (Klass and Hirsh, 1976; Kenyon et a;., 1993). The main glyoxylate shunt enzyme in *C. elegans*, encoded by *gei-7* (Tsuboi et al., 2002), is malate synthase/isocitrate lyase, which cleaves isocitrate to form glyoxylate and succinate. Glyoxylate can be used to produce malate, while succinate can be converted to oxaloacetate. Both malate and oxaloacetate are gloconeogenic and can be used to produce carbohydrates. When mitochondria are not functional, the glyoxylate shunt may be helpful because it allows the use of lipid break-down for generating carbohydrates, which can then undergo glycolysis and produce a net yield of ATP. Knock-down of *gei-7* reduced *daf-2* life span by about 30% (Murphy et al., 2003). GEI-7 function is mostly found in embryos and larvae (Wadsworth and Riddle, 1989). We analyzed *gei-7* expression across all developmental stages by serial analysis of gene expression (McKay et al., 2003) and found that *gei-7* expression occurs mostly between the first and fourth larval stages (L1-L4), with a peak in L3 worms and low levels at the other larval stages (Fig. 3.3a). We therefore decided to study *gei-7* expression in different genetic backgrounds in L4 animals, when the expression of the gene is minimal. *gei-7* transcriptional levels were upregulated only slightly in *misc-1* mutants compared to wild type (Fig. 3.3b), but *gei-7* expression in one of the best studied long-lived Mit mutants, *clk-1(qm30)* (Wong et al., 1995), was up-regulated four-fold (Fig. 3.3b). We then tested mutants in another Mit gene, *isp-1(qm150)* (Feng et al., 2001), encoding a subunit of complex III of the ETC. Also in this case, expression of *gei-7* was upregulated four-fold, as it was in the similarly long-lived *daf-2(e1370);isp-1(qm150)* (Feng et al., 2001) double mutant (Fig. 3.3b). Robust activation of the glyoxylate shunt seems to be associated with increased life span in mitochondrial mutants.

Knock-down of *gei-7* decreases the life span of *cyc-1* Mit mutants (Cristina et al., 2009). We knocked-down *gei-7* in wild type, *misc-1(tm2793)* and *clk-1(qm130)* worms. *gei-7* RNAi did not affect life span in wild type and *misc-1* mutants (data not shown). However, *gei-7* RNAi reduced the life span of *clk-1* mutants, impacting especially their maximum life span, compared to control-fed animals (Fig. 3.3c; Appendix 2, Table 1). The small effect of *gei-7* RNAi on *clk-1* life span might be explained by the low efficiency of *gei-7* knock-down achieved (44%; see Appendix 2, Figure S5).

Although there is scant information on the existence of a functional glyoxylate pathway in humans (Davis and Goodman, 1992), the general consensus is that this pathway is not functional in mammals. The effects of ETC mutations in patients with mitochondrial metabolic syndromes suggest that, unlike *C. elegans*, humans do not have the option of switching their metabolism to a mitochondrion-independent pathway of energy production. This is an important caveat in the use of *C. elegans* as a model to study the effects of mitochondrial function on aging or disease. Life span experiments performed in *gei-7* genetic backgrounds might provide information more relevant to mammals, since this mutation would remove the compensatory mechanism that underlies longevity in *C. elegans* mitochondrial mutants but that are not relevant to mammals.

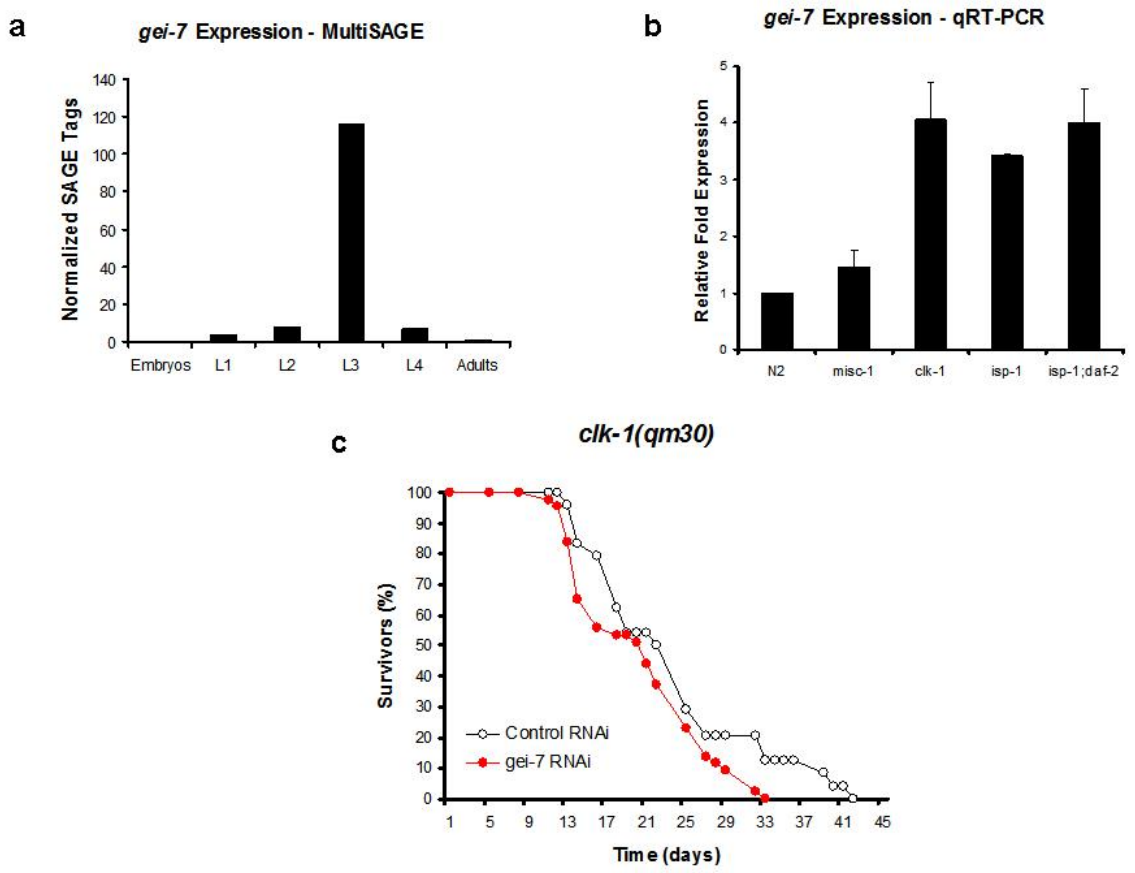

Figure 3.3 | Activation of the glyoxylate shunt in mutants with defective mitochondrial electron transport.
a, Expression levels of *gei-7*, according to SAGE transcript levels. Expression of this gene, which encodes the only enzyme of the *C. elegans* glyoxylate shunt, peaks at the L3 stage and is barely detected in young adults. **b,** Transcript levels of *gei-7* in N2, *misc-1* and the long-lived mitochondrial mutants *clk-1(qm30)*, *isp-1(qm150)* and *daf-2(e1370);isp-1(qm150)*. *gei-7* transcripts are increased in all mitochondrial mutants (*clk-1*, $P = 0.001$; *isp-1*, $P = 0.000154$; *isp-1;daf-2*, $P = 0.018$), compared to control, but not in *misc-1* (t-test $P = 0.06$). These data point to a prominent role for the glyoxylate pathway in providing an alternative source of energy when mitochondrial function is compromised. For this experiment, RNA was extracted from L4 animals by the TRIZOL (Invitrogen, Carlsbad CA, USA) method and used to make cDNA with SuperScript II Reverse Transcriptase (Invitrogen). Amplification of target genes was done with iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules CA, USA) using an AB 7500 Fast Real-Time PCR System with standard settings. Data analysis was performed with the 7500 Fast System software. **c,** *gei-7* RNAi decreases *clk-1* maximum life span. This experiment was performed as described in Fig. 3.2b, but at 20°C.

3.1 Methods

misc-1(tm2793) was backcrossed to N2 three times before use. Animals were grown under standard conditions (Brenner, 1974), unless otherwise specified. RNAi constructs (Kamath et al., 2003) were verified by PCR amplification and restriction digest. The *gei-7* RNAi construct was also sequenced. RNAi experiments were performed according to standard protocols (Timmons and Fire, 1998; Kamath et al., 2001). Life span experiments were performed as previously described (Kenyon et al., 1993). Statistical analyses were performed with the statistical package GraphPad Prism 5.02.

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3.3 Acknowledgments

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4 EFFECTS OF A *CAENORHABDITIS ELEGANS* DAUER PHEROMONE ASCAROSIDE ON PHYSIOLOGY AND SIGNAL TRANSDUCTION PATHWAYS [1]

4.1 Introduction

Development of the free-living soil nematode *Caenorhabditis elegans* includes an embryonic phase, four larval stages (L1-L4), and the adult. Under conditions unfavorable for reproductive growth, i.e., low food supply, high temperature, and/or high population density, *C. elegans* can enter the dauer stage as an alternative to the L3 (Cassada and Russell, 1975). Dauer larvae are a non-feeding diapause state that can survive for months by utilizing fat stores for energy. Once environmental conditions improve, they begin to feed, molt to the L4, and then to the adult (Riddle and Albert, 1997).

High population density is signaled by the concentration of a constitutively secreted pheromone (Golden and Riddle, 1984a). If the ratio of pheromone to food is high during the L1 and L2 stages, the larvae adjust their metabolism to accumulate fat in preparation for a possibly prolonged period without feeding. An accurate environmental assessment (precise threshold) is highly adaptive. An unnecessary developmental arrest substantially delays reproduction, whereas failure to disperse when necessary negatively impacts progeny survival.

The pheromone was shown to induce dauer formation by affecting several signal transduction pathways. Mutations in some of the genes in these pathways lead to constitutive dauer formation (Daf-c), whereas mutations affecting other pathway components impede entry into dauer, a phenotype called dauer-defective (Daf-d) (Riddle and Albert, 1997). The genes were placed in pathways based on epistatic relationships [reviewed in Riddle and Albert (1997)]. Two parallel signal transduction pathways, the *daf*-7/TGF-β (Transforming Growth Factor-β)

[1] A version of this chapter has been published. Gallo, M. and Riddle, D.L. (2009) Effects of a *Caenorhabditis elegans* dauer pheromone ascaroside on physiology and signal transduction pathways. *J. Chem. Ecol.* **35**: 272-279. Reprinted with kind permission from Springer Science+Business Media.

pathway and the *daf-2*/*IGF1R* (Insulin-like Growth Factor 1 Receptor) pathway, were defined, converging on the DAF-12 nuclear hormone receptor (Snow and Larsen, 2000; Jia et al., 2002).

The dauer pheromone was initially identified as a mixture of hydroxylated short-chain fatty acids or bile salts (Golden and Riddle, 1984b). Recently, the four major components of this pheromone were purified from extracts and synthesized (Jeong et al., 2005; Butcher et al., 2007, 2008). Biological activity of these structurally related ascarosides (Fig. 4.1) was demonstrated by measuring entry into the dauer stage on agar plates in the presence of limited food. One of the pheromone components with the trivial name daumone (Jeong et al., 2005) occurs at about one-tenth of the concentration of the two major components (Butcher et al., 2007). We used synthetic daumone to compare its modes of action with natural dauer pheromone extracts with the aim of uncovering the physiological effects and the mechanisms by which daumone induces dauer formation.

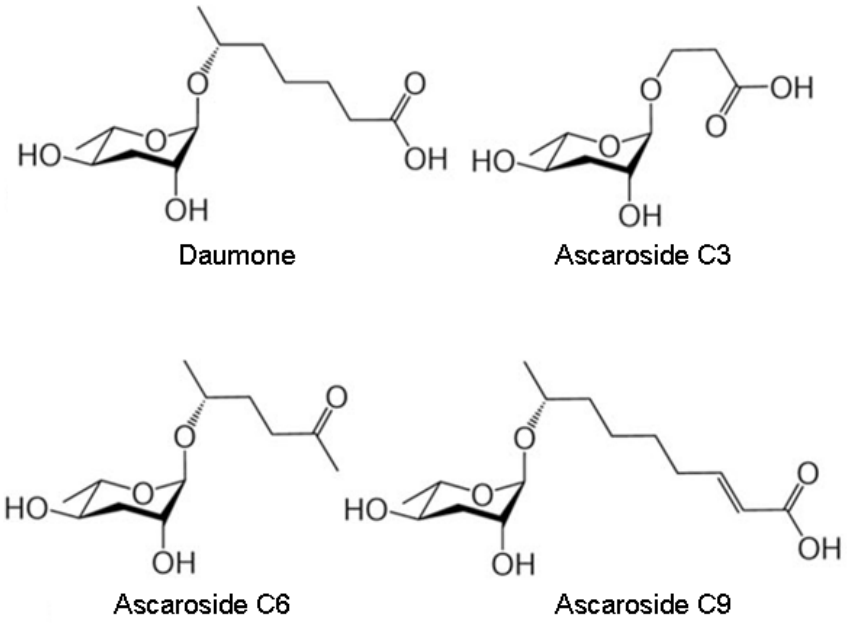

Fig. 4.1 Structures of the four known components of the dauer pheromone (adapted from Butcher et al., 2008). They are all derivatives of the 3,6-dideoxyhexose ascarilose. Our studies focus on the compound known as daumone.

4.2 Methods and materials

Caenorhabditis elegans strains used Wild-type N2, *mIs7[daf-7*p::*gfp*::*daf-7* 3' UTR *rol-6(su1006)]* (DR 2021), *zIs356[daf-16*p::*daf-16*::*gfp]* (TJ356) (Henderson and Johnson, 2001), *gpa-3(pk35)*, *daf-3(mgDf90)*, *daf-16(mgDf47)*, *daf-8(m85);daf-3(e1376)*, *daf-12(m20)*, *daf-14(m77);daf-3(e1376)*, *dpy-14(e188)*. Strains were cultured by using standard techniques (Brenner, 1974).

Dauer induction assays Assays were performed as described by Jeong et al. (2005). In brief, synthetic daumone (KDR Biotech, Seoul, Korea) was dissolved in 95% ethanol. Plates were prepared by adding daumone to NG agar (Brenner, 1974) without peptone to a final concentration of 384 μM. A total volume of 3 ml of NG plus daumone was dispensed in each 50-mm diam. plate. Control plates were prepared by adding 95% ethanol equal to the volume used to deliver daumone to the experimental plates. Heat-killed *Escherichia coli* OP50 (160 μg) were added to the plates and allowed to dry. Five adult worms were placed on each plate and allowed to lay eggs for 5 hr before being removed. The plates were then incubated at 25°C for 70-72 hr and dauer formation was scored visually with a stereomicroscope.

Pheromone plates Pheromone extracts were prepared as described by Golden and Riddle (1984a). Pheromone plates were prepared with pheromone at a final concentration of 3x (three times the concentration found in starved liquid culture media) following established protocols (Golden and Riddle, 1984c). Control plates were made by adding a volume of M9 buffer equivalent to the volume of pheromone added on the experimental plates.

DAF-16 nuclear localization Five *daf-16p*::*daf-16*::*gfp* (TJ356) adults were placed on daumone or control plates and allowed to lay eggs at 25°C. Nuclear localization was assessed after 14 hr by observing GFP fluorescence at 660x with the aid of a Zeiss Stemi SV 11 Apo

stereomicroscope. Embryos or L1 larvae were scored as either having DAF-16 nuclear localization or as intermediate/cytoplasmic localization. *P*-values were calculated by using the χ^2 test. Embryonic DAF-16 nuclear localization was similarly assessed on pheromone plates, with the exception that three gravid adults were placed on each plate and allowed to lay 30-50 eggs.

Daumone toxicity Triton X-100 (Sigma) was used to permeabilize the nematode cuticle. Before seeding plates with OP50, 300 μl of a stock 10% solution of Triton X-100 were added to plates to reach a final concentration of 1% (Rogalski et al., 1990). Control plates were made by adding 300 μl of distilled water instead of detergent. 10 to 15 gravid *dpy-14(e188)* adults were added per plate and incubated at 25°C. Survival was assessed at 16 and 24 hr. Statistical analysis was performed with the log-rank test available with the statistical package R.

4.3 Results

4.3.1 ***Daumone reduces TGF-β signalling*** TGF-β signaling promotes reproductive growth, whereas its down-regulation results in dauer formation (Ren et al., 1996). Exposure to dauer pheromone extracts prevents transcription of the TGF-β ligand *daf-7* (Ren et al., 1996), which is solely expressed in the amphid chemosensory neurons ASI. These are a pair of bilaterally symmetrical ciliated neurons that function to inhibit dauer entry (Bargman and Horvitz, 1991). To test whether daumone possesses similar properties to whole dauer pheromone extracts, we exposed a strain carrying an integrated *daf-7* promoter::GFP fusion (*daf-7*p::*gfp*) to 384 μM daumone. Reporter fluorescence gradually decreased from the L1 to the pre-dauer L2d (Fig. 4.2A-D), and was completely abrogated in dauer larvae formed in response to daumone.

Although the decreased promoter activity observed in our transgenic larvae recapitulated the effects of whole dauer pheromone extracts (Ren et al., 1996), there was also an obvious difference. About 25% of the larvae treated with daumone expressed the *daf-7*p::*gfp* reporter not only in the ASI neurons, but also in another amphid neuron (Fig. 4.2E). Expression was transient just prior to dauer formation and was absent in dauer larvae. The cell body of this neuron is just posterior to the midline of the second bulb of the pharynx, a location characteristic of the ciliated amphid neuron ASJ, which is required for exit from the dauer stage (Bargman and Horvitz, 1991). There have been no previous reports of *daf-7* expression in the ASJ neurons.

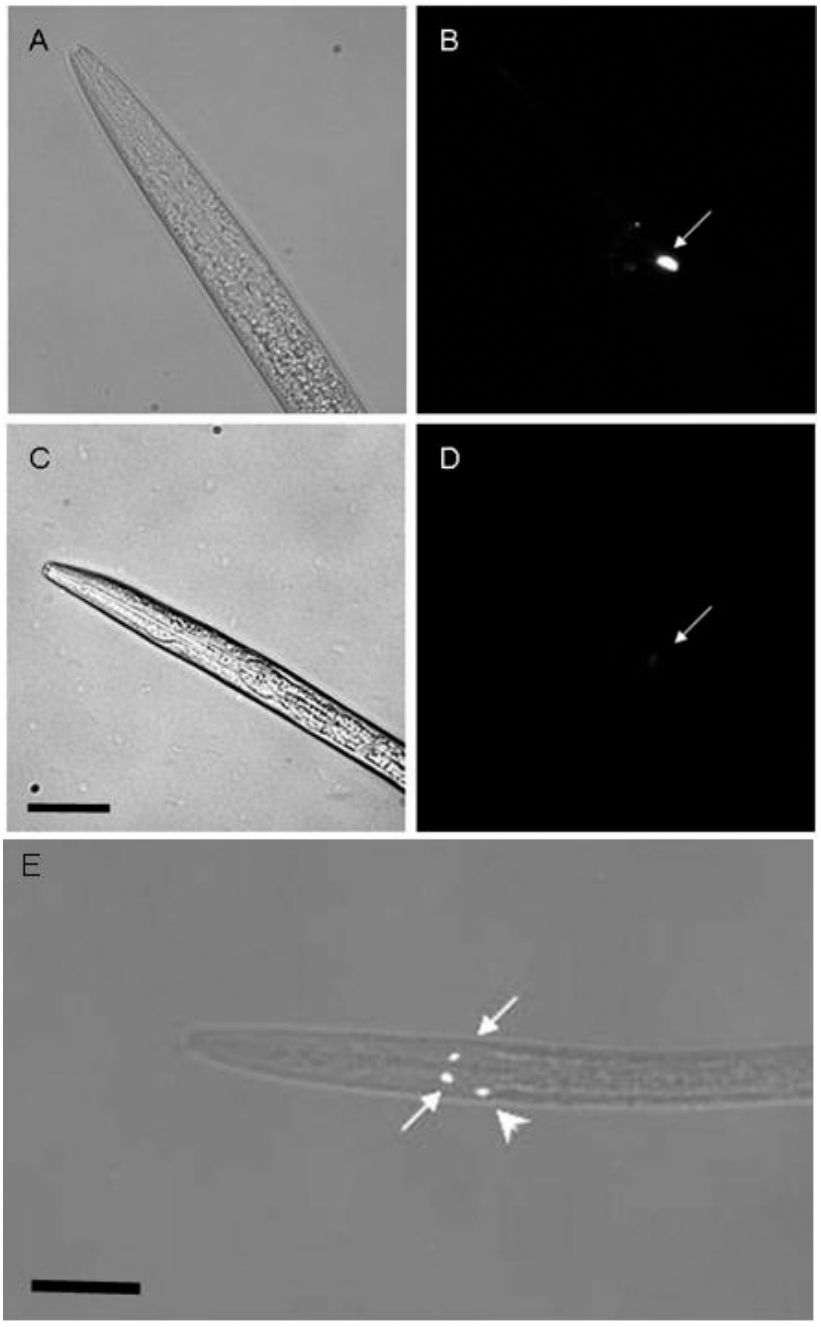

Fig. 4.2 (A-D) Daumone decreases expression of *daf-7*, the gene encoding a *C elegans* TGF-β ligand. Adult hermaphrodites carrying an integrated *daf-7p::gfp::daf-7* 3′ UTR construct (DR2021) were placed on control or daumone plates and were allowed to lay eggs overnight. After 14 hr of incubation, larvae were scored for GFP fluorescence with a UV stereomicroscope. (A) DIC and (B) fluorescent image of a control L2 larva. Control larvae showed strong expression of the GFP reporter construct in the ASI neurons. (C) DIC and (D) fluorescent image of a larva hatched in daumone. Larvae exposed to daumone showed little or no GFP reporter expression. (E) Daumone induces expression of *daf-7* in the ASJ neurons. *daf-7* is normally expressed in the ASI neurons (arrows). However, ~25% of DR2021 L2d worms treated with daumone expressed *daf-7* in one of the two ASJ neurons (arrowhead). The pre-dauer L2d shown is representative of the subpopulation expressing the reporter in ASJ. Scale bars, 50 μm.

4.3.2 ***Daumone induces DAF-16 nuclear localization in embryos*** Dauer induction involves down-regulation of *daf-7*/TGF-β and *daf-2/IGF1* signaling. Mutations that disrupt either pathway result in constitutive dauer formation. Insulin/IGF1 signaling inhibits the FOXO transcription factor DAF-16 (Lin et al., 1997). When insulin signalling is reduced, DAF-16 is not phosphorylated by the upstream insulin pathway component AKT-1 and is free to enter the nucleus (Henderson and Johnson, 2001; Paradis and Ruvkun, 1998) to regulate the transcription of genes involved in dauer formation and adult longevity [reviewed in Jensen et al. (2006)]. If daumone reduces insulin signalling, exposure to daumone should result in the nuclear localization of DAF-16. To test this, we exposed a transgenic line carrying an integrated DAF-16::GFP translational fusion (Henderson and Johnson, 2001) to daumone. We scored animals at different developmental stages as having nuclear or cytoplasmic/intermediate DAF-16 localization.

The decision to enter the dauer stage is taken during the L1 and the L2 (Golden and Riddle, 1984a) and indeed nuclear localization was stronger in daumone-treated L1s (Fig. 4.3) than in L1s grown on control plates, although this difference did not reach statistical significance ($P = 0.228$). Surprisingly, daumone induced strong DAF-16 nuclear localization in embryos ($P < 0.001$). It did not do so in adults (data not shown). If embryonic DAF-16 nuclear localization is a requirement for dauer formation, it should be elicited by exposure to natural pheromone extracts as well. We treated DAF-16::GFP worms with pheromone extracts at a concentration sufficient to induce 61% dauer formation in N2. However, we observed no difference in embryonic nuclear localization in worms treated with pheromone compared to control ($P = 0.64$).

DAF-16 nuclear localization can be achieved by repression of the insulin pathway, or by activation of a stress response pathway parallel to the insulin pathway. This pathway is mediated by the sirtuin SIR-2.1, which binds to DAF-16 and translocates to the nucleus (Henderson and Johnson, 2001; Berdichevsky et al., 2006). The DAF-16 nuclear localization in embryos raised the possibility this was a response to stress (embryos do not make the decision

to enter the dauer stage and natural pheromone does not induce DAF-16 nuclear localization). The only difference between treatment and control plates was the presence of daumone. Hence, daumone may have toxic effects on *C. elegans* at the concentration used (384 μM) (Jeong et al., 2005). Pheromones are usually active in the nM concentration range, or a few hundred molecules per square centimeter (Dulac and Torello, 2003), much less than the working concentration of daumone required to induce dauer formation in the bioassay (Jeong et al, 2005).

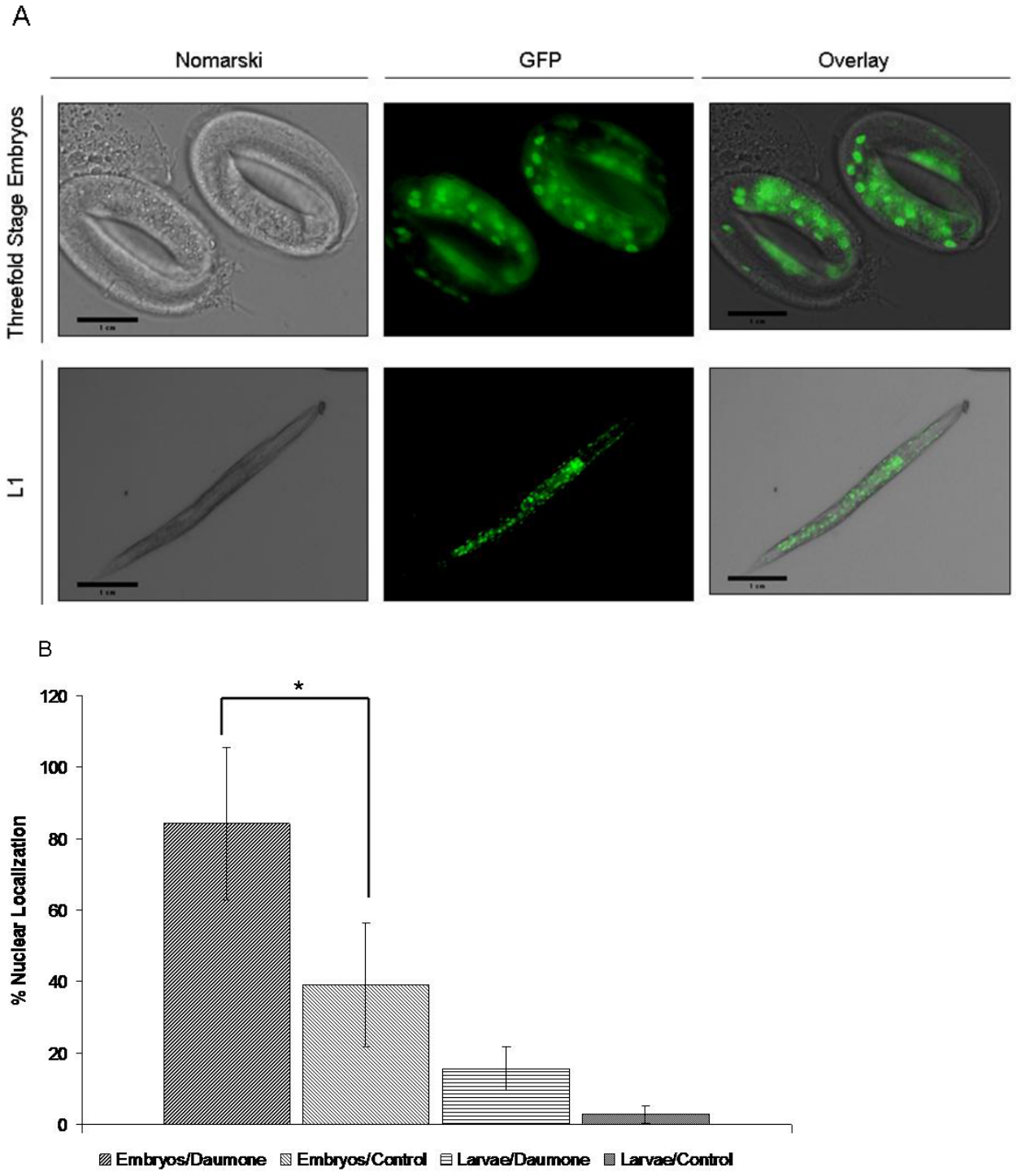

Fig. 4.3 Daumone induces DAF-16 nuclear localization in embryos. (A) A representative embryo and a representative L1 larva with DAF-16 nuclear localization upon daumone treatment. Strain TJ356 carries an integrated DAF-16::GFP translational fusion construct. (B) Quantification of DAF-16 nuclear localization in embryos and L1s. Animals were described as having nuclear localization if DAF-16 was observed predominantly in the nuclei, otherwise they were scored as intermediate/cytoplasmic localization. *P*-values were calculated with a χ^2 test. *Statistically significant difference. Error bars represent ± standard deviation (s.d.).

4.3.3 ***Daumone toxicity*** Mutations in the collagen gene *dpy-14* (DumPY) result in a defective cuticle that allows normally excluded substances to leak into mutant worms (Gallo et al., 2006). We used this strain as a sensitized background to perform a daumone resistance assay. To further permeabilize the cuticle of these mutants, we added 1% Triton X-100 to the plates. This concentration of Triton X-100 does not affect animal growth and reproduction (Rogalski et al., 1990). Detergent-treated *dpy-14* adults started dying within 16 hr of daumone exposure, as judged by absence of pharyngeal pumping and lack of response to prodding (Fig. 4.4). Dpy-14 adults on control plates [where daumone was replaced with 95% ethanol (Jeong et al., 2005)] did not die in the first 24 hr of daumone treatment. Hence, *dpy-14* adults exposed to daumone had a lower survival rate than controls (P = 0.0089 by log-rank test). If adults with a Rol-6 (roller) phenotype were placed on daumone plates, they also died or looked very sick within a few hours. *rol-6* encodes a cuticular collagen and mutations in this gene result in a cuticle twisted in a right-handed helix (Kramer et al., 1990).

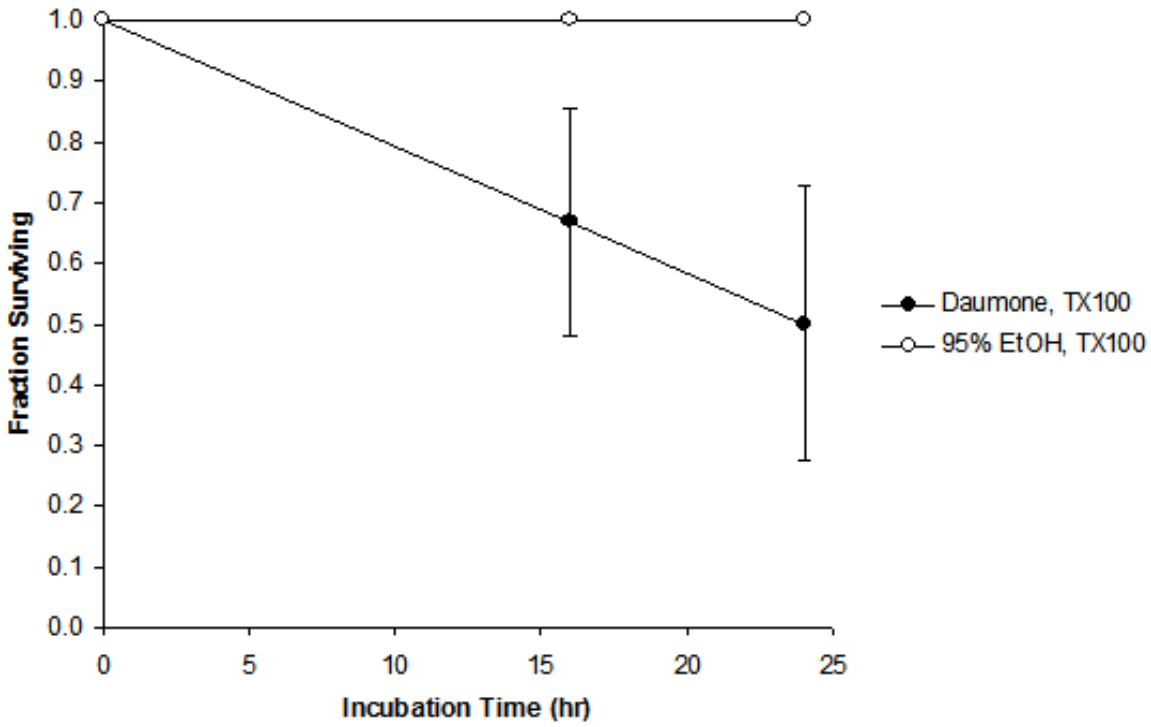

Fig. 4.4 Daumone is toxic to *dpy-14* mutants with a defective cuticle. This survival assay was performed in duplicate with a total of 18 treated and 10 untreated adults. Daumone is lethal to *dpy-14* adults at the concentration used, whereas none of the unexposed mutant adults died. Error bars represent ± standard error of the mean (s.e.).

4.3.4 ***Genes required for daumone-induced dauer entry*** Since the DAF-16 nuclear localization in embryos and L1 larvae could be a response to stress, we wanted to know what pathways might be required for dauer induction with daumone. We employed mutants that down-regulate the *daf-2/IGFR1*-insulin and *daf-7*/TGF-β pathways in dauer induction assays with daumone. The results point to an important role for the Smad protein DAF-14 in relaying the daumone signal (Fig. 4.5). The *C. elegans* TGF-β pathway [reviewed in Riddle and Albert (1997)] includes the DAF-7 ligand, the receptor heterocomplex formed by DAF-1 and DAF-4 and the two receptor Smads (R-Smads) DAF-8 and DAF-14. Activation of these R-Smads results in reproductive growth, whereas mutations that decrease their function result in a Daf-c phenotype. DAF-8 and DAF-14 antagonize the Smad family member DAF-3, which functions with the Sno/Ski family protein, DAF-5, to promote dauer formation. Mutations in DAF-3 and DAF-5 result in a Daf-d phenotype.

The downstream Daf-d mutant *daf-3* suppresses the *daf-8* and *daf-14* Daf-c mutations. Nevertheless, daumone induces dauer formation in such genetic backgrounds (Fig. 4.5). A failure to respond to daumone indicates that *daf-12* and *daf-14* are required for daumone induction. By contrast, *gpa-3, daf-3, daf-8* and *daf-16* are not required. Mutants in *daf*-3 (n = 38) or *daf-8; daf-3* double mutants (*N* = 78) responded to 384 µM daumone similar to wild-type, forming 50-60% dauer larvae. However, when DAF-14 function was reduced, as in our *daf-14; daf-3* double mutants, dauer formation in response to daumone was severely compromised (5.3%, *N* = 86). This level of dauer formation is actually comparable to that of a *daf-12* (Daf-d) mutant exposed to daumone (6.2% dauer formation, *N* = 103). Our results are therefore consistent with genetic data suggesting that the *daf-2/IGF1R* and TGF-β pathways converge on *daf-12* (Riddle and Albert, 1997).

One unexpected result was produced by the *gpa-3* (G-protein subunit α) knock-out mutant (Fig. 4.5). GPA-3 is a candidate for relaying the dauer pheromone signal from the pheromone receptors to downstream effectors (Lans et al., 2004), including DAF-11. *gpa-3* and the *daf-11* trans-membrane guanylyl cyclase have been shown to interact at the genetic level and have

been placed in a pathway both in parallel and partially upstream of the TGF-β pathway (Zwaal et al., 1997; Birnby et al, 2000). *daf-11* mutations result in decreased cGMP levels and constitutive dauer formation (Birnby et al., 2000). cGMP is used as a signalling molecule to modulate many cellular functions through the activation of kinases, other nucleotide cyclases, cyclic nucleotide phosphodiesterases and cGMP-gated ion channels [reviewed in Goy (1991)].

The *gpa-3(pk35)* knock-out strain has a Daf-d phenotype when exposed to pheromone extracts (Zwaal et al., 1997). However, exposure of the Daf-d *gpa-3(pk35)* nulls to daumone resulted in 87% dauer formation (*N* = 60), a significantly higher dauer formation rate than *gpa-3(pk35)* grown on control plates without daumone (28%, *N* = 31). This result suggests that dauer pheromone and daumone induce dauer formation by partially different pathways. The pheromone requires *gpa-3* for dauer induction, but daumone does not.

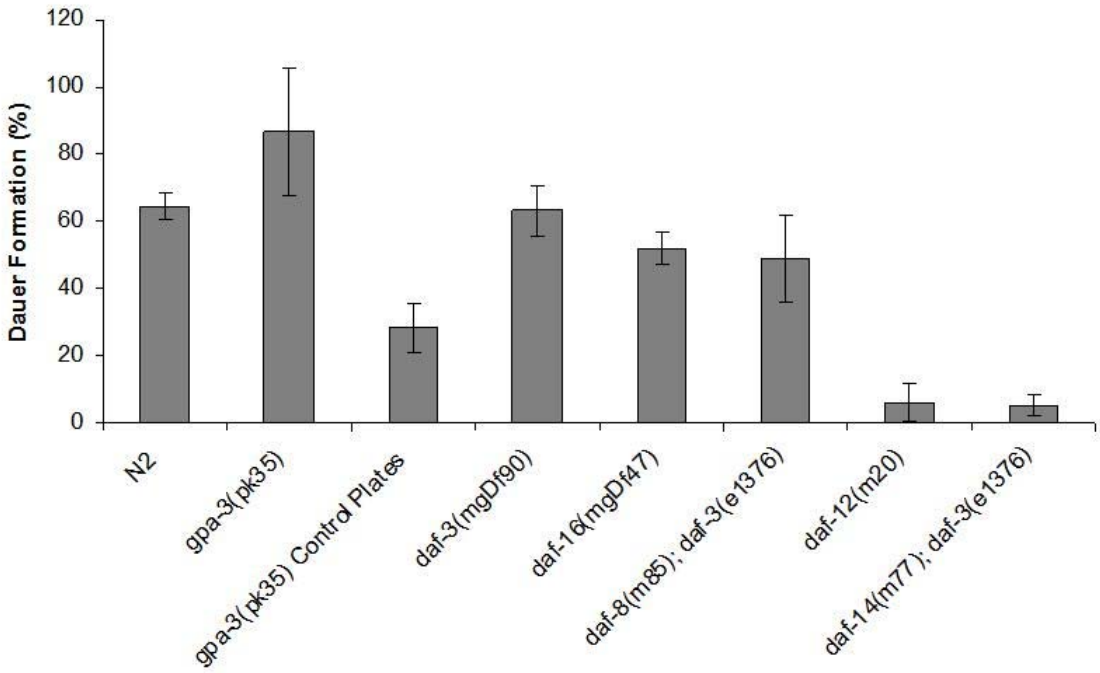

Fig. 4.5 Response of Daf mutants to daumone. Selected Daf-d mutants in the *daf-2/IGF1R* and TGF-β pathways were used in dauer induction assays with daumone. N2 with daumone treatment and *gpa-3* on daumone assay plates without daumone were used as controls. Bars represent ± standard deviation (s.d.).

4.4 Discussion

This work describes the effects of daumone on *C. elegans* physiology and signal transduction pathways. The optimal concentration for dauer induction assays proposed by Jeong et al. (2005) is about 1400-fold higher than the concentration of this compound in culture media (Butcher et al., 2007). Butcher et al. (2007) determined the dose-response effect of daumone on dauer formation. From that study and ours, it seems that high concentrations of daumone (384 μM) are required to produce dauer arrest in the absence of the most potent dauer-inducing ascarosides. At this level of exposure, daumone has a lethal effect on animals with permeable cuticles and results in embryonic nuclear localization of DAF-16, suggesting that it may induce dauer formation via a stress response pathway. DAF-16 activity is regulated by insulin signaling via AKT kinases and by a parallel stress response pathway involving SIR-2.1 (Henderson and Johnson, 2001; Berdichevsky et al., 2006).

Mutations in *daf-7* result in DAF-16 nuclear localization (Lee et al., 2001), so it is possible that the nuclear localization we observed was partially due to a daumone-induced decrease in *daf-7* gene function. However, DAF-16 nuclear localization in embryos seems to be primarily caused by daumone toxicity. A fluorescent derivative of daumone has been reported to penetrate the egg shell and enter the embryo (Baiga et al., 2008). Our data suggesting that daumone is toxic are in agreement with recent findings of Kim and Paik (2008), who used daumone to induce dauer formation in N2 and to keep them in the dauer stage for increasing lengths of time. They observed that the longer the exposure to daumone, the more severe were the developmental and reproductive defects in post-dauer animals. Such defects were not observed in starvation-induced dauer larvae (Klass and Hirsh, 1976).

In spite of its toxic effects, the use of daumone has proven useful for dissecting the functional relationship between the three major ascarosides in the dauer pheromone. Our experiments suggest that daumone does not act on all the canonical pathways for dauer entry. For instance, daumone does not require *gpa-3* to induce dauer entry, although this gene is

necessary for dauer induction by natural dauer pheromone extracts (Zwaal et al., 1997). This result suggests that daumone is not sensed by the ciliated neurons, where *gpa-3* is expressed, but instead it uses an alternative path into the animal. In fact, *daf-10* mutants are Daf-d, but form 82% dauer larvae at 25°C when exposed to daumone (Jeong et al., 2005). *daf-10* mutants have defective intraflagellar transport and their cilia – rarely formed - are not functional (Qin et al., 2001). *daf-10* does not form dauer larvae when exposed to natural pheromone extracts (Golden and Riddle, 1984c). All together, these data suggest that daumone (at the concentration used) does not require the ciliated neurons as does the natural dauer pheromone. Daumone may enter through the cuticle, as this ascaroside is lethal to animals with a permeable cuticle.

Mutations in the FOXO transcription factor *daf-16*, which is the main effector of insulin/IGF1 signaling, do not prevent dauer formation in response to daumone, nor do mutations in members of the TGF-β pathway. When *daf-16* is mutated, *daf-3(+)* in the TGF-β pathway may be sufficient for dauer entry in response to daumone. Likewise, when *daf-3* is mutated, *daf-16(+)* may be sufficient to mediate dauer formation. However, DAF-14 is necessary for daumone-mediated dauer entry, suggesting that DAF-14 is required by DAF-16 and DAF-3 to induce dauer formation. When DAF-14 and DAF-3 have reduced function (as in our *daf-14(m77); daf-3(e1376)* double mutant), DAF-16 is not able to induce dauer formation. Interaction between SMAD and FOXO transcription factors has been reported in human cells (Seoane et al., 2004), and was also proposed for DAF-16 and DAF-3 under dauer-inducing conditions in *C. elegans* (Ogg et al., 1997). It is possible that daumone enhances DAF-16/DAF-3 interaction in a DAF-14-dependent manner. The interactions proposed here explain what we observe when animals are treated with daumone, and do not necessarily correspond to the pathways normally used for dauer entry.

In conclusion, daumone, a minor component of the dauer pheromone, needs to be used in synthetic forms at concentrations so high to induce dauer formation that it becomes toxic. It does not fully recapitulate the events that normally lead to *C. elegans* dauer formation. Our

results suggest that dauer formation may be partially induced in response to toxins, in addition to the environmental cues already known (pheromone, food, and high temperatures). The pheromone cue may normally involve the orchestrated action of daumone, the two major ascarosides, and other pheromone components found in the natural dauer pheromone extracts by Butcher et al. (2007) that trigger the complex signalling processes revealed by the genetics. Different ascarosides may activate different sensory receptors, and at physiological concentrations the behavioral response may result from parallel activities. The chemical composition of the pheromone may contain specific information about environmental status.

4.5 Acknowledgments We thank Victor Jensen, Nigel O'Neil and Donha Park for helpful discussions. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). This work was supported by the Cordula and Gunter Paetzold University Graduate Fellowship and a Michael Smith Foundation for Health Research Senior Graduate Studentship to M.G. and the Canadian Institutes of Health Research (MOP 79458) to D.L.R.

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5 DISCUSSION

5.1 Novel functions for the 2-oxoglutarate carrier

On the basis of their high amino acid sequence identity (72%), we identified the *C. elegans* orthologue of human OGC and named it MISC-1. MISC-1 and OGC are also best BLAST reciprocal matches. In addition to the sequence similarity, we were able to show that MISC-1 and OGC have similar functions in *C. elegans* and mammalian cells, thus the two proteins match the definition of orthologues. Importantly, we showed that MISC-1 and OGC have functions other than transporting α-ketoglutarate across the inner mitochondrial membrane. Transport of α-ketoglutarate was what allowed purification of OGC in the first place (Bisaccia et al., 1985). However, we found that this mitochondrial carrier has important functions in determining the equilibrium of fusion and fission events in mitochondria, induction of apoptosis and insulin secretion in *C. elegans* and in mammalian cell systems. Furthermore, our studies are the first to show that MISC-1/OGC is an important regulator of oxidative stress response *in vivo*.

It was known that mammalian OGC is responsible for the import of mitochondrial GSH (Chen and Lash, 1998; Chen et al., 2000). It was also known that over-expression of rat *OGC* in NRK-52E cells had a protective effect against chemically-induced apoptosis (Xu et al., 2006). However, this work is the first to demonstrate a role for OGC in oxidative stress and apoptosis in a whole organism. Also, unlike previous reports, we studied the effects of *absence* of OGC on whole animals as well as single cells.

5.2 MISC-1/OGC and oxidative stress

We showed that absence of MISC-1 in *C. elegans* causes the animals to be extremely sensitive to ROS production induced by exposure to Juglone. This sensitivity was probably the result of high levels of physiological ROS in unstressed *misc-1* knock-out animals, compared to wild type. Yet the life span of *misc-1* knock-down and knock-out animals was similar to that of

control worms. This result is of importance, because the oxidative stress theory of aging predicts a negative correlation between ROS levels and life span. However, this theory cannot be upheld in view of the results we obtained studying *misc-1*. *C. elegans* has been used extensively as a model organism to study the relationship between oxidative stress and aging. The discovery that mutants in some genes had increased life spans and improved ability to withstand oxidative stress seemed to support the oxidative stress theory of aging. However, these stress assays involved exposing worms to Juglone and recording their ability to survive. This assay was not informative on the physiological levels of ROS in the animals. It was at best an assay for the level of activation of detoxification pathways.

This work is the first to directly measure ROS levels in *C. elegans* mutants for a ROS detoxification gene, providing data that contradict the oxidative stress theory of aging. *misc-1* mutants have two-fold higher ROS levels than wild type, yet have a similar life span. Our results support previous findings by other groups showing that genetic inactivation of superoxide dismutase (*sod*) genes do not have an effect on *C. elegans* life span. However, these studies inferred that ROS levels would increase in *sod* mutants without directly measuring their effect. Another study measured ROS levels, although not in a stress response mutant. Tavernarakis' group measured ROS levels in *daf-2* mutants and found them to be lower than in wild type (Artal-Sanz and Tavernarakis, 2009). However, *daf-2* is not a gene directly involved in ROS detoxification. This result could mean that mutations in *daf-2* activate stress response pathways with ROS detoxification roles. This explanation has been proposed before, in view of the fact that *daf-2* mutations de-repress the transcription factor DAF-16, which targets a subset of genes putatively involved in stress response (Henderson and Johnson, 2001). More direct measurements of ROS levels in animals carrying mutations in ROS detoxifying genes are required to prove or disprove the oxidative stress theory of aging.

5.3 The role of mitochondria in *C. elegans*

The study of *misc-1* has enabled us to gain a better understanding of the relationship not only between ROS levels and aging, but also between mitochondrial function and aging. Several papers showed that ETC mutants or RNAi against genes encoding ETC components extend *C. elegans* life span (Dillin et al., 2002; Lee et al., 2003; Rea et al., 2007). These findings were at odds with results from mammals, where ETC mutations generally are lethal [reviewed in (Wallace, 1999)]. Our studies showed that *misc-1* knock-out mutants are less sensitive to ETC inhibition by NaN_3 treatment than wild type. This result suggested that *misc-1* mutants are less dependent on mitochondrial function for survival than is wild type. We speculated that *misc-1* and ETC mutants might activate compensatory mechanisms to balance their reduced mitochondrial function.

The long-lived mutant *daf-2* has increased expression of *gei-7*, which codes for the only enzyme in the *C. elegans* glyoxylate shunt, which provides ATP in a mitochondrion-independent way. The finding was interesting because mammals do not have a functional glyoxylate shunt. We investigated the possibility that the compensatory mechanism acting in *misc-1* and long-lived mitochondrial mutants was activation of the glyoxylate shunt. We found slightly increased levels of *gei-7* expression in *misc-1* mutants. However, *gei-7* was expressed at four-fold higher levels in three long-lived mitochondrial mutants, compared to wild type. We hypothesize that the slight increase in *gei-7* expression in *misc-1* mutants is enough to allow normal development in these mutants. In fact, *misc-1* RNAi or knock-out worms do not have any gross morphological phenotype, including developmental time, size and fat deposition (Appendix 3). On the other hand, the four-fold increase in *gei-7* expression in *clk-1*, *isp-1*, and *daf-2;isp-1* mutants provides for more than the basic developmental requirements and has a positive effect on life span. All three mitochondrial mutants we tested live about twice as long as wild type. We therefore propose that the levels of *gei-7* expression are predictors of life span extension in *C. elegans*.

Among animals, nematodes are apparently unique in having a functional glyoxylate shunt. The other organisms known to have this pathway are bacteria and plants. *C. elegans* might therefore be less dependent on mitochondrial function for energy production than higher animals. *C. elegans* is viable even if its mitochondrial genome contents were reduced by genetic means to 4% of wild type (Bratic et al., 2009). We suggest caution in interpreting aging data deriving from the study of *C. elegans* mitochondrial mutants. Reproducing experiments in other organisms might be required before generalizing conclusions.

5.4 MISC-1/OGC is a novel modulator of apoptosis

Although it was known that over-expression of *OGC* conferred resistance to chemically-induced cell death, no data were available for its role in apoptosis under physiological conditions. We showed that lack of MISC-1 in *C. elegans* and of OGC in mouse cells results in apoptosis. However, we did not elucidate the exact mechanisms by which lack of MISC-1/OGC results in apoptosis. Our experiments show that MISC-1/OGC interacts with ANT and CED-9/Bcl-x_L in *C. elegans* and in mammalian cells. It is known that ANT and Bcl-x_L are components of the MPTP. We therefore conclude that MISC-1/OGC is also a component of the MPTP.

The MPTP was originally implicated in regulation of necrosis, although its involvement in apoptosis was recently also demonstrated (Nazareth et al., 1991; Zamzami et al., 1995). It is thought that opening of the MPTP affects the inner mitochondrial membrane the same way that MOMP affects the outer mitochondrial membrane. MPTP opening could allow cytochrome c, Ca^{2+} and other pro-apoptotic factors to leave the cristae and relocate to the intermembrane space. From here, these factors can then exploit MOMP to enter the cytosol.

Under physiological conditions, the MPTP behaves like a low-affinity Ca^{2+} channel. It is possible that efflux of mitochondrial Ca^{2+} via MPTP opening might play a bigger role in apoptosis than previously expected. We hypothesize that MISC-1/OGC regulates MPTP activity. OGC is inhibited by high concentrations of mitochondrial Ca^{2+}, as assessed by its ability to transfer α-ketoglutarate across the inner mitochondrial membrane (Contreras and

Satrústegui, 2009). Inhibition of MISC-1/OGC might result in conformational changes that de-stabilize the MPTP and induce its opening. Alternatively, it is possible that high matrix Ca^{2+} converts MISC-1/OGC from a metabolic carrier to a Ca^{2+} channel, contributing to the Ca^{2+} efflux function of the MPTP under apoptogenic conditions. A third possibility is that changes in metabolic rate affect the number of MISC-1/OGC molecules associated with the MPTP in the mitochondria. Absence or reduced levels of MISC-1/OGC might have destabilizing effects on the MPTP and cause its opening.

These hypotheses to explain the effects of MISC-1/OGC on the MPTP impinge on the ability of MISC-1/OGC to "sense" mitochondrial metabolic needs. Ca^{2+} is an important molecule in mitochondria and is needed for the activation of several key metabolic enzymes. α-ketoglutarate is an important intermediate of the Krebs cycle and of glutamate metabolism. Both Ca^{2+} and α-ketoglutarate interact with OGC. The fact that MISC-1/OGC physically interacts with the MPTP sheds new light on the integration of metabolic and cell survival signals.

So far, my discussion focused on the possible role of MISC-1/OGC in mediating Ca^{2+} fluxes. This is because Ca^{2+} flux from intracellular stores (mainly mitochondria and the ER) has been shown to be important to both apoptosis and insulin secretion, and these two phenomena are affected by lack of MISC-1/OGC. Furthermore, MISC-1/OGC participates in a complex with Bcl-x_L, and Bcl-2 proteins have been shown to affect Ca^{2+} homeostasis and insulin secretion (Lam et al., 1994; Zhou et al., 2000). However, other factors could be involved in MISC-1/OGC-mediated apoptosis.

An obvious pro-apoptotic factor that could leak upon MPTP opening is cytochrome c. We cannot rule out that MISC-1/OGC-mediated apoptosis involves efflux of cytochrome c via MPTP opening in mammals, but this is certainly not the case in *C. elegans*. The involvement of cytochrome c in *C. elegans* apoptosis has never been proven, perhaps not surprisingly. While cytochrome c binding to Apaf1 is required for assembly of the apoptosome to take place, *C. elegans* CED-4 activation does not involve cytochrome c. However, MISC-1/OGC-mediated apoptosis might require the efflux of any one of the other pro-apoptotic factors - including AIF

and EndoG, to name but a few – or all of them. Further work will be required to fully elucidate the mechanisms underlying MISC-1/OGC-mediated apoptosis.

5.5 MISC-1/OGC and mitochondrial fragmentation

Another process regulated by MISC-1/OGC that we found to be phylogenetically conserved is mitochondrial fragmentation. Knock-down of both *misc-1* in worms and *OGC* in human cells resulted in mitochondrial fragmentation. In mammals, whether mitochondrial fragmentation causes or follows apoptosis is still a matter of debate. On the other hand, *C. elegans* has been the only organism thus far where it has been conclusively demonstrated that mitochondrial fragmentation alone can induce apoptosis (Jagasia et al., 2005). *C. elegans* is a good model system to study the phenomenon of mitochondrial fusion and fission. Apoptosis in the adult can be observed in the germline, since the soma is fully post-mitotic. Using this system it is therefore possible to study the effects of a genetic or molecular manipulation on mitochondrial morphology in the soma and on cell death in the germline. We did this for *misc-1*, and our results indicate that *misc-1* RNAi causes mitochondrial fragmentation in the soma (specifically, the muscle) and apoptosis in the germline. Therefore, *misc-1* down-regulation caused mitochondrial fission even in a tissue that cannot undergo apoptosis. These findings point to a role for MISC-1/OGC in stabilizing the mitochondrial membrane.

This role is further corroborated by comparing muscle transmission electron micrographs of *misc-1* knock-out animals and wild type. We observed several defects in the inner mitochondrial membrane of *misc-1* animals, including fewer, disorganized and blebbing cristae. In order for mitochondrial fusion to occur, four steps need to happen: contact of the outer membranes, fusion of the outer membranes, contact of the inner membranes and fusion of the inner membranes. It is possible that the destabilization of the inner mitochondrial membrane due to lack of MISC-1 disrupts the third and/or fourth steps, thereby favouring mitochondrial fission over fusion.

The intermembrane junctions, where the MPTP is localized, may play an important role in the control of the mitochondrial fusion/fission equilibrium. In *C. elegans*, CED-9 physically interacts with FZO-1/Mfn1,2, a protein required for mitochondrial fusion (Rolland et al., 2009). Similarly, Bcl-2 and Bcl-x_L might interact with the mitochondrial fusion/fission machinery. We hypothesize that the MPTP might participate in the regulation of mitochondrial fragmentation through its interaction with anti-apoptotic Bcl-2-family proteins. Our data on the role of MISC-1/OGC in mitochondrial fission and its interaction with the MPTP and CED-9/Bcl-x_L suggest that the relative abundance of MISC-1/OGC in the inner mitochondrial membrane might affect the interaction of CED-9/Bcl-x_L with the mitochondrial fusion machinery. Whether the mitochondrial fragmentation phenotype observed upon *misc-1* and *OGC* knock-down is due to the direct interaction of MISC-1/OGC with the fission/fusion machinery or is attributable to changes in mitochondrial metabolism and/or mitochondrial membrane potential needs to be further investigated.

5.6 Lack of MISC-1/OGC increases insulin secretion

misc-1 RNAi increased insulin secretion in *C. elegans*, but it is unclear whether this is the result of metabolic changes caused by lack of MISC-1/OGC or the consequence of differential regulation of its molecular partners. We favour the hypothesis that lack of MISC-1/OGC affects the activation of Bcl-2-family proteins in their function of controlling intracellular Ca^{2+} stores and insulin secretion. This view is upheld by our pull-down results, which showed a physical interaction between MISC-1 and CED-9 in *C. elegans* and OGC and Bcl-x_L in mammalian cells. However, we cannot discount the possibility that other mechanisms of insulin secretion are affected by lack of MISC-1/OGC.

For instance, it may be possible that lack of MISC-1/OGC alters the balance of metabolites that participate in the Krebs cycle. Most of these metabolites have been identified as insulin secretagogues (MacDonald et al., 2005). Interestingly, one of the most effective secretagogues is α-ketoglutarate (MacDonald, 1982), the main substrate of OGC. Mutations that result in

constitutively active forms of the glutamate dehydrogenase enzyme, which converts glutamate to α-ketoglutarate, have been found in patients affected by hyperinsulinism/hyperammonemia (Stanley et al., 1998). Therefore, alteration of α-ketoglutarate concentration due to aberrant MISC-1/OGC levels could result in higher insulin secretion.

Similarly, mitochondrial Ca^{2+} homeostasis may be involved in the insulin secretion phenotype. Reports have suggested that the MPTP functions as a low affinity Ca^{2+} channel [reviewed in (Crompton et al., 2002)] and our results suggest that MISC-1/OGC regulates MPTP activity. It can be hypothesized that absence of MISC-1/OGC increases the MPTP-mediated outward Ca^{2+} flux from the mitochondrial matrix. Ca^{2+} is an important regulator of several mitochondrial metabolic enzymes, many of which indirectly affect insulin secretion. Decreased matrix Ca^{2+} and increased cytoplasmic Ca^{2+} upon MPTP opening could both contribute to insulin release, in line with the current understanding of the process of insulin secretion from mammalian β-cells.

It was interesting to notice that a relatively modest increase in insulin secretion in a *misc-1* knock-out was sufficient to suppress the Daf-c phenotype of *daf-2/IGF1R* mutants. This suppression points to the sensitivity of the IIS pathway to insulin-mediated regulation of development in worms. Moreover, our data suggest that the increased insulin secretion observed in *misc-1* mutants has a potent effect on germline stem cell proliferation. In fact, germline stem cell proliferation in *misc-1* adults was five-fold higher than in wild type. The positive effect of insulin secretion on germline proliferation is conserved from *C. elegans* (Michaelson et al., 2010) to mammals (Burks et al., 2000). It would be interesting to investigate a possible role of *OGC* on mammalian stem cell proliferation.

In conclusion, based on the role of *misc-1* in insulin secretion in *C. elegans*, we would predict that mammalian OGC suppresses insulin secretion. Knocking-down mammalian *OGC* should therefore result in higher levels of insulin secretion. However, contrary to our predictions, a recent report showed that knock-down of *OGC* results in lower insulin secretion (Odegaard et al., 2010). It is possible that metabolic regulation of insulin signalling is not

entirely congruent in *C. elegans* and mammals. A better understanding of the physiological regulation of insulin secretion in *C. elegans* – including which tissues produce insulin and which ones are targeted by insulin –will be required in order to use this model organism for the study of insulin-related disorders.

5.7 Daumone-induced dauer formation

Dauer formation in *C. elegans* is regulated by multiple signals. One of them is the dauer pheromone, whose concentration is a measure of population density. We studied the physiological effects of one component of the dauer pheromone, the ascaroside with the trivial name daumone (Jeong et al., 2005). Daumone is a minor component of the dauer pheromone at 20°C, but it is synthesized in higher amounts at higher temperatures (Butcher et al., 2008). We found that the high concentrations of purified daumone necessary to induce dauer larvae have deleterious effects on animal physiology. Specifically, a molecular marker of cellular stress (DAF-16::GFP) was activated upon daumone exposure. Furthermore, permeabilization of the cuticle allowed daumone entry into the worm with lethal effects.

We showed that daumone is not sensed by the canonical amphidial neuron pathway requiring *gpa-3*. This result suggests that daumone is not sensed like the other dauer pheromone components. This hypothesis is strengthened by the observation that exposure to daumone causes a *daf-7::gfp* reporter to be expressed ectopically in the ASJ neurons. Therefore daumone does not behave like a dauer-inducing ascaroside. Daumone might be a minor pheromone component used by the worm to assess levels of stressors in its environment (*e.g.* high temperature). As stated above, daumone itself seems to function as a stressor. Our studies of daumone indicate that exposure to stressors can induce dauer formation in a way that is partially independent of the canonical pathways for dauer formation.

We should also consider the possibility that daumone is simply an accidental by-product of pheromone biosynthesis. All dauer-inducing ascarosides known to date share the same basic chemical structure and differ only for the number of carbon atoms in their side chain (Jeong et

al., 2005; Butcher et al., 2007; Butcher et al., 2008; Srinivasan et al., 2008). Even if daumone is indeed a by-product, it is still possible that during evolution *C. elegans* adapted to exploit this molecule as an indicator of a stressful environment. Indeed, similar mechanisms might explain the existence of several dauer-inducing ascarosides, some of them being produced under specific environmental circumstances (Butcher et al., 2008) and some being used also as sexual attractants (Srinivasan et al., 2008).

Further studies of the interaction between pure ascarosides and the dauer gene network will tell us how many of these molecules exploit the canonical dauer pathways. The dauer pheromone could be seen as a cocktail of molecules that accurately reflect complex environmental conditions.

5.8 Significance

Our work has shed new light on the mechanisms of action of a dauer-inducing ascaroside, daumone, and a dauer-inhibiting gene, *misc-1*. Our experiments indicate that daumone, although a component of the dauer pheromone, is able to induce dauer formation only at non-physiological concentrations, thereby causing adverse effects on the animals. We propose that daumone is not a functionally fundamental dauer ascaroside, but it may modulate the activity of other dauer ascarosides or be a by-product of pheromone biosynthesis.

We have shown that mutations in the gene *misc-1/OGC* can suppress the dauer formation phenotype of a *daf-2/IGF1R* mutant by increasing insulin secretion. This increased insulin secretion is consistent with the increased germline stem cell proliferation observed in *misc-1* mutants. Our studies showed that MISC-1/OGC is not only involved in the regulation of insulin secretion, but also in mitochondrial fragmentation and apoptosis. We showed that these functions of MISC-1/OGC are phylogenetically conserved from *C. elegans* to mammals.

Our data provide a new paradigm for the interplay of metabolism and programmed cell death. The study of MISC-1/OGC shows that metabolism affects cell survival decisions in a more direct way than previously thought, by the direct physical interaction of important

metabolic proteins with members of the apoptotic machinery. We propose that MISC-1/OGC functions as a hub to integrate metabolic status and cell survival decisions through its physical interaction with the MPTP and Bcl-x_L.

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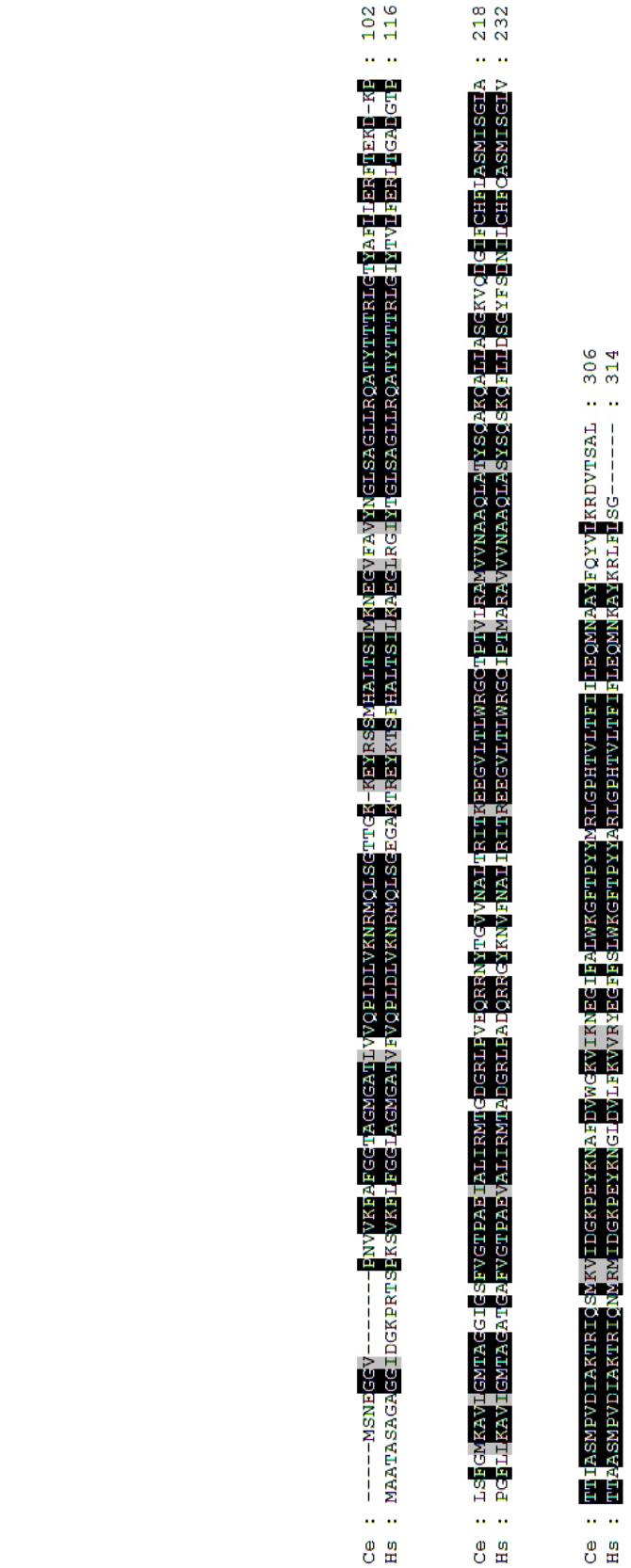
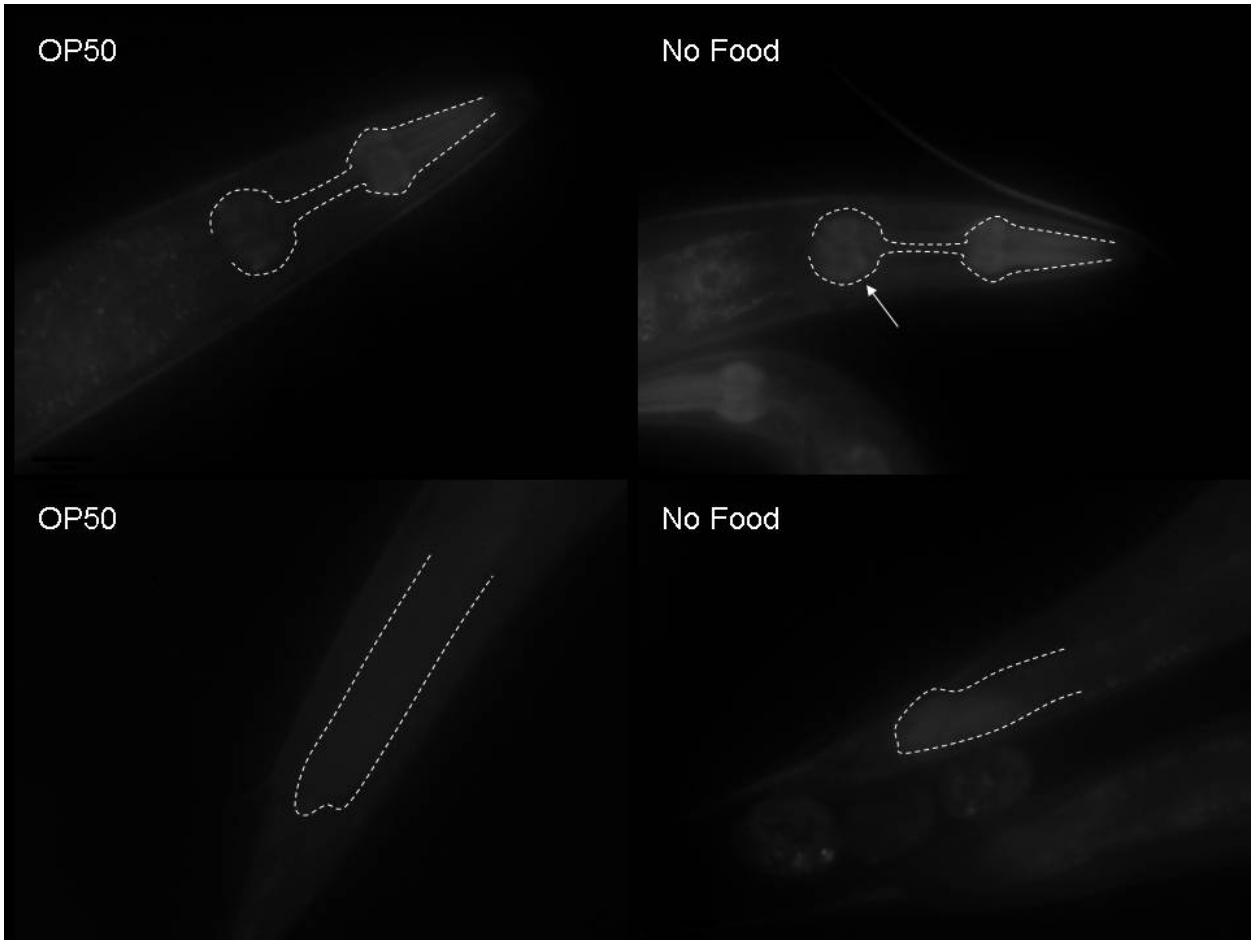
Appendix 1 – Supplementary information for Chapter 2

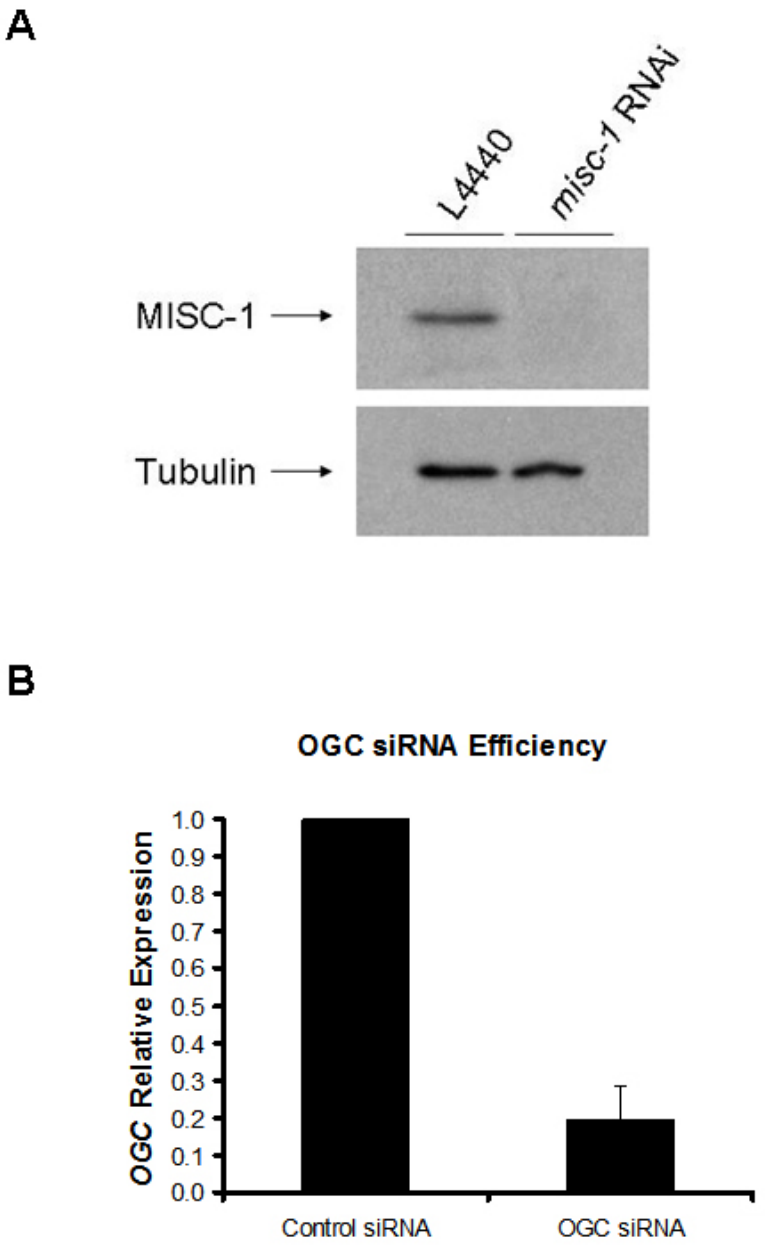
Supplementary Figure S1. Amino acid conservation between *C. elegans* MISC-1 and human OGC. MISC-1 and OGC share 72% identity and 80% similarity (e-value = $8e^{-105}$) at the amino acid level. The two proteins are best reciprocal matches. When doing BLAST similarity searches with the MISC-1 sequence, the second best human match is the dicarboxylate carrier, with an expect value of $2e^{-51}$, amino acid identity of 39% and similarity of 57%. BLAST similarity searches with the human OGC amino acid sequence against the *C. elegans* proteome showed the second best match as the uncharacterized protein K11G12.5, predicted to be a malate carrier. In this case, the expect value was $8e^{-50}$, amino acid identity was 40% and similarities were 57%. Identical amino acids are shown in black, similarities are shown in grey.

Supplementary Figure S1

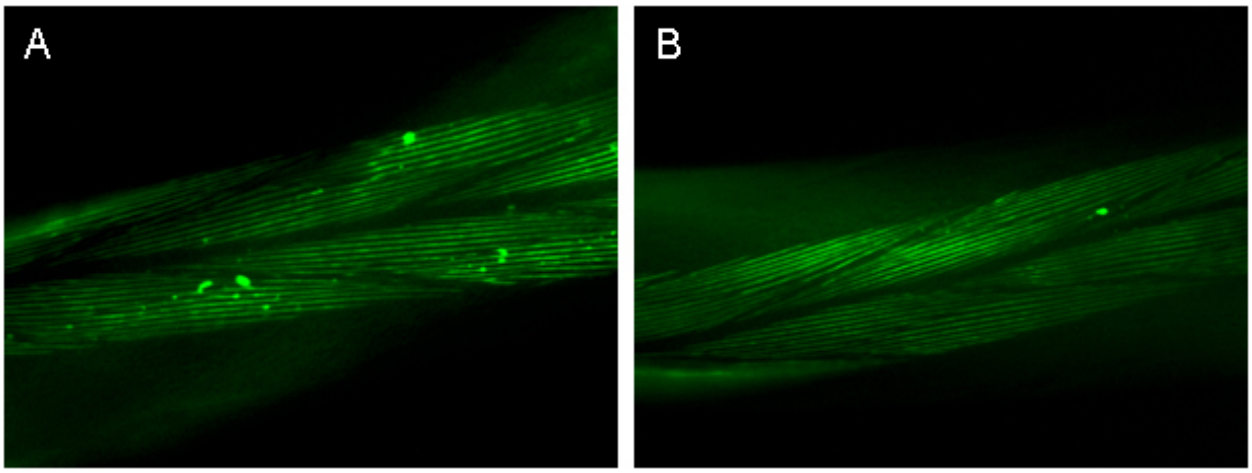




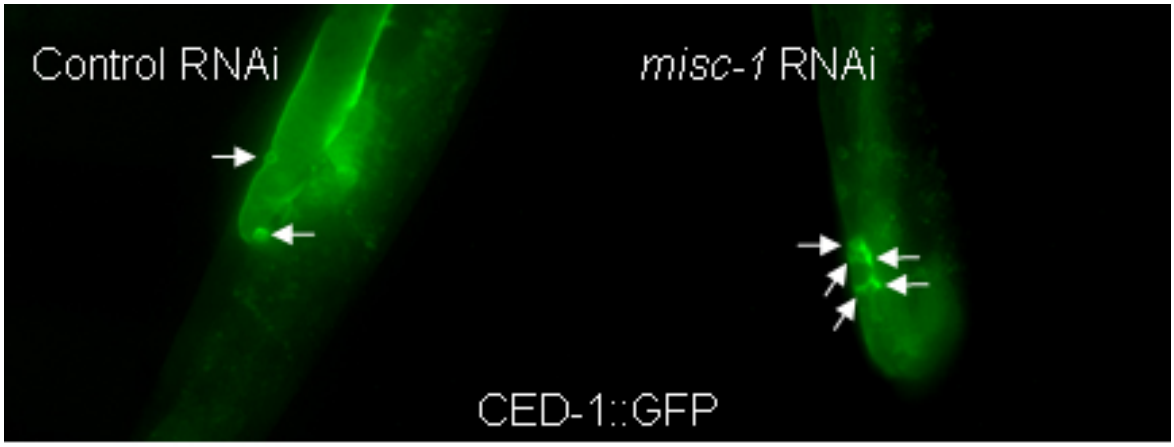
Supplementary Figure S2. Starvation increases MISC-1 expression. We observed an increase in MISC-1::GFP reporter expression in animals that were placed for 24 h on plates that contained no food, compared to animals that were fed *ad libitum* on plates streaked with *E. coli* OP50. The increase in reporter expression upon starvation was especially noticeable in the posterior bulb of the pharynx (outlined in upper right panel) and in the posterior intestine (outlined in lower right panel).



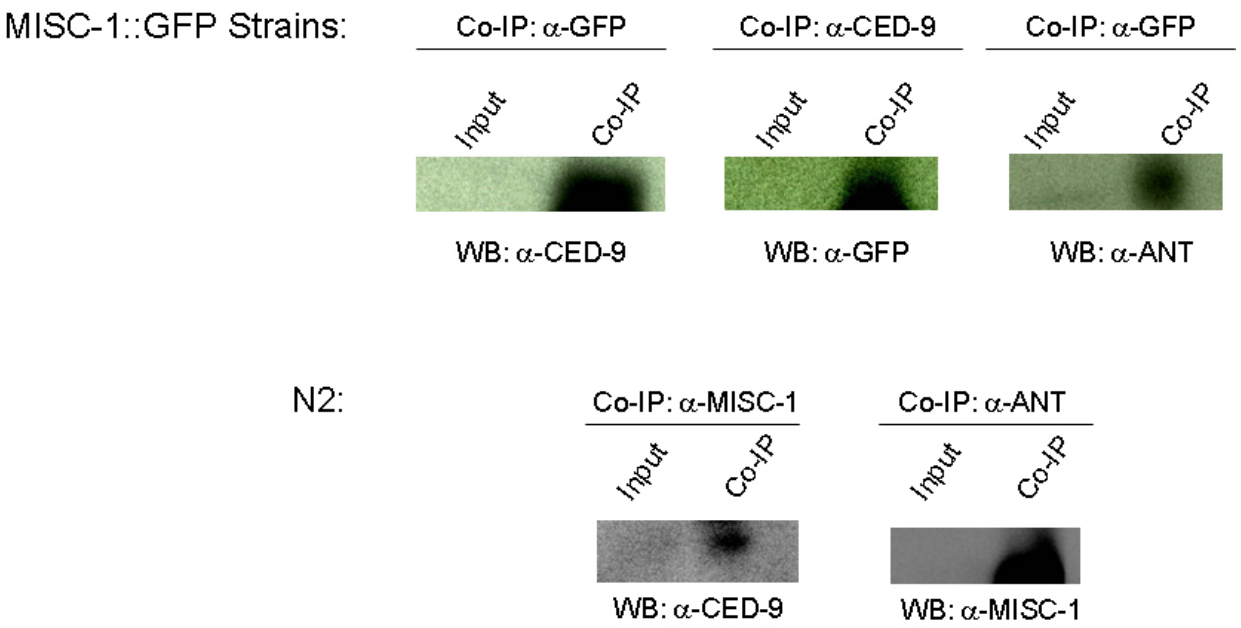
Supplementary Figure S3. Evaluation of *misc-1* and *OGC* knock-down .
(A) Synchronized L1 larvae were treated with either empty vector control (L4440) or *misc-1* RNAi vector until day 1 of adulthood. We performed a Western blot on the proteins extracted from these strains with our MISC-1 antibody. The results show a ~84% down-regulation of MISC-1 levels upon *misc-1* RNAi treatment. (B) Quantitative Real-Time PCR (qRT-PCR) was performed to assess the extent of *OGC* knock-down in HEK293 cells treated with *OGC* siRNA. As a control, we transfected cells with a mammalian X-Scramble siRNA vector. *OGC* expression levels were normalized to *Actβ*. Our results indicate that *OGC* siRNA reduced target gene expression by ~80%. Error bars: ±S.D.



Supplementary Figure S4. *misc-1* knock-down does not affect muscle morphology. Control RNAi and *misc-1* RNAi on a transgenic strain carrying a *myo-3*p::*myo-3*::*gfp*. This transgene is expressed in muscles and provides a tool to assess muscle morphology. These confocal images show that muscle morphology in *misc-1* RNAi-treated worms is indistinguishable from that of control RNAi-treated worms. The mitochondrial fragmentation phenotype observed in Figure 2B is therefore caused by a specific effect of *misc-1* RNAi on mitochondria, not on muscle structure.



Supplementary Figure S5. *misc-1* RNAi does not compromise the ability of the somatic gonad to remove apoptotic corpses. Control RNAi and *misc-1* RNAi treatment on a transgenic strain carrying CED-1::GFP are shown. This fluorescent reporter allows visualization of the apoptotic corpses being engulfed and removed by the somatic sheath cells (arrows). We observed a two-fold increase in the number of engulfing events per gonad arm in worms treated with *misc-1* RNAi, compared to control. This result suggests that the increase in apoptotic events observed in Fig. 4A-B was due to an effect of *misc-1* on apoptosis, and was not caused by a defect in the mechanism of cell corpse removal.

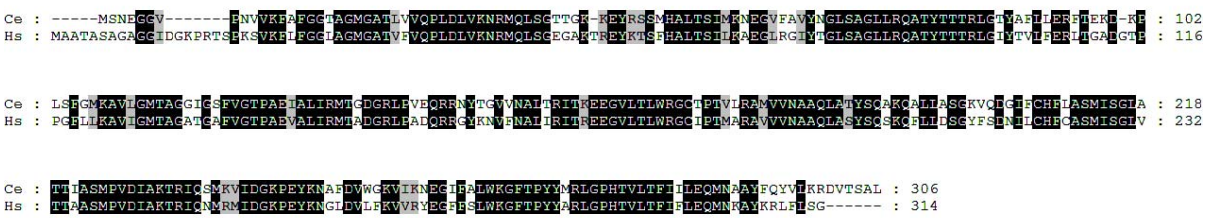


Supplementary Figure S6. MISC-1 is a component of the MPTP in *C. elegans*. Co-immunoprecipitation experiments performed in a transgenic line expressing MISC-1::GFP (upper panel) or in wild type N2 (lower panel). MISC-1 was shown to interact with CED-9 and ANT. The latter is an integral component of the MPTP. Our data suggest that MISC-1 is a novel component of the MPTP and therefore an important player in the induction of apoptosis. Antibodies used: α-CED-9: sc-33737, Santa Cruz Biotechnology, Santa Cruz CA, USA; α-GFP: ab6556, Abcam Inc., Cambridge MA, USA. See Methods section for other antibodies.

Appendix 2 – Supplementary information for Chapter 3

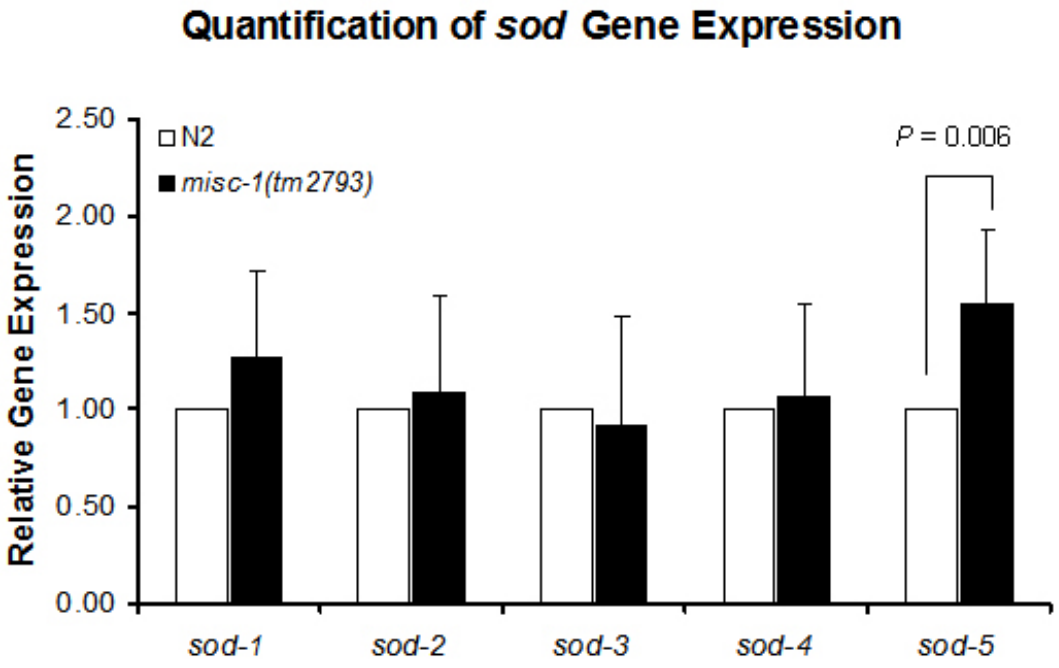
Supplementary Table 1

Trial #	Genotype	RNAi Treatment	N	Mean Life-Span (days)	Log-Rank *P*
1	N2		75	14.0	-
	misc-1		73	15.0	0.0211
2	N2		92	11.6	-
	misc-1		95	12.7	0.0844
1	*daf-2(m41)*		82	23.7	-
	daf-2(m41);misc-1(tm2793)		86	23.8	0.4700
2	*daf-2(m41)*		78	18.1	-
	daf-2(m41);misc-1(tm2793)		93	20.9	0.0614
1	*eat-2(ad465)*	Control	75	12.6	-
	eat-2(ad465)	*misc-1*	78	13.6	0.3998
2	*eat-2(ad465)*	Control	62	14.4	-
	eat-2(ad465)	*misc-1*	67	16.6	0.0001
1	*clk-1(qm130)*	Control	24	23.5	-
	clk-1(qm130)	*gei-7*	43	21.0	0.0562

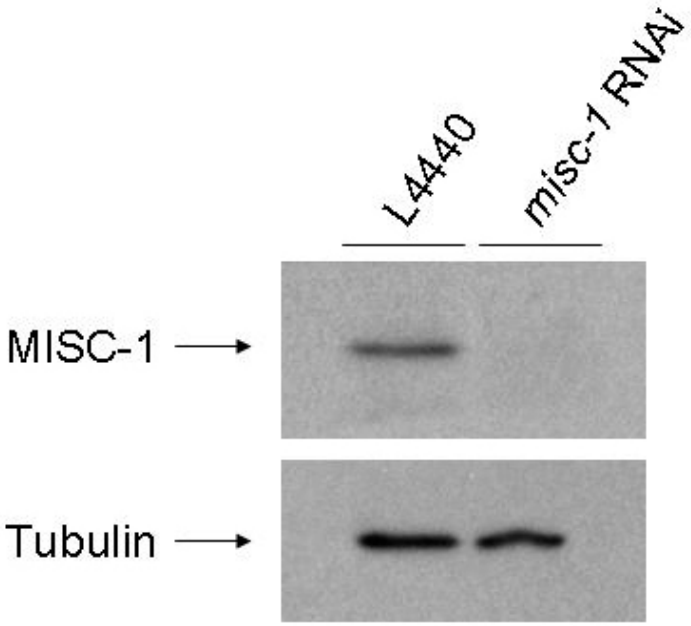


Supplementary Figure S1 | Amino acid conservation between *C. elegans* MISC-1 and human OGC.
MISC-1 and OGC share 72% amino acid identity and 80% similarity (e-value = $8e^{-105}$). The two proteins are best reciprocal matches. When doing BLAST similarity searches with the MISC-1 sequence, the second best human match is the dicarboxylate carrier (e-value=$2e^{-51}$) with an amino acid identity of 39% and similarity of 57%. BLAST similarity searches with the human OGC amino acid sequence against the *C. elegans* proteome, the second best match (e value=$8e^{-50}$) was the uncharacterized protein K11G12.5, predicted to be a malate carrier, with amino acid identity of 40% and similarity of 57%.

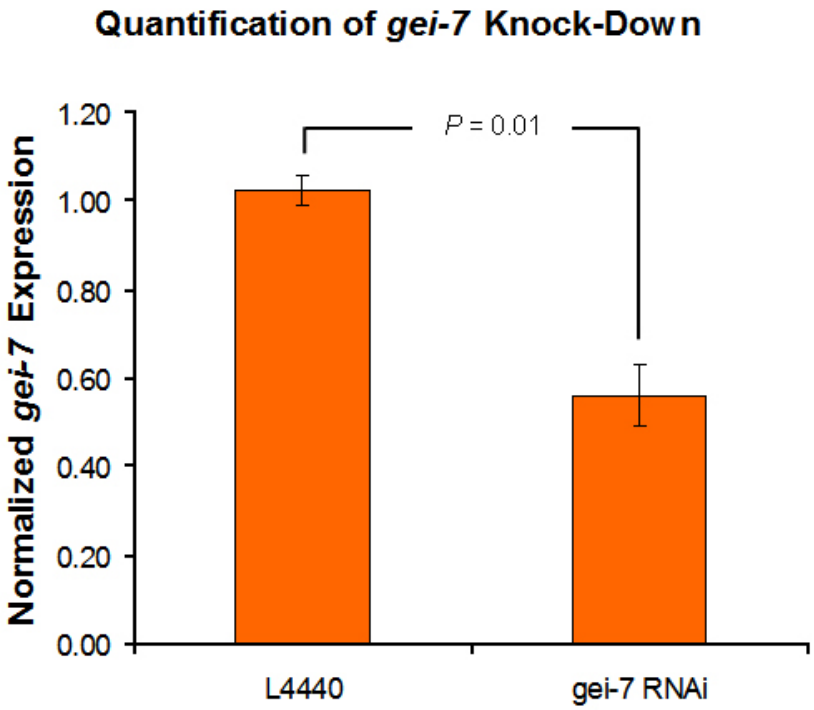
Supplementary Figure S2 | *misc-1(tm2793)* is a knock-out allele.
a, We obtained the *misc-1(tm2793)* allele from the *C. elegans* knock-out consortium. *tm2793* is a 203-bp deletion (red bar) that removes part of the first intron and the first half of the second exon of *misc-1*. **b,** *misc-1(tm2793)* animals do not express the MISC-1 protein. We generated a rabbit polyclonal antibody that reacts with *C. elegans* MISC-1 (α-MISC-1; GenScript, Piscataway, NJ, USA). The sequence of the antigen used to generate the MISC-1 antibody was CRIQSMKVIDGKPEY. Western Blot (WB) analysis shows that α-MISC-1 recognizes a 33 kDa protein in N2, but this protein is absent from *misc-1* protein lysates. WB employing an antibody against α-tubulin (Sigma-Aldrich,) shows that the two lanes contained comparable levels of protein.



Supplementary Figure S3 | *sod-5* transcripts are up-regulated in *misc-1* mutants. We assayed transcript levels for all five *sod* genes in synchronized N2 and *misc-1* day-one adults. Results are shown from three biological replicates per strain. Of the five *sod* transcripts, the only one that showed up-regulation in *misc-1* compared to control was *sod-5* (t-test P = 0.006), which encodes one of the two cytoplasmic SODs. Target gene transcripts were normalized against endogenous levels of *act-2* mRNA.

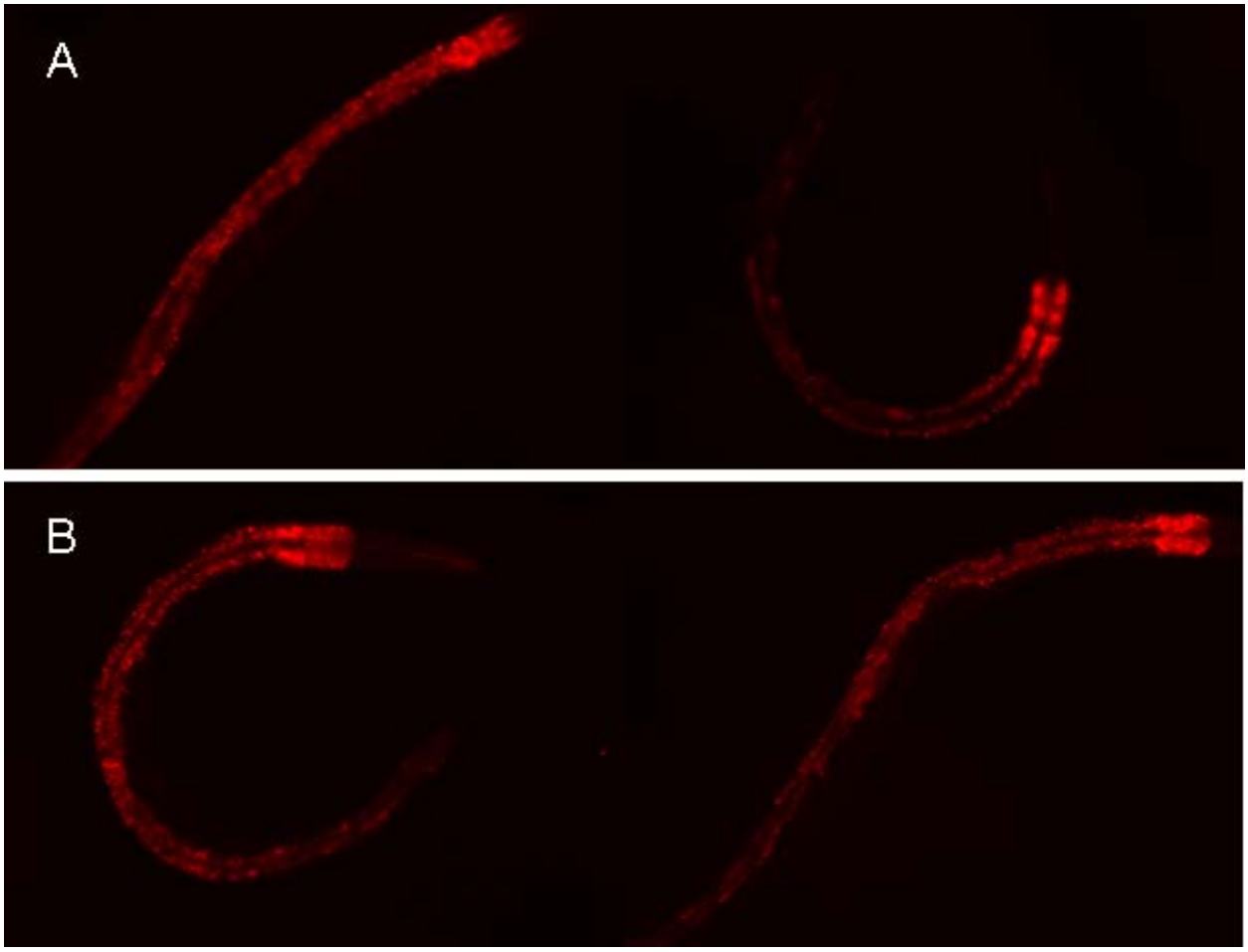


Supplementary Figure S4 | Knock-down efficiency for the *misc-1* RNAi construct.
We assessed *misc-1* RNAi efficiency by comparing MISC-1 protein levels in *fer-15(b26)* worms treated with either empty L4440 vector or *misc-1* RNAi. Animals were exposed to RNAi by feeding synchronized L1 larvae at 25°C. On day one of adulthood, the worms were harvested and protein levels were assessed by Western blot. Equimolar concentrations of protein lysates were loaded. Tubulin was used as an internal loading control. The *misc-1* RNAi construct was verified by PCR amplification and restriction digest with at least two different restriction enzymes. The empty L4440 vector was verified by PCR amplification. *misc-1* RNAi resulted in almost complete suppression of MISC-1 synthesis. Analysis of the Western blot with ImageJ indicates that *misc-1* RNAi reduces MISC-1 protein levels by 86.8% compared to control.



Supplementary Figure S5 | Knock-down efficiency for the *gei-7* RNAi construct. Synchronized *fer-15(b26)* animals were exposed to RNAi by feeding from the L1 through day-one of adulthood. Animals were fed *E. coli* HT115 carrying either empty RNAi vector (L4440) or *gei-7* RNAi. The *gei-7* RNAi construct was verified by PCR amplification, restriction digest with at least two restriction enzymes and by sequencing. The L4440 vector was verified by PCR amplification. *gei-7* RNAi resulted in ~44% decrease in target gene mRNA. (For methods, see the legend of Figure 4.)

Appendix 3 – Fat staining of *misc-1* mutants



Fat staining. Fat was stained in wild-type N2 (A) and *misc-1* knock-out mutants (B) with Nile Red (N1142, Invitrogen), according to established protocols [Ashrafi *et al.* (2003) Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. Nature *421*: 268-372]. Images were takes with the Rhodamine filter. No obvious differences in fat accumulation could be observed between wild type and *misc-1* mutants.

Appendix 4 – Targets of DAF-16 involved in *Caenorhabditis elegans* adult longevity and dauer formation[1]

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Targets of DAF-16 involved in *Caenorhabditis elegans* adult longevity and dauer formation

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Abstract

The Forkhead Box O transcription factor DAF-16 regulates genes affecting dauer larva formation and adult life span. Expression profiling and genome-wide searches for DAF-16 binding sites in gene regulatory regions have identified thousands of potential DAF-16 targets. Some of these genes have been shown to alter longevity when their expression is attenuated by RNAi treatment. DAF-16 also associates with other transcription factors, allowing combinatorial modulation of gene expression. Although extensive descriptions of the gene network regulated by DAF-16 have been attempted, there remain many gaps in the understanding of how DAF-16 regulates dauer formation and longevity.

Keywords: Life span; DAF-16; FOXO; Nematode aging; Stress resistance; Dauer larvae; *Caenorhabditis elegans*

1. Introduction

DAF-16 is the target of the insulin/IGF-1 pathway in *Caernorhabditis elegans*, and is required for the life span extension phenotype of many of the insulin/IGF-1 pathway mutants. DAF-16 activity is also required to enter the dauer juvenile stage, a long-lived state of diapause formed under stress conditions, overcrowding, or lack of food (Larsen et al., 1995; Ogg et al., 1997).

The *daf-16* (dauer formation) gene was identified by mutations that prevented dauer formation in response to starvation. DAF-16 activity is required for the constitutive dauer formation and the extended adult longevity of *daf-2* (insulin/IGF-1 receptor) mutants (Larsen et al., 1995). There is considerable interest in identifying the downstream targets of DAF-16 responsible for the associated life span and dauer phenotypes, and several groups have undertaken large-scale expression profiling to identify such targets. We will discuss these genome-wide studies, as well as protein interaction data for DAF-16, and summarize what has been learned so far about the mechanisms of aging.

2. Gene expression profiling to identify DAF-16 targets

Several groups have used genome-wide approaches to identify possible DAF-16 targets. Transcriptional profiling has employed Serial Analysis of Gene Expression (SAGE) (Halaschek-Wiener et al., 2005) or printed DNA microarrays (McElwee et al., 2003; Murphy et al., 2003).

Halaschek-Wiener et al. (2005) compared SAGE libraries for *daf-2* mutants at days 6 and 10 of adulthood to *daf-2*(+) controls. The rationale for this study was that in a *daf-2* mutant background *daf-16* would be constitutively active, so a change in transcript levels in the *daf-2* libraries for any given gene would suggest regulation mediated by DAF-16 or other outputs of the insulin/IGF-1 pathway. In aging *daf-2* adults, DAF-16 was responsible for the up-regulation of genes encoding heat shock proteins – specifically the *hsp-16* and *hsp-12* families of molecular

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[1] A version of this appendix was published. Jensen, V.L., Gallo, M., Riddle, D.L. (2006) Targets of DAF-16 Involved in C. elegans Adult Longevity and Dauer Formation. *Exp. Gerontol.* **41**: 922-927. Reprinted with permission from Elsevier. All rights reserved.

chaperones – together with collagen genes, genes involved in lipid metabolism and genes encoding ribosomal proteins. One of the most abundant transcripts in these *daf-2* aging adults was encoded by *tts-1* (transcribed telomere-like sequence), a gene encoding an RNA of unknown function (Jones et al., 2001; Halaschek-Wiener et al., 2005).

Transcript abundance dropped in *daf-2* day-6 adults (compared to controls) for genes involved in stress response (cold-shock genes, some *hsp* genes and some free radical scavengers, notably *sod-1*), and in the metabolism of lipids, DNA and RNA, proteins and carbohydrates. However, transcription levels for metabolic genes were back at control levels by day 10. The *fat* gene family (encoding fatty acid desaturases) was slightly up-regulated in *daf-2* day-10 adults.

The observed decrease in transcripts for stress response genes in *daf-2* day-6 libraries may seem to contradict the notion that DAF-16 increases longevity by up-regulating genes involved in oxidative stress. However, as Halaschek-Wiener et al. (2005) pointed out, it is likely that the metabolic repression observed at day 6 of adulthood would lower production of reactive oxygen species (ROS), thereby lowering the demand for expression of stress response genes.

A similar increase in transcription of *hsp* genes was observed in microarray studies. McElwee et al. (2003) analyzed gene expression in *daf-2* and *daf-16;daf-2* animals employing cDNA arrays. Transcripts for HSP-70 and HSP-16 families of heat-shock proteins displayed up-regulation that was DAF-16-dependent. This study showed that DAF-16 does not affect transcript levels for genes involved in protein synthesis or degradation, in apparent disagreement with the SAGE results of Halaschek-Wiener et al. (2005).

Transcripts of genes involved in mitochondrial electron transport did not vary in the presence or absence of DAF-16 (McElwee et al., 2003). On the contrary, transcripts for the manganese-containing super-oxide dismutase *sod-3*, the product of which is localized to the mitochondria and is responsible for ROS removal (Hunter et al., 1997), were elevated 10-fold in *daf-2*. These data suggest that the Age phenotype of *daf-2* mutants may not be due to reduced levels of ROS production, but rather to increased efficiency in removal of ROS from the mitochondria. Elevation of transcripts for genes involved in detoxification pathways was also found using microarrays printed with PCR-amplified genomic DNA fragments (Murphy et al., 2003). All together, this lends support to the oxidative damage theory of aging (Finkel and Holbrook, 2000).

These studies suggest that transcripts elevated in long-lived mutants are either directly or indirectly regulated by DAF-16. They provide lists of candidates for the effector genes specifying the physiological mechanisms, by which *C. elegans* cells extend their survival. Although most of the candidate genes have yet to be experimentally verified, the lists provide a tool for unearthing human orthologs that might be effectors of longevity.

Overall, the genome-wide studies have revealed similarities in the expression profiles of dauer larvae and long-lived adults. A common denominator for these two long-lived stages seems to be DAF-16, and this is reinforced by the presence of DAF-16 binding sites in genes similarly regulated in these two stages. On the other hand, dauer larvae and long-lived mutants also differ in some of the ways they metabolize their energy stores. The SAGE data indicate that transcripts involved in anaerobic metabolism are up-regulated in dauer larvae (Holt and Riddle, 2003), but not in *daf-2* mutant adults (Halaschek-Wiener et al., 2005).

3. Genome-wide screens for DAF-16 binding sites

DAF-16 binding elements (DBEs) have been found in many genes showing expression differences associated with dauer entry and adult longevity. Electrophoretic mobility shift assays revealed a 5′-TTGTTTAC-3′ DBE core sequence (Furuyama et al., 2000). This consensus is recognized by the three DAF-16 mammalian orthologs (FOXO4, FOXO1A and FOXO3A) as well, and is consistent with the known degenerate sequence (TRTTTAY) recognized by Forkhead transcription factors.

DBEs are typically about 1 kb upstream of the DAF-16 target gene initiation codon. They are present in genes that are upregulated or downregulated (Murphy et al., 2003) as a result of *daf-2* RNA interference (RNAi) (Fire et al., 1998), suggesting that DAF-16 may affect transcription both positively and negatively. Another sequence over-represented in promoter regions of genes controlled by DAF-16, dubbed DAF-16 Associated Element (DAE), is 5′-CTTATCA-3′. The DAE was found to be associated with the DBE in various combinations. Murphy et al. (2003) suggested that this sequence might be a binding site for an unidentified transcription factor, which would co-operate with DAF-16 in gene regulation.

A recent chromatin immuno-precipitation (ChIP) study (Oh et al., 2006) indicated that DAE sites may be bound directly by DAF-16, because it co-immuno-precipitated with DNA sequences containing various combinations of DBE and DAE, DBE only or DAE only. However, the ChIP data may contain a large number of false positives, including *Escherichia coli* sequences (44% of the total hits) from contaminating intestinal bacteria.

DBE and DAE elements are found at varied positions upstream and downstream of genes. In fact, Oh et al. (2006) identified motifs bound by DAF-16 up to 4000 nt 5′ and 5560 nt 3′ of the predicted translational start site. They also showed that some genes that possess putative DAF-16 binding sites are not actually bound by the transcription factor. Either this site is not functional in those genes, or chromatin structure may hinder DAF-16 binding to some of its targets under specific physiological conditions.

The ChIP experiments indicate that there are at least 103 potential DAF-16 target genes in the *C. elegans* genome.

They participate in several biological functions that are important for regulation of dauer formation and adult longevity. Specifically, 24% of the DAF-16 target genes are involved in intra- or extra-cellular signaling, 17% in development, 10% in transcription/translation, 7% in metabolism, 4% in detoxification and stress response, 2% in apoptosis, while 36% of the genes have an as yet unknown function.

With the abundance of new data on potential DAF-16 targets, there has been a refinement of the consensus sequence for its binding motifs. The DBE is now considered to be the canonical DAF-16 binding site, its sequence being GTAAAC/TAA or TTG/ATTTAC. A new consensus sequence for the DAE has also been obtained: TGATAAG or CTTATCA (Oh et al., 2006).

4. RNAi treatment of putative DAF-16 targets

With the large number of putative DAF-16 target genes, there is a need to identify the genes responsible for the phenotypes attributed to DAF-16 activity. Several groups have used RNAi to this end. Murphy et al. (2003) showed that RNAi treatment for genes containing the DBE and DAE motifs can alter life span. Inclusion of previously identified DAF-16 targets *sod-3* and *mtl-1* (metallothionein, which functions in heavy metal detoxification) in their gene set containing DBE/DAE motifs and exhibiting RNAi phenotypes strengthens their hypothesis that their potential longevity altering genes are downstream of DAF-16. McElwee et al. (2003) attenuated expression (via RNAi) of 35 potential DAF-16 targets, which contained the DBE. They found previously identified longevity associated genes (including *sod-3*, *ins-7* and *daf-16*) and a new one, a protease possibly involved in lysosomal proteinase activation (ZK384.3). Three of the 20 most abundant dauer SAGE tags identified in Jones et al. (2001), F38E11.2/*hsp 12.6*, F36D3.9, and T25D10.3, were among the 20 most up-regulated genes in *daf-2* mutant adults.

RNAi treatment of several putative direct DAF-16 target genes (identified via ChIP with anti-DAF-16 antibodies) alter life span (Oh et al., 2006). Of the 33 selected target genes, seven altered life span, nine altered fat storage, and nine affected dauer formation. Four genes fit into two of those three categories, and one gene, *zfp-1*, had decreased life span and fat accumulation as well as enhanced dauer formation (included in all three categories). A unique aspect of this experiment was the analysis of other phenotypes in addition to adult longevity. It would be of interest to see if any of the genes identified in the other studies have effects on fat storage and dauer formation.

The use of expression profiling technologies (SAGE and DNA microarrays) combined with motif finding, ChIP and RNAi can be a powerful way to identify targets of DAF-16. Expression studies have shown that there are potentially thousands of genes that are directly or indirectly controlled by DAF-16 activity. Some of these genes are involved in longevity, stress resistance and/or thermotolerance. An analysis of the Gene Ontology (GO) terms for all of the putative DAF-16 targets shown to have an effect on dauer formation, fat storage and/or longevity is shown in Fig. 1. Much additional work will be required to complete the entire downstream regulatory network, because a large, diverse gene set is involved. Longevity may be controlled by many pathways working in parallel, including germline signaling (Berman and Kenyon, 2006) and TOR (Jia et al., 2004) pathways. Cross-talk between these pathways may coordinate major life history traits (e.g., dauer formation, reproduction and longevity) in response to the environment (Antebi et al., 2000; Jia et al., 2004).

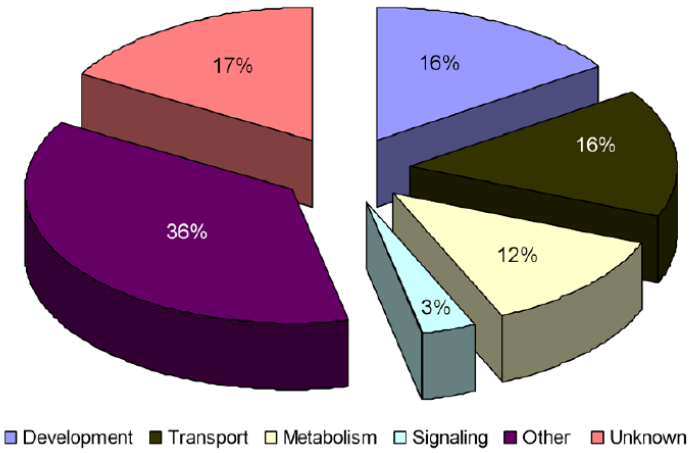

Fig. 1. GO Classifications for putative DAF-16 Targets. The pie chart shows the classification of genes possessing DAF-16 binding elements and with proven effect on life-span, fat storage and/or dauer formation resulting from RNAi treatment (McElwee et al., 2003; Murphy et al., 2003; Oh et al., 2005). The total number of genes considered was 90.

5. Interacting proteins

DAF-16 acts as a "master switch" in the dauer and longevity pathways. It is negatively regulated by *daf-2*/insulin-like signaling via PI 3 Kinase/AKT phosphorylation of Thr54, Ser240, Thr242 and Ser314. Any three of the four residues (conserved in mammalian DAF-16 orthologs) can be phosphorylated by AKT (Brunet et al., 1999; Guo et al., 1999; Lin et al., 2001). Phosphorylation inhibits DAF-16 function. This negative regulation is at least in part mediated by 14-3-3 proteins (Cahill et al., 2001).

In dimeric form (Cahill et al., 2001), 14-3-3 proteins bind their targets by recognizing phospho-serine/threonine motifs. Consequently, it is hypothesized that recruitment of 14-3-3 proteins to phosphorylated DAF-16 results in its exclusion from the nucleus, thereby inhibiting it from performing its regulatory functions. Cahill et al. (2001) showed that DAF-16 phosphorylation induced binding of 14-3-3 dimers *in vitro*. However, in the monomeric form, 14-3-3 could also bind DAF-16 in a phosphorylation-independent manner (Shen et al., 2003).

Binding of 14-3-3 proteins can be disrupted by phosphorylation at Ser196 in the DAF-16 forkhead domain. Interestingly, this phosphorylation was shown to be performed by CST (*C. elegans* Sterile 20) kinases, CST-1 and/or CST-2, in response to oxidative stress (Lehtinen et al., 2006). DAF-16 could be phosphorylated both *in vitro* and in 293T cells by the mammalian CST ortholog MST1. Furthermore, RNAi-mediated CST downregulation in worms led to a decreased lifespan, while CST-1 overexpression resulted in increased lifespan in a DAF-16-dependent manner. The authors proposed that CST phosphorylation of DAF-16 inhibits its interaction with 14-3-3 proteins, thereby allowing DAF-16 to localize to the nucleus and perform its function as transcriptional regulator. This work on CST/MST1 kinases enriched our knowledge on the ways in which DAF-16 activity is modulated and, importantly, further suggests that longevity pathways involved in aging are conserved from worms to mammals.

A recent report showed that SMK-1 – the only *C. elegans* homolog of mammalian SMEK1 (suppressor of MEK null) – acts as a coregulator of DAF-16 (Wolff et al., 2006). This was implied by the requirement of *smk-1* for DAF-16-mediated upregulation of *sod-3*, *ctl-1* and *lys-8* and for DAF-16-mediated downregulation of *daf-15*. *smk-1* was also required for DAF-16-dependent resistance to oxidative and UV stress and innate immune responses, but it was not required for the functions of DAF-16 in dauer development and reproduction. How SMK-1 physically interacts with DAF-16 and performs its coregulatory role is not clear yet. On the basis of conserved domains in SMK-1, however, it has been proposed that SMK-1 interacts directly with DAF-16 to regulate its transcriptional activity. Alternatively, it is possible that SMK-1 acts as a scaffolding protein, linking DAF-16 to phosphatases that ultimately regulate this TF by dephosphorylation.

6. DAF-16 combinatorial functions

DAF-16 may work in conjunction with other transcription factors (Fig. 2). In mammals, β-catenin can bind to the DAF-16 ortholog FOXO. Loss of BAR-1 (*C. elegans* β-catenin) reduces DAF-16 activity, suppresses dauer formation, and reduces longevity in *daf-2* mutants (Essers et al., 2005). Mutating *bar-1* also affects DAF-16-dependent *sod-3* expression. An overexpressing allele of *bar-1* was shown to enhance dauer formation in *daf-2* mutants but not in wild type. This suggests that activated DAF-16 may be required for BAR-1 activity in dauer formation. Hence, the interaction between BAR-1 and DAF-16 is responsible for some of the downstream influence of DAF-16.

The DAF-16 mammalian ortholog FOXO3a interacts with RUNX3, a RUNT related transcription factor (Yamamura et al., 2006). RUNX3, like β-catenin, is involved in apoptosis and the cell cycle (Essers et al., 2005; Yamamura et al., 2006). These functions have also been associated with DAF-16 (Furuyama et al., 2004). Mutating the *C. elegans* ortholog of RUNX3 (*rnt-1*) results in missing copulatory rays in males and variable abnormal hypodermal V and T cell divisions in hermaphrodites (www.wormbase.org).

The tissue specificity of DAF-16 function in longevity has been tested by Libina et al. (2003). When *daf-16* was expressed under control of a gut-specific promoter, it partially rescued the Age phenotype in *daf-16* mutants. By contrast, expressing DAF-16 in the muscles or neurons had no significant effect on longevity (despite DAF-16 expression in these tissues in wild-type worms). Only partial rescue of the Age phenotype may be an artifact of DAF-16 expression from an extrachromosomal transgene, or to combinatorial effects of DAF-16 with other transcription factors. DAF-16 may have different functions in different tissues as a result of interactions with different transcription factors.

Taken together, the protein interaction and tissue-specific expression data suggest that DAF-16 is not working alone in dauer induction or longevity. DAF-16 may have other interacting partners in addition to β-catenin/BAR-1 and RUNX3/RNT-1. Further study on DAF-16 interactions may identify more of its partners and their combinatorial effects on downstream gene expression.

7. Dauer longevity

Though dauer larvae are the longest lived stage in the *C. elegans* life cycle, genetic modification of dauer longevity has not yet been investigated. Perhaps this is due to the time required to measure the life span of dauer larvae. However, knowledge of what factors affect the longevity of dauer larvae would be valuable for two reasons: (1) *daf-2* mutant adults and dauer larvae have a similar expression pattern with regard to longevity genes (McElwee et al., 2004), so factors affecting dauer

longevity may relate to adult longevity, and (2) since *C. elegans* dauer larvae are analogous to the infective stage of parasitic nematodes, there is potential application to parasite control in knowing what factors are involved in survival of the infective stage. Better knowledge of infectious nematodes may have an important impact on human health due to the high number of infections worldwide (up to 1/4 of the human population) (Capello, 2004). Several studies have examined gene expression of dauer larvae, but this is just a glimpse into a cross section of dauer life (Holt and Riddle, 2003; Wang and Kim, 2003; Jones et al., 2001).

8. Dauer induction

As in dauer longevity, the genes downstream of DAF-16 in dauer induction have not been thoroughly studied. There are some advantages to dauer formation when searching for DAF-16 targets because the dauer/non-dauer switch provides a phenotype that can be scored in single animals, unlike longevity, which requires assay of a population. Any genetic effect on dauer formation may also show a longevity effect. Oh et al. (2006) using mixed-stage *daf-2* cultures, identified nine genes affecting dauer formation, which have nearby DAF-16 binding elements. Repeating this experiment on dauer or pre-dauer larvae should enrich for DAF-16 targets involved in dauer induction.

9. Conclusions

DAF-16, which is itself negatively regulated by phosphorylation, works with other transcription factors to control a downstream network of genes (Fig. 2). DAF-16 binds to at least two different promoter sequence motifs (DBE and DAE). Many potential target genes for DAF-16 have been identified via genome-wide screens, though only a few have been verified to have effects on longevity, fat storage or dauer formation. This work is an important step in defining the physiological mechanisms by which life span is regulated in a genetically tractable model. At this point, there is no reason to believe that the cellular physiology of mammalian aging will be fundamentally different from invertebrate models. There is also value in further understanding the role of DAF-16 in dauer formation and dauer longevity. Much more work needs to be done to verify direct vs. indirect gene targets, but the genome-wide approaches should lead us to better understand the transcriptional hierarchy in the target tissues, which in turn will allow us to tease apart the tissue specific responses from the systemic effects modulating longevity.

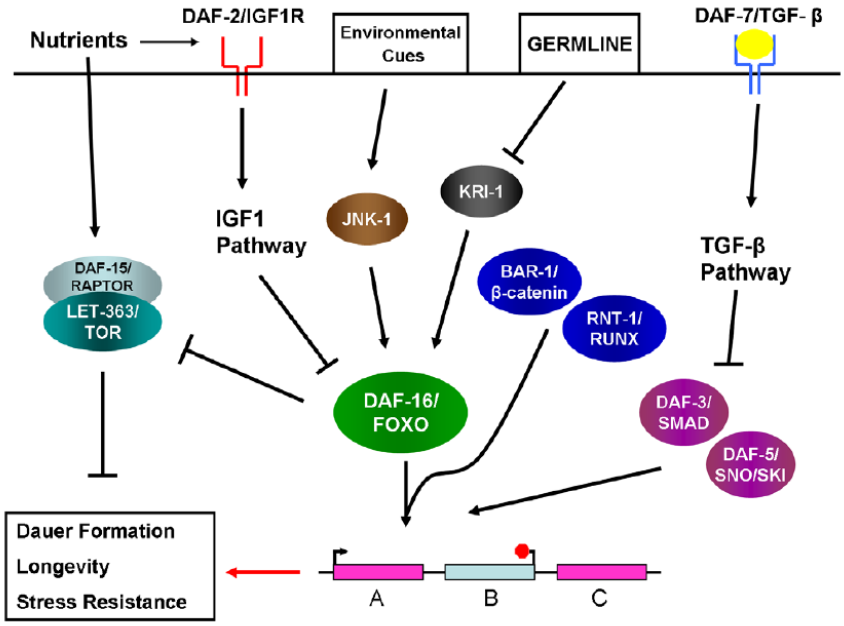

Fig. 2. Simplified model for the DAF-16 regulatory network. The insulin/IGF1 pathway negatively regulates DAF-16. Upon activation, DAF-16 negatively regulates TOR pathway members, among other genes, to further induce dauer formation and longevity. The Jun-N-terminal kinase (JNK) pathway responds to environmental cues (i.e. stress, heat, etc., . . .) and JNK-1 phosphorylates DAF-16 promoting its nuclear localization (Oh et al., 2005). In the absence of germline, KRI-1 signaling positively regulates DAF-16 (Berman and Kenyon, 2006). Output from the TGF-β pathway cooperate with DAF-16 (sometimes at the same promoter) to regulate dauer formation (Ogg et al., 1997). BAR-1/β-catenin and RNT-1/RUNX bind DAF-16 to co-regulate downstream targets. Integration of upstream signals on DAF-16, combined with its combinatorial interaction with different transcription factors, produce different effects on downstream targets – up-regulation (gene A) or down-regulation (gene B) – and have no effect on regulation of non-target genes (gene C).

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Appendix 5 – Regulation of metabolism in *Caenorhabditis elegans* longevity[1]

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MINIREVIEW

Regulation of metabolism in *Caenorhabditis elegans* longevity

Marco Gallo[1] and Donald L Riddle[*1,2]

See research article http://www.biomedcentral.com/1741-7007/8/14

Abstract

The nematode *Caenorhabditis elegans* is a favorite model for the study of aging. A wealth of genetic and genomic studies show that metabolic regulation is a hallmark of life-span modulation. A recent study in *BMC Biology* identifying metabolic signatures for longevity suggests that amino-acid pools may be important in longevity.

Aging in *Caenorhabditis elegans* seems to be intimately tied to the modulation of metabolism. The first genes identified for their ability to affect life span were *age-1*, which encodes a phosphatidylinositol kinase [1], and *daf-2* (constitutive dauer formation), which encodes a receptor tyrosine kinase similar to the mammalian insulin and insulin-like growth factor 1 (IGF1) receptors [2]. Although complete loss of *daf-2* function is lethal [3], partial loss of function extends adult life by two- to threefold. *daf-2* and *age-1* specify steps in the insulin/insulin-like signaling (IIS) pathway, thereby influencing metabolism. IIS signaling inactivates the FOXO (forkhead box O) transcription factor DAF-16, which controls genes that mediate a wide variety of functions, including metabolism, innate immunity, stress response, and translation (reviewed in [4]). The modulation of life span by the IIS pathway seems to result from an intricate network of physiological changes primarily mediated by unregulated DAF-16 activity, including major shifts in both energy and fat metabolism.

In addition to the IIS pathway, two other main groups of genes have profound effects on life span and are linked to metabolic control: genes involved in dietary restriction and mitochondrial function. Dietary restriction has been associated with increased life span in a wide variety of species (including mammals) and appears to be universally associated with longevity (reviewed in [5]). Increased longevity resulting from mutations in genes that regulate mitochondrial function is more problematic, although, at least superficially, it is related to metabolic slow-down. Work in nematodes and other invertebrates seems to support the notion that reducing the rate of oxidative phosphorylation (OXPHOS) leads to increased life span in two major ways: by decreasing the metabolic rate, and by reducing the level of reactive oxygen species, which are an obligate byproduct of OXPHOS. However, mutations that reduce mitochondrial function are beneficial for *C. elegans* longevity, but have deleterious and often lethal effects in mammals [6]. The reason for this discrepancy is not understood.

Given the large number of genes that have been shown to regulate life span through different pathways, it is reasonable to ask whether there are any physiological changes conducive to longevity that are common in all long-lived mutants. A recent metabolomic study on five different long-lived mutants published in *BMC Biology* by Fuchs *et al.* [7] suggests that the answer to this question may be yes. Fuchs *et al.* report that mutants carrying three different alleles of *daf-2*, one allele of the insulin-like gene *daf-28* or one allele of *ife-2* (translational initiation factor 4E) have similar metabolic signatures, which cluster away from those of wild-type worms. These metabolic signatures are consistent with previous findings that assessed gene expression in *daf-2* mutants [8]. In particular, they showed that long-lived *daf-2* worms upregulate the glyoxylate cycle, gluconeogenesis and starch metabolism.

Fuchs *et al.* [7] detected a common metabolic signature for IIS mutants and *ief-2*. All five of the long-lived mutants had increased pools of amino acids, especially of the branched-chain amino acids isoleucine, leucine and valine, together with phenylalanine and tyrosine. A similar increase in branched-chain amino acids has been found in animals carrying mutations in components of

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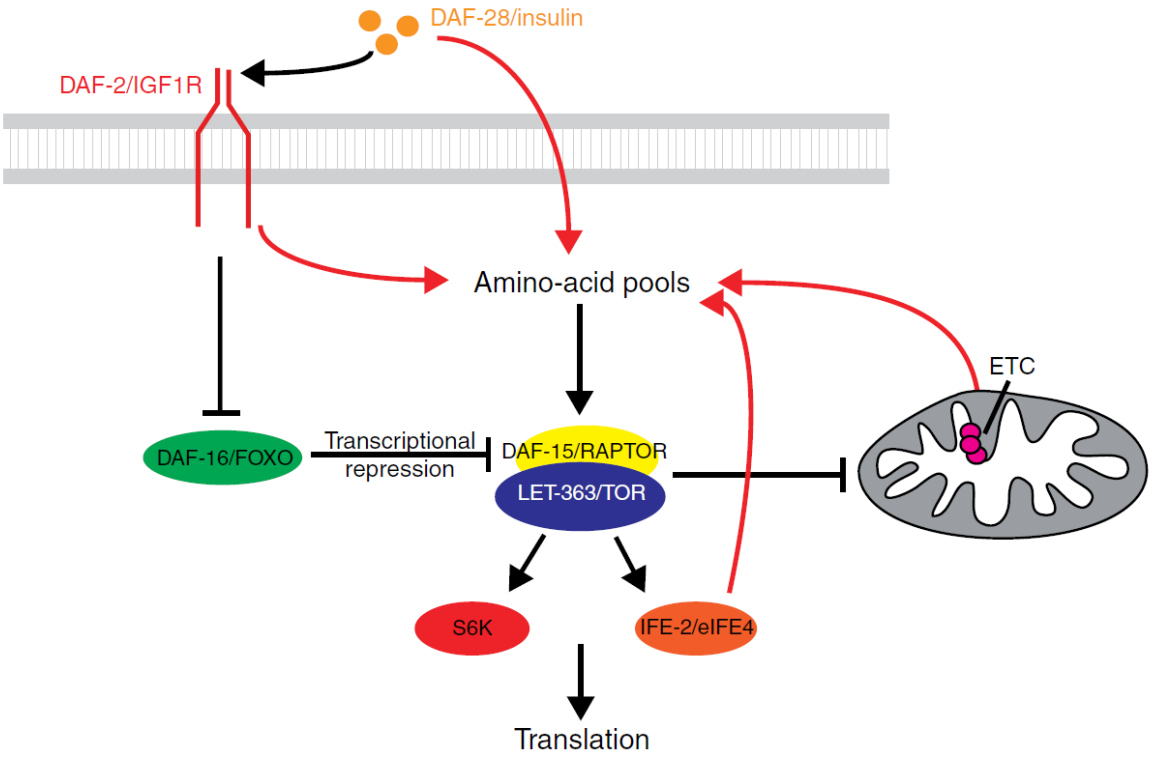

Figure 1. Possible link between accumulation of amino-acid pools, mitochondrial function, and the insulin and TOR pathways in *C. elegans* longevity. Black lines indicate the activating or repressive function of members of different signal transduction pathways. Amino-acid pools and other nutrients activate TOR signaling, which then results in increased translation following activation of the protein kinase S6K and the translation initiation factor eIFE4 and inhibition of mitochondrial biogenesis. However, mutations in DAF-2/IGF1R result in the activation of DAF-16, which represses *daf-15* transcription to reduce TOR signaling. Red arrows indicate the effects of mutations on cellular amino-acid pools. Specifically, Fuchs *et al.* [7] show that mutations in *daf-2/IGF1R*, *daf-28/insulin* and *ife-2/eIFE4* cause an increase in amino acid levels, especially branched-chain amino acids. Falk *et al.* [9] showed that mutations affecting complexes I, II and III of the electron transport chain (ETC), including some that increase life span, also result in accumulation of branched-chain amino acids. Mutations that confer longevity through different pathways share the metabolic signature of increased branched-chain amino-acid pools.

the mitochondrial electron transport chain (ETC) complexes I, II and III [9]. At least some of these ETC mutants are long-lived. The increase in branched-chain amino acids in long-lived worms is intriguing for two reasons. First, it opens the possibility that protein metabolism plays an important role in life-span determination. Second, branched-chain amino acids are known to stimulate protein synthesis and inhibit protein degradation in higher eukaryotes [10], a phenomenon mediated by the TOR (target of rapamycin) pathway.

Dietary restriction and metabolism

Although it might seem reasonable to assume that dietary restriction exerts its life-prolonging effects by reducing metabolic function, recent reports argue to the contrary (reviewed in [11]). Worms on a dietary restriction regimen induced genetically (by *eat* mutations), or by dilution of nutrients, or by exposure to axenic medium (medium that supports growth without bacteria), actually had increased metabolic rates, as measured by oxygen consumption and heat production. The mechanism by which dietary restriction regulates life span in worms is therefore not clear. While it seems to act by regulating the insulin pathway in *Drosophila* and rats, the IIS pathway is not responsible for dietary-restriction-induced longevity in *C. elegans*.

The most likely pathway exploited by dietary restriction in *C. elegans* is the TOR pathway. The physiological role of TOR kinase is to sense nutrient levels - such as cellular amino-acid pools - and to regulate transcription and protein biogenesis and degradation accordingly. TOR exists in two highly conserved protein complexes: TORC1, which regulates cell growth, protein synthesis and autophagy; and TORC2, which regulates cytoskeletal reorganization [12]. Both complexes regulate the metabolic state of *C. elegans*. TOR activates the ribosomal p70 S6 kinase (S6K) and the translation initiation factor eIF4E. The latter is encoded by *ife-2* in *C. elegans*, and a

mutant in this gene was studied in the metabolomic analysis performed by Fuchs *et al.* [7]. It was previously observed that knockdown of *TOR/let-363* pathway in *C. elegans* results in an almost twofold increase in life span [13]. Although not mentioned by Fuchs *et al.* [7], their metabolic profiles fit very well with a model whereby TOR modulates life span via *ife-2*: downregulation of TOR increases longevity; *ife-2* is an effector of the TOR pathway and animals carrying a mutation in this gene also live longer; and *ife-2* mutants accumulate pools of amino acids that are known to induce protein biogenesis and inhibit protein degradation. These data naturally lead to the speculation that the longevity of IIS mutants is at least partially derived from downregulation of TOR.

Mutation of DAF-15/RAPTOR, an activator of TOR, results in increased *C. elegans* life span [13]. Interestingly, *daf-15* is directly regulated by DAF-16, the ultimate effector of the IIS pathway. It was therefore proposed that mutations reducing IIS signaling (such as *daf-2* mutations) activate DAF-16, which then represses *daf-15* to decrease the function of TOR and enhance longevity (Figure 1). The increased amino-acid pools found by Fuchs *et al.* [7] in the IIS mutants *daf-2* and *daf-28* could be explained by the consequent downregulation of TOR, which in turn would result in decreased translation and consequent accumulation of amino-acid pools. Mutations in *ife-2* would also result in increased amino-acid pools.

Branched-chain amino acids and longevity

Fuchs *et al.* [7] may, in fact, hold a clue to one of the mysteries of the aging field: why do translation-defective mutants live longer? If translation mutants such as *ife-2* accumulate amino acids, they would mimic the conditions arising in mitochondrial ETC mutants and IIS pathway mutants. TOR pathway mutants would be predicted to have very similar metabolic profiles to ETC, *daf-2* or *ife-2* mutants. Analyzing the metabolomes of TOR mutants and translation-defective mutants could therefore shed some light on this problem.

In conclusion, the belief that decreased metabolism leads to longevity is, so far, a generalization that extends beyond the current evidence. We know that genes involved in metabolic control, such as *daf-2*, regulate life span, but we do not know if overall metabolism is downregulated in these mutants [11]. For instance, *daf-2* mutants exhibit decreased carbohydrate metabolism, but gene-expression data suggest that lipid utilization pathways are actually upregulated in these mutants [8].

Life-span-prolonging effects of downregulating protein synthesis might be specific to *C. elegans* and other invertebrates. The soma of the adult nematode is post-mitotic, and metabolic control might have different effects in *C. elegans* (where adult cell and tissue replacement does not occur) from those in higher eukaryotes, where compromised cells can be eliminated by apoptosis and replaced. *C. elegans* cells might have a higher tolerance for cellular insults and decreased metabolism. It is conceivable that translation of new proteins and other cell-maintenance processes may be more important to *C. elegans* than to higher organisms, as *C. elegans* somatic cells cannot be replaced.

Although the role of metabolism in aging is not straightforward, the metabolomics of longevity mutants may provide some answers. It is interesting to notice that several classes of long-lived mutants - mitochondrial ETC mutants, IIS mutants, and translation mutants - all have increased levels of branched-chain amino acids [7,9]. Metabolomic profiles of TOR pathway mutants, dietary restriction mutants and other translation mutants could reveal an under-appreciated function of amino-acid metabolism in longevity.

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