Suppressor analysis of the *clk-1* mutants of *Caenorhabditis elegans*

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Summary

clk-1 encodes a hydroxylase that is necessary for ubiquinone (UQ) biosynthesis. *clk-1* mutants do not synthesize UQ, but instead accumulate the precursor demethoxyubiquinone (DMQ). When fed on bacteria that synthesize UQ the mutants are viable but display slow development, behaviours and aging. However, they arrest development when fed on UQ synthesis-deficient bacteria. I have taken a genetic suppressor approach to investigate the causes of the various phenotypes as well as of the dietary requirements of the *clk-1* mutants.

We identified two classes of mutants that suppress the defecation phenotypes of *clk-1*. All of these "*dsc*" mutants suppress the lengthened cycle of *clk-1*. Class I mutants also restore the ability to react normally to changes in temperature whereas the Class II mutants do not. The characterization of the Class I mutants suggests that part of the phenotype of *clk-1* is due to an alteration of lipid metabolism, likely the level of lipid or lipoprotein oxidation. *dsc-4* encodes the worm homolog of the Microsomal Triglyceride Transfer Protein (MTP), a protein required for the formation of low density lipoproteins (LDL) in vertebrates, and whose absence in people leads to abetalipoproteinemia. *dsc-3* appears to be allelic to *tat-2*, which encodes a type IV P-type ATPase that is related to a family of human aminophospholipid transporters that includes ATP8B1/FIC1, whose inactivation results in cholestatic liver disease. *dsc-3* and *dsc-4* appear to affect distinct aspects of lipid metabolism. A general link between the Class II mutants and *clk-1* remains elusive. *dsc-1*, a Class II gene, encodes a paired-like homeodomain transcription factor that is necessary for the GABA sensitivity of enteric muscles.

We also identified 9 *clk-1(e2519)*-specific suppressors, which suppress most Clk phenotypes, including their requirement for dietary UQ. Our analysis of these suppressors reveals that it is the lack of UQ rather than the presence of DMQ that is responsible for most phenotypes. In

addition, they allowed us to show that most Clk phenotypes can be uncoupled from each other. We cloned six suppressors and all encode missense tRNA(Glu) suppressor genes. To my knowledge, these represent the first missense tRNA suppressors identified in any metazoan.

Résumé

Le gène *clk-1* encode une hydroxylase qui est nécessaire à la biosynthèse de l'ubiquinone. Plutôt que de synthétiser de l'ubiquinone, les mutants *clk-1* accumulent son précurseur, la démethoxyubiquinone. Ces mutants sont viables quand ils sont cultivés en présence de bactéries capables de synthèse d'ubiquinone, bien que leur développement, leurs comportements et leur vieillissement soient ralentis par rapport au type sauvage. Par contre, ces mutants subissent un arrêt du développement quand ils sont cultivés avec des bactéries déficientes en ubiquinone. J'ai choisi une approche basée sur la recherché de suppresseurs génétiques pour étudier les mécanismes à la base des phénotypes des mutants *clk-1*, y compris leurs besoins en ubiquinone alimentaire.

Nous avons identifié deux classes de mutants qui suppriment certains aspects de la défécation anormale des mutants *clk-1*. Tous ces mutants, qui sont appellés "*dsc*", suppriment la lenteur du cycle de défécation de *clk-1*. Par contre, seuls les mutants de la Classe I rétablissent aussi la capacité de réagir normalement à des changements de température. La caractérisation des mutants de la Classe I suggère qu'une partie du phénotype de *clk-1* est due à une altération du métabolisme des lipides, probablement au niveau de l'oxydation des lipides ou des lipoprotéines. *dsc-4* encode l'homologue chez le nématode de la 'Microsomal Triglyceride Transfer Protein' (MTP), qui est nécessaire pour la formation des lipoprotéines à faible densité (LDL) chez les vertébrés, et dont l'absence chez l'homme mène à l'abetaprotéinémie. *dsc-3* se présente comme allélique à *tat-2*, qui code pour une ATPase type P, du type IV, qui s'apparente à une famille humaine de transporteurs des aminophospholipides qui inclue la protéine ATP8/FIC1, dont l'inactivation mène à un type de maladie cholestatique du foie. Il apparaît donc que *dsc-3* et *dsc-4* touchent à des aspects différents du métabolisme des lipides. Un lien général entre les mutants de Classe II et *clk-1* n'est

pas encore apparent. *dsc-1*, un gène de Classe II, code pour un facteur de transcription à homéodomaine du type 'paired' qui est nécessaire à l'établissement de la sensibilité des muscles entériques au neurotransmetteur GABA.

Nous avons aussi identifié 9 mutants qui suppriment la plupart des phénotypes Clk, y compris les besoins en ubiquinone alimentaire, mais uniquement dans le cas de l'allèle *clk-1(e2519)*. Notre analyse de ces suppresseurs révèle que c'est le manque d'ubiquinone plutôt que la présence de DMQ qui est responsable de la plupart des phénotypes Clk. De plus, ces suppresseurs nous ont permis de montrer que la plupart des phénotypes Clk sont indépendants les uns des autres. Nous avons clonés 6 suppresseurs et tous codent pour des gènes d'ARN de transfert(Glu) avec des mutations suppressives de mutations faux-sens. A ma connaissance, c'est la première fois que de tels suppresseurs ont été identifiés chez un métazoaire.

Acknowledgements

Firstly, I would like to thank my supervisor Siegfried Hekimi. Throughout the course of my studies Siegfried has been a supportive and encouraging teacher, a mentor in the true sense of the word, and an inspiring example of what a scientist and a supervisor should be. I have benefited immensely from the countless hours he has spent with me discussing my project, preparing manuscripts and teaching me how to prepare talks. I greatly admire his drive and his originality. I am especially grateful for the efforts he has made to teach me how and why one should persevere in Science.

I owe thanks to many members of the lab for helping me out at so many levels. For teaching me how to pick, cross and love worms, I would like to thank Stephanie Felkai and Claire Bénard. For introducing me to the basics of worm genetics I would like to thank Bernard Lakowski.

For help with molecular biology, I am indebted to Claire Bénard. Claire has taught me everything that I know about molecular biology and I aspire to her level of experimental greatness.

For successful collaborations in the lab, I would like to thank Yukimasa Shibata and Phuong Anh (Phanh) Thi Nguyen. Working with Yukimasa on the *dsc* mutants taught me the benefits of scientific collaboration. He has shown me, by example, what attitude one should have towards one's colleagues and one's superiors, and also towards one's own work. I am grateful to have had the opportunity to work with him and to learn from him. I have also benefited from working alongside Phanh the last few years. All of her hard work, as well as her interest in the growth suppressors helped to move the project forward.

I would like to thank Jinliu Feng and Ambereen Hussain for helping to isolate *dsc* mutants. I would especially like to thank David Shanks for his efforts to try to find *dsc-1* suppressors and also for helping to isolate *clk-1* growth suppressors. I would also like to thank Tanya Rodrigues and Darius Camp for isolating their own *clk-1* suppressor mutants. Indeed their

mutants have helped us to make better sense of the mutants I have been working on.

I would like to thank my supervisory committee members, Joseph Dent and Thomas Bureau for their support and guidance.

For reagents I would like to thank Theresa Stiernagle/Caenorhabditis Genome Centre (strains), Andrew Fire (*gfp* vectors), Alan Coulson/Sanger Center (cosmid clones), Shohei Mitani/National Bioresource Project for the Nematode (*dsc-1* and *tat-2* deletion strains) and Erik Jorgensen (*exp-1* clones).

I would also like to acknowledge that over the course of my degree I have received support from a Faculty of Graduate Studies Fellowship and a Mary Louise Taylor McGill Major Fellowship.

For friendship, I would like to thank all of the original Bio girls (Inge, Gen, Leslie, Allison, Julia, Victoria and Kristen). I am glad that we have, more or less, managed to stay in touch since graduation. You have each in your own way been inspiring examples, showing me the different career paths one can successfully follow. I would also like to thank Claire. I cannot tell you how much I valued and enjoyed all of our walks home as well as our long conversations in and out of the lab. Marie-Annick and Vad, I am truly grateful for your friendship these last years. I have greatly enjoyed spending time with you (especially on Thursday evenings), and learning from your interesting and informed points of view. Gen and Mathieu, I could not have asked for better friends. You have been warm, generous, and unbelievably positive. I cannot say enough how I have appreciated knowing that I could always count on both of you.

Finally, I would like to thank my parents and my sister for always being encouraging and supportive of my decisions. You have even been remarkably understanding about my needing to spend more weekends and holidays with my worms than with you. I am sorry that I am still not coming home yet, but I promise to come home eventually.

Preface

This thesis is presented in accordance with the manuscript-bases thesis guidelines. It consists of an Introduction (Chapter 1), which gives a review of the relevant literature and states the rationale and objectives of the study, 5 research chapters (Chapters 2 to 6) and a summary and conclusion chapter (Chapter 7). Each research chapter contains sections equivalent to an Abstract, Introduction, Material and Methods, Results, and Discussion; each chapter contains its own Reference section.

This thesis is partially based on published manuscripts. Chapters 2, 3 and 5 have already been published, so these chapters are simply re-formatted versions of the published manuscripts; Chapter 6 is a version of a manuscript that has been submitted for publication; Chapter 4 contains unpublished material.

The thesis has been prepared entirely by me. The chapters have also been largely written by me, with the exception of Chapter 3, which is based on a multi-authored manuscript for which I am not the first-author. As this chapter as well as chapters 2, and 6 are or will be multi-authored papers, I have outlined in detail below my contributions as well as the contributions of each of the co-authors involved. Contributors that are not authors are thanked in the Acknowledgements sections. The contributions of my supervisor, Dr. Hekimi, are not spelled out for each chapter as his contribution was the same for all chapters: he was involved at all levels, contributed ideas, suggestions and insight. He was also involved in the preparation of all manuscripts. To acknowledge the collective effort of all authors and contributors involved, the pronoun "we" is used throughout the thesis.

Contributions of Co-authors

Chapter 1: Introduction

In this chapter I review the relevant literature, mostly related to *clk-1*. Portions of the chapter are reproduced with permission from:

Branicky R., Bénard C., Hekimi S. (2000) *clk-1*, Mitochondria and Physiological Rates. BioEssays. 22(1): 48-56.

This was an "ideas" paper that was a collaboration between myself, Claire Bénard and Dr. Hekimi. I was responsible for writing the original draft of the paper, and for working with the co-authors on subsequent versions. Claire contributed significantly to the planning of the paper, the editing of the manuscript, the preparation of the figures and the referencing of the paper. For the other major topics covered in the thesis (defecation, lipids and tRNA genes in *C. elegans*), the literature is reviewed in the corresponding chapter (Chapters 2 and 5, Chapter 4 and Chapter 6, respectively).

Chapter 2: The Screen for *dsc* Mutants

This chapter is a re-formatted version of:

Branicky R., Shibata Y., Feng J., Hekimi S. (2001) Phenotypic and suppressor analysis of defecation in *clk-1* mutants reveals that reaction to changes in temperature is an active process in *Caenorhabditis elegans*. Genetics. 159(3): 997-1006.

This paper was a collaboration between myself, Yukimasa Shibata and Jinliu Feng. I isolated most of the mutants myself and helped Jinliu to isolate some others. The initial mapping and characterization of the mutants was carried out by me. I worked together with Yukimasa on the subsequent mapping and characterization of the mutants. I wrote the manuscript.

Chapter 3: *dsc-4*

This chapter is a re-formatted version of:

Shibata Y., Branicky R., Oviedo-Landaverde I., Hekimi S. (2003) Redox regulation of germline and vulval development in *C. elegans.* Science. Dec 5;302(5651):1779-82.

The first author, Yukimasa Shibata, was the major contributor of the data presented in this paper. I was involved in the cloning of *dsc-4*, and contributed to the phenotypic analyses presented. As Yukimasa had left the lab before this paper was submitted for publication, I also contributed significantly to the writing of the paper, including the preparation of the actual text as well as the analysis and presentation of the results. Irene Oviedo-Landaverde constructed some of the clones that were used during the course of the study.

Chapter 4: *dsc-3*

This chapter reports my unpublished analysis of the *dsc-3* mutants. I worked together with Yukimasa on the mapping of *dsc-3*, and Yukimasa constructed the *dsc-3 dsc-4* double mutant strains, which were used for some of the analyses.

Chapter 5: *dsc-1*

This chapter is a re-formatted version of:

Branicky R., Hekimi S. Specification of Muscle Neurotransmitter Sensitivity by a Paired-like Homeodomain Protein in *C. elegans.* Development. Nov;132(22):4999-5009.

Chapter 6: Growth Suppressors of *clk-1*

This chapter is a re-formatted version of the submitted manuscript:

Branicky R., Nguyen P.A.T., Hekimi S. Uncoupling the pleiotropic phenotypes of the *Caenorhabditis elegans clk-1* mutants with tRNA missense suppressors.

This paper reports the isolation, characterization and cloning of growth suppressors of *clk-1*. I isolated the UQ+ growth suppressors myself, and

supervised an undergraduate student (David Shanks) to isolate the UQ-suppressors. I worked out the heritability, as well as the linkage and mapping strategies for the mutants, which allowed for their eventual molecular identification. I carried out the majority of the mapping for the cloned suppressors. I also carried out all of the initial phenotypic characterization, and devised the experimental methods to carry out much of the later characterizations. I wrote most of the paper.

Phuong Anh collaborated with me on some of the mapping and the phenotypic characterization. She also carried out the linkage analyses for a number of mutants that are not described in detail in the paper. She sequenced the mutants to identify their mutations. She also carried out much of the later phenotypic analyses, particularly the embryonic development and aging experiments and the phenotypic characterization on the different bacterial strains. We collaborated on developing the protocol for the various steps of the HPLC analyses, which she subsequently carried out herself.

Claims for Originality

In this thesis I report on the isolation, as well as the phenotypic, genetic and molecular characterization of suppressors of *clk-1*.

In Chapter 2, I report on the isolation of defecation suppressors of *clk-1*, which we call the *dsc* mutants. We isolated and genetically mapped 8 mutations, which fall into 5 complementation groups. Four of these complementation groups define new genes, which we named *dsc-1* to *dsc-4*; the fifth corresponds to *dec-7*, a gene that had previously been identified. We demonstrate that the defecation cycle is not temperature compensated, a finding that corrects a misconception in the field. We also show that *clk-1* mutants have two defecation defects. As had been previously described, they have an increased cycle length, and, as we showed, they also cannot adjust their cycle length in response to temperature changes, which was a novel finding. We also present some unique defecation phenotypes for the *dsc-2* and the *dec-7* mutants, which have not been reported for any other mutant.

In Chapters 3, 4, and 5, I present further characterization of *dsc-4*, *dsc-3* and *dsc-1*. In Chapter 3, I present our characterization of *dsc-4*. We show that *dsc-4* encodes the worm homolog of the Microsomal Triglyceride Transfer Protein (MTP). We also show that, in addition to suppressing the defecation phenotypes of *clk-1*, *dsc-4* suppresses the slow germline development of *clk-1*. Based on the interactions between *clk-1*, *dsc-4*, *vit-5* (a worm apoB homolog) and *sod-1* (an extracellular superoxide dismutases) we propose for the first time, that the rate of germline development in *C. elegans* is affected by the levels of a low density lipoprotein (LDL)-like lipoprotein particle. In addition, we suggest that, as in vertebrates, this LDL-like lipoprotein can exist in both native and oxidized forms in *C. elegans*, and that *clk-1* mutants have lowered levels of oxidized LDL because they have lower levels of cytoplasmic ROS. We also show that both the *let-60*/Ras and Inositol Triphosphate (IP_3)

pathways of signal transduction can affect the rate of germline development. Finally, by showing that *clk-1* suppresses the Multiple Vulvae (Muv) phenotype of activated *let-60*/Ras via lowering cytoplasmic ROS, we demonstrate that ROS levels can affect signal transduction in vivo, and alter developmental outcomes.

In Chapter 4, I present our characterization of *dsc-3*, which appears to be allelic to *tat-2*, which encodes a type IV P-type ATPase that is likely to be an aminophospholipid transporter. We show that the *dsc-3* mutants are hypersensitive to lowered levels of cholesterol in the media, and can be rescued by increased levels of cholesterol, which suggests that DSC-3 somehow regulates internal levels of cholesterol, possibly through an involvement in cholesterol absorption. Together with the identity of *dsc-4*, as well as interactions between *clk-1* and cholesterol, this demonstrates that *clk-1* affects some aspects of lipid metabolism in worms, likely by altering its level of oxidation.

In Chapter 5, I present our characterization of *dsc-1*. *dsc-1* mutants have a shortened defecation cycle length and are defective for the last step of the defecation behaviour, the expulsion step. We show that *dsc-1* encodes a Paired-like homeodomain transcription factor (a class of transcription factors studied in *C. elegans* for their roles in neuronal specification), which is expressed and required in the enteric muscles for the proper regulation of the cycle length and for the expulsion step. At present, only one neurotransmitter receptor, the GABA-gated cation channel EXP-1, is known to be required in these muscles for their function. We show that DSC-1(+) is required in these muscles for the expression of EXP-1, and that the defecation defects of the *dsc-1* mutants are due to the loss of EXP-1 expression. Thus, *dsc-1* encodes the first Paired-like homeodomain transcription shown to be required for muscle specification in *C. elegans*. We also show for the first time that the enteric muscles play a role in regulating the defecation cycle length.

In Chapter 6, I present our analysis of *clk-1* growth suppressors. These mutants were identified in screens for suppressors of the slow growth phenotype as well as of the requirement for dietary ubiquinone (UQ). We show that these mutations suppress most Clk-1 phenotypes and re-establish a minute amount of UQ biosynthesis. This same amount of UQ is insufficient when obtained from the diet. Thus, we show for the first time that wild-type rates of development and behaviour can be sustained by vanishingly small amounts of UQ, but only if it is synthesized endogenously. We cloned six suppressors and all encode $tRNA^{Glu}$ genes that carry mutations in their anticodons. To my knowledge, these represent the first missense suppressor tRNAs identified in any metazoan by classical forward genetics.

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Chapter 1:

Introduction

C. elegans as a model to study quantitative phenotypes

Sydney Brenner proposed the small, self-fertilizing hermaphroditic nematode *Caenorhabditis elegans* as a model to study behaviour using two main approaches: by dissecting the genetic specification of the nervous system and by describing the ultrastructure of the nervous system (Brenner, 1974). He chose *C. elegans* because of its short life cycle and overall amenability to genetic analysis and also because of its relatively simple nervous system. Over the last 40 years, the development and function of the nervous system has been intensely studied in this model. Hundreds of nervous system as well as behavioural mutants have been described, as has the connectivity of the entire nervous system (White et al., 1986). However, *C. elegans* has also proven to be an ideal model for the study of diverse aspects of developmental biology, including embryogenesis and cell fate specification, sex determination, and apoptosis.

One general characteristic of *C. elegans* that has allowed for the identification of numerous behavioural, as well as developmental mutants, is that many aspects of worm biology are precisely timed. For instance, the cell cycles in early embryos take 20 minutes at 20°C (Sulston et al., 1983), the worm completes its development from egg to adult almost invariably in 50 hours at 25°C (Wood et al., 1980), the worm reaches a maximum egg-laying rate of 6 eggs/hour (Wong et al., 1995), and lives an average of 14.5 days at 20°C (Klass, 1977). Pharyngeal pumping, necessary for food intake, occurs every 230 msec (Avery and Horvitz, 1989; Raizen et al., 1995), the period of the defecation cycle is 45 to 50 sec at 20°C (Thomas, 1990), and worms move 36 waves forward at 20°C before reversing and then moving backward (Croll, 1975). These features are so stereotyped in wild type worms that mutants exhibiting abnormal rates can be easily spotted. Thus, mutants displaying prolonged cell cycles (e.g. (O'Connell et al., 1998)), slowed development (e.g. (Feng et al., 2001)), altered egg-laying rates (e.g. (Adachi and Kagawa, 2003)),

altered lifespans (e.g. (Ishii et al., 1998; Kenyon et al., 1993; Lakowski and Hekimi, 1998), irregular pharyngeal pumping rates (e.g. (Avery, 1993)), abnormal defecation cycle lengths (e.g. (Iwasaki et al., 1995)), altered reversal frequencies (e.g. (Brockie et al., 2001)), as well as other abnormal developmental and behavioural rates, have all been isolated and studied. That the timing of these features can be modified by mutations underscores the fact that the determination of physiological rates has a genetic basis. Given the genetic tractability of *C. elegans*, it is possible to use this model system to study the molecular genetic basis of physiological rates.

Maternal-effect mutations

Many different types of mutations have been screened for in *C. elegans*. One class of mutations that have been identified are maternal-effect mutations, that is, mutations for which the maternal genotype influences the zygotic phenotype. This is the case for genes for which the mother deposits some form of gene product, be it transcript, proteins, or other factor, into the oocyte. These mutations can be either strictly maternal or non-strict. For a strictly maternal mutation, the phenotype is solely dependent on the maternal genotype: for a recessive mutation, homozygous mutants from a heterozygous mother will display a wild-type phenotype (a "maternal-rescue" effect) and heterozygous mutants derived from crossing a homozygous mother with wild-type males will display a mutant phenotype. These genes tend to act very early during embryogenesis. For a non-strict mutation, wild-type copies of the gene in either the mother or the zygote are sufficient for a wild-type phenotype: homozygous mutants from a heterozygous mother will display a wild-type phenotype (a "maternal-rescue" effect) and heterozygous mutants derived from crossing a homozygous mother with wild-type males will display a wild-type phenotype (a "zygotic-rescue" effect). These genes would also be expected to act during embryogenesis, possibly around the time that

transcription from the zygotic genome begins (Edgar et al., 1994; Newmann-Smith and Rothman, 1988). There are also some, but relatively few cases of paternal-effect mutations (e.g. (Hill et al., 1989; O'Connell et al., 2001)), as the sperm contributes the haploid complement of chromosomes to the zygote, but relatively little additional material, aside from the centrioles.

A large number of maternal-effect mutations affecting early embryogenesis have been identified, many of which are lethal. For example, Kemphues et al identified 54 mutations, defining 29 genes, in a screen for maternal-effect lethal (or *mel*) mutations on linkage group II (Kemphues et al., 1988). Similarly in a screen for maternal-effect embryonic lethal mutations on linkage group III, Gonczy et al identified 48 mutations in 34 distinct genes that were required for cell division in one cell-stage embryos (Gonczy et al., 1999). Moreover, Wood et al showed that of 24 temperature-sensitive embryonic-lethal mutants, 21 showed some type of maternal effect (Wood et al., 1980). Similarly, Isnenghi et al showed that of 30 temperature-sensitive mutants defining genes essential for embryogenesis (or *emb* genes), 28 had a maternal effect (Isnenghi et al., 1983).

However, there are also some cases of maternal-effect mutations that do not affect embryogenesis *per se*. For example, the maternal-effect sterile mutants *mes-2*, *-3*, *-4* and *-6*, which are involved in silencing of the X-chromosome, result in a grandchildless phenotype. That is, the first generation of homozygous mutants are fertile but produce sterile progeny due to the necrotic cell death of germ cells during larval development (Fong et al., 2002; Garvin et al., 1998). Mutations in the genes *fem-1*, *-2* , *-3* and *tra-3*, which are required for masculinization of the soma and germline in males and of the germline in hermaphrodites, also display maternal-effects (Hodgkin, 1986; Hodgkin and Brenner, 1977), as do mutations in *age-1*, which encodes a component of the insulin-like signaling pathway and affects stress resistance, dauer formation, and

lifespan (Friedman and Johnson, 1988; Morris et al., 1996). These and other maternal-effect mutants raise some interesting questions. For example, if a mutation can be fully rescued zygotically, why does the mother contribute gene product?

In order to identify new classes of maternal-effect mutations, Hekimi et al systematically screened for maternal-effect mutations that were not required for viability (Hekimi et al., 1995). In this screen 41 maternal-effect mutations in 24 different genes were identified. These mutations fell into a number of different phenotypic classes, such as Maternal-effect Uncoordinated (Mau), Maternal-effect Morphologically Abnormal (Mal), Maternal-effect Uncoordinated and Morphologically abnormal (Mum), Maternal-effect Dumpy (Mad), Maternal-effect Uncoordinated and Dumpy (Mud), and abnormal function of biological Clocks (Clk). With few exceptions all mutations were non-strictly maternal, that is, they could be both maternally and zygotically rescued.

The *clk* mutants

The Clk phenotype is characterized by an alteration in the timing of developmental and behavioural features (Branicky et al., 2000; Hekimi et al., 1995; Wong et al., 1995). *clk* mutants display an average lengthening of the durations of embryonic development and post-embryonic development, an average slowing of the swimming, pharyngeal pumping and defecation behaviours, an increased lifespan and a profound maternal effect. In the initial viable maternal-effect screen, mutations in three *clk* genes, *clk-1*, *-2*, and *-3*, were identified (Hekimi et al., 1995). Based on the similarity in phenotype, *gro-1,* a gene identified by other means (Hodgkin and Doniach, 1997), is also considered to be a Clk mutant (Lakowski and Hekimi, 1996). Recently, Meng repeated the viable maternal-effect screen and identified seven additional *clk* mutants (*clk-4* to *clk-10*) (Meng, 2000). Interestingly, although an increased lifespan was not part of the screening criteria for either of the maternal-effect screens, all *clk* mutants more or

less display this phenotype. This suggests that maternal-effect mutations that produce slow rates of behaviour and development will also be accompanied by, or possibly even lead to, an increased lifespan.

An initial question was whether all *clk* genes have similar cellular functions. The detailed phenotypic and molecular analyses of four of the *clk* genes (*clk-2*, *gro-1*, *clk-8* and *clk-1*) suggests that this is not the case, but rather that, disruption of very different processes can produce a Clk phenotype. However, it is not presently clear for any of the mutants why the disruption of its respective function leads to a Clk phenotype. Thus, it is possible that, at some level, all *clk* mutants affect a common process, which ultimately produces the Clk phenotype (see Chapter 7: Discussion).

clk-2

The *clk-2* gene is defined by two temperature-sensitive alleles, *qm37,* which was identified by Hekimi et al (Hekimi et al., 1995), and *mn159*, which was isolated by Hartman in a screen for radiation sensitive mutants and originally defined the *rad-5* gene, and was later found to be allelic to *clk-2* (Ahmed et al., 2001; Hartman and Herman, 1982). At permissive temperatures, the mutants are viable and display the typical Clk phenotype, however at the restrictive temperature, they display embryonic lethality. Embryonic development strictly requires the activity of maternal *clk-2(+)* during a narrow time window before the 4-cell stage, and likely relies on maternally deposited *clk-2* transcript (Benard et al., 2001). Thus, although the mutants can display a viable maternal-effect phenotype, *clk-2* is actually an essential gene. Consistent with this, *clk-2(RNAi)* also leads to embryonic lethality at all temperatures (Benard et al., 2001).

clk-2 is homologous to *TEL2,* an *Saccharomyces cerevisiae* gene that regulates telomere length and participates in silencing at subtelomeric regions (Benard et al., 2001; Runge and Zakian, 1996). *clk-2* has also been shown to affect telomere length in worms, and could possibly also alter gene expression, although this has not been tested directly (Benard

et al., 2001). In addition, *clk-2* appears to act in some sort of cell cycle checkpoint in the germline that is required for responding appropriately to damage (Ahmed et al., 2001). It is not presently clear whether *clk-2* is involved in a specific DNA damage checkpoint or in responding to cellular damage more generally (Benard and Hekimi, 2002). The human orthologue, *hclk2*, appears to be involved in similar processes, namely telomere length regulation and responding to cellular damages (Jiang et al., 2003).

gro-1

gro-1 mutants display the typical Clk phenotype, that is, slow development, slow behaviours, an increased lifespan (although the effect is only moderate) and a maternal effect (Lakowski and Hekimi, 1996; Lemieux et al., 2001). Most features can be rescued equally zygotically and maternally, however wild-type rates of embryogenesis strictly require maternal *gro-1(+)* (Lemieux et al., 2001). *gro-1* encodes a highly conserved enzyme, isopentenylpyrophosphate:tRNA transferase (IPT). This enzyme catalyzes the transfer of an isopentenyl moiety to the adenosine adjacent to the 3' end of the anticodon of tRNAs whose anticodons terminate in U. Generally, tRNA modifications act to maintain the correct reading frame of the translational machinery, and thus to maintain the fidelity and efficiency of protein translation (Bjork et al., 1999; Persson, 1993; Urbonavicius et al., 2001).

This enzyme has been shown to affect various biological features in different organisms. The *Escherichia coli* orthologue *miaA* has been shown to affect growth rate and also the rate of GC to TA spontaneous transversions (Connolly and Winkler, 1989; Diaz et al., 1987). The activity of the *S. cerevisiae* orthologue MOD5 has been shown to affect the efficiency of suppression of nonsense mutations (Dihanich et al., 1987). Recently the human orthologue TRIT1 has been suggested to be a candidate tumor suppressor in human lung cancer, which, by maintaning

translational fidelity and efficiency, may negatively regulate carcinogenesis (Spinola et al., 2005).

Similar to the situation with the yeast orthologue *MOD5,* the *gro-1* transcript contains two in frame ATG codons; initiation at the first results in a protein that localizes to the mitochondria, whereas initiation at the second results in cytoplasmic and nuclear localization (Lemieux et al., 2001; Najarian et al., 1987). Only the mitochondrially-localized form can rescue the *gro-1* mutant phenotypes, suggesting that, like Mod5p, GRO-1 exists in two forms, one of which is required for the modification of mitochondrial tRNAs (Lemieux et al., 2001). However, and although it is the basis of the phenotype, the lack of *gro-1(+)* activity in the mitochondria must result in a subtle defect as the mutant does not appear to have measurably impaired mitochondrial function. That is, the mutant appears to have essentially wild-type respiration levels, ATP levels and metabolic capacities (Braeckman et al., 2002; Braeckman et al., 1999).

clk-8/tpk-1

clk-8 was isolated in the screen by Meng (Meng, 2000). The phenotype is qualitatively the same as the other *clk* mutants, but, as compared to the original 4 *clk* mutants, the developmental rates are less affected in *clk-8* mutants, while the behavioural rates, particularly that of pharyngeal pumping, are more severely affected. The *clk-8* mutants also exhibit a greater lifespan increase (de Jong et al., 2004; Meng, 2000). As for the other *clk* mutants, there is also a maternal effect, but this has not been studied in detail.

clk-8 encodes thiamine pyrophosphokinase (TPK), and has thus been renamed *tpk-1* (de Jong et al., 2004). TPK is required for the conversion of thiamine (Vitamin B1) into the biologically active form, thiamine pyrophosphate (TPP). TPP is an essential co-factor for more than 24 enzymes, including pyruvate dehydrogenase, which is required for ATP production via the Krebs cycle and enzymes involved in lipid and

carbohydrate catabolism, and which participate in the biosynthesis of cellular constituents including neurotransmitters, reducing equivalents used in oxidant stress defenses and pentoses that are used for nucleic acid synthesis. Animals cannot synthesize thiamine but extract it from their food. In humans, thiamine deficiencies can lead to cardiovascular, musculature and most prominently, neurological defects (Haas, 1988; Singleton and Martin, 2001). Worms also appear to require thiamine from their bacterial food source. Wild-type worms grown on thiamine deficient bacteria (*thiE*$^-$) display severe *tpk-1*-like phenotypes and sterility in the second generation; the *tpk-1* mutants are hypersensitive to the *thiE*$^-$ bacteria as they become sick and lay only dead eggs in the first generation. Moreover, the *tpk-1* mutants can be rescued by exogenous TPP but not thiamine, consistent with their phenotype being due to the lack of TPP (de Jong et al., 2004).

It is presently not clear how the absence of *tpk-1* leads to all of the phenotypes. Oxygen consumption appears to be intact in the mutant, suggesting that the overall phenotype is not mediated by decreased electron transport or ATP synthesis (de Jong et al., 2004). However de Jong *et al.* showed that the basis of the slow pharyngeal pumping is the frequent failure of the excitatory postsynaptic potential produced by the pacemaker neuron MC to trigger muscle contractions. This defect could be due to decreased neurotransmitter release or decreased muscle excitability, both of which are consistent with the defects caused by thiamine deficiencies in humans.

clk-1

Of all the *clk* mutants, *clk-1* has been studied in greatest detail. Mutations in *clk-1* affect the timing of numerous traits, including embryonic and post-embryonic development, cell cycles in early embryos, egg production rate, life span, and the mean cycle lengths of the defecation, pharyngeal pumping and swimming rhythmic behaviours. For almost all phenotypes,

the missense mutant *clk-1(e2519)* has a weaker phenotype than the null mutant *clk-1(qm30)* (Wong et al., 1995).

clk-1 as a regulator of timing

For a number of reasons, it is unlikely that the average slowdown observed in *clk-1* mutants is the non-specific result of an overall sickness. This is likely true for all the *clk* mutants, but especially for *clk-1*, which, unlike the others, does not appear anatomically sickly or abnormal in any way. In fact, *clk-1* mutants appear healthy, attain normal body size and colouration, are very active, exhibit the full repertoire of normal behaviours, display neither embryonic nor post-embryonic lethality, and live longer than the wild type. Rather, several properties of *clk-1* mutants suggest that the average slowdown is the result of an inability to appropriately set the rate of physiological processes. Firstly, the slowdown observed in *clk-1* mutants, of both developmental and behavioural features, is often accompanied by an increase in variability, both within and among animals (Wong et al., 1995). This is well illustrated by the defecation cycle: the mean cycle length of wild-type animals is 50.8 seconds with a standard deviation of 5.6 seconds, while *clk-1(qm30)* mutants have a mean cycle length of 92.4 seconds with a standard deviation of 15.0 seconds. Moreover, at least for embryonic development, timing appears to be completely deregulated in the mutants (Wong et al., 1995). Wild-type animals have a normal distribution of developmental times, centered around a single modal value. In contrast, the distribution of missense mutant *clk-1(e2519)* animals is broad, and there is no defined distribution at all in *clk-1(qm30)* null mutants. In fact, the rate of embryogenesis appears to be randomized over a 24-hour range, with a small percentage of *clk-1* embryos developing even faster than the wild type.

Consistent with a role for *clk-1* in setting the rates of various features, overexpression of *clk-1(+)* can accelerate the defecation, pharyngeal pumping and aging rates. Between day 1 and day 4 of adult life, there is a dramatic increase in the defecation cycle length of wild-type

animals, but a much lesser increase in *clk-1* mutants. Expression of a CLK-1::GFP fusion protein fully prevents this slowdown in a large subset of transgenic animals. Indeed, 24% of transgenic animals on day 4 are as fast as they were on day 1, while less than 3% of the wild-type animals remain fast on day 4 (Felkai et al., 1999). In addition. overexpression of *clk-1(+)* using a heat shock promoter can rescue the slow pumping rate of *clk-1* mutants, and causes a significant percentage of animals to pump even faster than the wild type (Burgess, 2002). Finally, overexpression of *clk-1(+)* leads to a significantly reduced life span. The short-lived transgenic animals do not appear to be sick or otherwise different from the wild type (except for defecation on day 4) suggesting that the decrease in life span is the result of an increased rate of aging, and not the result of a novel, life-shortening pathology. Moreover, introduction of an *e2519::gfp* transgene, which expresses a mutant form of the *clk-1* gene, had no effect on life span, which strongly suggests that overexpression is not deleterious *per se*, but that overexpression of functional *clk-1* gene product accelerates aging.

clk-1 mutants cannot adjust to changes in temperature

In addition, *clk-1* mutant embryos cannot adjust to changes in temperature (Wong et al., 1995). For both the wild-type and *clk-1* mutants, the duration of embryonic development is dependent upon temperature. However, *clk-1* mutants develop, on average, more slowly than the wild type at all temperatures and are more strongly affected by temperature than are wild-type embryos. A striking difference between the wild-type and *clk-1* mutants is their reaction to changes in temperature. Two-celled embryos were dissected from animals raised at 15°C, 20°C, and 25°C, and were then all transferred to 20°C, at which temperature the length of embryogenesis was measured. For wild-type embryos, the shift to 20°C resulted in a length of embryogenesis typical of 20°C, regardless of the temperature experienced before the embryo was shifted. In contrast, the

length of embryonic development of the *clk-1* embryos at 20°C depended on the temperature experienced before the shift. For example, *clk-1(qm30)* embryos originating from animals raised at 15°C, develop slower at 20°C than embryos which originate from animals raised at 20°C. Even more dramatically, embryos originating from animals raised at 25°C are able to develop faster at 20°C than embryos that originated from animals raised at 20°C. Indeed, at 20°C, these embryos develop as fast as if they were still at 25°C. These results indicate that, unlike the wild type, *clk-1* embryos have difficulty adjusting to changes in temperature. This suggests that in poikilothermic animals, the adaptation of developmental rates to rapid changes in temperature is an active process rather than only the passive result of an overall speed-up or slow-down of biochemical reactions. It implies the existence of cellular mechanisms that sense changes in temperature and rapidly translate that information into a developmental rate that is optimal at that temperature. Given that *clk-1* mutants cannot properly adjust to changes in temperature, the wild-type activity of *clk-1* could be involved in such a process. Possibly there are other phenotypes that require *clk-1(+)* in order to respond to changes in temperature or other environmental conditions that require *clk-1(+)* in order to be translated into the appropriate physiological response.

There is a profound maternal rescue of all *clk-1* phenotypes

Given the nature of the features affected in *clk-1* mutants, it is likely that *clk-1* is required throughout the lifetime of the worm. Developmental and behavioural rates depend on environmental conditions, and need therefore to be readjusted throughout the animal's life. Moreover, Northern analysis of wild-type worms reveals the presence of *clk-1* transcripts at all stages of development (J.-C. Labbé and Hekimi, unpublished result), also implying a continuous requirement for *clk-1*. Surprisingly, all phenotypes, including those measured in adults, like behaviour and life span, are rescued when

homozygous mutants originate from a heterozygous mother that carried a wild-type copy of the gene (Burgess et al., 2003).

One possible explanation for the maternal rescue is that *clk-1* product is required throughout development, but only in small amounts, and that the maternally provided product lasts until adulthood. Indeed, we have has recently shown that maternally-rescued *clk-1* embryos, that is homozygous mutant embryos derived from a heterozygous mother, contain a small amount of maternally derived CLK-1 protein, which persists throughout development and appears to be sufficient to confer an essentially wild-type phenotype (Burgess et al., 2003). Only by developmentally arresting these embryos for a number of days is the maternally-derived protein depleted, and the Clk-1 mutant phenotypes expressed.

clk-1 is required for ubiquinone biosynthesis

clk-1 encodes a mitochondrial protein that is highly conserved, structurally and functionally, among eukaryotes (Ewbank et al., 1997; Jonassen et al., 1996; Marbois and Clarke, 1996; Vajo et al., 1999). CLK-1 is a hydroxylase (Stenmark et al., 2001) that is required for the hydroxylation of 5-demethoxyubiquinone to 5-hydroxyubiquinone, which is converted by another enzyme, COQ-3, into ubiquinone (UQ, also called coenzyme Q, CoQ). UQ is a prenylated benzoquinone lipid that is involved in numerous reactions in the cell (see below), including as a transporter of electrons between complexes I and II to III of the mitochondrial respiratory chain. In the absence of the CLK-1 protein, worms and mice are devoid of UQ (Jonassen et al., 2001; Levavasseur et al., 2001; Miyadera et al., 2001a) and instead accumulate the UQ precursor, demethoxyubiquinone (DMQ) (Levavasseur et al., 2001; Miyadera et al., 2001a). However, respiration is only slightly affected in *clk-1* mutants (Braeckman et al., 2002; Braeckman et al., 1999; Felkai et al., 1999; Levavasseur et al., 2001; Miyadera et al., 2001b), suggesting that DMQ can partially substitute for UQ in vivo. In addition, in vitro studies have shown that DMQ can function as an electron

transporter in the mitochondrial electron transport chain of worms, although it is less efficient at accepting electrons from complex II than from complex I (Miyadera et al., 2001b). Despite the fact that DMQ appears capable to function as an electron transporter, it cannot entirely substitute for UQ. *mclk1* (mouse *clk-1*) knockout mutants arrest development at midgestation (Levavasseur et al., 2001) and worm *clk-1* mutants require UQ from their bacterial food source in order to proceed through development to reproductive adulthood (Hihi et al., 2002; Jonassen et al., 2001). Indeed, *clk-1* mutants fed on mutant bacteria that are defective in ubiquinone biosynthesis (*ubiB*$^-$ or *ubiG*$^-$ mutants) arrest development, eventually growing up to become sterile adults (Burgess et al., 2003).

Ubiquinone has multiple roles

Ubiquinone is found in most, if not all, cellular membranes. It is involved in a number of reduction-oxidation (or redox) reactions in the cell. It can cycle between a reduced (UQH_2) state, and an oxidized state but can also exist in other, shorter-lived redox states. As described above, UQ is well-known for its role in the mitochondrial electron transport chain, where is acts as an electron shuttle. UQ is reduced at complex I (NADH-UQ reductase) and complex II (succinate-UQ reductase) and then transfers its electrons to complex III (ubiquinol-cyctochrome c reductase), which regenerates oxidized UQ. UQ is also believed to act as an electron transporter at the plasma membrane (Santos-Ocana et al., 1998b; Sun et al., 1992), as well as in lysosomes (Gille and Nohl, 2000). It has been demonstrated that plasma membrane electron transport is involved in the stabilization of ascorbate (see below), however the function of the lysosomal system is currently unclear.

UQ also acts as a co-factor for a number of other enzymes and cellular processes. For example, it has been shown to be required for the function of uncoupling proteins (UCP) (Echtay et al., 2001; Echtay et al., 2000). The UCPs are inner-mitochondrial membrane proteins that translocate protons into the mitochondria, thereby disrupting the proton

gradient across the membrane and uncoupling oxidative phosphorylation from ATP production, resulting in the production of heat rather than energy. UQ has also been show to be required for the regulation of the mitochondrial permeability transition pore (PTP) (Fontaine et al., 1998). The PTP is an inner-mitochondrial complex that regulates the permeability of the membrane to ions and solutes. PTP opening leads to the collapse of the proton-motive force, disruption of ionic homeostasis, mitochondrial swelling, and massive ATP hydrolysis. It is believed to be a key early event in apoptosis and necrotic cell death (Kim et al., 2003; Zamzami and Kroemer, 2001), but also may act more generally in mitochondrial calcium signaling (Ichas et al., 1994). UQ has also been shown to be a co-factor for other enzymes, such as dihydroorotate dehydrogenase (Hines et al., 1986). This enzyme is involved in the *de novo* biosynthesis of pyrimidine nucleotides, which is a redox reaction that is linked to mitochondrial electron transport.

UQ has also been shown to have some interesting functions in *E. coli*. The Arc two-component system comprising the ArcB sensor kinase and the ArcA response regulator, modulates gene expression in response to redox conditions of growth (Lin and Luchi, 1991). Under aerobic conditions, quinones, including UQ become oxidized which serves as a signal of the physiological redox state to ArcB (Georgellis et al., 2001). In addition, it has been shown that UQ is required for disulfide bond formation, an oxidative reaction that is critical for the structure and stability of some proteins. Ubiquinone is a direct electron acceptor of DsbB, a protein required for *de novo* disulfide bond formation; DsbB is activated by reducing UQ (Bader et al., 1999; Bader et al., 2000).

Intriguingly, UQ acts as both an antioxidant and a prooxidant; that is, it acts to both scavenge free radicals (or reactive oxygen species, ROS) and, in some redox states, promotes ROS production. In its reduced form (UQH_2), it is a very effective antioxidant, whose major role is in the prevention of the initiation and/or propagation of lipid peroxidation. Lipid

peroxidation is a chain reaction that involves the reaction of free radicals with unsaturated fatty acids, which, upon reacting with oxygen and other free radicals, generate a lipid peroxide. The lipid peroxide itself is then capable of propagating the chain reaction. UQH_2 is capable of reducing fatty acid radicals directly, to prevent lipid peroxidation in cellular membranes (Ernster and Forsmark-Andree, 1993; Forsmark et al., 1991; Poon et al., 1997) and in lipoprotein particles (Alleva et al., 1995; Ingold et al., 1993; Stocker et al., 1991). It also acts indirectly to prevent lipid peroxidation by regenerating antioxidants such as tocopherol (vitamin E) (Mukai et al., 1990) and ascorbic acid (vitamin C) (Santos-Ocana et al., 1998a).

In addition to acting as an antioxidant, the reactions in which UQ participates in the mitochondrial respiratory chain are likely the major sources of ROS production in the cell (Turrens, 1997). UQ and UQH_2 can be converted into the semiquinone radical (UQH·), which is a prooxidant. Ubisemiquinone can react with molecular oxygen to form the superoxide radical ($\cdot OH_2$). Superoxide molecules are highly toxic to proteins, especially those containing iron-sulfur clusters (Flint et al., 1993). Furthermore, the detoxification of superoxide by the superoxide dismutases, results in the production of hydrogen peroxide, from which the highly toxic hydroxyl radical (·OH) can be formed. These reactive oxygen species cause extensive oxidative damage to biological macromolecules, including DNA, proteins and lipids. However, it is also important to note that, in addition being toxic molecules, ROS are important signaling devices (Droge, 2002; Finkel, 2001) (and see Chapter 7: Discussion). Thus, altering redox states can have profound biological effects.

Mutants deficient in ubiquinone biosynthesis

UQ biosynthesis has been studied extensively in *E. coli* and *S. cerevisiae*. Nine genes specifically required for ubiquinone biosynthesis have been identified in both bacteria and yeast (reviewed in (Meganathan,

2001),(Johnson et al., 2005)). In bacteria these genes are called *ubi* genes (*ubiA* to *ubiH* and *ubiX*) and in yeast they are called *coq* genes (*coq1* to *coq9*). The corresponding genes have also been named *coq* genes in worms, (*coq-1* to *coq-8*; no *coq9* homologue has yet been identified) and in mammals (only counterparts to *coq3* and *coq7* have been identified). *clk-1* is homologous to yeast *coq7* and carries out the same enzymatic step as *ubiF* in *E. coli*, which, based on sequence comparison, lack a *clk-1* homologue, (Ewbank et al., 1997; Jonassen et al., 1996; Stenmark et al., 2001).

Mutants that are defective in ubiquinone biosynthesis in both bacteria and yeast cannot respire. Thus, they are unable to grow on non-fermentable carbon sources such as succinate while maintaining their ability to grown normally on fermentable carbon sources such as glucose (reviewed in (Meganathan, 2001)). Ubiquinone deficiencies also produce pleiotropic phenotypes; some are due to the lack of respiration while others are specific to the lack of UQ, and are likely due to alterations in ROS metabolism. For example, *E. coli ubi-* mutants display resistance to heat and some antibiotics, as well as sensitivity to visible light, and to oxidative stress induced by H_20_2, Cu^{2+}, and paraquat (reviewed in (Soballe and Poole, 1999); (Soballe and Poole, 2000)). *S. cerevisiae coq* mutants have been shown to be sensitive to polyunsaturated fatty acids, which are prone to autooxidation (Do et al., 1996; Poon et al., 1997). Moreover, ubiquinone deficient *Schizosaccharamyces pombe* mutants are hypersensitive to oxidative stress produced by H_20_2 as well as Cu^{2+}, and require antioxidants such as α-tocopherol or glutathione for growth on minimal carbon sources (Saiki et al., 2003; Uchida et al., 2000).

There are relatively few ubiquinone-deficient animal models. However, given that animals are not facultative anaerobes, animal mutants that are completely devoid of ubiquinone would be expected to be inviable. In *C. elegans*, mutants exist for *clk-1*, *coq-3* and *coq-4*. As described in detail above, *clk-1* mutants exhibit a very pleiotropic

phenotype that includes slow growth, behaviours and aging when grown on UQ-replete bacteria, but are inviable on UQ-deficient bacteria. In contrast, both *coq-3* and *coq-4* mutants are inviable, even when grown on UQ-replete bacteria (Han, 2001; Hihi et al., 2002). For *coq-3*, this is presumably because the COQ-3 enzyme is a methyltransferase that acts at two steps of the UQ biosynthetic pathway, one of which is earlier than the step at which CLK-1 acts (Poon et al., 1999). Thus, although it has not been established which UQ intermediate the *coq-3* mutants accumulate, they would not be able to produce DMQ. This suggests that, at least in worms, DMQ can partially substitute for endogenous UQ, and that exogenously provided UQ cannot entirely substitute for endogenously synthesized UQ. The *coq-4* situation is likely similar to that of *coq-3*, although the exact biochemical function of COQ-4 as well as the enzymatic steps at which it acts are not presently known, in particular because there is no homologue in *E. coli* (Belogrudov et al., 2001; Marbois et al., 2005). The intermediate that the *coq-4* mutants accumulate has also not been determined.

In mice, the only UQ-deficient mutants that exist are the *mclk1* knockout mice. As described above, these mutants are inviable, arresting development at midgestation (Levavasseur et al., 2001). However, ES cells derived from these mice exhibit several phenotypes indicative of altered ROS metabolism, including lowered levels of ROS, resistance to the ROS-generating compound menadione and reduced levels of oxidized lipids and damaged DNA. It has recently been shown that mice that are heterozygous for the *mclk1* knockout allele, although grossly normal by several measures, exhibit a dramatically increased lifespan (Liu et al., 2005). Given the phenotypes of the ES cells and the link between ROS levels and lifespan in several systems, this could be due to lowered levels of ROS in the heterozygotes. Taken together with the bacteria and yeast data, and the phenotype of *clk-1*, this suggests that altering ubiquinone levels is likely to alter redox physiology, but given the multiple roles of

ubiquinone, including as a pro-oxidant and as an antioxidant, it is not clear in which direction the levels will be shifted, and what other pleiotropic effects will be produced.

Rationale and objectives of the thesis

At present, it is not clear how the absence of endogenous UQ production relates to the Clk-1 mutant phenotypes. DMQ can partially replace UQ in the electron transport chain and *clk-1* mutant mitochondria contain some bacterially-derived UQ (Jonassen et al., 2003; Jonassen et al., 2002). Possibly, neither DMQ nor bacterially-derived UQ can replace endogenously synthesized UQ for some of its other numerous functions, whose impairment is responsible for some aspects of the phenotype. Alternatively, it could be that some aspects are not due to the absence of UQ, but rather to the presence of DMQ. DMQ is generally not present in significant quantities in the wild type and it has different redox properties than UQ (Miyadera et al., 2002). A change in balance between UQ and DMQ could result in an alteration of redox state, which could contribute to some of the mutant phenotypes. Finally, it remains possible that *clk-1* has other functions, in addition to the biosynthesis of ubiquinone. This is suggested by the observation that *clk-1(e2519)* mutants have a weaker phenotype than *clk-1(qm30)* mutants, yet neither produce any UQ. However, unlike *clk-1(qm30)* which is null, the *clk-1(e2519)* mutant produces a full length protein, which could retain some activity (Hihi et al., 2003).

One way to address these questions is to identify gene activities that are necessary for *clk-1* mutations to affect particular features, or in other words, to identify mutations that can suppress some of the Clk-1 phenotypes. The goal of this thesis has been to identify mutations that suppress specific aspects of the Clk-1 phenotype, with the hope that their study will allow us to understand the function of CLK-1 and the phenotypic consequences of losing of *clk-1(+)* activity.

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Chapter 2:
The Screen for Defecation Suppressors of *clk-1* (*dsc* mutants)

Phenotypic and suppressor analysis of defecation in *clk-1* mutants reveals that reaction to changes in temperature is an active process in *Caenorhabditis elegans*

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Abstract

Mutations in the *Caenorhabditis elegans* maternal-effect gene *clk-1* affect cellular, developmental, and behavioral timing. They result in a slowing of the cell cycle, embryonic and post-embryonic development, reproduction and aging, as well as of the defecation, swimming, and pharyngeal pumping cycles. Here, we analyze the defecation behavior in *clk-1* mutants, phenotypically and genetically. When wild-type worms are grown at 20° and shifted to a new temperature, the defecation cycle length is significantly affected by that new temperature. In contrast, we find that when *clk-1* mutants are shifted, the defecation cycle length is unaffected by that new temperature. We carried out a screen for mutations that suppress the slow defecation phenotype at 20° and identified two distinct classes of genes, which we call *dsc* for defecation suppressor of *clk-1*. Mutations in one class also restore the ability to react normally to changes in temperature, while mutations in the other class do not. Together, these results suggest that *clk-1* is necessary for readjusting the defecation cycle length in response to changes in temperature. On the other hand, in the absence of *clk-1* activity, we observe temperature compensation, a mechanism that maintains a constant defecation period in the face of changes in temperature.

Introduction

Mutations in the *Caenorhabditis elegans* gene *clk-1* are highly pleiotropic, affecting the rates of physiological traits that occur over a wide range of timescales (WONG *et al.* 1995). They result in a mean lengthening of the cell cycle of early embryos, embryonic and post-embryonic development, as well as the defecation, swimming, and pharyngeal pumping cycles of adults. *clk-1* mutations also affect reproductive features, like the egg-production rate and self-brood size, which are both reduced, and lead to an increased life span.

A number of observations suggest that the phenotypes of *clk-1* mutants are the result of an inability to appropriately set the rate of physiological processes (BRANICKY *et al.* 2000; WONG *et al.* 1995). One example is that many of the features affected by *clk-1* mutations are more variable, in addition to being slower on average. For instance, although the average length of embryogenesis of *clk-1* mutants is slower than that of the wild type, some *clk-1* embryos can develop faster than wild-type embryos, while others take more than two times longer, which suggests that timing is deregulated in the mutants (WONG *et al.* 1995). Also, we have observed that *clk-1* mutant embryos are unable to properly adjust their rate of development in response to changes in temperature. Briefly, when wild-type embryos are cultured to the 2-cell stage at a particular temperature, and are then transferred to a new temperature, they immediately develop at a rate corresponding to that new temperature. In contrast, when *clk-1* mutant embryos are transferred to a new temperature, the rate of development at the new temperature is strongly influenced by the temperature experienced before the shift (WONG *et al.* 1995). This suggests that *clk-1* might also be needed to re-set physiological rates in response to changes in temperature. Finally, all of the phenotypes affected in *clk-1* mutants can be maternally rescued, that is, homozygous mutant progeny issued from a heterozygous hermaphrodite are phenotypically wild-type. This maternal rescue extends

to adulthood, such that all adult behaviors, and even the long life of *clk-1* mutants are rescued (HEKIMI *et al.* 1995; WONG *et al.* 1995). We have interpreted this to mean that *clk-1* affects a regulatory process that is somehow involved in setting physiological rates in the worm (BRANICKY *et al.* 2000; FELKAI *et al.* 1999; WONG *et al.* 1995). In the presence of maternally supplied *clk-1* product, timing can be set appropriately early in development so that homozygous *clk-1* mutants can subsequently develop and behave like the wild type.

clk-1 encodes a mitochondrial protein that is highly conserved, structurally and functionally, among eukaryotes (EWBANK *et al.* 1997; JONASSEN *et al.* 1996; PROFT *et al.* 1995; VAJO *et al.* 1999), and encodes a putative hydroxylase (STENMARK *et al.* 2001) that is required for the biosynthesis of ubiquinone, (UQ, also called coenzyme Q, CoQ), a prenylated benzoquinone lipid that functions as a transporter of electrons in complexes II and III of the respiratory chain. Mutants of the yeast homologue of *clk-1*, *coq7,* do not produce UQ, and therefore cannot grow on non-fermentable carbon sources (MARBOIS and CLARKE 1996). Mitochondria isolated from *clk-1* mutants also do not contain detectable levels of UQ but instead accumulate the UQ biosynthetic intermediate, demethoxyubiquinone (DMQ) (MIYADERA *et al.* 2001). In *clk-1* mutants this compound functions as an electron carrier, such that the mitochondria can maintain respiration despite the complete absence of UQ (FELKAI *et al.* 1999; MIYADERA *et al.* 2001). However, DMQ cannot entirely substitute for UQ as *clk-1* mutants cannot complete development when they are fed *E. coli* strains that do not produce UQ (JONASSEN *et al.* 2001).

It is, however, not clear how the absence of UQ relates to the other mutant phenotypes as there is no correlation between this biochemical phenotype and the severity of the overall phenotype. Indeed, the quinone phenotype is identical for all three known *clk-1* alleles (*e2519, qm30* and *qm51*): UQ is totally absent from mitochondria in all three cases, and all three accumulate the same amount of DMQ. Yet, most of the features

affected in *clk-1* mutants are slowed down much more severely in the putative null alleles *qm30* and *qm51*, than they are in the partial loss of function allele *e2519* (Felkai et al. 1999; Wong et al. 1995). Also, by various measures of energy metabolism in intact worms, *clk-1* mutants have been shown to have metabolic capacities and ATP levels comparable to the wild type (Braeckman et al. 1999). Together, these observations suggest that much of the phenotype of *clk-1* mutants may not be the direct consequence of an absence of UQ in mitochondria or a decreased level of energy production.

One of the features affected in *clk-1* mutants is the defecation cycle. In *C. elegans*, defecation is effected by a stereotyped Defecation Motor Program (DMP). The DMP consists of three distinct steps: the posterior body muscle contraction (pBoc), the anterior body muscle contraction (aBoc), and the expulsion (Exp), which consists of the enteric muscle contractions (EMC) (THOMAS 1990). In the presence of adequate food, the defecation cycle period of 56 seconds is regular in single animals over time and among animals, with a standard deviation of only a few seconds. In addition to its tight periodicity, the defecation cycle has other properties that suggest that it might be controlled by an endogenous "clock". For example, the phase of the cycle can be reset by lightly touching the animal, and the rhythm is maintained even in the absence of expression of the DMP (LIU and THOMAS 1994).

The periodicity of the defecation cycle can be altered by mutations in at least 13 genes (Dec phenotype). These mutations fall into two major classes: short Dec (Dec-s), for mutations that decrease the cycle length, and long Dec (Dec-L), for mutations that increase the cycle length (IWASAKI *et al.* 1995). The molecular identification of the Dec-L gene, *dec-4* (*lef-1/itr-1*), as the inositol triphosphate receptor (IP3 receptor), a protein involved in regulating intracellular calcium levels, suggests that calcium oscillations contribute to the regulation of the rhythm. Indeed, DAL SANTO *et al.* (1999) showed that calcium levels peak in the intestine just prior to the first

muscle contraction of the DMP, and that expression of the IP3 receptor in the intestine was sufficient for normal rhythm generation. Although it is not yet clear how the different Dec genes might be interacting to regulate the defecation cycle, the molecular characterization of two other genes, *flr-1* and *unc-43/dec-8*, also support roles for calcium and the intestine in rhythm regulation. *flr-1* mutants, originally identified on the basis of their resistance to fluoride (KATSURA *et al.* 1994), have, among other defecation phenotypes, a very short defecation cycle length (IWASAKI *et al.* 1995). *flr-1* encodes an ion channel of the degenerin/epithelial sodium channel superfamily, which is expressed only in the intestine from embryos to adults (TAKE-UCHI *et al.* 1998). Mutations in *unc-43* result in multiple behavioral defects including defecation phenotypes (LIU and THOMAS 1994; REINER *et al.* 1999). Loss-of-function mutations result in an increased frequency of defecation, usually the result of a repetition of the DMP ~13 sec after the initiation of the primary motor program, whereas the gain of function mutation results in a decreased frequency of defecation. *unc-43* encodes the *C. elegans* CaM Kinase II, which is widely expressed in neurons, muscles, and the intestine (REINER *et al.* 1999).

Here, we have analyzed the defecation behavior of *clk-1* mutants, with particular emphasis on the response of the mutants to changes in temperature. We find that when wild-type worms are grown at 20° and then shifted to a new temperature, the defecation cycle length is significantly affected by that new temperature. In contrast, when *clk-1* mutants are shifted, the defecation cycle length is the same at the new temperature as it is at 20°. We reasoned that if *clk-1* is actively involved in regulating the timing of the defecation cycle it might work through other gene products. We carried out a screen for suppressor mutations at 20° and identified ten mutations, which correspond to seven different complementation groups. We find that the suppressor mutations fall into two distinct classes. In addition to suppressing the slow defecation at 20°, one class also suppresses the inability of *clk-1* mutants to adjust their

defecation cycle length after a temperature shift, while the other class does not. One general conclusion that can be drawn from this study is that adjusting the rate of defecation in response to changes in temperature is an active process in *C. elegans,* which requires *clk-1*.

Materials and Methods

General methods and strains: Most strains were derived from the wild-type *C. elegans* N2 Bristol strain and were cultured as described (BRENNER 1974). The wild-type RW7000 strain was used for some of the linkage analyses, using sequence-tagged sites (STS). All animals were grown at 20° unless otherwise indicated. The genes, alleles, and STSs used in this work are as follows:
LGI: *bli-4(e937)*, *stP124*; LGII: *rol-6(e187)*, *rol-1(e91)*, *unc-52(su250ts)*, *maP1*; LGIII: *daf-2(e1368)*, *dpy-17(e164)*, *clk-1(qm30)*, *unc-32(e189)*, *dec-7(sa296)*, *vab-7(e1562);* LGIV: *flr-3(ut9)*, *dpy-9(e12)*, *unc-33(e204)*, *unc-5(e53)*, *unc-31(e928)*, *dpy-4(e1166sd)*, *sP4;* LGV: *unc-34(e315)*, *dpy-11(e224)*, *stP192*; LGX: *lin-15(n765)*, *flr-4(ut7)*, *unc-3(e151)*, *lon-2(e678)*, *unc-2(e55)*, *stP103*.

Isolation of suppressor mutations: *clk-1(qm30)* animals were mutagenized with 25 mM ethyl methane sulfonate (EMS), as described (SULSTON and HODGKIN 1988). Groups of five mutagenized hermaphrodites (P0) were plated on 60-mm petri dishes and left to self-fertilize. Groups of 25 F1 animals were transferred to 90-mm plates as young adults and were left to lay eggs for ~24 hours. F2 animals were scored for one defecation cycle each at 20°. Based on the assumption that specific suppressor mutations would not cause morphological or other behavioral defects, only wild-type looking animals were scored. A maximum of 50 F2 animals were scored from each plate to minimize the

probability of scoring multiple worms carrying the same mutation. Animals that had a defecation cycle length of less than 65 seconds were picked to 60-mm plates, singled, and left to self-fertilize. The progeny (F3) of the singled candidate worms were scored for defecation; only those strains that had a significant proportion of fast defecating worms in the F3 generation were kept for further genetic and phenotypic analysis. A total of 5421 F2 animals were screened, a number equivalent to 2134 haploid genomes (ELLIS and HORVITZ 1991).

Mapping of suppressor mutations: Mutations were linked to chromosomes in one of three ways. For mutations that could be mapped on wild-type (*clk-1(+)*) backgrounds, linkage analysis was performed either by using the DA438 strain (for *qm133, qm141*) (AVERY 1993), or by using the wild-type RW7000 strain and testing linkage to STSs by PCR analysis (for *qm142, qm183*) as described (WILLIAMS 1995). For mutations that had less obvious phenotypes on wild-type backgrounds or that could not be linked to an STS, linkage to each chromosome was tested separately, using strains that contained visible marker mutations in the *clk-1(qm30)* background (for *qm178*, *qm179*, *qm180*, *qm182*, and *qm184*). Formal linkage analysis was not needed for *qm166,* as it was found to be tightly linked to *clk-1(qm30)*. Once linkage to a chromosome was established, mutations were mapped more precisely using 2- and 3-point mapping strategies.

To avoid marker effects, for most 3-point mapping experiments, homozygous recombinant progeny were isolated from F2 recombinant animals, and were crossed with homozygous mutant males. Defecation was scored in the F1 cross-progeny. Because *qm133* has other phenotypes that could be scored in addition to defecation cycle length, including a thin, starved appearance, all mapping of *qm133* was done using conventional methods (by singling recombinant F2 animals and scoring for the presence of ¼ *qm133* animals in the F3 generation). All

mapping of *qm133*, *qm141*, and *qm183* was done with strains in a *clk-1(+)* background and was scored at 20°; all mapping of *qm179*, *qm180*, *qm182*, and *qm184* was done with strains in a *clk-1(qm30)* background, and was scored at 25°. Mapping of *qm142* was also done in a *clk-1(qm30)* background, but was scored at 20°. As *qm142* has a dominant effect, it was mapped by crossing homozygous recombinant progeny with *clk-1(qm30)* males and scoring the F1 animals after 48hr after they had reached adulthood, when the dominant effect of *qm142* is strongest. The mapping data has been submitted to WormBase (http://www.wormbase.org/), and the critical data are summarized in Table 2.2.

Complementation tests: Complementation tests were performed when different mutations appeared to map to the same genetic region, or when a mutation mapped to a region where a Dec gene had previously been mapped. Generally, males homozygous for one mutation were mated to hermaphrodites homozygous for the other mutation, and defecation was scored in the trans-heterozygous F1 animals. In this manner it was found that: *qm166* and *qm178* fail to complement each other, and the previously identified mutant *dec-7(sa296)*, which are therefore all likely to be allelic. Similarly, *qm179*, *qm184* and *qm180* all fail to complement each other, and are therefore all likely to be allelic. *qm133* maps in the region of *flr-4*, but complements *flr-4(ut7)*, suggesting that they define distinct genes. Similarly, *qm182* maps in the region of *flr-3*, but complements *flr-3(ut9)*, suggesting that they define distinct genes.

Behavioral analyses: Defecation was scored in hermaphrodites on their first day of adulthood at 20°, unless otherwise indicated. The defecation cycle length was defined as the duration between the pBoc steps of two consecutive defecations. Each animal was scored for five consecutive cycles (six consecutive pBocs), and the mean and standard deviation was

calculated. To prevent the animals from being heated by the microscope lamp during the scoring session, the plates were placed on 'heat sinks' (petri dishes filled with water) and animals were only scored from them for a maximum of 15 minutes.

Temperature shift experiments: Animals were grown at 20° and transferred to either 15° or 25° as young adults. Animals were then scored at 15° or 25°, 2-6 hours after being transferred to that temperature. As it was difficult to maintain the temperature of the plates at 15°, animals were only scored for three consecutive cycles at this temperature, and plates were only kept on the microscope for as long as was required to score one animal.

***qm142* time course studies:** To generate heterozygous *dsc-2(qm142)/+* animals, *dsc-2(qm142) dpy-11(e224)* animals were mated with N2 males. To generate *qm30; qm142/+* animals, *clk-1(qm30); unc-5(e53)* hermaphrodites were mated with *clk-1(qm30); dsc-2(qm142)* males. Late L4 stage, F1 generation animals were picked to plates and examined 3 hours later. Animals that had molted to adults during this period were used for the experiment and were considered to be 1.5 hr old adults at the end of the interval. The sample size of each genotype for each time point is ~10. The same sets of animals were scored at the different time points.

Statistical analyses: We performed 2-sample Student's t-tests, taking into account the unequal variances of the samples. We tested whether N2 and *clk-1(qm30)* mutants were different when grown and scored at 15° and 25° than when they were grown and scored at 20°. Highly significant differences ($p<0.05$) were found for both N2 and *clk-1(qm30)* at 20° vs at 15°. Significant differences were not detected for N2 at 20° vs at 25° ($p=0.40$) or for *clk-1(qm30)* at 20° vs at 25° ($p=0.27$). We also tested whether N2 and *clk-1(qm30)* mutants were different when grown at 20°

and scored at 15° and 25° than when they were grown and scored at 20°. Highly significant differences ($p<0.001$) were found all comparisons except for *clk-1(qm30)* at 20° vs at 15° ($p=0.29$) and at 20° vs at 25° ($p=0.99$). We also tested whether each suppressor mutation had a significant effect on the defecation cycle by comparing, at every temperature, each *clk-1(qm30)* double mutant strain with *clk-1(qm30)*, and every *clk-1(+)* mutant strain with *clk-1(+)*. Highly significant differences (generally $p<0.001$) were found for all comparisons except: *clk-1(qm30) dec-7(qm166)* vs *clk-1(qm30)* at 15° ($p=0.34$) and at 25° ($p=0.97$); *clk-1(qm30) dec-7(qm178)* vs *clk-1(qm30)* at 15° ($p=0.83$) and at 25° ($p=0.15$); *clk-1(qm30); dsc-3(qm184)* vs *clk-1(qm30)* at 15° ($p=0.80$); *clk-1(qm30); dec(qm183)* vs *clk-1(qm30)* at 20° ($p=0.12$) and at 25° ($p=0.31$).

Results

***clk-1* mutants have a lengthened defecation cycle that is not affected by changes in temperature:** Defecation in *Caenorhabditis elegans* is achieved by the periodic activation of a stereotyped motor program. In wild-type animals, the defecation cycle length is 56 seconds, with a standard deviation of only 3.4 sec (at 20°). As previously described (FELKAI *et al.* 1999; WONG *et al.* 1995), in *clk-1* mutants the defecation cycle is both increased in length and more irregular: in *clk-1(qm30)* animals, the cycle length is 88 sec, with a standard deviation of 14 sec, and in the weaker allele *clk-1(e2519),* it is 77 sec, with a standard deviation of 7 sec (at 20°) (Figure 2.1, Table 2.1).

To examine the effect of temperature on the defecation cycle length of wild-type and *clk-1* mutant worms, we raised the worms for two generations at 15° or 25° and scored them at the temperature at which they had been raised (Figure 2.1A). We found that *clk-1* mutants are slower than the wild type at all temperatures and that both genotypes have

significantly longer cycles when grown and scored at 15° than when grown and scored at 20°. In contrast, when wild-type or mutant worms are grown and scored at 25° the defecation rates are not significantly different from those at 20°. These results agree with, and extend, those of IWASAKI *et al.* (1995).

To examine the effect of temperature shifts on the defecation cycle length of wild-type and *clk-1* mutant worms, we raised the worms at 20° and shifted them to 15° and to 25°, and scored them at that new temperature (Figure 2.1B). We found that when wild-type animals are shifted to either 15° or 25°, the defecation cycle length is significantly affected by the change in temperature. When the worms are transferred from 20° to 25°, the mean cycle length is decreased by 13 sec; when the worms are transferred from 20° to 15°, the mean cycle length is increased by 26 sec. This adjustment happens very rapidly. For example, we found that when wild-type worms are scored 5 minutes after they are transferred to 25°, they are already as fast as they are when scored 2-6 hours later (data not shown). Note that the defecation cycle of wild-type worms raised and scored at 25° (Figure 2.1A) is in fact slower than when the worms are raised at 20° and then shifted to 25° for scoring (Figure 2.1B). This suggests that the worms have become adapted or acclimated to the higher temperature during development and are thus less affected when scored at that temperature. However, whether the worms are raised at 15° or 20° does not appear to affect the length of the defecation cycle when it is scored at 15°.

In contrast to the observations with the wild type, we found that *clk-1* mutants are unable to re-adjust the length of their defecation cycle after they are shifted to a new temperature. When *clk-1* mutants are transferred from either 20° to 25° or from 20° to 15° there is no change in the mean cycle length (Figure 2.1B, Table 2.1). This suggests that after the mutants have become adapted to 20°, they cannot re-adjust the defecation cycle

length upon a temperature shift, indicating that *clk-1(+)* activity is required for this adjustment to occur.

A screen for suppressors of *clk-1*: In order to identify genes that interact with *clk-1* to regulate the defecation cycle length, we carried out a screen to isolate mutations that could suppress *clk-1*, that is, mutations that could restore the length of the defecation cycle of *clk-1* mutants to that of the wild type. *clk-1(qm30)* worms were mutagenized with EMS and second generation (F2) animals, were directly scored for one defecation cycle each at 20°. Animals that had a cycle length of less than 65 sec were kept for further analysis. In this manner, we screened 5421 F2 animals (an equivalent of ~2134 haploid genomes) and identified eight suppressor mutations, which correspond to five different complementation groups. Seven of these mutations are recessive and one mutation, *qm142*, has dominant effects (described in detail below). Based on the mapping and complementation tests we performed (see Materials and Methods and Table 2.2), it is likely that these mutations define four new complementation groups, which we have called *dsc* for defecation suppressor of *clk-1*. Two mutations, *qm166* and *qm178*, are alleles of the previously identified Dec-s gene *dec-7*. From our screen, we also isolated two other recessive mutations, *qm141* and *qm183*, which affect defecation, but which do not suppress *clk-1(qm30)* (discussed below). None of the mutations can suppress other *clk-1* phenotypes, such as slow growth or pharyngeal pumping (data not shown).

Analysis of suppressor mutants: We have analyzed the phenotype of these suppressor mutants in a number of different ways (Table 2.1). We re-isolated all of the mutations on a wild-type (*clk-1(+)*) background to determine how the mutations affect defecation in the presence of *clk-1(+)* activity. To see if the suppressors could also suppress the inability of *clk-1* mutants to react to changes in temperature, we scored every *clk-1(+)* and *clk-1(qm30)* strain at 20°, as well as after shifts to 15° and 25°. We also

re-isolated the mutations on the background of *clk-1(e2519)*, which has partial *clk-1* activity.

There are two classes of suppressors: By analyzing the effect of the mutations on the *clk-1(qm30)* background, we find that the suppressors fall into two distinct classes, based on their differential abilities to suppress *clk-1(qm30)* after temperature shifts, particularly to 25° (Figure 2.2). The Class I mutants *dsc-3(qm179, qm180, qm184)* and *dsc-4(qm182)* strongly suppress *clk-1(qm30)* at 20° as well as after shifts to 25° (Figure 2.2A and 2.3A). At 25°, *dsc-3(qm179),* the strongest mutant in this respect, can even shorten the defecation cycle length of *clk-1(qm30)* mutants to less than the wild type length. Thus, the profile of defecation in the Class I *dsc; clk-1(qm30)* double mutants at the different temperatures is just like that of the wild type: slowest at 15°, fastest at 25°. Therefore, these mutants can suppress the long defecation cycle of *clk-1(qm30)* mutants when grown and scored at 20°, as well as the temperature insensitivity when grown at 20° and then shifted to another temperature.

In contrast to Class I mutants, the Class II mutants suppress only weakly (*dsc-1(qm133)* and *dsc-2(qm142)*), or not at all (*dec-7(qm166, qm178)*), after a temperature shift. This suggests that although these mutants can suppress the long defecation cycle of *clk-1(qm30)* mutants at 20°, they cannot suppress the inability of *clk-1(qm30)* mutants to readjust their defecation cycle length after a temperature shift. *dec-7*, however, might be a special case, as the mutants' slow mean defecation cycle after shifts to 15° or 25° could be due to an inability to carry out the DMP, rather than to intrinsically slow cycling (see below).

The phenotypes of Class I and Class II mutants are much more similar on the wild-type background than on the *clk-1(qm30)* background, although the Class I mutants generally have weaker effects on the wild type than the Class II mutants, particularly at 15° and 20° (Figure 2.2B). All the mutations significantly decrease the length of the defecation cycle

at all temperatures and both the Class I and Class II mutants react to changes in temperature like the wild type (i.e. defecation is fastest at 25°, slowest at 15°). This indicates that the lack of effect of the Class II mutants at 25° on the *clk-1(qm30)* background cannot be due to any of the *dsc* mutations being intrinsically temperature sensitive. Overall, the observation that on the *clk-1(qm30)* background there are two distinct classes of interactions suggests that the genes of the different classes interact with *clk-1* in different ways.

The effect of the *dsc* mutations on the *clk-1(e2519)* background: We re-isolated the *dsc* mutations on the background of *clk-1(e2519)*, which has a weaker phenotype than *clk-1(qm30)*, to see if they could speed up *clk-1(e2519)* more than *clk-1(qm30)*. Although we did consistently find that the defecation cycle lengths of the *clk-1(e2519); dsc* double mutants was shorter than that of the *clk-1(qm30); dsc* double mutants, the difference was generally very small (Table 2.1; see below for a description of the special case of *dsc-5(qm141)*). The exact significance of this is unclear at present, however, it could suggest that *clk-1* also has effects on the defecation cycle that do not depend on the *dsc* genes. Possibly, part of the residual activity of *e2519,* which allows the mutants to have a faster defecation cycle than *qm30* mutants, acts in such a *dsc* gene-independent fashion.

Mutations that cannot suppress *clk-1(qm30)*: While screening for suppressors, we isolated a number of candidate strains from which we could not subsequently isolate mutations that strongly suppress *clk-1(qm30)*. In two cases, however, the candidate strains did segregate mutations that significantly affect the rate of defecation. One of these mutations, *dsc-5(qm141)*, has a strong effect on the defecation cycle of wild-type worms at all temperatures, as well as on *clk-1(e2519)* mutants, but only a very weak, but significant, effect on *clk-1(qm30)* mutants at all

temperatures. This suggests that *dsc-5(qm141)* might be acting largely through *clk-1*, as it affects the null mutant (*qm30*) only slightly, whereas it fully suppresses the partial loss of function mutation (*e2519*). The other mutation we isolated, *qm183*, also has significant effects on the defecation cycle of wild-type worms at all temperatures, but it cannot suppress either *clk-1(e2519)* or *clk-1(qm30)* mutants. It is clear, therefore, that the slow defecation of *clk-1(qm30)* mutants cannot simply be suppressed by every mutation that decreases the defecation cycle length of the wild type. In fact, some mutations may require full (i.e. *qm183*) or at least partial (i.e. *qm141*) *clk-1(+)* activity in order to affect the defecation cycle.

The *dsc-2(qm142)* mutation has a semi-dominant, time-dependent effect: We found that the strength of the *qm142* mutant phenotype changes with the age of the animal, in both heterozygotes and homozygotes, albeit at very different rates (Figure 2.4). We performed a time course study, in which we scored defecation in the same animals at different time points after they had molted to adults. In a *clk-1(qm30)* background, the homozygous *dsc-2(qm142)* animals are almost as slow as the *clk-1(qm30)* animals 2 hours after molting to adults, but by 8 hr, the defecation cycle length is restored to the wild-type length, and by 18 hr, the defecation cycle length is significantly shorter than that of the wild type. The defecation cycle length of *clk-1(qm30)*; *dsc-2(qm142)*/+ animals is very similar to that of *clk-1(qm30)* animals until about 40 hr. In fact, it takes about 48 hours after molting to adults for the defecation cycle length of the *dsc-2(qm142)*/+ heterozygous animals to become as fast as the age matched wild-type animals. As the *dsc-2(qm142)*/+ heterozygotes never become as fast as the homozygotes at any time point, and take longer than the homozygotes to speed up significantly, the effect of the *qm142* mutation is incompletely dominant over the wild-type allele. This is also confirmed by observations of *qm142* heterozygotes and homozygotes on the wild-type background (data not shown), although the effects are

much less dramatic. One way in which the *dsc-2(qm142)* allele could have this semi-dominant time-dependent effect is that the mutation results in a protein that can interfere with the function of the wild-type DSC-2 protein. An accumulation of the mutant product with time could increase the severity of the mutant phenotype.

Mutations in *dec-7* result in multiple discrete defecation cycle lengths: We characterized all the suppressor mutants by analyzing the mean defecation cycle length of a number of animals that had each been scored for five defecation cycles (Table 2.1). We also calculated the standard deviations of individual animals. We noticed that animals carrying *dec-7* mutations in a *clk-1(qm30)* background have very high standard deviations at 15° and 20° but not at 25° (see Figure 2.3B for the example of *dec-7(qm166)* at 20° and 25°). To analyze this variability further we plotted the frequency of single defecation cycle lengths of *clk-1 dec-7* animals at four different temperatures (Figure 2.5). At all temperatures, there is only one frequency peak for *clk-1(qm30)* mutants, but there are two peaks for both *clk-1 dec-7* double mutant strains at the three temperatures below 25° (Figure 2.5A,B,C). One of the peaks occurs at a cycle length that is two times that of where the other peak occurs. At 25°, however, there is only one peak, which coincides with the *clk-1(qm30)* peak (Figure 2.5D).

One interpretation of this pattern is that *clk-1* and/or *dec-7* have a role in coupling the activation of the defecation motor program (DMP) to the cycle, such that the coupling increasingly fails in *clk-1 dec-7* double mutants with increasing temperature. This would result in double cycle lengths and could mean that at 25° every cycle observed is actually a double cycle. Multiple discrete cycle lengths are not observed in *dec-7* mutants on a *clk-1(+)* background or in other Class II mutants, and thus, this phenomenon appears to be specific to *clk-1 dec-7* mutants.

Discussion

Mutations in *clk-1* affect numerous features of the worm including the rates of rhythmic behaviors, as well as growth and reproductive features (BRANICKY *et al.* 2000; WONG *et al.* 1995). Results from earlier experiments, which looked at the effect of temperature shifts on the growth rate of embryos, suggested that *clk-1* mutants are impaired in their ability to sense or react to changes in temperature (WONG *et al.* 1995). Here we have focused on the defecation cycle to further explore this phenomenon.

Reaction of defecation to changes in temperature: When we examined the reaction of wild-type animals to changes in temperature we found that when they are raised at 20° and are shifted to either 15° or 25° the defecation cycle length is profoundly altered by the shift. When shifted to 15°, the defecation cycle is significantly lengthened, and when shifted to 25°, the defecation cycle is significantly shortened. The specifics of assay conditions or temperature-shift protocol may affect these results: one previous report found little change in cycle length with temperature (LIU and THOMAS 1994) but results similar to those reported here have also been obtained recently by K. Iwasaki and J. Thomas (personal communication). Our findings do, however, indicate that there is a temperature compensation mechanism. Indeed, we have found that, in contrast to the wild type, *clk-1* mutants do not adjust the length of their defecation cycle when they are shifted to a new temperature. Because worms are poikilothermic animals and the rate of the biochemical reactions that underly physiological processes must be temperature-dependent, this implies that there is a temperature compensation mechanism that can maintain the cycle length even at different temperatures. In summary, our findings suggest that the reaction to temperature we observe in the wild type is an active process that requires *clk-1* activity. In the absence of *clk-1* activity, we can observe a

temperature compensation mechanism, which presumably also acts in the wild type, such that the wild-type condition appears to be a combination of temperature compensation and active adjustment.

Suppressors of the defecation phenotypes of *clk-1* mutants: We reasoned that if *clk-1* is involved in regulating the timing of the defecation cycle it might work through other gene products. In order to identify such genes we carried out a screen for mutations that could suppress *clk-1(qm30)* at 20°. We identified eight suppressor mutations, which fall into five different complementation groups. We found that these mutations also significantly decrease the defecation cycle length of the wild type and *clk-1(e2519)* mutants at 20°. If we consider the degree to which the mutations decrease the cycle length in the different backgrounds, (i.e. the percentage by which the mutation decreases the cycle length in each background), all of the *dsc* mutations (except *dsc-5(qm141)* and *dec(qm183)*) have the greatest effect on the *clk-1(qm30)* background and the weakest effect on the wild-type background (Table 2.1). In some cases, this is very striking. For example the *dsc-3* mutants decrease the rate of defecation ~25% on the *clk-1(qm30)* background, but only ~5% on the wild-type background. This effect is so small that these mutations could probably not have been found in a wild-type background. On the other hand, only the *dec-7(qm166)* mutation affects the wild type to the same degree as it affects *clk-1*. It is therefore not surprising that *dec-7* was the only gene we identified that was also found in a previous screen aimed at finding mutations that affect the defecation cycle length in a wild-type background (IWASAKI *et al.* 1995).

When we examined how defecation in the *dsc* or *dec; clk-1(qm30)* double mutants is affected by changes in temperature, we found that the suppressors fall into two distinct classes. Class I mutants completely suppresses the defecation phenotypes of *clk-1* mutants, such that they suppress *clk-1* at 20° and after a switch to a new temperature. Double

aimed at finding mutations that affect the defecation cycle length in a wild-type background (IWASAKI *et al.* 1995).

When we examined how defecation in the *dsc* or *dec; clk-1(qm30)* double mutants is affected by changes in temperature, we found that the suppressors fall into two distinct classes. Class I mutants completely suppresses the defecation phenotypes of *clk-1* mutants, such that they suppress *clk-1* at 20° and after a switch to a new temperature. Double mutants carrying Class I mutations have a profile of defecation very much like that of the wild type with respect to temperature: shorter after a shift to 25° and longer after a shift to 15°. In contrast, although the Class II mutants suppress as well as the Class I mutants when grown and scored at 20°, they cannot overcome the inability of *clk-1* mutants to react to changes in temperature. After a switch to 15° or 25° these mutations cannot, or only very poorly, suppress the slow defecation cycle. The existence of the Class II mutants therefore indicates that the slow defecation and the inability to adjust the cycle length in response to changes in temperature are separable phenotypes of *clk-1* mutants.

Although it is not yet clear how the Class I mutants restore the reaction to temperature, one interesting possibility is that these genes are actually components of the temperature compensation mechanism. These mutations might therefore be acting by abolishing the mechanism of temperature compensation observed in the absence of *clk-1*. In this model, the reaction to temperature observed in the *clk-1; dsc* double mutants might be a more passive (thermodynamic) reaction than the combination of compensation and active adjustment we believe to be the wild-type condition. This would imply that the combined mechanisms of temperature compensation and active adjustment increase the defecation cycle length from what it would be if it was determined passively by temperature. A mechanism that prevents the organism from functioning permanently at the highest possible rate at a given temperature might be adaptive.

Towards understanding the pleiotropic *clk-1* phenotype: Although *clk-1* affects ubiquinone biosynthesis (JONASSEN *et al.* 2001; MIYADERA *et al.* 2001; STENMARK *et al.* 2001), it is not clear how this is linked to the other phenotypes of *clk-1* mutants. Indeed, *clk-1* mutant mitochondria can function properly using DMQ, the biosynthetic intermediate they accumulate, and the severity of the ubiquinone phenotype is not correlated with the severity of the organismal phenotypes. The results presented here also support the notion that the phenotype of *clk-1* mutants, specifically the slow defecation phenotype, cannot simply be explained by a lack of ubiquinone. Indeed it is unlikely that the suppressors, which do not affect any other phenotype, act by overcoming the ubiquinone deficiency in only those cells that are responsible for the slow defecation cycle of the *clk-1* mutants. One possibility is that *clk-1* functions in other processes in addition to the biosynthesis of ubiquinone. Alternatively, it could be that although DMQ can partially replace UQ in the respiratory chain, it is unable to replace UQ for some of its other functions, whose resulting impairment is responsible for some aspects of the *clk-1* phenotype, such as the slow and temperature-invariant defecation cycle we have described here. UQ is found in almost all biological membranes (DALLNER and SINDELAR 2000), and is known to be a co-factor of the uncoupling proteins (UCP) in the mitochondria (ECHTAY *et al.* 2001; ECHTAY *et al.* 2000), to regulate the permeability transition pore (FONTAINE *et al.* 1998), and to function in plasma membrane and lysosomal oxido-reductase systems (GILLE and NOHL 2000; SANTOS-OCANA *et al.* 1998). Identifying the molecular nature of the *dsc* genes and their cellular and intracellular localization should thus help us understand the basis of the *clk-1* phenotypes and the regulation of the defecation cycle in general.

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Table 2.1:Quantitative phenotypic analysis of suppressor mutations on wild-type and *clk-1* backgrounds.

Mutation	Length of Defecation Cycle (sec)						
	Wild type			*clk-1(qm30)*			*clk-1(e2519)*
	15°	20°	25°	15°	20°	25°	20°
+	82.8 ± 9.2	55.9 ± 3.4	42.5 ± 2.5	90.7 ± 6.0	88.3 ± 13.5	88.4 ± 13.8	77.1 ± 6.7
dsc-1(qm133)	62.3 ± 9.6	46.9 ± 4.5	33.4 ± 4.0	76.7 ± 10.6	64.7 ± 12.2	72.9 ± 18.4	62.2 ± 12.9
dsc-2(qm142sd)	61.1 ± 5.0	45.1 ± 15.5	31.2 ± 3.7	76.7 ± 21.1	60.0 ± 14.0	74.6 ± 10.0	58.8 ± 6.7
dec-7(qm166)	55.1 ± 3.2	35.6 ± 3.2	34.1 ± 4.7	86.2 ± 22.2	58.9 ± 27.8	88.6 ±13.2	ND
dec-7(qm178)	ND	ND	ND	91.8 ± 23.9	63.2 ± 24.0	92.3 ± 11.4	ND
dsc-3(qm179)	75.6 ± 4.4	51.9 ± 2.8	35.3 ± 3.5	76.8 ± 9.1	63.0 ± 6.2	41.6 ± 4.3	56.4 ± 3.0

dsc-3(qm180)	74.4 ± 5.8	52.7 ± 2.9	33.7 ± 2.9	85.7 ± 9.2	68.5 ± 6.9	60.0 ± 20.7	66.7 ± 5.1
dsc-3(qm184)	73.9 ± 4.7	53.0 ± 3.2	37.0 ± 2.2	90.3 ± 5.3	66.0 ± 10.3	53.3 ± 7.6	61.4 ± 5.7
dsc-4(qm182)	68.1 ± 5.6	47.8 ± 2.5	33.9 ± 2.0	75.8 ± 7.5	60.7 ± 6.3	48.0 ± 7.7	60.2 ± 3.4
dsc-5(qm141)	64.7 ± 8.1	46.3 ± 7.0	36.5 ± 1.8	79.5 ± 6.9	75.5 ± 9.6	74.8 ± 7.4	56.7 ± 5.1
dec(qm183)	69.7 ± 5.2	49.9 ± 3.0	33.3 ± 1.2	102.2 ± 11.6	84.8 ± 12.2	84.4 ± 13.9	83.1 ± 15.8

Animals were raised at 20° and scored at 15°, 20°, or 25°. The numbers given are the means ± the standard deviations of animals that had each been scored for 5 consecutive defecation cycles at 20° and 25°, and for 3 consecutive cycles at 15°. The sample sizes are: for N2 and *clk-1(qm30)* strains at 20° n ≥ 50; for N2 and *clk-1(qm30)* strains at 15° and 25° n ≥ 25; for *clk-1(e2519)* strains n ≥ 25. As *dec-7* is tightly linked to *clk-1*, the defecation cycle lengths of some *dec-7* strains are not determined (ND). Phenotypic analyses of the *clk-1(+) dec-7(qm166)* strain were carried out in the background of the *dpy-17(e164)* mutation, which does not affect defecation.

Table 2.2: Summary of genetic mapping of mutants isolated in the suppressor screen.

Mutation	Genetic Mapping Data[a]
dsc-1(qm133) X	[*unc-3 lin15/dsc-1*]
	unc-3 (15/51) *dsc-1* (36/51) *lin-15*
	complements *flr-4(ut7)*
dsc-2(qm142d) V	[*clk-1; unc-34 dpy-11/dsc-2*]
	unc-34 (6/41) *dsc-2* (35/41) *dpy-11*
dsc-3(qm179) IV	[*clk-1; unc-33 dpy-4/dsc-3*]
	unc-33 (17/61) *dsc-3* (44/61) *dpy-4*
	fails to complement *qm180* and *qm184*
dsc-4(qm182) IV	[*clk-1; dpy-9/dsc-4*]
	0/40[b]
	complements *flr-3(ut9)*
dsc-5(qm141) II	[*rol-1unc-52/dsc-5*]
	rol-1 (7/37) *dsc-5* (30/37) *unc-52*
dec(qm183) X	[*lon-2 unc-2/dec*]
	lon-2 (3/45) *dec* (42/45) *unc-2*

dec-7(qm166) III	fails to complement *dec-7(sa296)* and *qm178*

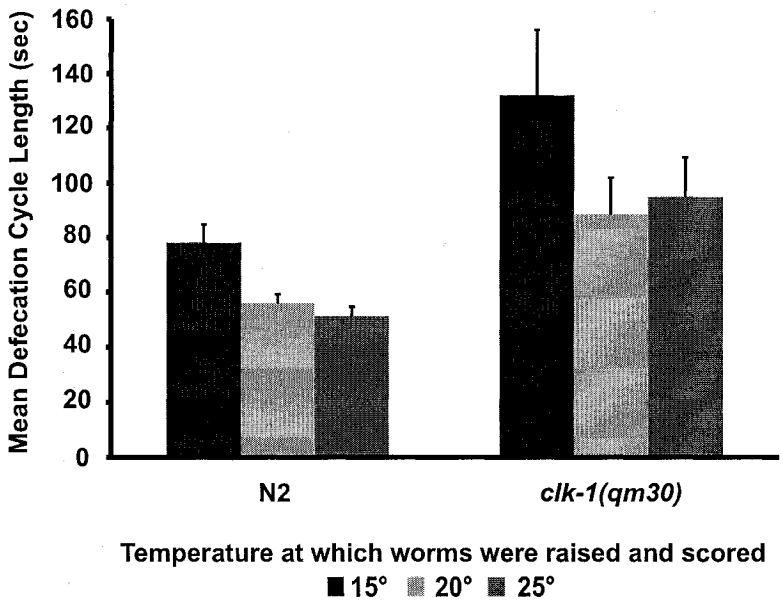
[a] The genotypes given in square brackets are those of the F1 animals whose descendants were scored to obtain 2- and 3- factor mapping data.

[b] Non-Dpy F2 progeny were scored for the presence of the *qm182* mutation; the denominator represents the number of *qm182* animals that were isolated and the numerator represents the number of *qm182* animals that were also heterozygous for the *dpy-9* mutation.

Figure 2.1: The effects of temperature on the defecation cycle length of the wild type (N2) and *clk-1* mutants.

The bars represent the means of animals that had each been scored for 5 consecutive defecation cycles at 20° and 25°, and for 3 consecutive cycles at 15°. The error bars represent the standard deviations of the means. The sample sizes are: for 20° $n \geq 50$; for 15° and 25° $n \geq 25$. **(A)** Animals were raised at 15°, 20°, and 25° and were scored at the temperature at which they had been raised. The numerical values are: for N2 at 15°, 20°, and 25°, respectively, 78.0 ± 6.7, 55.9 ± 3.4, 51.2 ± 3.4; for *clk-1(qm30)* at 15°, 20°, and 25°, respectively, 131.9 ± 24.0, 88.4 ± 13.5, 94.8 ± 14.3. **(B)** Animals were raised at 20° and scored at 15°, 20°, and 25°. The numerical values can be found in Table 2.1.

A


Mean Defecation Cycle Length (sec)
160
140
120
100
80
60
40
20
0
N2
clk-1(qm30)
Temperature at which worms were raised and scored
15°
20°
25°

B

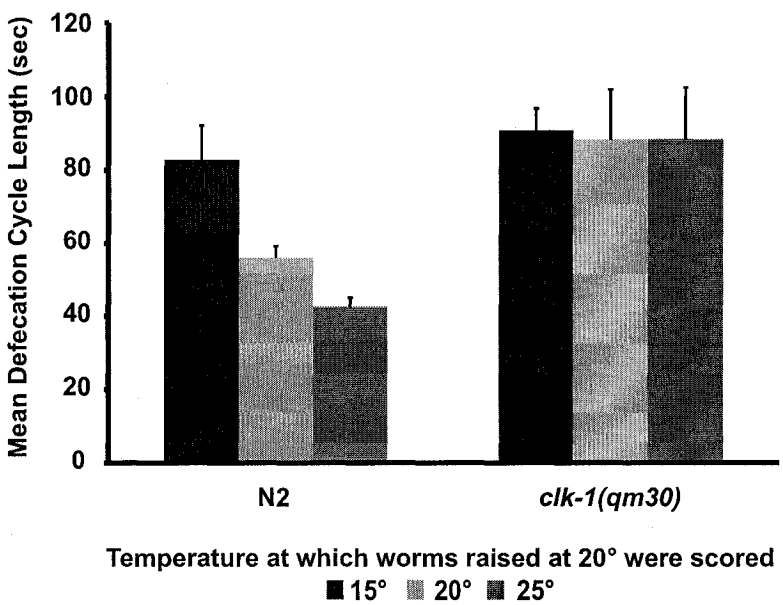
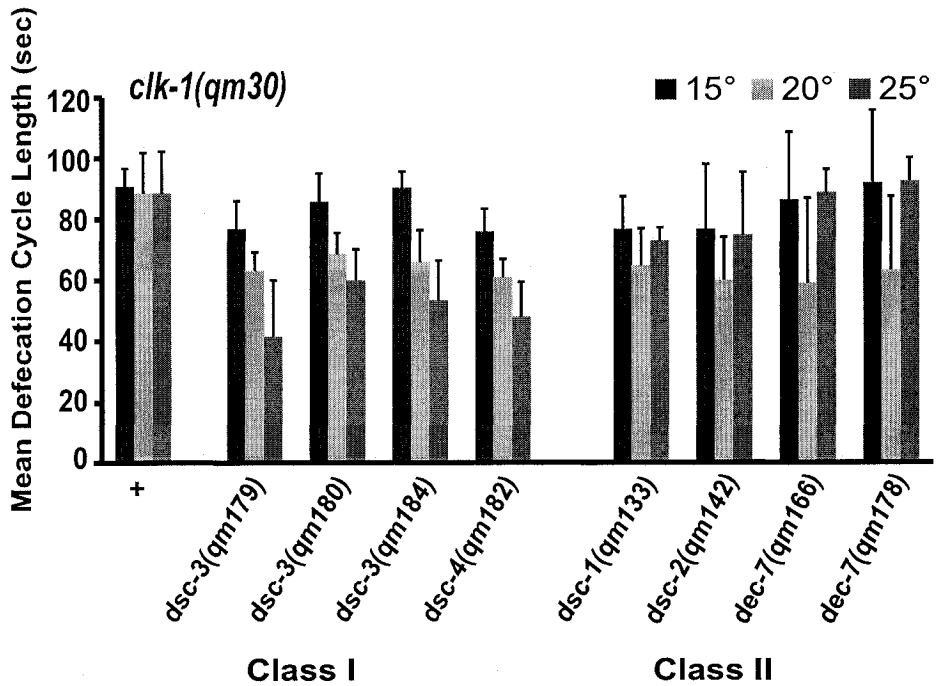

Mean Defecation Cycle Length (sec)
120
100
80
60
40
20
0
N2
clk-1(qm30)
Temperature at which worms raised at 20° were scored
15°
20°
25°

Figure 2.2: There are two classes of *clk-1* suppressor mutants. Animals were raised at 20° and scored at 15°, 20°, and 25°. The bars represent the means of animals that had each been scored for 5 consecutive defecation cycles at 20° and 25°, and for 3 consecutive cycles at 15°. The error bars represent the standard deviations of the means. The sample sizes are: at 20° $n \geq 50$; at 15° and 25° $n \geq 25$. **(A)** Suppressor mutations on the *clk-1(qm30)* background. The Class I mutations suppress the increased cycle length of *clk-1(qm30)* mutants and restore the ability to react to changes in temperature. The Class II mutations suppress the increased cycle length at 20° but do not restore the ability to react to changes in temperature. **(B)** Suppressor mutations on the wild-type background. The Class I mutants generally have weaker effects than the Class II mutants, particularly at 15° and 20°, but both classes of mutants react to changes in temperature like the wild type. The numerical values can be found in Table 2.1.

A


clk-1(qm30)
15°
20°
25°
Mean Defecation Cycle Length (sec)
120
100
80
60
40
20
0
+
dsc-3(qm179)
dsc-3(qm180)
dsc-3(qm184)
dsc-4(qm182)
dsc-1(qm133)
dsc-2(qm142)
dec-7(qm166)
dec-7(qm178)
Class I
Class II

B

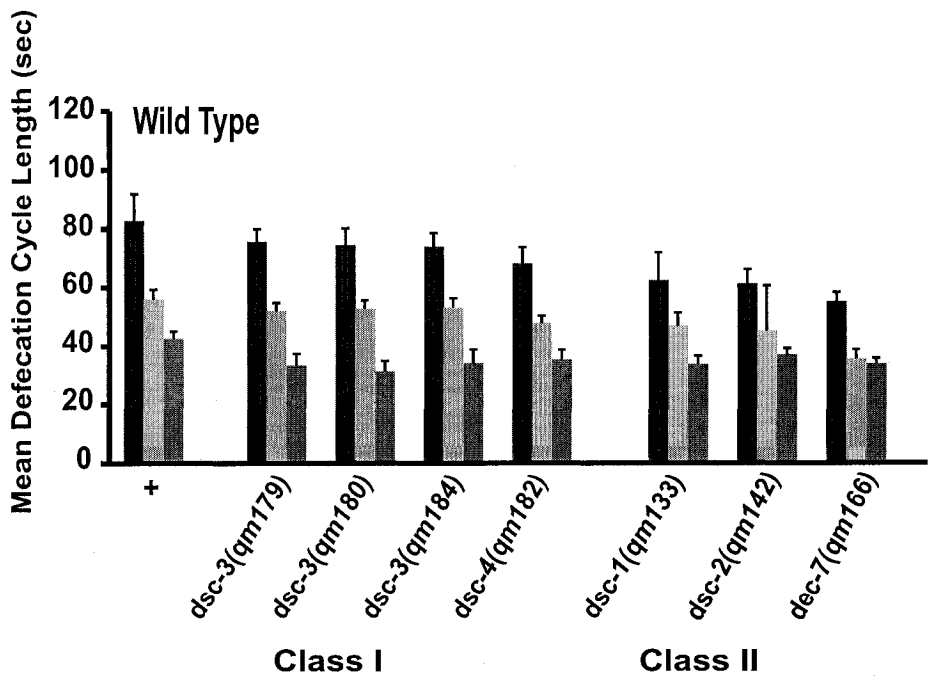
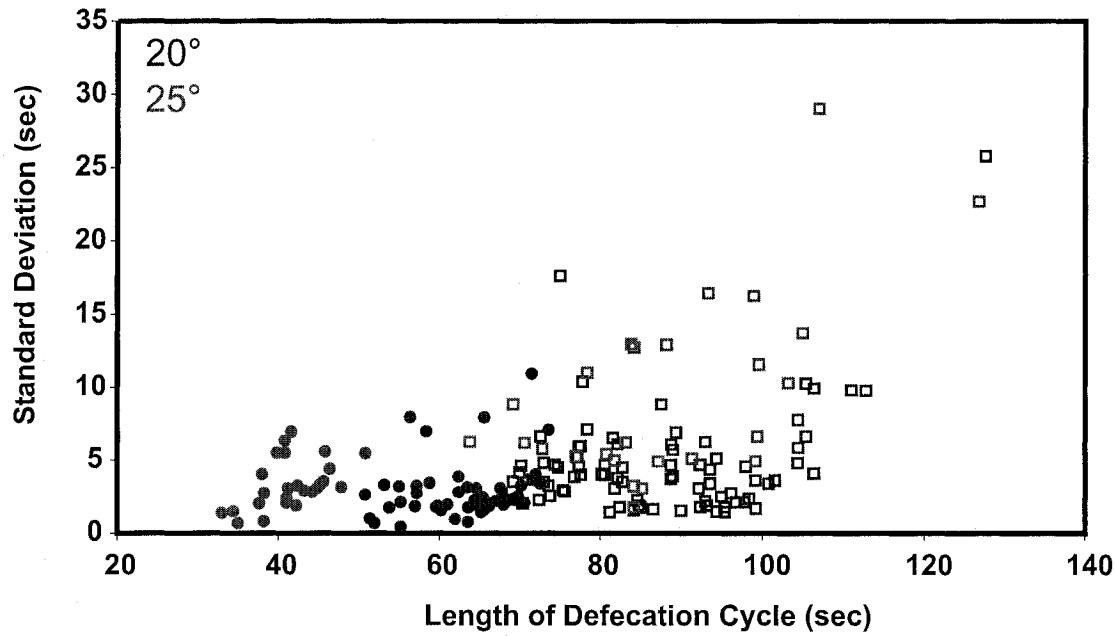

Wild Type
Mean Defecation Cycle Length (sec)
120
100
80
60
40
20
0
+
dsc-3(qm179)
dsc-3(qm180)
dsc-3(qm184)
dsc-4(qm182)
dsc-1(qm133)
dsc-2(qm142)
dec-7(qm166)
Class I
Class II

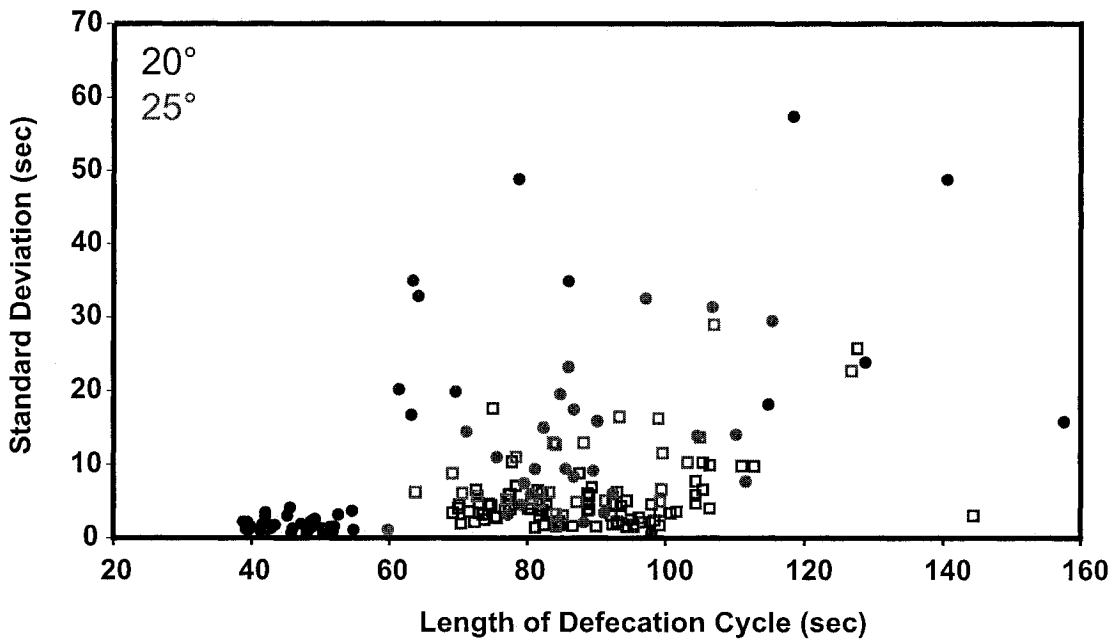
Figure 2.3: Examples of Class I and Class II suppressors.
Each point represents the mean (on the ordinate) and standard deviation (on the abscissa) of one animal, scored for 5 consecutive defecation cycles. **(A)** *dsc-3(qm179)* is an example of a Class I mutant: it suppresses *clk-1(qm30)* at 20° and after a shift to 25°, such that in the double mutant, defecation is faster at 25° than at 20°. **(B)** *dec-7(qm166)* is an example of a Class II mutant: it suppresses *clk-1(qm30)* at 20°, but cannot suppress after a shift to 25°, such that the defecation rate of the double mutant at 25° is not faster than the defecation rate of *clk-1(qm30)*.

A



□ *clk-1(qm30)* ● *clk-1(qm30); dsc-3(qm179)* □ *clk-1(qm30)* ● *clk-1(qm30); dsc-3(qm179)*

B



□ *clk-1(qm30)* ● *clk-1(qm30) dec-7(qm166)* □ *clk-1(qm30)* ● *clk-1(qm30) dec-7(qm166)*

Figure 2.4: The severity of phenotype of *dsc-2(qm142)* mutants is semi-dominant and time-dependent.

A time course study of mutants heterozygous and homozygous for the *dsc-2(qm142)* mutation in the *clk-1(qm30)* background. Animals were scored at different time points after having molted to adults (at time 0 hr). Each point represents the mean of ~10 animals, scored for 3 consecutive defecation cycles at 20°; the error bars represent the standard errors of the means. *clk-1* designates the *clk-1(qm30)* allele, *dsc-2* the *dsc-2(qm142)* allele, and + the *dsc-2(+)* allele.

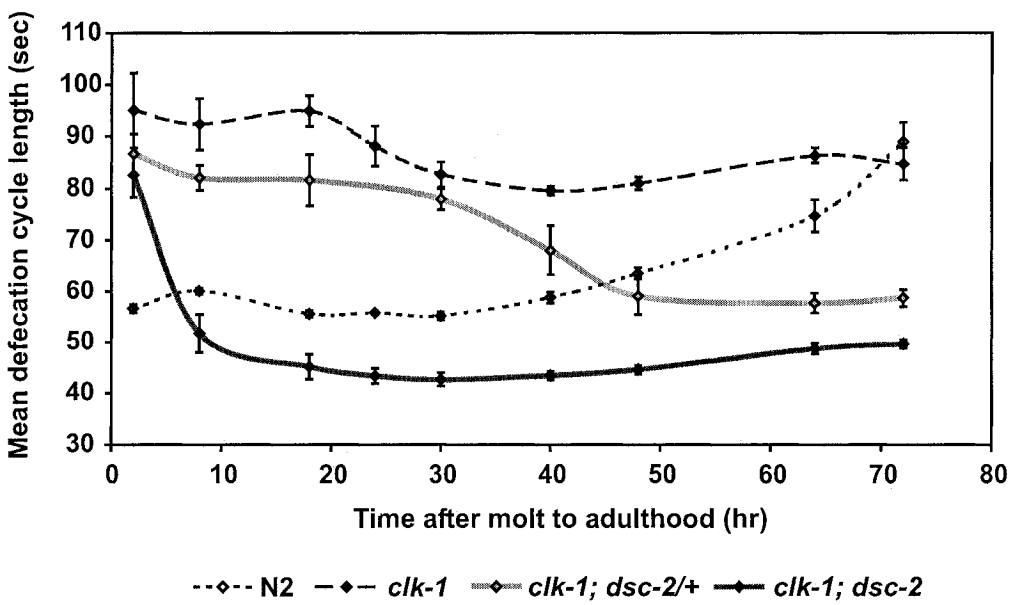
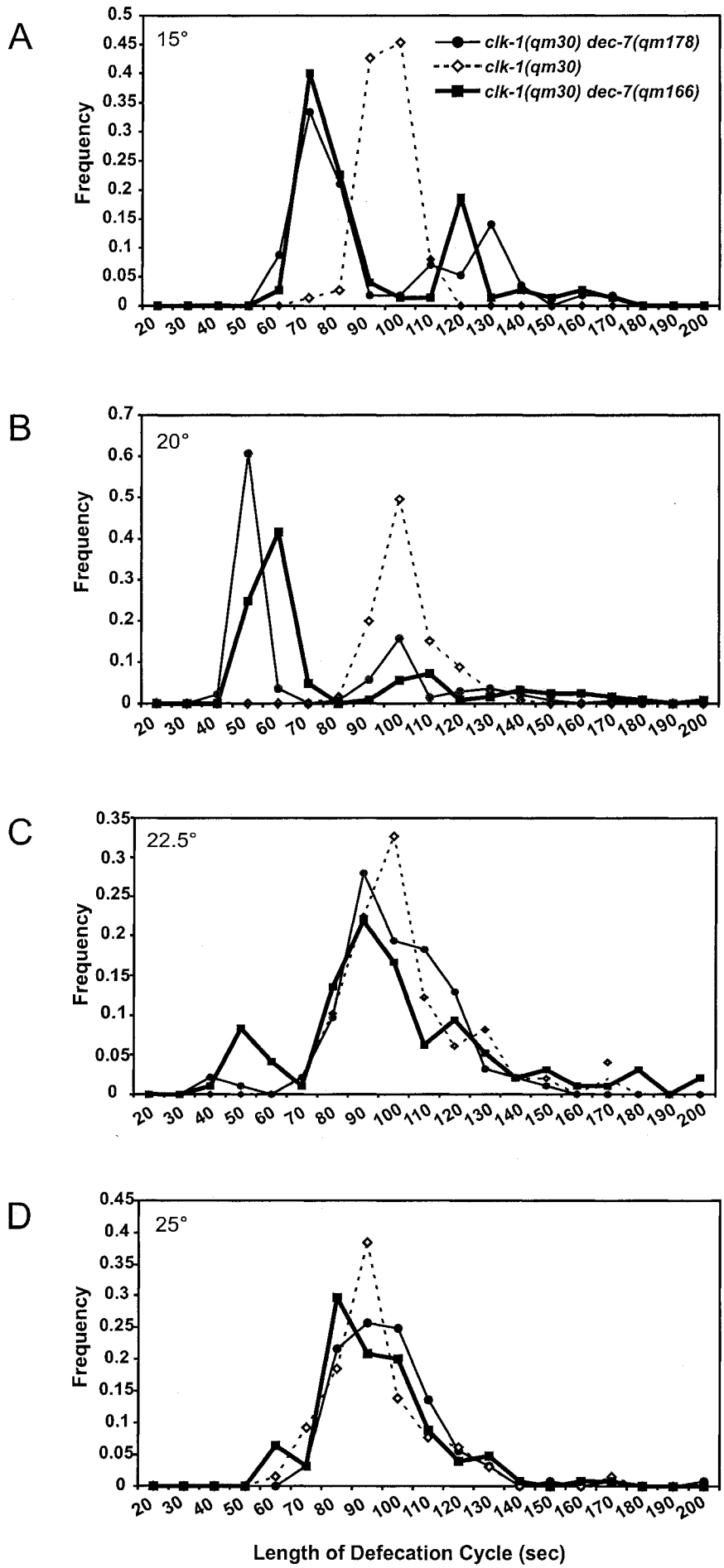

Mean defecation cycle length (sec)
110
100
90
80
70
60
50
40
30
0
10
20
30
40
50
60
70
80
Time after molt to adulthood (hr)
N2
clk-1
clk-1; dsc-2/+
clk-1; dsc-2

Figure 2.5: Mutations in *dec-7* result in multiple discrete defecation cycle lengths.

An analysis of the frequency of single defecation cycle lengths of *dec-7* mutants in the *clk-1(qm30)* background. The sample sizes for all genotypes at 15°, 20°, and 25° are $n \geq 25$, and at 22.5° n = 10-25. At all temperatures, there is only one frequency peak for *clk-1(qm30)* mutants. At 15° **(A)**, 20° **(B)**, and 22.5° **(C)** there are two frequency peaks for both *clk-1(qm30) dec-7(qm166)* and *clk-1(qm30) dec-7(qm178)* double mutants, the second peak occurring at a cycle length double that of the first peak. At 25° **(D)**, there is only one frequency peak for the *clk-1(qm30) dec-7* double mutants, which coincides with the *clk-1(qm30)* peak.


A
15°
clk-1(qm30) dec-7(qm178)
clk-1(qm30)
clk-1(qm30) dec-7(qm166)
Frequency
B
20°
Frequency
C
22.5°
Frequency
D
25°
Frequency
Length of Defecation Cycle (sec)

Connecting Statement: Bridging Chapters 2 and 3

In the previous chapter, I reported the isolation of the *dsc* mutants. In the following chapter, I report on the genetic, phenotypic and molecular characterization of the Class I *dsc* mutant, *dsc-4*. Our analysis revealed that, in addition to suppressing the defecation phenotypes of *clk-1*, *dsc-4* also suppresses the slow germline development. In fact, much of the anlysis presented in this chapter involves the slow germline development phenotype.

Chapter 3:

dsc-4

Redox regulation of germline and vulval development in *Caenorhabditis elegans*

Yukimasa Shibata, Robyn Branicky, Irene Oviedo Landaverde and Siegfried Hekimi

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Abstract

In vitro studies have indicated that reactive oxygen species (ROS) and the oxidation of signaling molecules are important mediators of signal transduction. We have identified two pathways by which the altered redox chemistry of the *clk-1* mutants of *Caenorhabditis elegans* acts in vivo on germline development. One pathway depends on the oxidation of an analogue of vertebrate low density lipoprotein (LDL) and acts on the germline through the ARK-1 kinase and inositol trisphosphate (IP_3) signaling. The other pathway is the oncogenic *ras* signaling pathway, whose action on germline as well as vulval development appears to be modulated by cytoplasmic ROS.

Introduction

Reactive oxygen species (ROS) are short-lived reactive molecules that can modify cellular components including nucleic acids, proteins and lipids. For example, the oxidation of low density lipoproteins (LDL) by ROS is one of the causative factors of atherosclerosis (*1*). ROS are toxic but the oxidation of macromolecules can also serve as a signalling device (*2*). Moreover, in vitro studies have shown that ROS act as intracellular messengers in signal transduction pathways, such as *ras* signaling (*3, 4*). However little is known about the effect of ROS on signal transduction in intact animals (*5*).

Ubiquinone (UQ or coenzyme Q) is a redox-active lipid that has numerous biochemical roles and is involved in the production of ROS. However, UQ is also an antioxidant that prevents the initiation and/or propagation of lipid peroxidation in cellular membranes (*6*). The *C. elegans clk-1* gene encodes a conserved enzyme that is necessary for UQ biosynthesis (*7*). In the absence of CLK-1, mutants accumulate demethoxyubiquinone (DMQ)(*8, 9*), which can partially replace UQ as an electron carrier (*8*). However, *clk-1* mutants require dietary UQ for their survival (*9, 10*).

clk-1 mutants show a highly pleiotropic phenotype that includes an average slowing down and deregulation of a number of physiological processes, including aging (*11*). Presumably, given that *clk-1* mutants obtain significant amounts of UQ from their diet (*12*), these phenotypes are due to the presence of DMQ, which might be a better antioxidant than UQ (*13*). Thus, the *clk-1* mutant phenotypes could be the consequence of altered redox signaling.

Results and Discussion

In addition to the previously described phenotypes, we find that somatic and germline development are desynchronized in *clk-1* mutants. In wild-type hermaphrodites, primary spermatocytes and sperm are observed at the late fourth larval stage (L4), and oogenesis commences shortly after the adult molt (Figure 3.1). However, the majority of *clk-1* mutants are either before or in the process of spermatogenesis at the adult molt (Figure 3.5A) and only 3% of the anterior gonads have initiated oogenesis at 6 hours after the adult molt (Figure 3.1B,C). Although the development of the germline of the posterior gonad of *clk-1* mutants is also delayed compared to that of the wild type, it is less affected than the anterior gonad. The delay in germline development also produces a delay in egg-laying (Figure 3.6A).

We find that the *dsc-4(qm182)* mutation, which was isolated as a suppressor of the slow defecation phenotype of *clk-1* mutants (*14*), also suppresses the germline phenotypes. Both *dsc-4(qm182)* and *dsc-4(RNAi)* suppress the delayed germline development (Figure 3.1B,C and 3.2A) and egg-laying (Figure 3.6A), but do not affect the rate of post-embryonic development (Figure 3.7), indicating that *dsc-4* acts by suppressing the slow development of the germline and not by slowing down somatic development.

We cloned *dsc-4* and found that it encodes an 892-residue protein similar, and probably orthologous, to the large subunit of the microsomal triglyceride transfer protein (MTP) (Figure 3.8)(*15*). MTP is an endoplasmic reticulum protein that is necessary for the secretion of apolipoproteinB (apoB)-containing lipoproteins, in particular LDL (*16*). Lipoproteins consist of a high molecular weight protein complexed to various lipids, including triglycerides, cholesteryl esters, cholesterol and phospholipids (*17*). apoB is the core component of LDL. In humans, mutations in the large subunit of MTP cause abetalipoproteinemia, a severe deficiency in LDL secretion (*18*). *dsc-4::gfp* transcriptional and rescuing translational reporter genes are expressed in the intestine from early embryogenesis, just after the beginning of elongation, throughout larval stages and adulthood (data not shown). In the worm, the intestine is the digestive organ

and the major secretory organ; in particular, it secretes the vitellogenins, which are apoB homologues.

The genome of *C. elegans* contains five apoB-like genes (*vit-2* to –6) (*19*). To determine whether altered secretion of apoB-dependent lipoproteins is responsible for the effect of *dsc-4* on *C. elegans* germline development we carried out RNAi against *vit-2*, *-5* and *-6*. Since the coding region of the *vit-5* RNAi clone used is 98% identical to both *vit-3* and *-4*, the *vit-5* RNAi treatment is expected to disrupt all three genes (Figure 3.9A). The effect of *vit-5(RNAi)* is as strong as that of *dsc-4(RNAi)* (Figure 3.2A), but not additive to it (Figure 3.9B). The effect of RNAi against *vit-2* and *-6* is extremely weak, and RNAi against any of the *vit* genes did not prevent egg production (data not shown). The *vit* genes were originally isolated as genes that encode yolk proteins. However, if *dsc-4* was required for yolk production, mutation of *dsc-4* should drastically reduce brood size like mutations in *rme-2,* which encodes a receptor for yolk proteins (*20*), but it does not. Thus, our results indicate that, in addition to their roles as yolk proteins, at least some of the VITs are also part of lipoprotein particles that resemble the apoB-dependent LDL particles found in vertebrates.

In mammals, cholesterol is a major constituent of lipoproteins, and reducing its intake or synthesis leads to reduced levels of LDL. Cholesterol depletion completely suppresses the slow germline development of *clk-1* mutants, but has only a mild effect on other genotypes such as *dsc-4* and *clk-1; dsc-4* (Figure 3.5B). This suggests that, as in mammals, reducing cholesterol intake reduces the secretion of LDL-like particles.

To test whether the germline phenotype of *clk-1* is caused by lower levels of ROS due to the presence of DMQ, we performed RNAi against the *C. elegans* superoxide dismutases, *sod-1 to –4*. Reducing the activity of these detoxifying enzymes will result in an elevation of the level of ROS. We found that *sod-1(RNAi),* but not RNAi against any of the other *sod* genes, suppresses the delayed egg production of *clk-1* mutants (Figure 3.6C), but has no effect on the wild type (Figure 3.6B). Moreover, *sod-1(RNAi)* is as efficient as *dsc-4(RNAi)* and *vit-5(RNAi)* in suppressing slow germline development (Figure 3.2A). As SOD-1 is a cytoplasmic Cu/Zn superoxide dismutase, these results indicate that the slow germline development of *clk-1* mutants is suppressed by an increase of

cytoplasmic superoxide, suggesting that this phenotype is due to low levels of ROS. The effect of *sod-1(RNAi)* is not additive to that of *dsc-4(qm182)* (Figure 3.6D), which suggests that the oxidation of LDL-like lipoproteins affects germline development in *C. elegans.* In addition, *sod-1(RNAi)* appears to act in the intestine (Figure 3.2B), the site of lipoprotein formation and secretion.

In conclusion, our results indicate that decreasing the oxidation of LDL-like lipoproteins slows down germline development (as observed in *clk-1* mutants), whereas decreasing the production of native LDL-like lipoproteins (as observed in *clk-1; dsc-4* mutants) can restore a normal rate of development. Thus, native lipoproteins inhibit, and oxidized lipoproteins stimulate germline development (Figure 3.4A). However, it is not clear yet whether oxidized LDL itself has an effect on the germline, as envisioned in Figure 3.4A, or whether the oxidation of LDL acts simply by reducing the level of native LDL because oxidized LDL is recognized as damaged and is removed.

Our studies provide an invertebrate model system to explore the biology of LDL-like lipoproteins, including their oxidation (Figure 3.4B). Many of the constituents and processes identified in vertebrate studies have been found to be present: assembly in the ER and its requirement for MTP activity, an apoB-like protein core for the particle, the importance of cholesterol in determining lipoprotein levels, oxidation and the involvement of UQ and SOD activity in regulating the level of oxidation (*21, 22*).

To identify the mechanism of action of lipoproteins on the germline, we focused on the ARK-1 kinase, which has been shown to affect the activity of the EGFR-like receptor LET-23 on ovulation (*23*), and is specifically expressed in the germline at high levels (*24*). We find that *ark-1(RNAi)* suppresses the slow germline development of *clk-1* mutants by acting in the germline (Figure 3.2C), and that its effect is not additive to that of *dsc-4(qm182)* (Table 3.1). Together these findings suggest that ARK-1 is normally activated by lipoproteins and inhibits germline development.

ARK-1 acts on LET-23, which stimulates both the IP_3 and *ras* signal transduction pathways (*25*). We find that both a gain-of-function allele of the IP3 receptor (*itr-1(sy328)*) and the *ras* gain-of-function allele *let-60(n1046)* moderately suppress the slow germline development of *clk-1* (Figure 3.2D,E).

However, the gonad function of *let-23* is believed to be *let-60ras* independent (*23*). We also find that *let-60* acts independently of the *dsc-4/ark-1* pathway in our system: the *let-60(gf)* mutation enhances the effect of *dsc-4(RNAi)* and this combination suppresses more strongly than any other condition (Figure 3.2F). This suggests that germline development is affected independently by both the *let-60ras* and *dsc-4/ark-1* pathways (Figure 3.4C).

The effect of *let-60(gf)* on the germline of *clk-1* mutants suggests that the *ras* pathway is down-regulated by *clk-1* mutations, which is consistent with the redox-sensitivity of *ras* signaling in in vitro cellular models (*4*). To test this further, we examined the effect of *clk-1* on the activity of *let-60* for vulva formation. In this pathway, *ras* activation inhibits transcription factors such as LIN-1, which itself inhibits vulva formation (*26*). Thus, *let-60(gf)* and *lin-1(lf)* mutants have multiple vulvae instead of only one (*27*). We found that *clk-1* strongly suppresses *let-60(gf)* but has no effect on *lin-1(e1026)*, suggesting that *clk-1* acts on the *ras* pathway upstream of *lin-1* (Figure 3.3A,B). We found that the effect of *clk-1* on the *ras* pathway is due to reduced redox signaling. Indeed, RNAi against *sod-1* and, to a lesser extent, *sod-4,* the extracellular Cu/Zn SOD, substantially restore vulva formation in *clk-1; let-60(gf)* mutants (Figure 3.3C). Neither the *sods* on *clk-1(+)* nor *dsc-4* on *clk-1;let-60* had a significant effect on vulva formation (data not shown). That the cytoplasmic and extracellular SODs have an effect is consistent with the cytoplasmic and membrane-associated localization of *ras*.

The phenotype of *clk-1* mutants is extremely pleiotropic, with most aspects of development, behavior and reproduction being slowed on average (*11*). We have found that at least one of these phenotypes could be explained by a reduction of the level of oxidation of LDL-like lipoproteins, and that *clk-1* affects the *ras* pathway by lowering cytoplasmic ROS levels. These findings provide a unique model to study the effect of redox signal transduction on the development of whole organisms and suggest a model for the *clk-1* pleiotropy, in which the complexity of the phenotype is due to the multiplicity of signaling roles that are carried out by the oxidative modification of cellular constituents.

References

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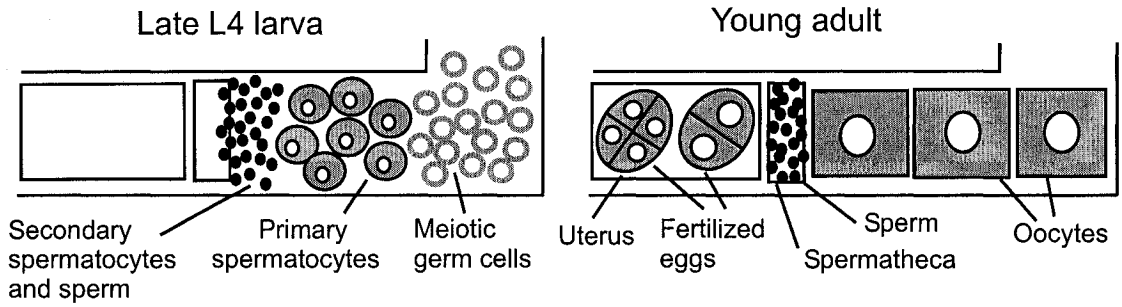
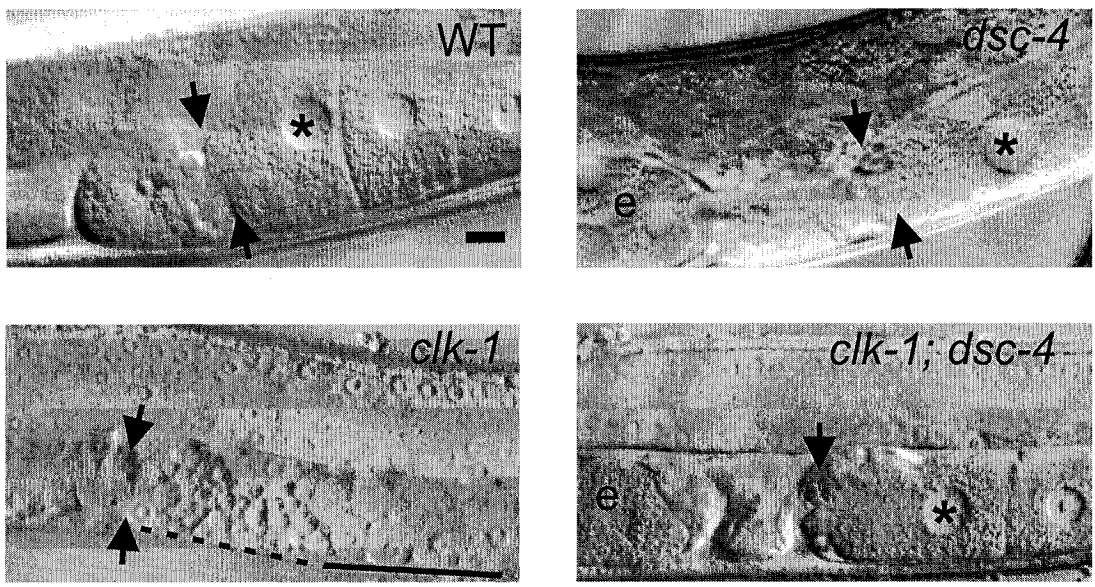
29. We thank Frédéric Bussière for technical assistance, Claire Bénard for reading the manuscript, A. Coulson, A. Fire and Y. Kohara for providing clones, and the Caenorhabditis Genetics Center (funded by NIH's NCRR), for providing strains. S.H. is a CIHR Scientist and R.B. is supported by a McGill Major Fellowship. The Genbank accession number for the *dsc-4* cDNA is AY428645.

Figure 3.1: The effects of *clk-1* and *dsc-4* on germline development.
(A) Schematic representation of the proximal portion of the gonad and germline of late L4 and young adult hermaphrodites. The gonad normally consists of 2 U-shaped arms (anterior and posterior) that join the centrally located uterus. Left is proximal (relative to the vulva) and right is distal. **(B)** The proximal end of the posterior germline at 6 hours after the adult molt. Left is anterior and top is dorsal. Asterisks indicate the nucleus of the most proximal oocyte; arrows indicate the proximal end of the germline; e indicates fertilized egg; the dotted line indicates a region where spermatogenesis is taking place and the solid line indicates a region containing primary spermatocytes. The bar is 10 μm. **(C)** The percentage of germlines at each of four different developmental stages is shown for each genotype at 6 hours after the adult molt (n≥30). The anterior (A) and posterior (P) gonad arms are presented separately.

A


Late L4 larva
Young adult
Secondary spermatocytes and sperm
Primary spermatocytes
Meiotic germ cells
Uterus
Fertilized eggs
Sperm
Spermatheca
Oocytes

B


WT
dsc-4
clk-1
clk-1; dsc-4
e
e

C

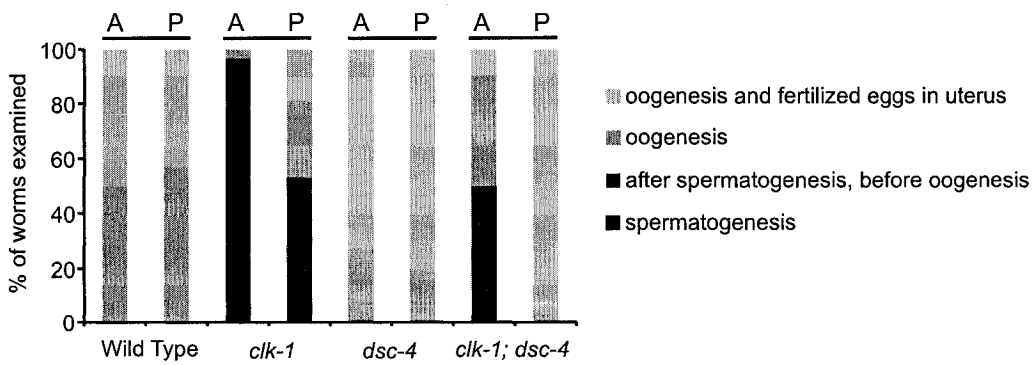

A P A P A P A P
100
80
60
40
20
0
% of worms examined
Wild Type
clk-1
dsc-4
clk-1; dsc-4
oogenesis and fertilized eggs in uterus
oogenesis
after spermatogenesis, before oogenesis
spermatogenesis

Figure 3.2: Genetic analysis of the mechanism of action of *dsc-4* on the germline development of *clk-1*.

The percentage of germlines observed to be at each of three different developmental stages at 1.5 hours after the adult molt is shown. The stages of development of the anterior (A) and posterior (P) gonad arms are presented separately. All RNAi experiments were carried out by the feeding method; controls were fed the HT115 bacteria containing the RNAi feeding vector, pPD129.36. **(A)-(C)** The effect of RNAi against various genes in the *clk-1(qm30)* or *clk-1(qm30); sid-1(qt2)* mutant backgrounds (n ≥ 30). The *sid-1* mutants were used to test whether the site of action of the RNAi is the intestine. As RNAi is not systemic in *sid-1* mutants (*28*), RNAi by feeding is confined to the intestine (*15*). **(D)** The effect of the *itr-1(sy328)* mutation on germline development (n ≥ 20). All genotypes include the *unc-24(e138)* mutation, which is linked to *itr-1*. **(E)** The effect of the *let-60(n1046)* gain-of-function mutation on germline development (n ≥ 30). **(F)** The effect of *dsc-4(RNAi)* on the germline development of *clk-1* and *clk-1; let-60* mutants (n ≥ 30).

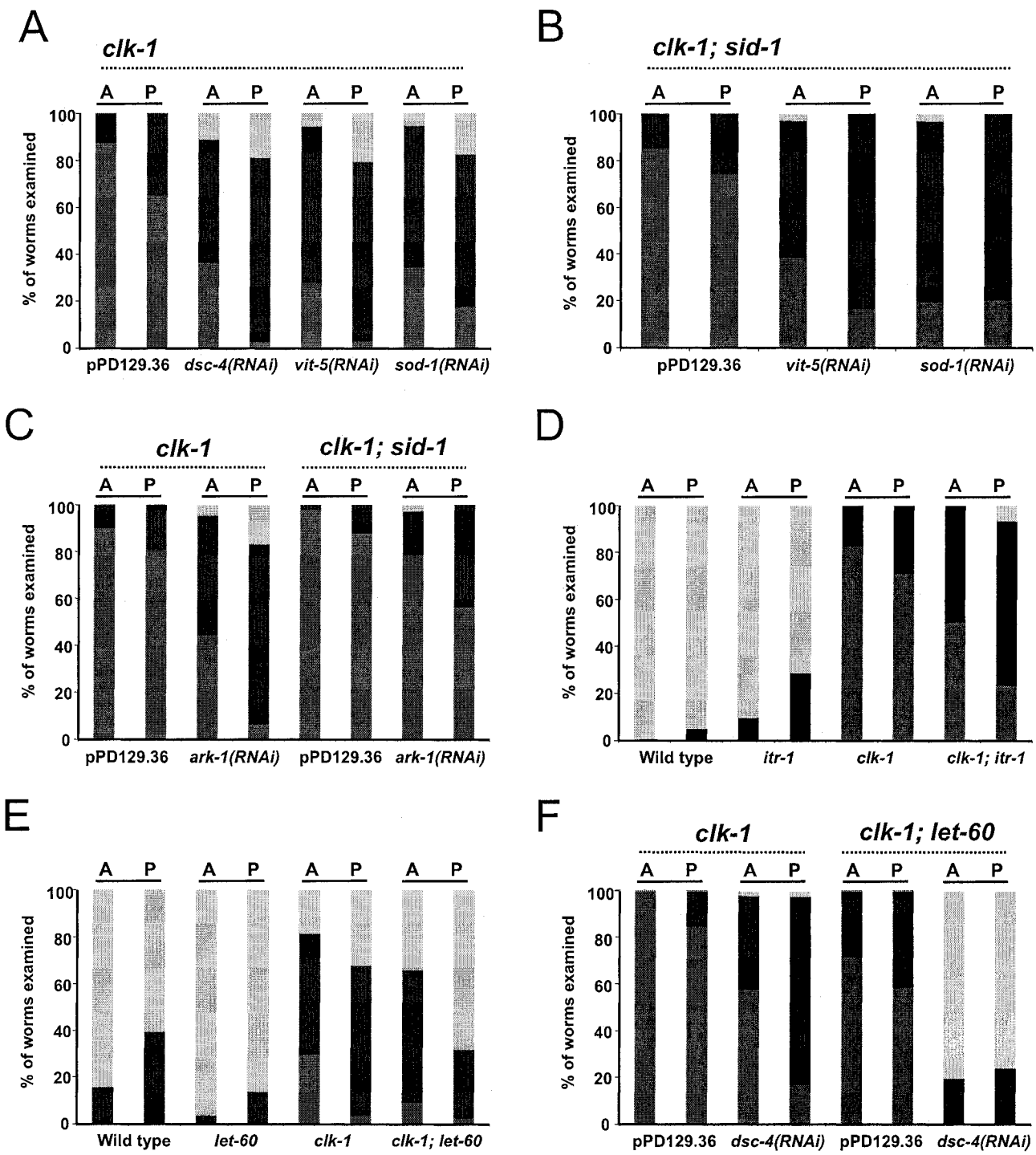
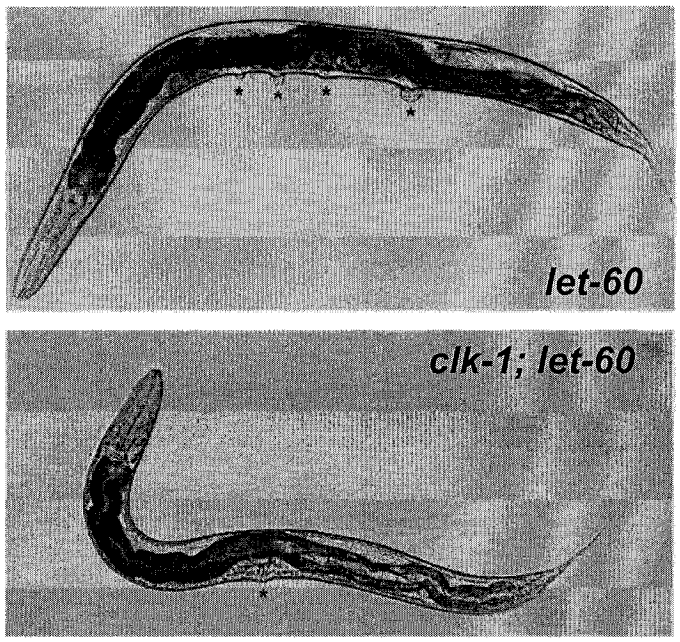
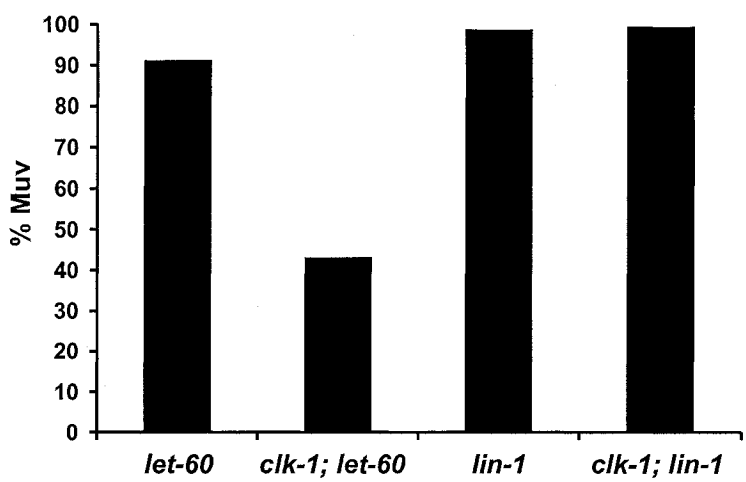

A
clk-1
A P A P A P A P
100
80
60
40
20
0
% of worms examined
pPD129.36
dsc-4(RNAi)
vit-5(RNAi)
sod-1(RNAi)
B
clk-1; sid-1
A P A P A P
pPD129.36
vit-5(RNAi)
sod-1(RNAi)
C
clk-1
clk-1; sid-1
A P A P A P A P
pPD129.36
ark-1(RNAi)
pPD129.36
ark-1(RNAi)
D
A P A P A P A P
Wild type
itr-1
clk-1
clk-1; itr-1
E
A P A P A P A P
Wild type
let-60
clk-1
clk-1; let-60
F
clk-1
clk-1; let-60
A P A P A P A P
pPD129.36
dsc-4(RNAi)
pPD129.36
dsc-4(RNAi)
after primary spermatocyte formation before oogenesis
primary spermatocyte formation
before primary spermatocyte formation

Figure 3.3: *clk-1* affects *ras* signaling during vulva formation.
(A) A *let-60(n1046gf)* mutant with multiple vulvae (indicated by asterisks) and a *clk-1(qm30); let-60(n1046gf)* mutant with a single vulva. **(B)** The multivulva phenotype (Muv) of *let-60(gf)* but not *lin-1(e1026)* is suppressed by *clk-1* ($n \geq 30$). **(C)** The effect of RNAi against the four superoxide dismutase (*sod)* genes on the Muv phenotype of *clk-1; let-60(gf)* mutants ($n \geq 750$).

A


let-60
clk-1; let-60

B


% Muv
100
90
80
70
60
50
40
30
20
10
0
let-60
clk-1; let-60
lin-1
clk-1; lin-1

C

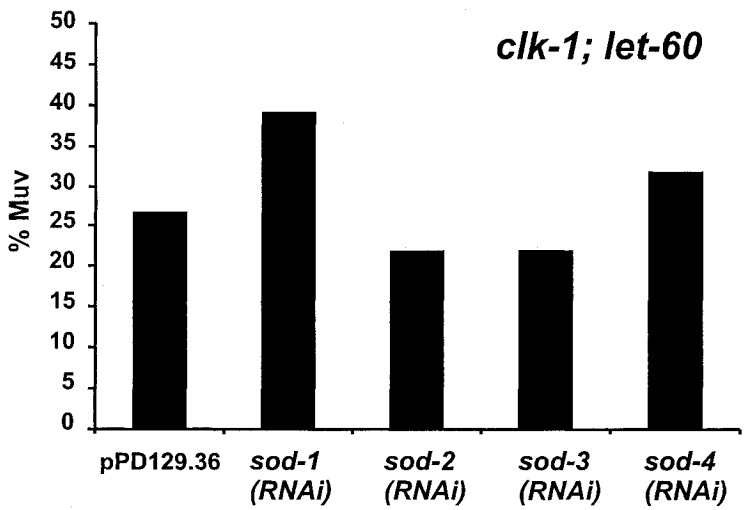
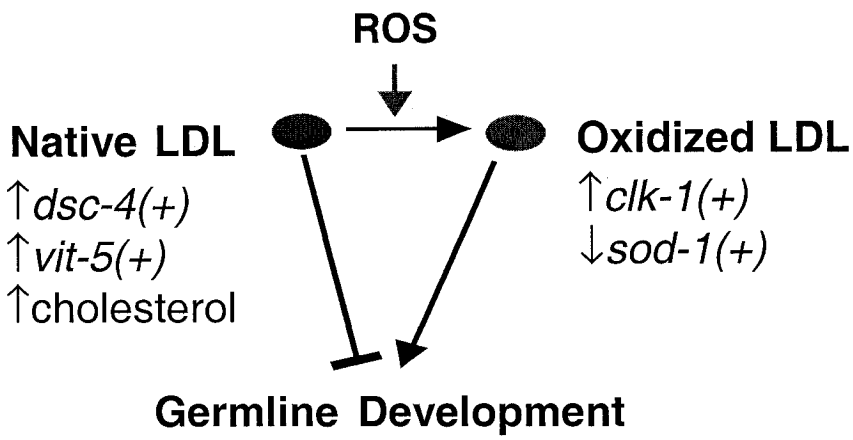

clk-1; let-60
% Muv
50
45
40
35
30
25
20
15
10
5
0
pPD129.36
sod-1 (RNAi)
sod-2 (RNAi)
sod-3 (RNAi)
sod-4 (RNAi)

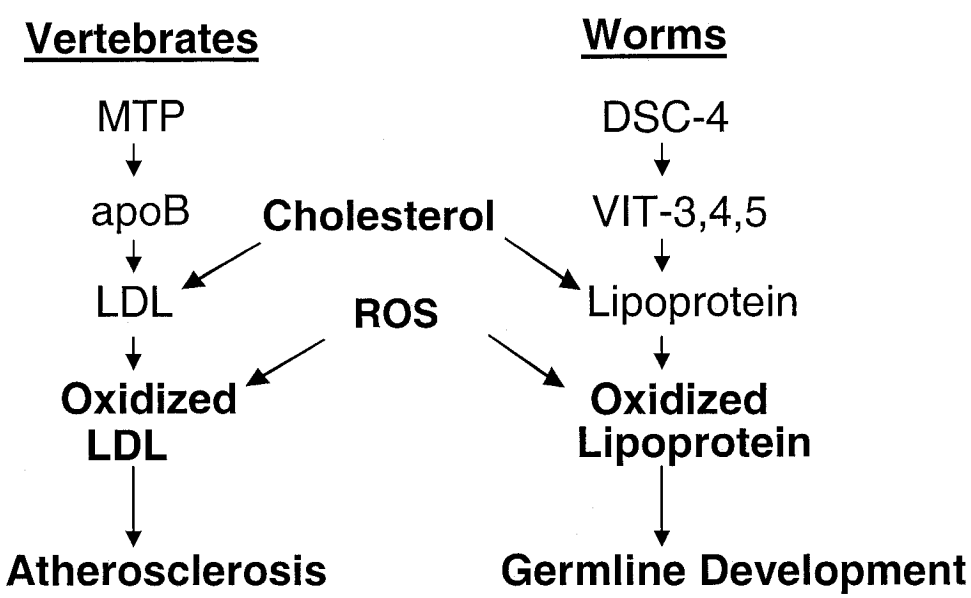
Figure 3.4: Genetic interactions and schematic representations of pathways affecting germline development.

(A) Factors affecting the relative abundance of native and oxidized LDL-like lipoproteins and their effects on germline development. **(B)** A comparison of the components in the LDL formation and oxidation pathways in vertebrates and worms. **(C)** A model of genetic interactions affecting germline development in *clk-1* mutants.

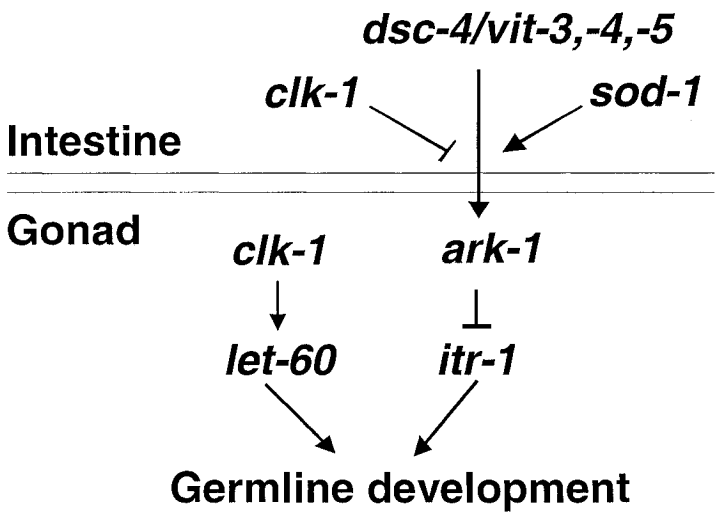
A


ROS
Native LDL
↑dsc-4(+)
↑vit-5(+)
↑cholesterol
Oxidized LDL
↑clk-1(+)
↓sod-1(+)
Germline Development

B


Vertebrates
Worms
MTP
apoB
LDL
Cholesterol
ROS
DSC-4
VIT-3,4,5
Lipoprotein
Oxidized LDL
Oxidized Lipoprotein
Atherosclerosis
Germline Development

C


dsc-4/vit-3,-4,-5
clk-1
sod-1
Intestine
Gonad
clk-1
ark-1
let-60
itr-1
Germline development

Supporting Material

Materials and Methods

Strains and culture methods

Animals were cultured at 20°C as described (*1*), and were fed the *E. coli* strain OP50 unless otherwise indicated. Cholesterol depletion experiments were performed on standard NGM plates seeded with OP50, but without any added cholesterol (NGM-C). Worms were cultured on the NGM-C plates for two or more generations before the rate of germline development was examined. For RNAi experiments, worms were cultured on NGM plates supplemented with 1 mM IPTG and 50 μg/mL ampicilin, and were fed the *E. coli* strain HT115 transformed with pPD129.36 derived plasmids. P0s were transferred to the RNAi plates as L4 larvae and the F1 generation was examined.

The wild-type strain was N2 (Bristol strain). The following mutations were used: *clk-1(qm30)* III; *dsc-4(qm182), dpy-9(e12), lin-1(e1026), unc-33(e204), lfe-1/itr-1/dec-4(sy328), unc-24(e138), let-60(n1046)* IV; *sid-1(qt2)* V.

Mapping, cloning and sequencing of dsc-4

dsc-4 had previously been mapped to the left arm of LG IV (-27.6) (*2*). Three-point mapping experiments with *unc-33 dpy-9* suggested that *dsc-4* was to the left or very close to *dpy-9:* in a cross between *clk-1; dsc-4* and *clk-1; unc-33 dpy-9*, 14/14 Unc non-Dpy picked up *dsc-4*, while 0/20 Dpy non-Unc picked up *dsc-4.* Two-point mapping experiments failed to separate *dsc-4* from *dpy-9* by recombination, suggesting that *dsc-4* is very close to *dpy-9,* however, as this has also been reported for other genes mapped to the left of *dpy*-9 (*3*), it is likely that recombination is suppressed in this region. 14 cosmids which roughly correspond to this genetic region were assayed for rescuing activity. They were divided into three pools (pool 1; C15E6, T05C7, B0545, B0312 and F52F6, pool 2; R02D3, T21D12, K02D7, F18F11, AH12, F40D2 and T07A9, pool 3; M04G7 and

M02G12) and each pool was injected into *clk-1(qm30); dsc-4(qm182)* mutants. For each pool, the total concentration of cosmids was 15 μg/mL and the concentration of the co-injection marker P_{ttx-3}*::gfp* (*4*) was 185 μg/mL. Pool 2 rescued the fast defecation of *clk-1(qm30); dsc-4(qm182)* mutants. Each cosmid in pool 2 was tested individually and the cosmid K02D7 was found to rescue the *clk-1(qm30); dsc-4(qm182)* mutants. PCR products that correspond to the predicted genes on K02D7 were tested individually and it was found that the PCR product corresponding to the predicted gene K02D7.4 (from 26654 to 34896 of K02D7) could rescue the *clk-1(qm30); dsc-4(qm182)* mutants (Figure 3.10). This PCR product was amplified from N2 genomic DNA by nested PCR and contains 1.6 kb of the region upstream of *dsc-4*. PCR products were injected at a concentration of 2 μg/mL with the co-injection marker P_{ttx-3}*::gfp* at a concentration of 190 μg/mL.

To confirm that *dsc-4* corresponds to the predicted gene K02D7.4, K02D7.4 was PCR amplified from *clk-1(qm30)* and *clk-1(qm30); dsc-4(qm182)* genomic DNA and both strands were sequenced. Upon comparison with published sequences, two missense mutations were found in the K02D7.4 gene amplified from *clk-1(qm30); dsc-4(qm182)* mutants. A C→T transition at position 354 of the cDNA results in a serine to phenylalanine substitution at position 62 of the protein; A G→A transition at position 605 of the cDNA results in an alanine to threonine substitution at position 146 of the protein (Figure 3.8A,C). These mutations were absent in the K02D7.4 gene amplified from *clk-1(qm30)* mutants.

We also examined the *K02D7.4(RNAi)* phenotype in *clk-1, dsc-4* and wild-type backgrounds. We found that RNAi directed against the K02D7.4 gene almost exactly phenocopies the *qm182* mutation in the *clk-1* background. The defecation rate as well as the egg-laying rate of *clk-1(qm30); K02D7.4(RNAi)* animals is similar to that of the *clk-1(qm30); dsc-4(qm182)* mutants. Finally, we found the effect of *K02D7.4(RNAi)* is not

additive to that of the *dsc-4* mutation for defecation or egg laying in the *clk-1* background (data not shown). Also, *K02D7.4(RNAi)* in *dsc-4* mutants did not cause any obvious enhancement of the phenotype. Together, these results demonstrate that K02D7.4 is indeed *dsc-4,* and that the *qm182* allele is likely a strong or complete loss-of-functional allele.

dsc-4 encodes an 892-residue protein, which is similar to the large subunit of the microsomal triglyceride transfer protein (MTP). DSC-4 has a single clear homologue in every animal species whose genome has been sequenced, but appears to be absent from plants and unicellular organisms. The identities between homologues extend across the entire sequence and are not confined to particular regions or domains, and the alignment of DSC-4 with vertebrate MTPs does not require the introduction of numerous gaps (Figure 3.8C). When DSC-4 is compared to the NCBI non-redundant protein database using PSI-blast, the 7 proteins with the highest scores are bona fide vertebrate MTPs.

Sequencing of the dsc-4 cDNA

The yk357a6 cDNA clone corresponds to the predicted gene K02D7.4, but the clone does not contain the full 5' end of the *dsc-4* cDNA. Using an SL1-specific primer and *dsc-4* gene-specific primers, the 5' end of the *dsc-4* cDNA was amplified from a first-strand cDNA library generated by the reverse-transcription of poly(A)$^+$ selected RNA isolated from mixed-stage wild-type animals using a poly-dT primer. Both strands were sequenced (Figure 3.8A,B).

RNAi

For the *dsc-4* RNAi clone, a *Hind*III-*Xho*I fragment of yk357a6 was cloned into the *Hind*III *Xho*I sites of pPD129.36. For *sod* RNAi clones, PCR products were amplified from a first-strand cDNA library (generated by the reverse transcription of total RNA isolated from mixed-stage N2 worms using random primers). The PCR products were cloned into the *Sma*I site of pPD129.36. The following regions were used: for *sod-1,* 17287-18262

of C15F1; for *sod-2,* 1216-2354 of F10D11; for *sod-3,* 14358-15759 of C08A9; for *sod-4,* 1253-2977 of F55H2.

For *vit* RNAi clones, PCR products were amplified from N2 genomic DNA, digested and cloned into pPD129.36. For *vit-2*, an internal *Eco*RI-*Sal*I fragment was cloned (24381-25252 of C42D8). For *vit-5,* a PCR product corresponding to 9271-10130 of C04F6 was cloned into the *Pst*I and *Xba*I sites. For *vit-6*, a PCR product corresponding to 5787-6851 of K07H8 was cloned into the *Xho*I and *Hind*III sites. All inserts were sequenced to confirm the identity of the *vit* gene cloned.

To make the *ark-1* RNAi clone, a PCR product corresponding to 6525-7045 of C01C7 was amplified from N2 genomic DNA and cloned into the *Bam*H1 site of pPD129.36.

Using sid-1: We used the *sid-1(qt2)* mutant to determine whether the site of action of some of the RNAi effects observed is the intestine. The *sid-1(qt2)* mutation disrupts the process that allows RNA interference to be systemic in *C. elegans* (*5*). In *sid-1* mutants, the effect of dsRNA is confined to the site of introduction of the dsRNA, while in the wild type the effect can spread from the tissue in which the dsRNA was introduced to most of the animal, including to the germline and then to the next generation. We reasoned that in *sid-1* mutants the effect of feeding dsRNA should be confined to the intestine. As expected, given that *unc-22* is required in muscle cells, *unc-22(RNAi)* produced it's characteristic uncoordinated movement phenotype in *clk-1*, but not in *sid-1; clk-1* mutants. Furthermore, C13G3.3 RNAi produces embryonic lethality in the progeny of treated animals in the *clk-1* but not in the *clk-1;sid-1* background. This means that *sid-1(qt2)* indeed prevents ingested dsRNA to have an effect in the germline. On the other hand, RNAi against *vit-5* was able to suppress the slow germline development in the double mutants (Figure 3.2B), as expected given that *vit-5* is expressed in the intestine.

RNAi experiments were performed as described (*6*). The controls were fed the HT115 bacteria transformed with the pPD129.36/L4440 vector. For all experiments in which we were testing the effect of RNA interference with gene action, the controls used for comparison were also cultured on the RNAi plates because we found that both germline and vulval development phenotypes were different on RNAi plates, as compared to normal NGM plates.

Phenotypic Analyses

Time course analysis of egg-laying rate: L4-stage animals were picked to plates and examined three hours later. Animals that had molted to adults during this period were used for the experiment and were considered to be 1.5 hours old at the end of the interval. At 24 hour intervals, animals were singled and allowed to lay eggs for four hours. The average number of eggs laid per hour per worm was calculated.

Rate of postembryonic development: Eggs were picked to plates and examined one hour later. Animals that had hatched during this period were used for the experiment. The percentage of animals that had reached adulthood by each time point was scored.

Developmental stage of the germline: Worms were synchronized at the final molt as for the time course analyses of egg-laying rate. The proximal portion of the germline was examined using DIC microscopy either immediately afterward (for examining 1.5h old adults), or 4.5 hours later (for examining 6h old adults).

Vulva Formation: Animals were examined under the dissecting microscope and were considered to be Muv if they had more than one vulva.

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4. O. Hobert *et al.*, *Neuron* **19**, 345-57 (1997).
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Table 3.1: The effect of *ark-1(RNAi)* on germline development.

Animals were examined at 1.5 hours after the adult molt. The stages of development of the anterior (A) and posterior (P) arms are presented separately.

Genotype + RNAi construct	Gonad Arm	Percentage of germlines at each developmental stage:				n
		Before primary spermatocyte formation	Primary spermatocyte formation	After primary spermatocyte formation before oogenesis	Oogenesis	
N2 + pPD129.36	A	0	58	42	0	36
N2 + pPD129.36	P	0	60	40	0	35
N2 + *ark-1*	A	0	56	44	0	36
N2 + *ark-1*	P	0	71	29	0	35
dsc-4(qm182) + pPD129.36	A	0	21	79	0	29
dsc-4(qm182) + pPD129.36	P	0	47	53	0	30
dsc-4(qm182) + *ark-1*	A	0	4	96	0	27
dsc-4(qm182) + *ark-1*	P	0	37	63	0	27
clk-1(qm30) + pPD129.36	A	90	10	0	0	29
clk-1(qm30) + pPD129.36	P	80	20	0	0	46
clk-1(qm30) + *ark-1*	A	44	51	5	0	43
clk-1(qm30) + *ark-1*	P	6	77	17	0	47
clk-1(qm30); dsc-4(qm182) + pPD129.36	A	0	0	100	0	40
clk-1(qm30); dsc-4(qm182) + pPD129.36	P	0	15	85	0	40
clk-1(qm30); dsc-4(qm182) + *ark-1*	A	0	5	95	0	40
clk-1(qm30); dsc-4(qm182) + *ark-1*	P	0	10	90	0	40
clk-1(qm30); sid-1(qt2) + pPD129.36	A	99	1	0	0	73
clk-1(qm30); sid-1(qt2) + pPD129.36	P	88	12	0	0	73
clk-1(qm30); sid-1(qt2) + *ark-1*	A	78	19	3	0	37
clk-1(qm30); sid-1(qt2) + *ark-1*	P	56	44	0	0	41

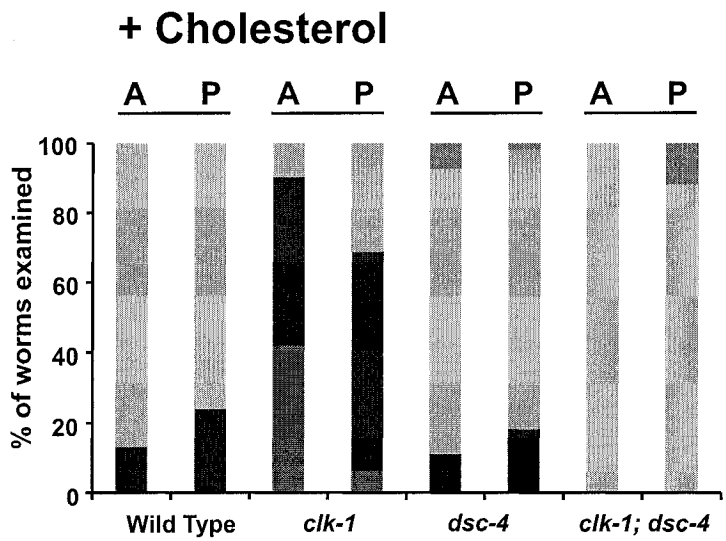
Figure 3.5: Both the *dsc-4(qm182)* mutation and reduced cholesterol intake suppress the slowed germline development of *clk-1* mutants.

The percentage of germlines observed to be at each of four different developmental stages at 1.5 hours after the adult molt. The stages of development of the anterior (A) and posterior (P) gonad arms are presented separately.

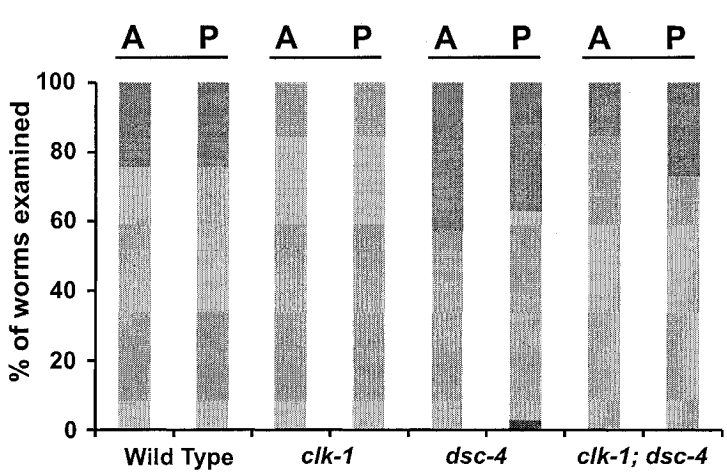
(A) Worms were cultured on NGM plates supplemented with cholesterol ($n \geq 30$).

(B) Worms were cultured on NGM plates not supplemented with cholesterol ($n \geq 19$).

A



B

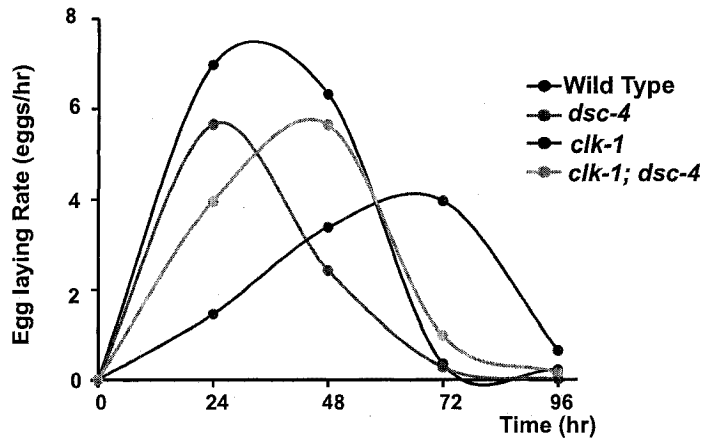


oogenesis
after primary spermatocyte formation before oogenesis
primary spermatocyte formation
before primary spermatocyte formation

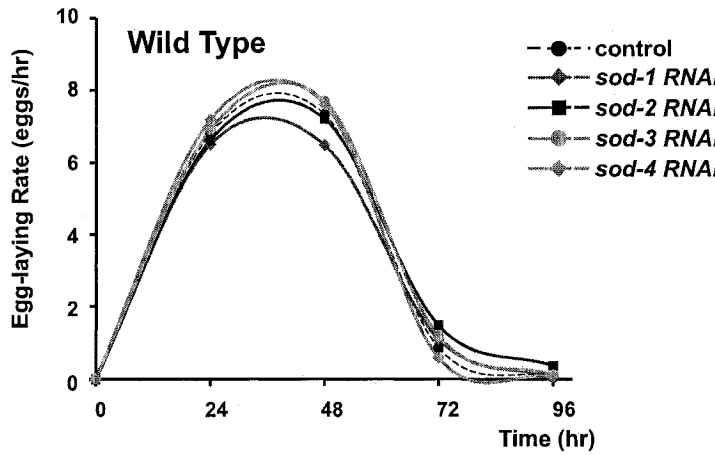
Figure 3.6: Time course analysis of egg-laying rate.

Worms were synchronized at the adult molt (Time 0) and the egg-laying rate (number of eggs laid per hour) was measured at 24h intervals. The genotypes are as follows: Wild type, N2; *dsc-4, dsc-4(qm182); clk-1, clk-1(qm30)*; and *clk-1; dsc-4, clk-1(qm30); dsc-4(qm182).* **(A)** The effect of *dsc-4* on the egg-laying rate of wild-type and *clk-1* mutant animals ($n \geq 30$). **(B)** The effect of RNAi interference against the *sod* genes on the egg-laying rate of wild-type worms ($n \geq 15$). The controls were fed on the HT115 bacteria transformed with the pPD129.36 vector. **(C)** The effect of RNAi interference against the *sod* genes on the egg-laying rate of *clk-1* mutants ($n \geq 20$). The controls were fed on the HT115 bacteria transformed with the pPD129.36 vector. **(D)** The effect of *sod-1(RNAi)* on the egg-laying rate of *dsc-4* mutants ($n \geq 23$). The non-RNAi controls were fed on the HT115 bacteria transformed with the pPD129.36 vector.

A


Egg laying Rate (eggs/hr)
Time (hr)
Wild Type
dsc-4
clk-1
clk-1; dsc-4

B


Egg-laying Rate (eggs/hr)
Time (hr)
Wild Type
control
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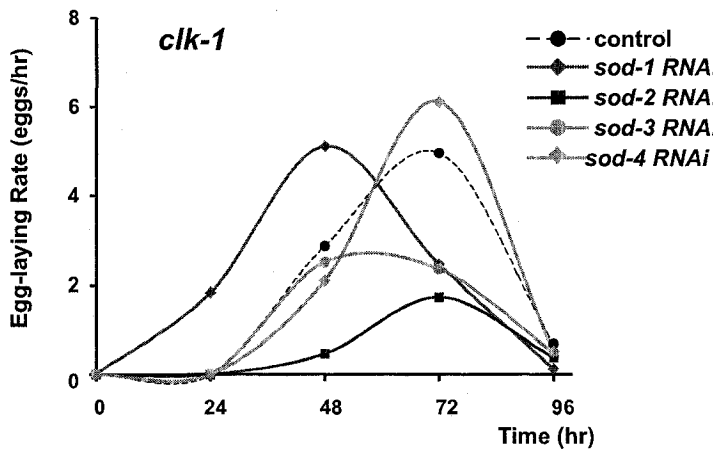
sod-2 RNAi
sod-3 RNAi
sod-4 RNAi

C


Egg-laying Rate (eggs/hr)
Time (hr)
clk-1
control
sod-1 RNAi
sod-2 RNAi
sod-3 RNAi
sod-4 RNAi

D

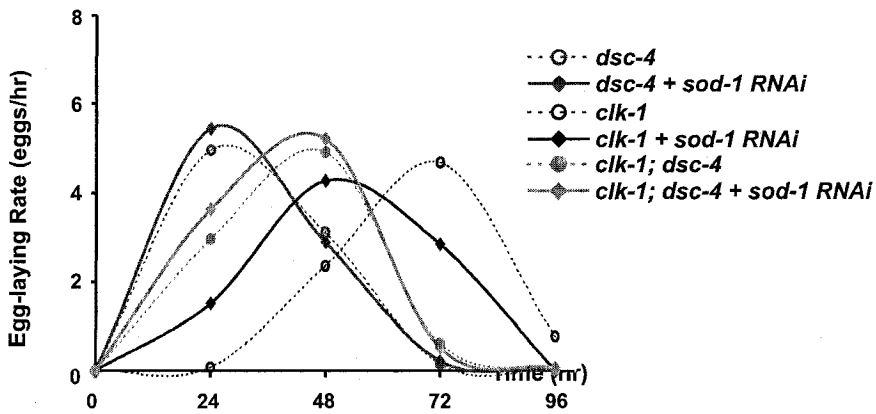

Egg-laying Rate (eggs/hr)
dsc-4
dsc-4 + sod-1 RNAi
clk-1
clk-1 + sod-1 RNAi
clk-1; dsc-4
clk-1; dsc-4 + sod-1 RNAi

Figure 3.7: The *dsc-4* mutation does not suppress the slow post-embryonic development of *clk-1* mutants.

The percentage of worms that have reached adulthood during each time interval is shown (n ≥ 50). *clk-1(qm30)* mutants as well as *clk-1(qm30); dsc-4(qm182)* double mutants all reach adulthood between 63 and 81 hours after hatching.

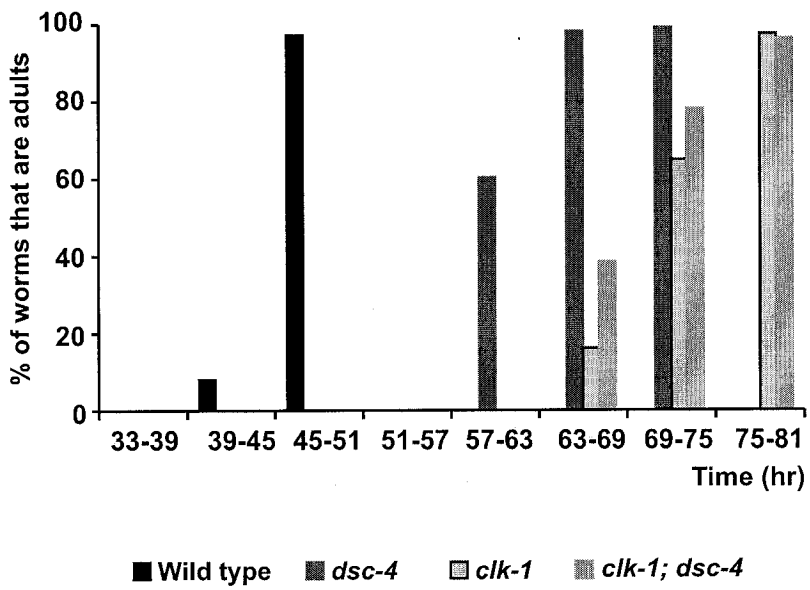

100
80
60
40
20
0
% of worms that are adults
33-39
39-45
45-51
51-57
57-63
63-69
69-75
75-81
Time (hr)
Wild type
dsc-4
clk-1
clk-1; dsc-4

Figure 3.8: *dsc-4* encodes a homologue of MTP.

(A) Sequence of the *dsc-4* cDNA. ^ indicates a predicted cleavage site by signal sequence. # indicates the mutation sites. The C354T mutation results in a Ser62Phe substitution and the G605A mutation results in an Ala146Thr substitution. The underlined residues correspond to the apoB binding domain, the dotted underlined residues correspond to the apoB and PDI binding domain, and the double underlined residues correspond to the lipid binding domain. The sequences of the 5' and 3'-UTRs have also been included. **(B)** The genomic structure of *dsc-4* gene. The filled-in and open boxes correspond to non-coding and coding regions, respectively. Sequencing of a cDNA as well as a PCR product amplified from a first-strand cDNA library (see Materials and Methods) revealed that the *dsc-4* message is SL1 trans-spliced and contains 11 exons. **(C)** Alignment of DSC-4 and vertebrate microsomal triglyceride transfer proteins (MTPs). Identical residues are highlighted in red and residues that are have >75% and >50% similarity are highlighted in blue and grey, respectively. The asterisks indicate the two mutation sites of *qm182*. A C $\rightarrow$ T transition at position 354 of the cDNA results in a serine to phenylalanine substitution at position 62 of the protein and a G $\rightarrow$ A transition at position 605 of the cDNA results in an alanine to threonine substitution at position 146 of the protein. The signal sequence is not shown.

A

```
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                      ^                                  #
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                                                                                  #
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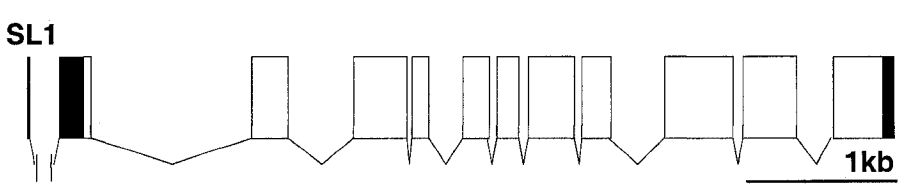

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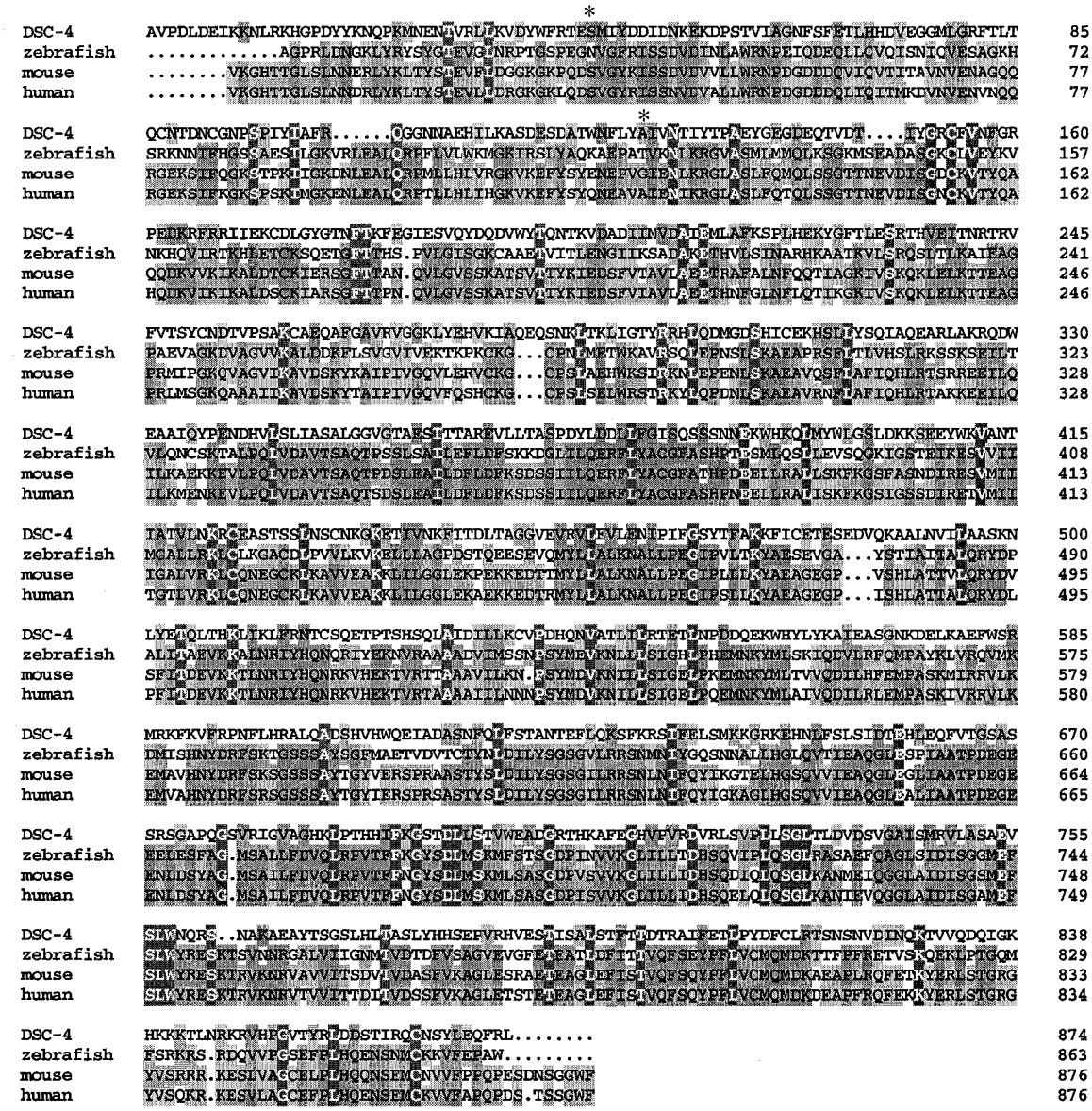
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DSC-4 PEDKRFRRIIEKCDLGYGTNFLKFEGIESVQYDQDVWYLQNTKVDADIIMVDAIEMLAFKSPLHEKYGFTLESRTHVEITNRTRV 245
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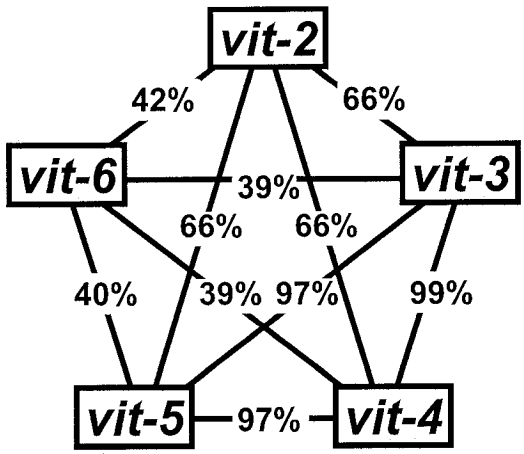
DSC-4 LYETQLTHKLIKIFRNTCSQETPTSHSQLAIDILLKCVFDHQNVATLLTRTETYNPDDQEKWHYLYKAIEASGNKDELKAEFWSR 585
zebrafish ALLTAEVEKALNRIYHQNQRIYEKNVRAAFADVIMSSNCSYMEVKNLLTSIGHLPHEMNKYMLSKIQDVLRFQMPAYKLVRQVMK 575
mouse SFLIDEVKKTLNRIYHQNRKVHEKTVRTTFAAVILKN.FSYMDVKNILTSIGELPKEMNKYMLTVVQDILHFEMPASKMIRRVLK 579
human PFLIDEVKKTLNRIYHQNRKVHEKTVRTAFAAIILNNNFSYMDVKNILTSIGELPQEMNKYMLAIVQDILRLEMPASKIVRRVLK 580
DSC-4 MRKFKVFRPNFLHRALQADSHVHWQEIADASNFQDFSTANTEFLQKSFKRSLFELSMKKGRKEHNLFSLSIDTEHLEQFVTGSAS 670
zebrafish DMISHNYDRFSKTGSSSAYSGFMAETVDVTCTYNIDILYSGSGVLRRSNMNYGQSNNALLHGLQVTIEAQGLESPIAATPDEGE 660
mouse EMAVHNYDRFSKSGSSSAYTGYVERSPRAASTYSIDILYSGSGILRRSNLNFQYIKGTELHGSCVVIEAQGLEGLIAATPDEGE 664
human EMVAHNYDRFSRSGSSSAYTGYIERSPRSASTYSIDILYSGSGILRRSNLNFQYIGKAGLHGSQVVIEAQGLEALIAATPDEGE 665
DSC-4 SRSGAPQCSVRIGVAGHKLPTHHDEKGSTDLLSTVWEADGRTHKAFECHVFVRDVRLSVPLLSGLTLDVDSVGALSMRVLASADV 755
zebrafish EELESFAG.MSALLFDVQLREVTFRFKGYSDLMSKMFSTSGDPINVVKGILLLIDHSQVIPLQSGLFASAEFCAGLSIDISGCMEF 744
mouse ENLDSYAG.MSALLFDVQLREVTFRFNGYSDLMSKMLSASGDPVSVVKGILLLIDHSQDIQLQSGLFANMEIQGGLAIDISGSMEF 748
human ENLDSYAG.MSALLFDVQLREVTFRFNGYSDLMSKMLSASGDPISVVKGILLLIDHSQELQLQSGLFANIEVQGGLAIDISGAMEF 749
DSC-4 SLWNQRS..NAKAEAYTSGSLHLTASLYHHSEPVRHVESTISATSTFTMDTRAIFETLPYDFCLRTSNSNVDINQKTVVQDQIGK 838
zebrafish SLYYRESKTSVNNRGALVIIGNMTVDTDFVSAGVEVGFELEATIDFTTIVQFSEYPFLVCMCMDKTTFPFRETVSKQEKLPTGQM 829
mouse SLYYRESKTRVKNRVAVVITSDVTVDASFVKAGLESRAFEAGIEFTSTVQFSQYPFLVCMCMDKAEAPLRQFETKYERLSTGRG 833
human SLYYRESKTRVKNRVTVVITTDITVDSSFVKAGLETSTEIEAGIEFISTVQFSQYPFLVCMCMDKDEAPFRQFEKKYERLSTGRG 834
DSC-4 HKKKTLNRKRVHEGVTYRLDDSTIRQCNSYLEQFRL..... 874
zebrafish FSRKRS.RDQVVFGSEFFLHQENSNMCKKVFEPAW..... 863
mouse YVSRRR.KESLVACCELPLHQQNSEMCNVVEPPQPESDNSGGWF 876
human YVSQKR.KESVLACCEFPLHQENSEMCKVVFAPQPDS.TSSGWF 876

Figure 3.9: RNA interference against *vit-3, -4,* and *-5* suppresses the slow germline development of *clk-1* mutants.

(A) A comparison of the identity between *vit* genes. The percentages of identical nucleotides between *vit* gene sequences are indicated. **(B)** The effect of *vit-5(RNAi)* on egg-laying rate. A time course experiment was performed with the wild type, *clk-1(qm30)* and *clk-1(qm30); dsc-4(qm182)* mutants treated with *vit-5(RNAi)* ($n \geq 27$). Since the coding region of the *vit-5* RNAi clone used is 98% identical to both *vit-3* and *-4*, the *vit-5* RNAi treatment is expected to disrupt all three genes. *vit-5(RNAi)* affects the egg-laying rate only in the *clk-1(qm30)* background. That the effect of *vit-5(RNAi)* is not additive to that of the *dsc-4* mutation suggests that the effect of the *dsc-4* mutation is due to reduced secretion of VIT-3, -4 and -5.

A


vit-2
42%
66%
vit-6
39%
vit-3
66%
66%
40%
39%
97%
99%
vit-5
97%
vit-4

B

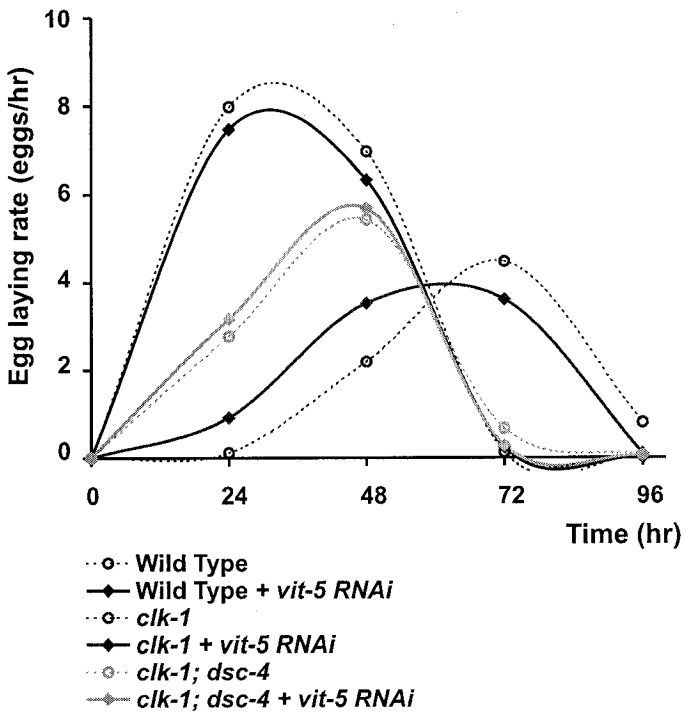
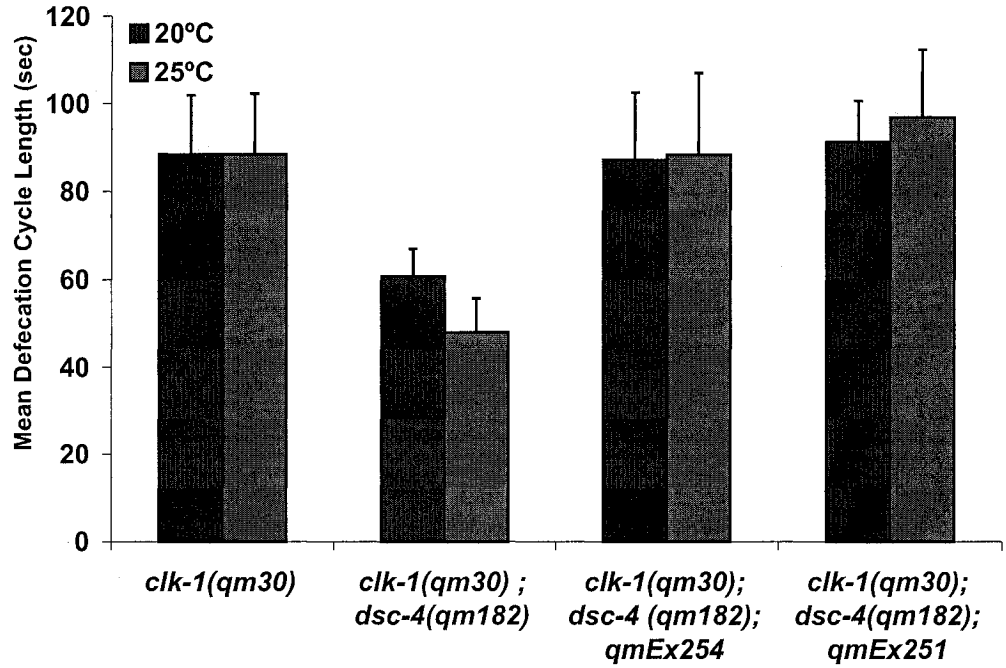
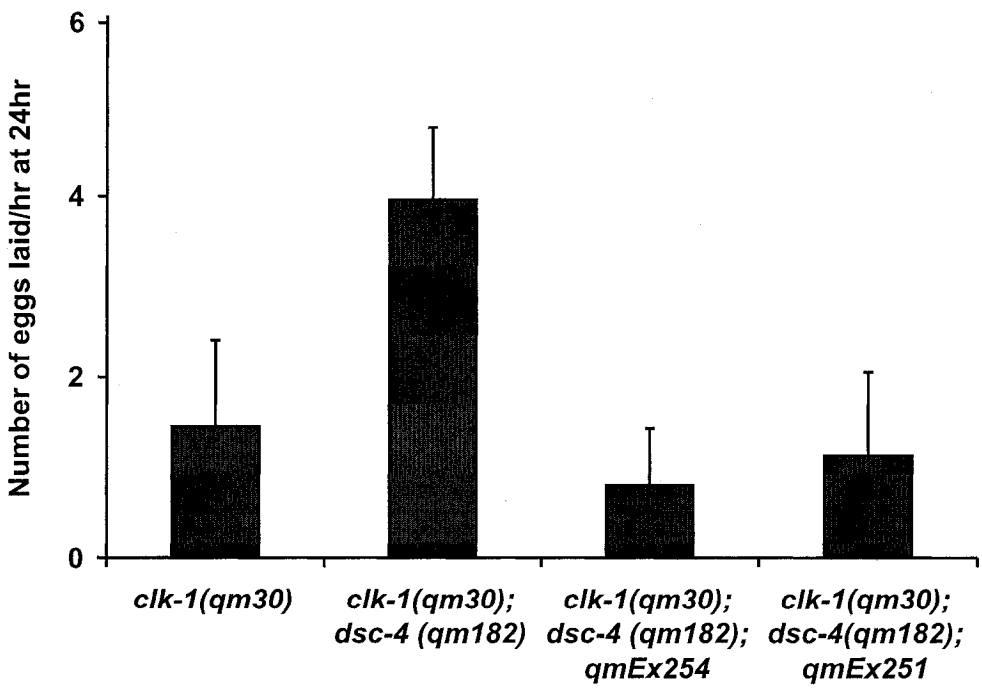

Egg laying rate (eggs/hr)
10
8
6
4
2
0
0
24
48
72
96
Time (hr)
Wild Type
Wild Type + vit-5 RNAi
clk-1
clk-1 + vit-5 RNAi
clk-1; dsc-4
clk-1; dsc-4 + vit-5 RNAi

Figure 3.10: K02D7.4 rescues both the suppression of slow defecation and slow germline development of *clk-1(qm30)* mutants conferred by *dsc-4(qm182).*

qmEx254 and *qmEx251* are two different extrachromosomal arrays containing the K02D7.4 gene. **(A)** Defecation. The *dsc-4(qm182)* mutation suppresses the slow defecation of *clk-1(qm30)* mutants at 20 and 25°C. Both extrachromosomal arrays can rescue this suppressing effect. Each bar represents the mean defecation cycle length of >15 animals scored for 5 consecutive defecation cycles at either 20 or 25°C; the error bars represent the standard deviations of the means. **(B)** Germline development. The *dsc-4(qm182)* mutation suppresses the delayed germline development of *clk-1* mutants, which results in an increased rate of egg laying shortly after the adult molt. Both extrachromosomal arrays rescue this effect. Each bar represents the mean egg-laying rate (number of eggs laid per hour) of > 30 animals at 24 hours after the adult molt; the error bars represent the standard deviations. That wild-type copies of K02D7.4 can rescue the phenotypes caused by the *dsc-4(qm182)* mutation indicates that *dsc-4* corresponds to K02D7.4.


120
100
80
60
40
20
0
20°C
25°C
Mean Defecation Cycle Length (sec)
clk-1(qm30)
clk-1(qm30) ; dsc-4(qm182)
clk-1(qm30); dsc-4 (qm182); qmEx254
clk-1(qm30); dsc-4(qm182); qmEx251


6
4
2
0
Number of eggs laid/hr at 24hr
clk-1(qm30)
clk-1(qm30); dsc-4 (qm182)
clk-1(qm30); dsc-4 (qm182); qmEx254
clk-1(qm30); dsc-4(qm182); qmEx251

Connecting Statement: Bridging Chapters 3 and 4

In the previous chapter, I reported the characterization of the Class I *dsc* mutant, *dsc-4*. In the following chapter, I report on the characterization of the other Class I *dsc* mutant, *dsc-3,* including its interactions with *dsc-4*.

Chapter 4:

dsc-3

Abstract

The *dsc-3* and *dsc-4* mutants were isolated in a screen for mutations that suppress the slow defecation phenotype of *clk-1*. Here we report our analysis of *dsc-3* and its interactions with *dsc-4*. We previously showed that *dsc-4* encodes the worm homolog of MTP, a lipid transfer protein necessary for lipoprotein metabolism. *dsc-3* appears to be allelic to *tat-2*, a type IV P-type ATPase. *tat-2* is related to a family of human aminophospholipid transporters that includes ATP8B1/FIC1, whose inactivation results in cholestatic liver disease. We show that *dsc-3* and *dsc-4* affect defecation by a similar mechanism, likely by affecting lipoprotein secretion. However, *dsc-3* also affects growth by a mechanism that is cholesterol-dependent but appears to be lipoprotein-independent. Thus, *dsc-3* and *dsc-4* appear to affect distinct but overlapping aspects of lipid metabolism. We speculate that DSC-3 is involved in cholesterol uptake. The identification of additional *dsc* mutants might help to identify other cellular processes that impinge on cholesterol and lipoprotein metabolism and to establish *C. elegans* models of important human diseases.

Introduction

Atherosclerosis is the primary cause of both heart disease and stroke, which are the major causes of death in Westernized countries. Atherosclerosis is a progressive disease, characterized by the accumulation of lipids in the large arteries (reviewed in (Lusis, 2000)). A major risk factor for atherosclerosis is elevated levels of plasma cholesterol. The levels of plasma cholesterol in the body are controlled by three different processes: absorption of dietary cholesterol, endogenous synthesis of cholesterol and biliary cholesterol excretion. The biliary system plays a key role in the whole-body sterol balance as it is the only system that can rid the body of cholesterol. Cholesterol can be excreted directly into bile, or first catabolised into bile acids, which are then secreted into bile and can be eliminated via the intestine (reviewed in (Lu et al., 2001)).

Although high levels of cholesterol are associated with disease states, cholesterol plays key roles in many cellular processes. It plays an essential role in membranes, where it both influences their physico-chemical properties, such as fluidity and ion permeability, but also helps to organize microdomains (the so-called rafts), which are specialized loci for protein sorting and signal transduction (Brown and London, 1998; Haines, 2001; Simons and Toomre, 2000). Cholesterol is also a precursor for important molecules such as steroid hormones, oxysterols, vitamin D, and, as mentioned above, bile acids.

In *C. elegans*, much less is known about how the levels of cholesterol are controlled and in what cellular processes cholesterol functions. However, it has been well established that nematodes are auxotrophic for sterols. Although they possess a number of enzymes that can modify sterols (reviewed in (Chitwood, 1999; Entchev and Kurzchalia, 2005)), they are incapable of *de novo* sterol biosynthesis, and therefore depend upon exogenous sterol for their growth and reproduction (Hieb and Rothstein, 1968). Unlike in mammals, which predominantly

accumulate cholesterol, the major sterol found in *C. elegans* is 7-dehydrocholesterol. Sterols such as cholesterol, ergosterol, sitosterol, or lathosterol, which can be converted into 7-dehydrocholesterol, as well 7-dehydrocholesterol itself, can all support growth (Chitwood et al., 1984; Merris et al., 2003).

Simply not adding sterols to the growth media has only a weak effect on development and growth, presumably because the various components of the media are products of animal and plant origin and likely contain trace amounts of cholesterol or other sterols. On this "cholesterol-depleted" media, some larvae have molting defects, reduced body sizes, gonadal migration defects, and exhibit uncoordinated movement (Shim et al., 2002). However, under these conditions the worms can be propagated indefinitely. Under stricter, presumably "cholesterol-free" conditions, worms can only be maintained for one generation. The first generation can develop from eggs into fertile adults, but the brood size is reduced. The second generation arrests at early larval stages and exhibits molting defects and partial dauer formation (Crowder et al., 2001; Matyash et al., 2004; Merris et al., 2004). The numerous effects of cholesterol deprivation have recently been reviewed in detail (Entchev and Kurzchalia, 2005; Kurzchalia and Ward, 2003).

There is much data suggesting that, like in humans, sterols are required in *C. elegans* for the production of steroid hormones. One type of hormone for which they are required is necessary to prevent entry into the dauer stage, an alternative larval stage into which *C. elegans* will enter in response to adverse environmental conditions. Mutations in *daf-9* and *daf-12*, which encode a cytochrome P450 related to those involved in the biosynthesis of steroid hormones in mammals and a nuclear hormone receptor, respectively, result in a constitutive dauer (Daf-c) phenotype, even under favorable conditions (Antebi et al., 1998; Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002). Mutation of *sdf-9*, a protein tyrosine phosphatase-like molecule that appears to potentiate *daf-9*, also results in

a Daf-c phenotype (Ohkura et al., 2003). The Daf-c phenotype of all three mutants is enhanced by lowering the media cholesterol (Gerisch et al., 2001; Jia et al., 2002; Ohkura et al., 2003), and both *daf-9* and *sdf-9* can be rescued by increasing the media cholesterol (Jia et al., 2002; Ohkura et al., 2003). Moreover, it has recently been shown that lipophilic extracts from wild-type but not *daf-9* mutants are capable of preventing dauer formation in a number of Daf-C mutants (Gill et al., 2004). Other researchers have partially purified a cholesterol-derived hormone, gramravali, which promotes reproductive growth under dauer-inducing conditions (Matyash et al., 2004).

Cholesterol also appears to be involved in the production of a hormone that regulates molting. The hormone ecdysone regulates molting in insects. Although no clear ecdysone receptor exists in *C. elegans*, cholesterol deprivation produces a molting defect similar to that observed by disruption of the nuclear hormone receptor *nhr-23*, the *C. elegans* homolog of a Drosophila orphan nuclear hormone receptor that is induced by ecdysone (Kostrouchova et al., 1998; Kostrouchova et al., 2001). Moreover, mutation of *lrp-1*, the worm homolog of megalin/gp330, a mammalian protein involved in the uptake of Vitamin D, results in a similar molting defect, which is enhanced by cholesterol deprivation (Yochem et al., 1999). Similar molting defects are caused by RNAi knockdown of the *dab-1* and *imp-2* genes, which have been speculated to be involved in lipoprotein receptor trafficking and steroid hormone production, respectively (Grigorenko et al., 2004; Kamikura and Cooper, 2003).

However, in contrast to most animals, *C. elegans* does not appear to require cholesterol in its membranes. As described above, only trace amounts of sterols in the media can support growth, suggesting that sterols do not need to be incorporated into every cellular membrane. In support of this, a direct quantification has revealed that there is ~20 times less cholesterol in the membranes of *C. elegans* embryos than in the membranes of vertebrate cells (Chitwood et al., 1984). Moreover, using

either the fluorescent cholesterol analogue dehydroergosterol (DHE), or the antibiotic filipin, which labels cholesterol, it has been shown that only a very small subset of cells contain cholesterol in their membranes (Matyash et al., 2001; Merris et al., 2003). Finally, the enantiomer of cholesterol cannot substitute for natural cholesterol to support growth of *C. elegans*, although the physical properties of membranes are generally not influenced by enantio-specific sterol-lipid interactions (Crowder et al., 2001).

Despite the differences between nematodes and other animals, there are a number of examples that suggest that *C. elegans* may be a useful model for studying many aspects of lipid biology, particularly those with disease relevance. For example, both McKay et al and Ashrafi et al (Ashrafi et al., 2003; McKay et al., 2003) have used an RNAi approach to screen for genes involved in fat storage, and in both cases, identified genes with clear mammalian homologs known to be involved in lipid metabolism. *ncr-1* and *ncr-2*, the worm homologs of NPC1, a human disease gene implicated in intracellular cholesterol trafficking, also appear to be involved in some form of cholesterol processing (Li et al., 2004; Sym et al., 2000). *dsc-4*, the worm homolog of the microsomal triglyceride transfer protein (MTP), a protein required for the assembly of apoB-containing lipoproteins in vertebrates, also appears to be involved in the secretion of a lipoprotein-like particle in worms (Shibata et al., 2003).

Here we report our analysis of *dsc-3,* and its interactions with *dsc-4.* Both genes were isolated in a screen for defecation suppressors of *clk-1.* As described above, *dsc-4* encodes the worm homolog of MTP, a lipid transfer protein necessary for lipoprotein metabolism. Here we present evidence that *dsc-3* is allelic to *tat-2,* a type IV P-type ATPase. *tat-2* is related to a family of human aminophospholipid transporters that includes ATP8B1/FIC1, mutation of which results in cholestatic liver disease. We show that *dsc-3* and *dsc-4* affect defecation by a similar mechanism, likely by affecting lipoprotein secretion. However, *dsc-3* also affects growth. The

mechanism by which it affects growth is probably lipoprotein-independent, although it is strongly affected by the availability of cholesterol. We speculate on possible functions of DSC-3, and on the mechanisms by which *dsc-3* suppresses *clk-1*.

Materials and Methods

Culture Methods and Strains

C. elegans were cultured under standard conditions at 20°C and were fed on the *E. coli* strain OP50. Unless otherwise indicated, the worms were cultured on NGM plates supplemented with the standard amount of cholesterol (5 µg/mL). "High cholesterol" plates were supplemented with 50 µg/mL cholesterol; "low cholesterol" plates were either supplemented with 0.5 µg/mL cholesterol or were not supplemented with any cholesterol, as indicated.

The mutations used in this study are as follows: *clk-1(qm30) III*; *dpy-9(e12)*, *dsc-4(qm182)*, *unc-33(e204)*, *dpy-13(e184)*, *dsc-3(qm179, qm180, qm184)*, *tat-2(tm1634)* (kindly provided by Shohei Mitani and the National Bioresource Project for the Nematode), *unc-5(e53)*, *unc-24(e138)*, *and dpy-4(e166) IV*.

Mapping Experiments

3-point mapping experiments were carried out essentially as described (Branicky et al., 2001). Briefly, to avoid marker effects, homozygous recombinant progeny were isolated from F2 recombinant animals, and were crossed with homozygous *dsc-3* mutant males. Defecation was scored in the F1 cross-progeny. All experiments were carried out in the *clk-1(qm30)* background.

Construction of the *dsc-4 dsc-3* double mutants

To construct the *dsc-4 dsc-3* double mutants, *dpy-9 dsc-3/+ dsc-3* males were crossed with *dsc-4 unc-24* hermaphrodites. F1's that produced both

Dpy and Unc progeny were selected (*dpy-9 + dsc-3 +/+ dsc-4 + unc-24*), and from their progeny, wild-type F2's that produced both wild-type and Dpy F3 progeny (*+ dsc-4 dsc-3/dpy-9 + dsc-3*) were selected, and the *dsc-4 dsc-3* recombinant chromosome was homozygosed. To construct the *clk-1; dsc-4 dsc-3* triple mutant, *clk-1; dpy-9 dsc-3/+ dsc-3* males were crossed with *clk-1; dsc-4 unc-33* hermaphrodites. F1's that produced both Unc and Dpy progeny were selected (*clk-1; dpy-9 + + dsc-3/+ dsc-4 unc-33 +*), and from their progeny, wild-type F2's that produced both wild-type and Dpy progeny were selected (*clk-1; + dsc-4 dsc-3/dpy-9 + dsc-3*), and the recombinant *dsc-4 dsc-3* chromosome was homozygosed. For both the double and the triple mutants, the presence of both the *dsc-3* and the *dsc-4* mutations were confirmed by complementation tests.

Defecation Analyses

Assays were performed essentially as described (Branicky et al., 2001). Animals were scored after shifts to 25°C, for 3 or 5 consecutive cycles, as indicated.

Cholesterol Experiments

Worms of the P0 generation were transferred to the experiment plates at the L4 stage. For the defecation assays, the F1's were switched to 25°C and scored 2-6 hours after switching. For the growth rate and larval arrest experiments, ~20 P0 worms were allowed to lay eggs for 6 to 8 hours and the growth of the F1's was monitored.

RNAi

A portion of the *tat-2* cDNA (from 11028 to 11731 of the coding region, relative to the ATG) was amplified from a cDNA library, which was generated from RNA extracted from mixed-stage worms and random hexamer primers ($pd(N)_6$, Amersham Biosciences), and cloned into the *Pst*I and *Nhe*I sites in the pPD129.36 vector (kindly provided by Dr. A. Fire). The portion of the cloned cDNA flanked by the T7 promoter sites was then amplified from the clone and the resulting PCR product was

used as template for in vitro transcription (Promega Ribomax). Double stranded RNA was injected into *clk-1(qm30)* animals at a concentration of ~1 μg/μL as described (Fire et al., 1998).

Results and Discussion

dsc-3 and *dsc-4* affect defecation by a common mechanism

The *dsc-3(qm179, qm180* and *qm184)* mutants were isolated in a screen for the slow defecation phenotypes of *clk-1(qm30)* (Branicky et al., 2001). Two classes of mutants were isolated from the screen. The Class I mutants suppress the lengthened cycle of *clk-1(qm30)* at 20°C and after shifts to 25°C. The Class II mutants only suppress at 20°C. The *dsc-3* mutants together with *dsc-4(qm182)*, define Class I.

We constructed *dsc-4 dsc-3* double and *clk-1; dsc-4 dsc-3* triple mutants to test whether the effects of the *dsc* mutations were additive to each other. As shown in Figure 4.1, the defecation phenotype is essentially the same for both *dsc-3(qm179)* and *dsc-4(qm182)* mutants. Furthermore, in both wild-type (N2) and *clk-1(qm30)* mutant backgrounds, their effects are not additive to each other, that is, double mutants are not more severely affected than the single mutants. Taken together, these two observations suggest that the *dsc-3* and *dsc-4* mutants likely affect defecation by a similar mechanism.

dsc-3 is allelic to *tat-2*

dsc-4 encodes the worm homolog of the Microsomal Triglyceride Transfer Protein (MTP) (Shibata et al., 2003), a protein known to be required for the formation of apoB-containing lipoproteins in vertebrates, including the low density lipoprotein (LDL). Based on a number of observations, we have suggested that *dsc-4* is also required for the formation of LDL-like lipoproteins in worms. We proposed a model based on LDL existing in both native and oxidized forms, with the native form being converted to the oxidized form through the action of ROS, and presumably eliminated as

damaged. We speculated that, as *clk-1* mutants have lower levels of ROS, they have higher levels of native LDL. The *dsc-4* mutation can compensate for this increase by lowering the primary production of LDL. Similarly, *dsc-3* might somehow, but maybe more indirectly, lower LDL levels, thereby suppressing *clk-1*.

We mapped *dsc-3* to position 1.34 between *dpy-13* and *unc-5* on LG IV (Table 4.1). We assayed the 8 cosmids that span this region (from ~1.07 to 1.56) for rescuing activity. None of the cosmids were able to rescue the mutants (Table 4.2), suggesting that the gene corresponding to *dsc-3* was not contained on any of these cosmids. In fact, there are 2 cosmid gaps in this region. The genome sequence in these regions was derived from YACs rather than cosmids.

Based on the idea that *dsc-3* could be involved in lipid metabolism like *dsc-4*, we identified a candidate gene in this region, *tat-2*, which was not entirely contained on any of the cosmids with which we had attempted transformation rescue. The *tat-2* gene is a very large gene, spanning over 13 kb, only a portion of which is contained in the H06H21 cosmid. Two lines of evidence suggest that *dsc-3* is indeed allelic to *tat-2*. Firstly, we found that *tat-2(RNAi)* could phenocopy the *dsc-3* mutants (Figure 4.2A). The defecation rate of the *clk-1(qm30); tat-2(RNAi)* mutants is indistinguishable from that of the *clk-1(qm30); dsc-3* mutants. In addition, *clk-1(qm30); tat-2(RNAi)* mutants show the same pale appearance that is observed in the *clk-1(qm30); dsc-3* mutants after shifts to 25°C (data not shown). Secondly, we found that the *tat-2(tm1634)* deletion phenocopies and fails to complement the *dsc-3(qm179)* mutation (Figure 4.2B). Together, these observation strongly suggest that *dsc-3* and *tat-2* are allelic. To confirm this, in the future we will need to identify the *dsc-3* lesions and/or rescue the *dsc-3* mutants with the *tat-2* gene.

tat-2, for Transbilayer Amphipath Transporters, encodes a Type IV, P-type ATPase, which is an ATP-dependent amino-phospholipid transporter (Tang et al., 1996). The closest worm homolog of *tat-2* is *tat-1*,

with which it shares 30% identity. In humans, *tat-2* is most homologous to a family of Type IV P-type ATPase genes (ATP8B1, ATP8B2, and ATP8B4, with which it shares 50%, 56% and 54% sequence identity, respectively) (Figure 4.3). On the other hand, sequence analysis suggests that *tat-1* is more similar to ATP8A1 and ATP8A2, a closely related family of Type IV P-type ATPases. For example, *tat-1* share 49% and 46% identity with ATP8A1 and ATP8A2, respectively, but only 36%, 34% and 37% with ATP8B1, ATP8B2, and ATP8B4, respectively. This suggests that *tat-2* is likely to be the worm ortholog of the ATP8B family.

Of these six human genes, only ATP8B1 has been studied extensively and in a disease context. ATP8B1 corresponds to the disease locus FIC1/PFIC1/BRIC (for Familial Intrahepatic Cholestasis/ Progressive Familial Intrahepatic Cholestasis/Benign Recurrent Intrahepatic Cholestasis), mutation of which produces cholestasis, a liver disease brought about by impaired bile flow (Bull et al., 1998). Bile is a complex fluid that contains bile acids, cholesterol, phospholipids and bilirubin. It is synthesized by hepatocytes and is then secreted across the canilicular membrane into the biliary tract and then into the intestine. Bile acids, which themselves are cholesterol derivatives, are essential for the digestion and absorption of lipids and lipid-soluble vitamins in the small intestine. Although a large amount of bile acids are secreted into the intestine, the vast majority are re-absorbed in the ileum and delivered back to the liver (reviewed (Trauner and Boyer, 2003)). Given the expression of ATP8B1 in both the canilicular membrane in the liver and the apical membrane of the intestine, this protein could function in both the transport of bile acids from the liver and their re-absorption in the intestine (Ujhazy et al., 2001). Other manifestations of the disease suggest that it may also have more general roles in mechanisms of secretion and absorption (Bull et al., 1998).

The exact biochemical function of this protein is currently not well understood. Several researchers have suggested that ATP8B1 is an

aminophospholipid flippase, whose activity maintains the asymmetric distribution of lipids in the plasma membrane (Bull et al., 1998; Daleke, 2003; Ujhazy et al., 2001). Another possibility is that it is not directly involved in the secretion or absorption of bile acids, but affects membrane composition, and by doing so affects the function of other transporters such as BSEP/ABCB11, a major bile acid transporter.

dsc-3 mutants are hypersensitive to low media cholesterol

Given the role of bile acid secretion in the absorption of lipids and the phenotypic similarities between *dsc-3* and *dsc-4*, we speculated that *dsc-3* may be involved in cholesterol absorption. One way to test whether a mutant is involved in some aspect of cholesterol metabolism, is to test whether the mutant is hypersensitive to lowering the amount of cholesterol in the growth media, or whether they can be rescued by increasing the amount of cholesterol in the growth media. For example, the constitutive dauer phenotype exhibited by mutants of the worm Niemann-Pick C genes (*ncr-1 ncr-2* double mutants) is partially rescued by adding 5 times the normal amount of cholesterol to their growth media (Li et al., 2004), whereas the recovery of *daf-9* mutants from the dauer stage is dramatically reduced by lowering media cholesterol to 1/100 of the normal amount (Jia et al., 2002).

As described in the Introduction, lowering cholesterol can produce numerous phenotypes including slow growth, larval arrest and dauer formation. We tested whether the growth rate of the *dsc* mutants was affected by lowering the level of cholesterol. We found that the growth rates of all genotypes, including that of the wild type, are greatly slowed down on plates to which no cholesterol has been added (data not shown). In contrast, by adding only 1/10 of the normal amount of cholesterol (0.5 µg/mL), most genotypes are only mildly affected. As shown in Figure 4.4, the wild-type is slowed down by about half a day. *clk-1, dsc-4* and *clk-1; dsc-4* mutants are similarly or even less affected. On the other hand, the *dsc-3* mutants are hypersensitive to the lowered amount of cholesterol,

exhibiting even slower growth and an increased percentage of dead and arrested larvae.

To further examine the larval arrest phenotype, we cultured worms on plates not supplemented with any cholesterol (Figure 4.5). As mentioned above, under these conditions all strains grow slowly, but, with the exception of strains that contain a *dsc-3* mutation, most animals were able to reach adulthood. Thus, the *dsc-3* mutation confers a hyper-sensitivity to lowered cholesterol, resulting in 35% to 80% larval arrest, depending on the genetic background. It is not yet clear whether the *dsc-4* mutation also confers some level of hyper-sensitivity as *dsc-4* has no effect by itself. In fact, only in one of several experiments (the data shown are for 2 that were precisely quantified), did the *clk-1; dsc-4* mutants exhibit substantial growth arrest. Furthermore, in this case the majority of larvae that were arrested were arrested as dauer larvae, whereas the *dsc-3* mutants tend to arrest as sickly L2 or L3 larvae. In addition, the *dsc-4* mutation appears to enhance the larval arrest of *dsc-3* only in the wild-type background. In conclusion, the effect of low cholesterol on *dsc-4* will need to be examined further. Taken together, these observations suggest that DSC-3 is involved in some aspect of lipid metabolism that is affected by the amount of cholesterol in the media. This could be consistent with a role for DSC-3 in lipid absorption. On the other hand, the *dsc-4* mutants are a lot less sensitive to the levels of cholesterol in the media, suggesting that, either lipoprotein secretion does not impact growth rate, or, that the amount of cholesterol in the media is not limiting for lipoprotein secretion. In fact, these considerations suggest that *dsc-3* regulates growth by a lipoprotein-independent mechanism.

High cholesterol can rescue the defecation phenotypes of both *dsc-3* and *dsc-4* mutants, but only in certain genetic backgrounds

As a next step, we tested whether *dsc-3* and *dsc-4* mutants could be rescued by increasing the amount of media cholesterol. We compared the defecation rate on plates containing 10 times the normal amount of media

cholesterol (50 µg/mL) to the rate on normal plates (Figure 4.6). (We tested a number of concentrations and found the effect to be maximal at 50 µg/mL; data not shown). In the wild-type background, only *dsc-3* mutants are rescued by increased amounts of cholesterol. In contrast, in the *clk-1* background, only the *dsc-4* mutants are partially rescued.

Although this may seem paradoxical at first, we propose the following model. We have previously speculated that the defecation rate is controlled by the level of lipoprotein secretion (Shibata et al., 2003). If this is true, there may be thresholds to the amounts of cholesterol that are required to stimulate or inhibit lipoprotein secretion. *clk-1* likely decreases lipid oxidation, which in turn may increase the lipid pool and stimulate lipoprotein secretion, thus resulting in slow defecation. We propose that increasing the amount of available cholesterol also increases lipoprotein secretion and thereby could results in even slower defecation. As described above, DSC-3 might be involved in cholesterol uptake, and *dsc-3* mutants might be cholesterol depleted. By increasing cholesterol in the media, the pool can be slightly increased. In *clk-1(+); dsc-3* mutants this stimulates lipoprotein secretion and slows down defecation. In the *clk-1(qm30); dsc-3* mutants, the pool is already larger, thanks to the action of the *clk-1* mutation, so the small amount by which the pool is increased by the additional cholesterol in the media is not sufficient to stimulate secretion. The situation with *dsc-4* is different. As *dsc-4* is directly involved in lipoprotein secretion, and *dsc-4* mutants have impaired lipoprotein secretion, we can expect that the threshold amount of lipid required to stimulate lipoprotein secretion is likely higher in this background. Consistent with this, we find that lipoprotein secretion can only be stimulated in *dsc-4* mutants by both removing the activity of *clk-1,* and increasing the amount of media cholesterol.

The functions of Class I *dsc* mutants

Taken together, these results are consistent with DSC-3 playing a role in cholesterol uptake. By influencing the amount of amount of cholesterol in

the pool, this in turn can affect the level of lipoprotein secretion and the defecation rate, in a manner similar to *dsc-4*. It is tempting to speculate that, like ATP8B1, DSC-3 could act via a worm bile analogue, perhaps by affecting membrane composition and in turn affecting bile transporters. Indeed, there are numerous bile transporter-related ABC transporters in *C. elegans*, some of which have been shown to be expressed in the intestine (Sheps et al., 2004; Zhao et al., 2004). Alternatively, as ATP8B1 may also have general roles in absorption and secretion, DSC-3 may affect lipid absorption more directly. One way to investigate the function of DSC-3 could be to directly measure the amounts of cholesterol and other lipids in the various mutant backgrounds.

We have previously shown that *dsc-4* is likely required for the formation of LDL-like lipoproteins in *C. elegans*. Reducing lipoprotein secretion appears to affect the defecation rate, most profoundly in the *clk-1(qm30)* background. Here we have shown that *dsc-3* acts in a different cellular process than *dsc-4*, but by affecting lipid levels in turn affects lipoprotein secretion and defecation. This suggests that other genes that can mutate to produce a Class I Dsc phenotype may participate in even additional cellular processes that impinge on lipoprotein metabolism. Given the role of lipoproteins in major human diseases, the identification of additional *dsc* mutants could help to establish important disease models in *C. elegans.*

Acknowledgements

I gratefully acknowledge Yukimasa Shibata, with whom I have collaborated on the mapping of *dsc-3*, and whom I thank for constructing the *dsc-4 dsc-3* double mutants. I also thank Tanya Rodrigues for help with some of the cholesterol experiments. The *dsc-3(tm1634)* deletion strain was kindly provided by Dr. Shohei Mitani and the National Bioresource Project for the Nematode.

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Table 4.1 Summary of Mapping Experiments Used to Genetically Position *dsc-3*

Mapping Experiment	Mapping Result	Genetic Position (95% Confidence Interval)
clk-1; dsc-3 X *clk-1; unc-33 dpy-4*	*unc-33* 33/114 *dsc-3* 81/114 *dpy-4*[1]	1.1 (-0.36 to 2.80)
clk-1; dsc-3 X *clk-1; unc-5 dpy-4*	0/28 Unc picked up *dsc-3* 32/32 Dpy picked up *dsc-3*[2]	left of unc-5
clk-1; dsc-3 X *clk-1; dpy-13 unc-5*	*dpy-13* 39/57 *dsc-3* 18/57 *unc-5*[3]	1.34 (1.07 to 1.56)

[1]Represents the pooled data for 3 independent experiments carried out with *dsc-3(qm179, qm180,* and *qm184)* mutants.

[2]Represents the pooled data for 2 independent experiments carried out with *dsc-3(qm179* and *qm180)* mutants.

[3]Represents the data for 1 experiment, carried out with the *dsc-3(qm179)* mutants.

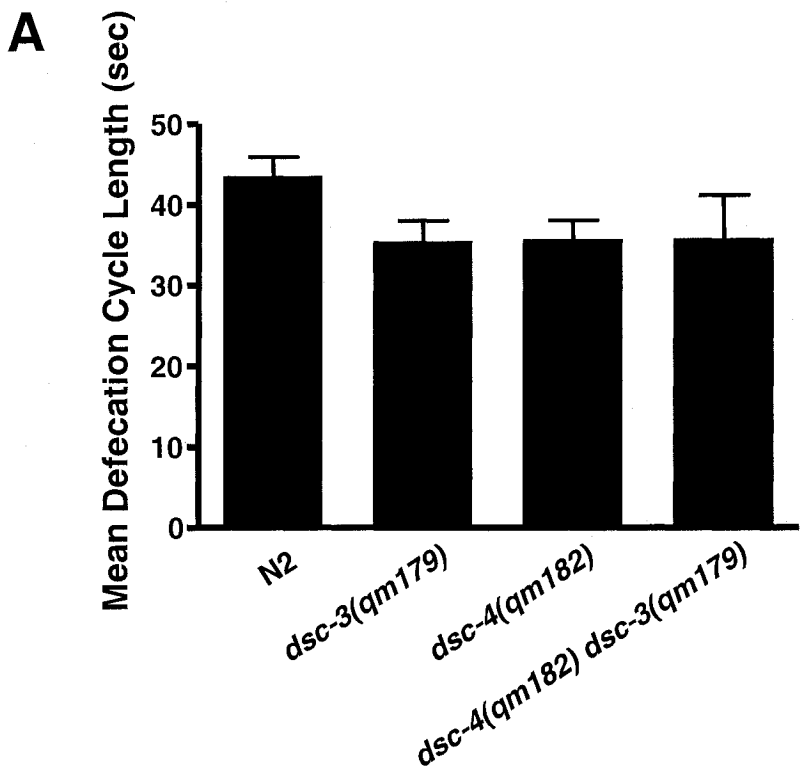
Table 4.2 Cosmid clones tested for rescuing activity in *dsc-3(qm179)* mutants

Injection Mix	DNA contents	Rescued Lines[1]
Mix 1	cosmids: E03H12, T28H11, C09B9, R13H9, C02B10 (3 ng/ul each) co-injection marker: *ttx-3*::*gfp* (185 ng/ul)	0/6
Mix 2	cosmid: T08B6 (5ng/ul) co-injection maker: *ttx-3*::*gfp* (195 ng/ul)	0/9
Mix 3	cosmids: R11E3, H06H21 (5 ng/ul each) co-injection maker: *ttx-3*::*gfp* (190 ng/ul)	0/2
Mix 3A Mix 3B	cosmid: H06H21 (5ng/ul) cosmid: R11E3 (5ng/ul) co-injection maker: *ttx-3*::*gfp*: 190 ng/ul	0/1 0/6

[1] The mixes were injected in *clk-1(qm30); dsc-1(qm179)* mutants. At least 5 F2 animals were scored for each line, at 25°C.

Figure 4.1:The *dsc-3* and *dsc-4* mutations likely affect defecation by the same mechanism.

The graphs represent the mean defecation cycle lengths of *dsc-3*, *dsc-4* and d*sc-4 dsc-3* double mutants in wild-type (N2) **(A)** and *clk-1* **(B)** backgrounds. Each bar represents the mean defecation cycle length of >15 animals. The error bars represent the standard deviations. Each animal was scored for 3 defecation cycles at 25°C. The effects of the *dsc-3* and *dsc-4* mutations are not additive to each other, suggesting that they affect the defecation rate by a similar mechanism.


A
Mean Defecation Cycle Length (sec)
50
40
30
20
10
0
N2
dsc-3(qm179)
dsc-4(qm182)
dsc-4(qm182) dsc-3(qm179)

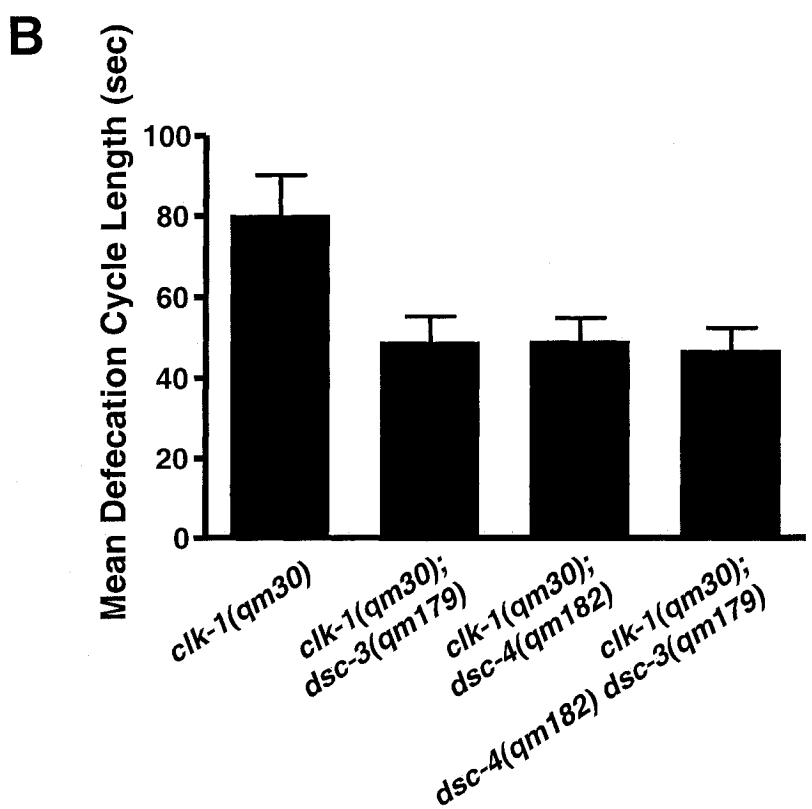

B
Mean Defecation Cycle Length (sec)
100
80
60
40
20
0
clk-1(qm30)
clk-1(qm30); dsc-3(qm179)
clk-1(qm30); dsc-4(qm182)
clk-1(qm30); dsc-4(qm182) dsc-3(qm179)

Figure 4.2: *dsc-3* is likely allelic to *tat-2*.

(A) *tat-2(RNAi)* phenocopies the *dsc-3 (qm179, qm180 and qm184)* mutants. Each bar represents the mean defecation cycle length of 25 animals scored for 3 to 5 consecutive defecation cycles at 25°C.The error bars represent the standard deviations. The RNAi control was injected with buffer. **(B)** The *tat-2(tm1634)* deletion phenocopies and fails to complement the *dsc-3(qm179)* mutation. The bars represent the mean defecation cycle length of 10 animals scored for 3 consecutive cycles each at 25°C. The error bars represent the standard deviations.

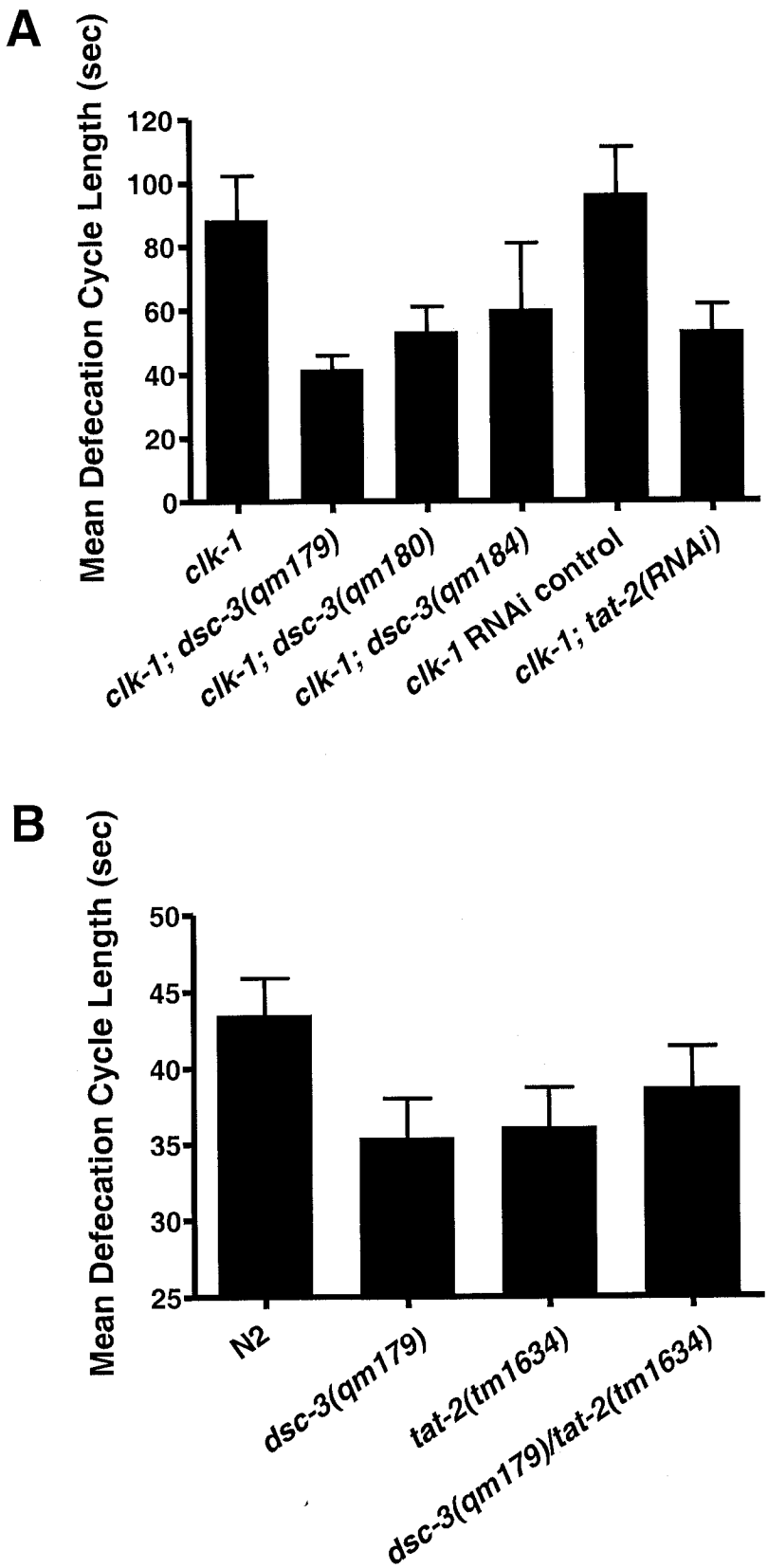

A
Mean Defecation Cycle Length (sec)
120
100
80
60
40
20
0
clk-1
clk-1; dsc-3(qm179)
clk-1; dsc-3(qm180)
clk-1; dsc-3(qm184)
clk-1 RNAi control
clk-1; tat-2(RNAi)
B
Mean Defecation Cycle Length (sec)
50
45
40
35
30
25
N2
dsc-3(qm179)
tat-2(tm1634)
dsc-3(qm179)/tat-2(tm1634)

Figure 4.3: Alignment of the amino acid sequences of the predicted gene *tat-2* and homologous Type IV P-Type ATPases from humans. Residues shaded in black are conserved in all 4 proteins; residues shaded in grey are conserved in only 3.The *tat-2* sequence is based on the predicted amino acid sequence from wormbase (www.wormbase.org) plus 92 additional amino acids (amino acids 154-245). These amino acids were identified by a tBlastn search of the worm genomic sequence using the sequence of the human ATP8B2 protein as a query. ATP8B1 (Accession NM_005603) corresponds to the disease locus FIC1/PFIC1/BRIC, and is homologous to three other human genes: ATP8B2 (Accession NM_020452), ATP8B3 (Accession AY302538), and ATP8B4 (Accession BC057236). ATP8B3 was not included in the alignment as the reported sequence appears to contain some errors. The ATP8B4 sequence is only partial. *tat-2* shares 50% identity with ATP8B1, 56% with ATP8B2 and 54% with ATP8B4.

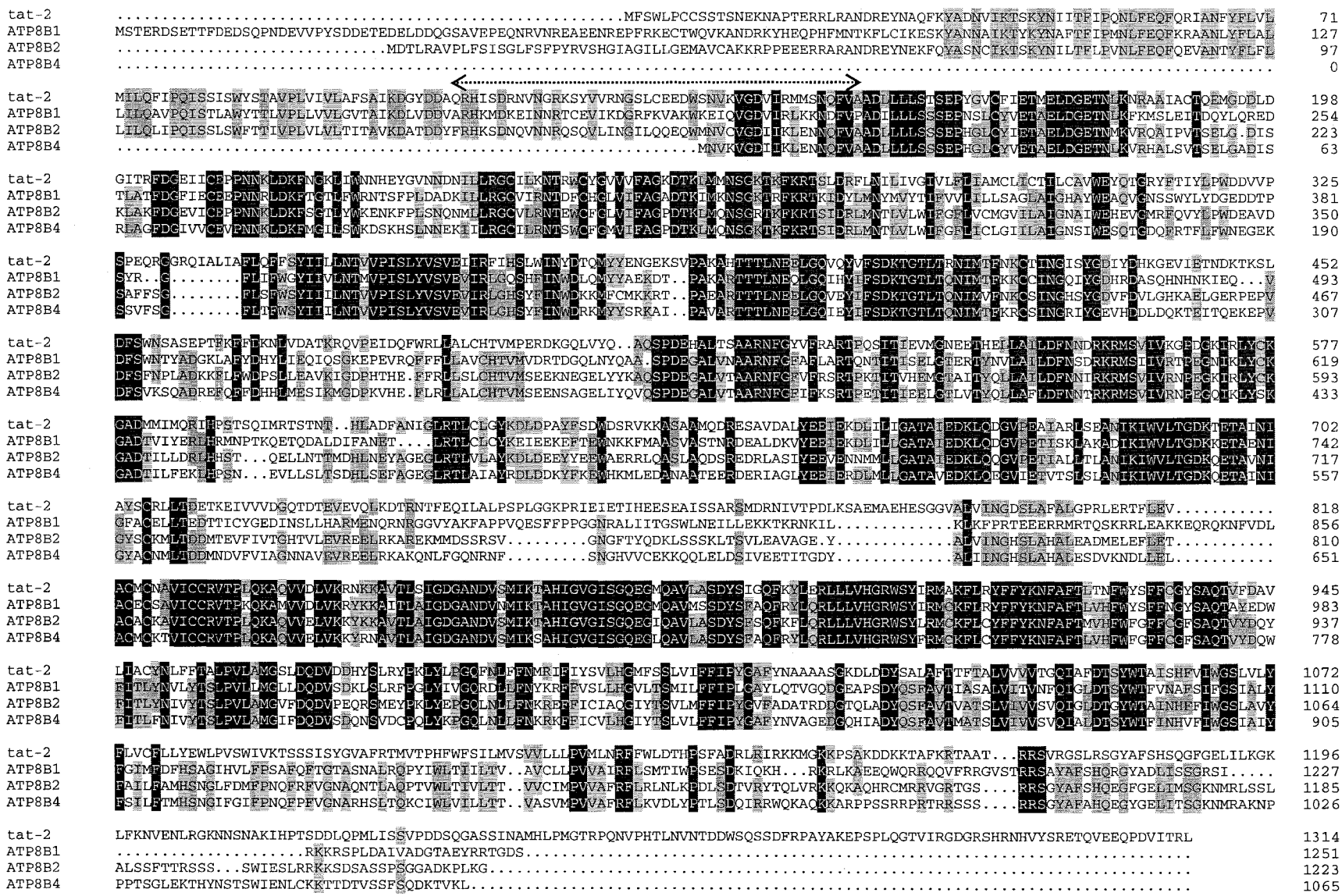

tat-2
ATP8B1
ATP8B2
ATP8B4

Figure 4.4:The *dsc-3* mutants are hypersensitive to low levels of media cholesterol I.

The graphs represent the rate of post-embryonic development of *dsc-3* and *dsc-4* mutants in wild-type (N2) and *clk-1* mutant backgrounds. Animals were monitored twice daily until they reached adulthood. The bars represent the percentage of worms that had reached adulthood by that time; the last bar represents the percentage of animals that arrested or died as larvae and therefore never reached adulthood. For each genotype >200 worms were scored for each condition. The *dsc-3* mutants are most affected by the reduced amount of cholesterol, exhibiting both slowed rates of development and an increased percentage of dead or arrested larvae.

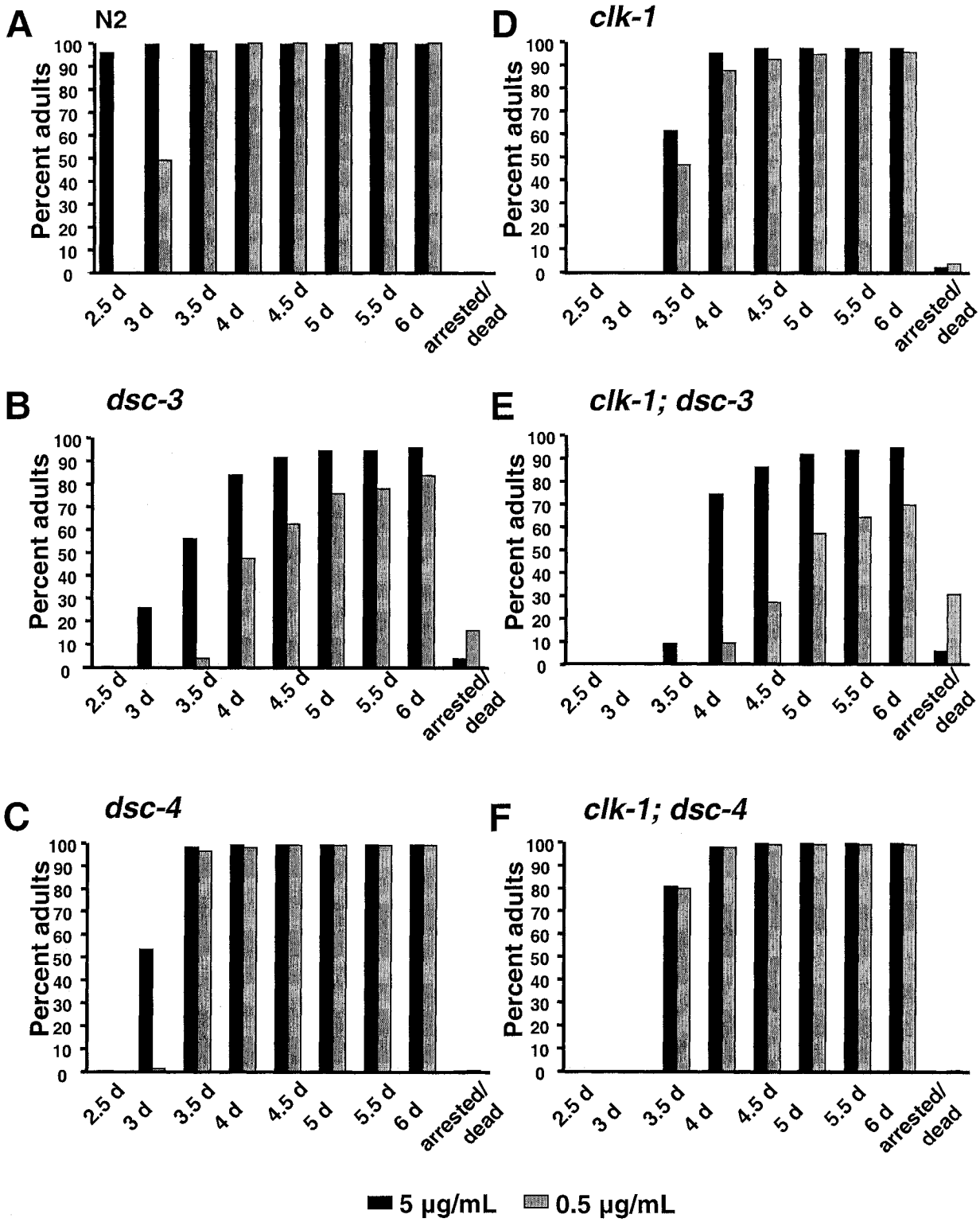

A
N2
D
clk-1
B
dsc-3
E
clk-1; dsc-3
C
dsc-4
F
clk-1; dsc-4
Percent adults
100
90
80
70
60
50
40
30
20
10
0
2.5 d
3 d
3.5 d
4 d
4.5 d
5 d
5.5 d
6 d
arrested/ dead
5 µg/mL
0.5 µg/mL

Figure 4.5: The *dsc-3* mutants are hypersensitive to low levels of media cholesterol II.

The graph represents the percentage of worms that arrest development on plates that are not supplemented with any cholesterol. The alleles used are: *clk-1(qm30)*, *dsc-3(qm179)*, *dsc-4(qm182)*. The data was pooled from 2 experiments for all genotypes except for the *dsc-4 dsc-3* double mutants, for which the data is from 1 experiment. For each genotype, >200 worms were scored for each condition, except for the *dsc-4 dsc-3* double mutants on 0µg/mL cholesterol, for which >100 worms were scored. The *dsc-3* mutation confers a hypersensitivity to low cholesterol in all genetic backgrounds tested. It is not clear whether the *dsc-4* mutation also confers some low level of hyper-sensitivity (see main text).

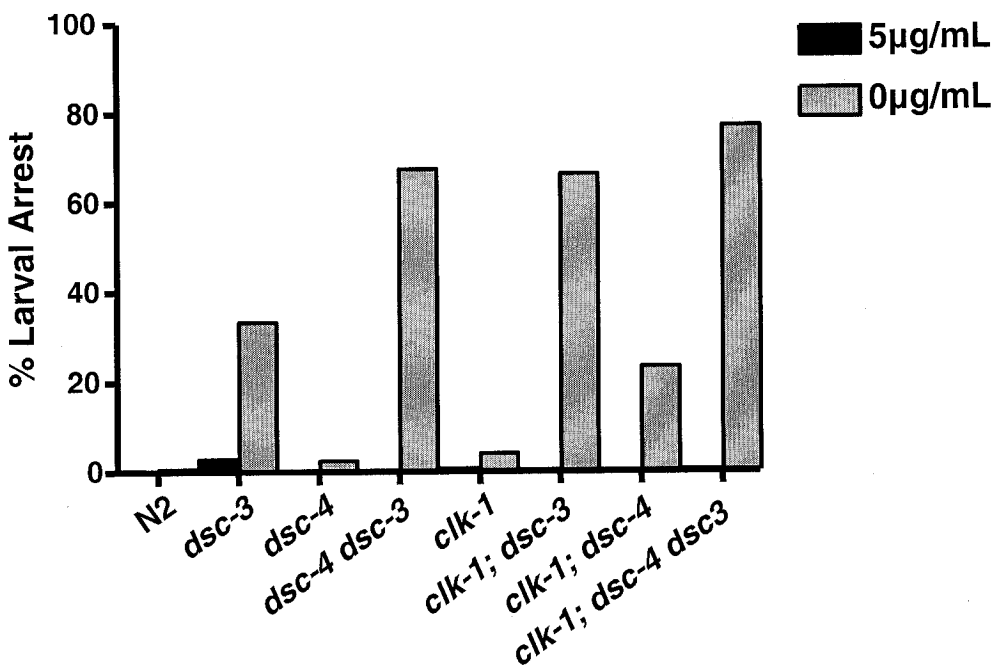
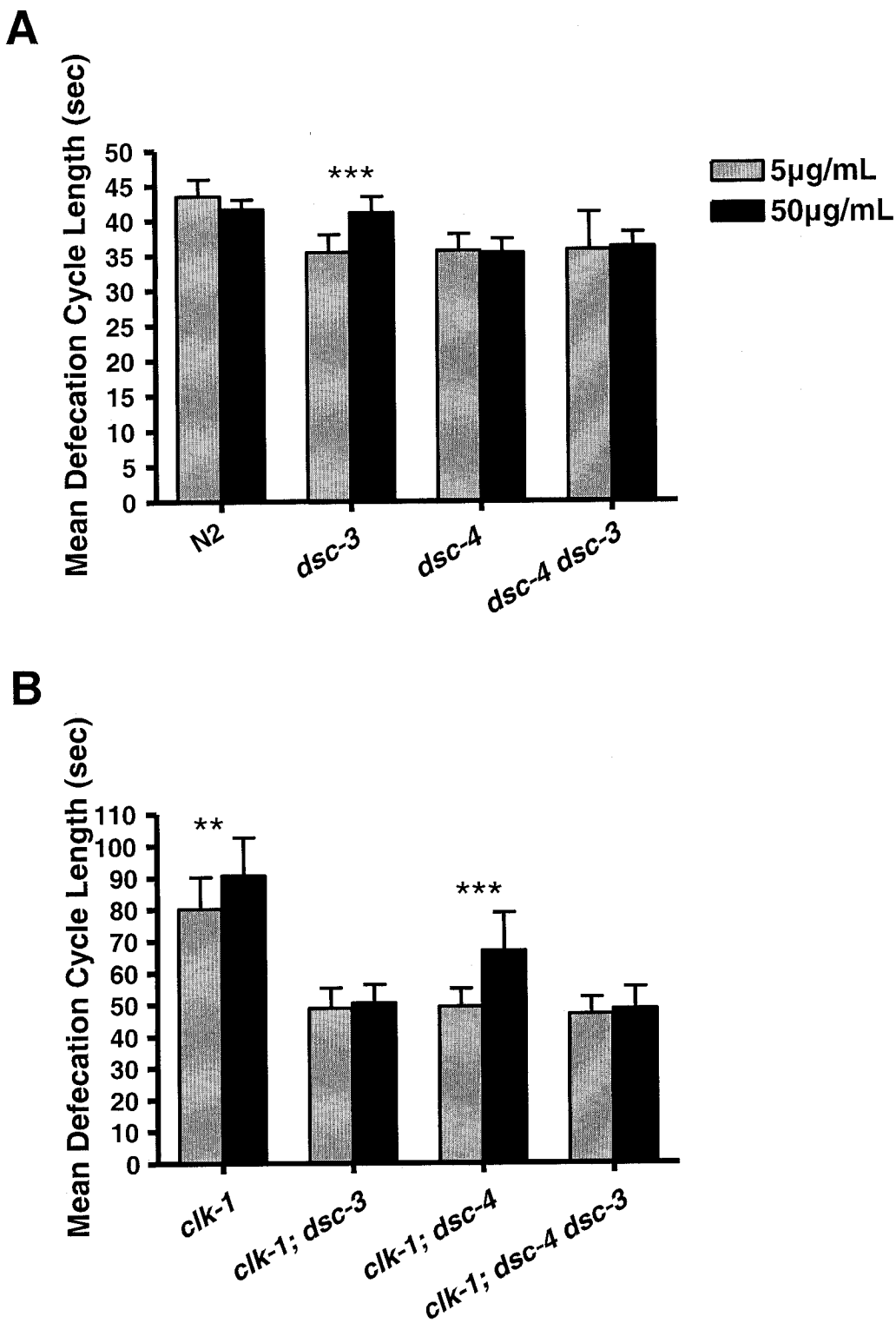

100
80
60
40
20
0
% Larval Arrest
5µg/mL
0µg/mL
N2
dsc-3
dsc-4
dsc-4 dsc-3
clk-1
clk-1; dsc-3
clk-1; dsc-4
clk-1; dsc-4 dsc3

Figure 4.6: Both the *dsc-3* and *dsc-4* mutations can be rescued by increasing the media cholesterol, but only in certain backgrounds.
The graphs represent the mean defecation cycle lengths of *dsc-3*, *dsc-4* and *dsc-3 dsc-4* double mutants in wild-type (N2) **(A)** and *clk-1* **(B)** backgrounds when grown on normal (5 µg/mL) and high (50 µg/mL) media cholesterol. Each bar represents the mean defecation cycle length of >15 animals. The error bars represent the standard deviations. Each animal was scored for 3 defecation cycles at 25°C. The asterisks indicate a statistically significant difference between 5 µg/mL and 50 µg/mL. ** indicates P<0.01; *** Indicates P<0.001. The *dsc-3* mutation is rescued by high cholesterol in a *clk-1(+)* background whereas the *dsc-4* mutation is partially rescued in the *clk-1(qm30)* background. The cycle length of *clk-1(qm30)* is further slowed down by increased cholesterol in the media.


A
Mean Defecation Cycle Length (sec)
50
45
40
35
30
25
20
15
10
5
0
***
5µg/mL
50µg/mL
N2
dsc-3
dsc-4
dsc-4 dsc-3
B
Mean Defecation Cycle Length (sec)
110
100
90
80
70
60
50
40
30
20
10
0
**
***
clk-1
clk-1; dsc-3
clk-1; dsc-4
clk-1; dsc-4 dsc-3

Connecting Statement: Bridging Chapters 4 and 5

In the previous chapter, I reported the characterization of the Class I *dsc* mutant, *dsc-3*. In the following chapter, I report on the characterization of the Class II *dsc* mutant, *dsc-1*. In addition to suppressing the slow defecation phenotype of *clk-1*, *dsc-1* mutations also affect one of the steps of the defecation behaviour, the expulsion step. Much of the analysis of *dsc-1* is related to this, as well as to the cycle length defect, in a *clk-1(+)* genetic background. Its interaction with *clk-1* is also briefly explored.

Chapter 5:

dsc-1

Specification of Muscle Neurotransmitter Sensitivity by a Paired-like Homeodomain Protein in *C. elegans*

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Summary

The effects of neurotransmitters depend on the receptors expressed on the target cells. In *C. elegans*, there are two types of GABA receptors that elicit opposite effects: excitatory receptors that open cation-selective channels and inhibitory receptors that open anion-selective channels. The four non-striated enteric muscle cells that are required for the expulsion step of the defecation behavior of worms are all sensitive to GABA: the sphincter muscle expresses a classical GABA-sensitive chloride channel (UNC-49) and probably relaxes in response to GABA, while the three other cells express a cation-selective channel (EXP-1) and contract. Here we show that the expression of the *exp-1* gene is under the control of *dsc-1*, which encodes a Paired-like homeodomain protein, a class of transcription factors previously associated with the terminal differentiation of neurons in *C. elegans*. *dsc-1* mutants have anatomically normal enteric muscles but are expulsion defective. We show that this defect is due to the lack of expression of *exp-1* in the three cells that contract in response to GABA. In addition, *dsc-1*, but not *exp-1*, affects the periodicity of the behavior, revealing an unanticipated role for the enteric muscles in regulating this ultradian rhythm.

Introduction

Homeodomain transcription factors are involved at numerous levels of animal development: while HOX genes specify the relative position of entire domains of gene expression in the antero-posterior axis, at the other extreme there are types of homeobox genes that appear to determine the expression of only a few genes in a single cell type. For example, in *Caenorhabditis elegans*, Paired-like homeodomain proteins have been found to be involved in the fine-tuning of neuronal differentiation, specifying such features as cell-type specific synaptic inputs (Miller et al., 1992; White et al., 1992; Winnier et al., 1999), neurotransmitter synthesis (Eastman et al., 1999; Jin et al., 1999), and axonal outgrowth (Brockie et al., 2001; Pujol et al., 2000).

By relating behavioral outputs to patterns of gene expression and the expression of unique differentiated cellular features, it is possible to study the molecular, genetic and cellular bases of behavior in *C. elegans*. For example, neural circuits regulating behaviors such as pharyngeal pumping, egg-laying, and locomotion have all been studied in this manner (reviewed in (Rankin, 2002; Whittaker and Sternberg, 2004)). The neuromuscular system that regulates the Defecation Motor Program (DMP) (Liu and Thomas, 1994) is also beginning to be studied at this level. The DMP consists of three distinct, stereotyped steps (Thomas, 1990): the posterior body muscle contraction (pBoc), the anterior body muscle contraction (aBoc), and the expulsion (Exp), which is the most complex step as it consists of the coordinated contractions of two specialized enteric muscles, the intestinal and anal depressor muscles, and probably the relaxation of a third enteric muscle, the sphincter. The defecation cycle period, which is the interval between two series of these three contractions, is very regular over time in single animals, and from animal to animal, but is modulated by sensory inputs such as touch (Liu and Thomas, 1994), food (Liu and Thomas, 1994), and temperature (Branicky et al., 2001).

Two GABAergic neurons, AVL and DVB, send processes to the enteric muscles (the intestinal, sphincter and anal depressor muscles) and are required for the proper execution of the DMP. Laser ablation studies have revealed that these two neurons are partially redundant for enteric muscle contractions. AVL, but not DVB, also appears to be required for the aBoc step (McIntire et al., 1993b). The pBoc step may be regulated by a non-neural pathway as neuronal ablations have failed to identify any neuron required for the pBoc (E. Jorgensen, personal communication). Interestingly, the neurotransmitter GABA, normally an inhibitory neurotransmitter, is an excitatory neurotransmitter for enteric muscle contraction. In fact, loss of the GABA biosynthetic enzyme glutamic acid decarboxylase (GAD), encoded by the *unc-25* gene, causes an expulsion defective (or Exp) phenotype, which can be rescued by exogenous GABA (Jin et al., 1999; McIntire et al., 1993a). In addition, mutations that disrupt GABA transport (*unc-47, gat-1*), and presynaptic GABA release (*unc-2*) also produce an Exp phenotype (Jiang et al., 2004; Mathews et al., 2003; McIntire et al., 1993a; McIntire et al., 1997). The excitatory effect of GABA on these muscles is mediated by *exp-1*, an unusual cation-selective GABA receptor, which is expressed in the intestinal and anal depressor muscles (Beg and Jorgensen, 2003). There is little known about the development and specification of these muscles, except that they are non-striated muscles that require Twist, encoded by the *hlh-8* gene, for their formation. Loss of *hlh-8* also produces an Exp phenotype, because these muscles are missing or are severely reduced in the mutant (Corsi et al., 2000).

In addition to mutants defective in the execution of the DMP steps, a number of mutants that affect the periodicity of the defecation cycle have been identified (i.e. (Branicky et al., 2001; Iwasaki et al., 1995)). In general, these mutants are distinct from those affecting the steps of the DMP, suggesting that the expression of the DMP is regulated by mechanisms that are distinct from those required for maintaining the periodicity of the DMP. The characterization of some of these genes has

shown that calcium dependent signals in the intestine play an important role in regulating the periodicity of the DMP. In particular, loss of *itr-1* (*dec-4/lef-1*), which encodes the worm inositol triphosphate receptor (IP_3 receptor) and regulates calcium release from the endoplasmic reticulum, slows down the defecation period (Dal Santo et al., 1999). Furthermore, the same authors showed that calcium levels peak in the intestine just prior to the first muscle contraction of the DMP, and that expression of the *itr-1* in the intestine is sufficient for normal rhythm generation. Moreover, mutations in *unc-43,* the *C. elegans* calcium dependent calmodulin Kinase II (CaM Kinase II), which is widely expressed in neurons, muscles, and the intestine, result in multiple behavioral defects including defecation phenotypes (Reiner et al., 1999).

The characteristics of several other mutants also point to the intestine as the main modulator of the DMP periodicity. For example, *flr-1* and *flr-4* mutants, originally identified on the basis of their resistance to fluoride (Katsura et al., 1994), have very short defecation cycle lengths (Iwasaki et al., 1995). Intestinal expression of both *flr-1,* which encodes an ion channel of the degenerin/epithelial sodium channel superfamily, and *flr-4*, which encodes a Serine/Threonine protein kinase, is sufficient to rescue their respective defects (Take-Uchi et al., 1998; Take-Uchi et al., 2005). The *elo-2* gene, which encodes a fatty acid elongation enzyme that is expressed in the intestine, also appears to be involved in regulating the defecation cycle length as RNAi-mediated suppression of *elo-2* results in a shortened defecation cycle length (Kniazeva et al., 2003). Finally, loss of function of *dsc-4*, which encodes the worm microsomal triglyceride transfer protein (MTP), and is likely required in the intestine for lipid transport, also causes a shortened defecation cycle length (Branicky et al., 2001; Shibata et al., 2003).

We have studied the altered defecation cycle of the *clk-1* mutants, in which a number of behavioral, developmental and physiological features are abnormal (Branicky et al., 2001; Wong et al., 1995). *clk-1* encodes a

highly conserved protein that is required for ubiquinone (UQ or CoQ) biosynthesis (Ewbank et al., 1997). We have analyzed the defecation behavior of *clk-1* mutants in some detail and found that in the mutants the cycle length is both increased and insensitive to changes in temperature. To investigate these defects further, we have carried out a screen to identify suppressor mutations (which we call *dsc*, for defecation suppressor of *clk-1*) and identified two classes of suppressor mutants. The class I *dsc* mutants (which includes *dsc-4*) suppress both the lengthened defecation cycle and the temperature insensitivity; the class II mutants (which includes *dsc-1*) suppresses only the lengthened defecation cycle, but does not restore normal reaction to changes in temperature (Branicky et al., 2001).

Here we report our characterization of *dsc-1*. We find that the *dsc-1* mutants have a shortened defecation cycle length and are also expulsion defective. We cloned *dsc-1* and found that it encodes a Paired-like homeobox transcription factor that is expressed in some sensory neurons and in the enteric muscles. *dsc-1* is the first homeobox gene of its class that is found to be expressed in, and important for the differentiation of, non-neuronal cells in *C. elegans*. We show that expression of *dsc-1* in the intestinal and anal depressor muscles alone is sufficient to rescue both the defecation cycle length and expulsion defects. However, a variety of experimental manipulations allow for the uncoupling of the effects of *dsc-1* on these two phenotypes, suggesting a previously unanticipated role for the enteric muscles in regulating the defecation cycle length. We also show that *dsc-1(+)* activity is required for the expression of *exp-1* in the intestinal and anal depressor muscles, and that the expulsion defect of the *dsc-1* mutant is due to loss of *exp-1* expression.

Materials and Methods

General methods and strains: Animals were cultured at 20°C as described (Brenner, 1974), and were fed the *E. coli* strain OP50. The *dsc-*

1(qm133) allele was isolated in an EMS mutagenesis screen (Branicky et al., 2001) and was outcrossed 5 times; the *dsc-1(tm241)* allele was obtained from the National Bioresource Project for the nematode in Japan, and was outcrossed 3 times.

The mutations used to refine the genetic position of *dsc-1* include *lin-15(n765)*, *flr-4(ut7)*, and *unc-7(e5)*. *dsc-1* was also mapped relative to the mnDF20 deficiency using the SP278 strain (mnDp1 (X;V)/+ V; mnDf20 X). Other mutations and strains used in this study include *exp-1(sa6) I*, EG1653 *oxIs22* [*Punc-49:UNC-49::GFP, lin-15(+)*] *II* (Bamber et al., 1999), *clk-1(qm30) III*, *unc-25(e156) III*, and *hlh-8(nr2061) X* (Liu et al., 1999).

Defecation was scored as described in Branicky et al., (2001). Each animal was scored for 5 consecutive cycles at 20°C. To quantify the Exp phenotype, animals were also scored for 5 consecutive defecation cycles, which includes 6 consecutive posterior body contractions (pBocs), and the number of pBocs followed by an expulsion was determined. The Student's t-test was used to determine statistical significance and was carried out using the Graphpad Prism 4.0 software.

Positional Cloning of *dsc-1*: *dsc-1* had previously been mapped to the right arm of LG X, between *unc-3* and *lin-15* (Branicky et al., 2001). Using 2-point, 3-point and deficiency mapping strategies, we further refined the genetic position of *dsc-1* and determined that it is in between the two cloned genes *pag-3* and *unc-7* (see www.wormbase.org). The 6 cosmids that correspond to this region were injected into the *dsc-1* mutants as a single pool, in pairs, and singly, and it was determined that the *dsc-1* mutants could be rescued by all injection mixes containing the C18B12 cosmid. The three predicted genes on the C18B12 cosmid were amplified from the cosmid by PCR and were tested for rescuing activity. A PCR product corresponding to the predicted gene C18B12.3 (from 30798 to 24611 on C18B12) was able to rescue the *dsc-1* mutants. All cosmids and PCR products were injected at a concentration of 50 ng/μl with the co-

injection marker *ttx-3::gfp* (Hobert et al., 1999) at a concentration of 150 ng/μl.

To determine the nature of the *qm133* mutation, genomic DNA was extracted from *dsc-1(qm133)* mutants and the predicted coding region was sequenced from both strands. We identified a G to A transition at position 695 of the coding region relative to the ATG (position 26250 of the C18B12 cosmid) resulting in an R to H substitution at residue 232 of the protein.

RT-PCR: First-strand cDNA libraries were generated by the reverse transcription of total RNA extracted from adult worms, using a polydT primer and reverse transcriptase. The *dsc-1* transcript was amplified from the library with primers corresponding to the predicted transcript and primers complementary to the polyA tail. The *dsc-1* transcript contains 5 exons and is 933 bp long. The 3'UTR is 284 bp long, and likely uses one of two possible AAUAAA polyadenylation signals, located 14 or 18 bases upstream of the cleavage site. The *dsc-1* transcript does not seem to be trans-spliced as the transcript could not be amplified using primers corresponding to either the SL1 or the SL2 splice-leader sequences, although there is a consensus trans-splice site located 14 bases upstream of the start codon.

Construction of plasmids and transgenic strains:

For transcriptional and translational *dsc-1:: gfp* reporter fusions, portions of the *dsc-1* promoter and genomic region were amplified from the C18B12 cosmid with primers containing synthetic *Pst*I and *Nhe*I sites, and were cloned into the *Pst*I and *Xba*I sites of the pPD95.77 vector, kindly provided by A. Fire. Unless otherwise indicated, constructs were injected into N2 and *dsc-1* at a concentration of 50 ng/µl along with the transformation marker pRF4 [*rol-6(su1006)*] or *ttx-3::gfp* at a concentration of 150 ng/µl.

Pdsc-1:: gfp: The insert contains bases 30284-28330 of C18B12, which includes ~2 kb upstream of the initiating ATG of *dsc-1* and the first 18 bp.

Pdsc-1::dsc-1::gfp: The insert contains bases 30284-25275 of C18B12, which includes ~2 kb upstream of the initiating ATG of *dsc-1* and the entire genomic sequence excluding the stop codon. For this construct, *gfp* expression could be observed in 4/4 lines, all of which were rescued. However, rescued lines also produced ~30% non-rescued transgenic worms.

NdE-box::dsc-1::gfp: The Nde box is a concatamerized element of the *ceh-24* promoter, that was previously found (Harfe and Fire, 1998) and has been used to drive expression specifically in the enteric muscles (Bulow et al., 2002). It was amplified from the pBH10.21 clone and was cloned into the *Pst*I and *Xba*I sites of the pPD95.77 vector. (The *Pst*I site was introduced in the primer and the *Xba*I site was internal to the PCR product.) The *dsc-1* cDNA, (-19 to +930) was amplified from a cDNA library with primers containing synthetic *Kpn*I sites, and was cloned into the *Kpn*I site of pPD95.77. This construct was injected into *dsc-1* at concentrations of 25 and 100 ng/µl along with the transformation marker *ttx-3::gfp* (at concentrations of 175 and 100 ng/µl). For this construct, *gfp* expression could only be observed in 3/5 transgenic lines; all 3 were rescued. However, rescued lines also produced ~30% non-rescued transgenic worms.

Pexp-1::gfp (pAB04): kindly provided by E. Jorgensen (Beg and Jorgensen, 2003).

NdE-box::exp-1: This fusion was created using a PCR approach essentially as described previously (Hobert, 2002); the construct is not a GFP fusion so as not to inferfere with the function of EXP-1. The Nde-box portion was amplified from the pBH10.21 clone and the *exp-1* gene (from -60 to +4128, which includes the UTRs) from genomic DNA. The fusion PCR product was injected in *exp-1* and *dsc-1* mutants at a concentration

of ~1ng/uL together with the transformation marker *ttx-3::gfp* at a concentration of 100 ng/μL. For both backgrounds 4/4 lines were rescued. Rescued lines produced ~10% non-rescued transgenic worms.

RNAi: A portion of the *dsc-1* cDNA (+33 to + 933) was amplified from a first-strand cDNA library with primers containing synthetic *Nhe*I and *Pst*I restriction sites and was cloned into the corresponding sites in the pPD129.36 vector, kindly provided by A. Fire. The cDNA flanked by the T7 promoter sites was then amplified from the clone and was used as the template for in vitro transcription (Promega Ribomax). Double stranded RNA was injected into wild-type animals at a concentration of ~1 μg/μL as described (Fire et al., 1998). The F1 progeny were scored for their defecation cycle length and number of pBocs associated with expulsions.

Results

dsc-1 mutants have a shortened defecation cycle and lack enteric muscle contractions

The *dsc-1(qm133)* mutant was identified in a screen for suppressors of the slow defecation phenotype of *clk-1* mutants (Branicky et al., 2001). In addition to decreasing the defecation cycle length of *clk-1* mutants, *dsc-1* also significantly shortens the defecation cycle length in a wild-type background (Figure 5.1A). In addition to affecting the periodicity of the cycle, *dsc-1* is also required for the proper execution of the defecation motor program. *dsc-1* mutants have normal posterior and anterior body muscle contractions, but lack the enteric muscle contractions (Figure 5.1B). As this step is necessary for the expulsion of the gut contents, *dsc-1* mutants also have a distended gut, as the gut lumen fills with bacterial debris (Figure 5.2B). This phenotype, termed the "Constipated" or Con phenotype, is common to most if not all expulsion defective mutants (Figure 5.2C,D; Hobert et al., 1999; McIntire et al., 1993b; Reiner and Thomas, 1995; Thomas, 1990).

dsc-1 encodes a Q50 Paired-like homeodomain protein

dsc-1 was previously mapped to LGX between *unc-3* and *lin-15* (Branicky et al., 2001). Using 2- and 3-point mapping strategies we determined that *dsc-1* is tightly linked to, and to the left of both *unc-7* and *flr-4*. Using the chromosomal deficiency *mnDf20*, which deletes *pag-3* but not *dsc-1*, we determined that *dsc-1* is right of *pag-3*. The 6 cosmids that cover the region between *pag-3* and *flr-4* were injected into *dsc-1* mutants as a single pool, then in pairs, and then singly. All injection mixes containing the C18B12 cosmid were capable of rescuing all phenotypes of the *dsc-1* mutants. Of the three predicted genes on the C18B12 cosmid, a PCR product corresponding to only one, C18B12.3, was capable of rescuing of *dsc-1* mutants (Figure 5.1).

We sequenced the coding region of C18B12.3 and found a G to A point mutation that results in an R to H substitution at residue 232 of the protein (Figure 5.3B). In addition, a strain harboring a deletion in the C18B12.3 gene, (*C18B12.3(tm241)*), phenocopies and fails to complement the *dsc-1(qm133)* mutant (Figure 5.1 and data not shown). Finally, injection of double-stranded RNA corresponding to the C18B12.3 coding region into wild-type animals also phenocopies the *dsc-1(qm133)* mutation (Figure 5.1). Together these observations indicate that *dsc-1* corresponds to the predicted gene C18B12.3 and suggest that the *dsc-1(qm133)* mutation may be a genetic null.

dsc-1 encodes a Q50 Paired-like (or Prd-like) homeodomain protein. Paired-class proteins are defined by the presence of a homeodomain (HD) that resembles that encoded by the *Drosophila prd* gene and are characterized by 6 diagnostic residues in the HD. Three sub-classes can be defined according to the residue at position 50 of the HD. The Pax or Prd-type genes are characterized by a Serine at position 50 and also contain a second DNA binding domain, the so- called Paired domain (PD). The other two sub-classes lack the PD (and are therefore Prd-like) and have either a Lysine (K_{50}) or a Glutamine (Q_{50}) at position 50,

the latter being considered to be the most ancestral of the three sub-families (reviewed in (Galliot et al., 1999)). Comparison between the homeodomain of DSC-1 with the consensus Q_{50} Prd-like homeodomain (Figure 5.3C) reveals that the DSC-1 HD contains only 4 of the 6 diagnostic residues for the Prd class, indicating that it is somewhat diverged. In addition, *dsc-1* does not appear to be orthologous to any of the more than 12 Q_{50} Prd-like sub-families already described, suggesting that *dsc-1* encodes a novel Q_{50} Prd-like protein. In addition, *dsc-1* does not appear to have orthologues in any species other than *C. briggsae*, suggesting that it might be a recently evolved, nematode-specific, or even *Caenorhabditis*-specific gene.

The severity of phenotype of the *qm133* mutant, which harbors an R→H substitution at position 53 of the homeodomain is the same as that of the *tm241* deletion mutant, which lacks the HD entirely (Fig 5.1). This suggests that the *qm133* mutation may entirely abolish the binding of the *dsc-1* HD to its target DNA. Indeed the Arg53 residue is one of the most highly conserved residues of the HD from a broad range of species and has been shown to contact the DNA phosphate backbone in the paired- and engrailed-DNA crystal structures (Kissinger et al., 1990; Wilson et al., 1995). In addition, it has been shown that mutations at position R53 of human Q_{50} Prd-like genes CHX10 and HESX1, which result in microphthalmia and septo-optic dysplasia, respectively, entirely abolish binding to target DNA in vitro (Dattani et al., 1998; Ferda Percin et al., 2000).

dsc-1 is expressed in enteric muscles

In *C. elegans*, there are 13 genes encoding Prd-like homeobox proteins (6 are K_{50} Prd-like and 7 are Q_{50} Prd-like genes), 8 of which (*unc-4*, *unc-30*, *unc-42*, *ceh-10*, *ceh-17*, *ceh-36*, *ceh-37*, and *ttx-1*) have been studied genetically, and have all been shown to be required for particular features of a subset of neurons (Altun-Gultekin et al., 2001; Baran et al., 1999; Eastman et al., 1999; Jin et al., 1994; Lanjuin et al., 2003; Miller et al.,

1992; Pujol et al., 2000; Satterlee et al., 2001; Svendsen and McGhee, 1995; White et al., 1992). Based on some of these analyses, it has been suggested that this class of homeoproteins may have essential roles in defining neuron subtype identity (Baran et al., 1999). In order to determine the focus of action of *dsc-1*, we examined the expression pattern of transcriptional and translational *gfp* reporter fusions.

We found that both the transcriptional and translational fusions are expressed in a subset of bilateral sensory neurons. Based on the positions of the cell bodies and processes we believe we have identified these neurons as AWA, AWB, AWC, ASE, FLP and PVD (Figure 5.4A,B,D,E). GFP expression could also occasionally be observed in a 6th pair of unidentified neurons, located to the anterior of AWC (not shown). As expected, we observed expression of the transcriptional fusion in both the cell bodies and axons, and in the cytoplasm as well as in the nucleus, whereas expression of the translational fusion was mostly confined to the nucleus.

In addition to expression in neurons, we also observed expression in the enteric muscles. Expression of the transcriptional fusion could only be observed in the sphincter muscle (Figure 5.4C), whereas expression of the translational fusion was observed in the nuclei of the two intestinal muscles (IM) and the anal depressor muscle (AD), but not in the sphincter. This suggests that sequences upstream and internal to the *dsc-1* gene might participate in a combinatorial code of expression that identifies as a group all the muscles that are involved in defecation, and that there are sequences in the coding region that prevent expression of *dsc-1* in the sphincter and promote expression of *dsc-1* in the IM and AD (see Discussion).

Expression of *dsc-1(+)* in two enteric muscles is sufficient to rescue the defecation defects of *dsc-1* mutants

In order to determine whether it was the expression of *dsc-1* in the neurons, the enteric muscles or both that was necessary for the defecation

phenotypes of *dsc-1* mutants, we used a synthetic promoter element (the so-called NdE-box), derived from the concatamerization of an element in the *ceh-24* promoter (Harfe and Fire, 1998) to specifically drive expression of the *dsc-1* cDNA in the intestinal and anal depressor muscles (Figure. 5.5A,B). Indeed we found that expression of *dsc-1(+)* in only these two muscle types was sufficient to rescue both the defecation cycle length and expulsion defects of *dsc-1* mutants. Moreover, we found that the degree of rescue obtained with this construct was the same as that obtained by a translational fusion that uses the *dsc-1* promoter and the entire genomic region containing *dsc-1* to drive *dsc-1* expression in both the enteric muscles and neurons. This suggests that, for the defecation phenotypes that we have been studying in the *dsc-1* mutants, *dsc-1(+)* is required only in the IM and AD. Consistent with this, we observed that *dsc-1(RNAi)* only knocks down the expression of *Pdsc-1::dsc-1::gfp* in these two enteric muscles and not in the *dsc-1* expressing neurons. However, *dsc-1(RNAi)* can completely phenocopy the *dsc-1(qm133, tm241)* mutations (Figure 5. 1), indicating that loss of *dsc-1* in only these two enteric muscles is responsible for the defecation defects in the mutants.

dsc-1(+) is required for the expression of *exp-1*

After having established that the expression of *dsc-1(+)* in the AD and IM is necessary for the regulation of the defecation cycle length and the expulsion step, we sought to determine the nature of the defect in the mutant muscles. Using a combination of polarized light microscopy, rhodamine-phalloidin staining and expression of a non-rescuing *dsc-1(qm133)::gfp* reporter, we could observe that the IM, AD, and sphincter muscles were all present in the mutants, and had an overall wild-type appearance (data not shown). Given the role of homeoproteins of the Prd-like class in defining neuron sub-type identity, we wondered whether *dsc-1* could have a similar role in these muscles. A number of genes affecting the expulsion step at the neuronal level, as well as some genes required for the development of the enteric muscles have been identified (Corsi et

al., 2000; Hobert et al., 1999; Jin et al., 1999; Mathews et al., 2003). However, only one gene, *exp-1*, has been shown to be required in these muscles for their function in the execution of the expulsion step. *exp-1* encodes an unusual cation-selective GABA receptor, which is expressed in the intestinal and anal depressor muscles as well as in a few other cells (Beg and Jorgensen, 2003).

We found that expression of an *exp-1::gfp* transcriptional reporter could not be detected in the IM and AD in either *dsc-1(qm133)* or *dsc-1(tm241)* backgrounds, while *gfp* expression could be observed when the same transgene was transferred into a *dsc-1(+)* background (Figure 5.6A,B and data not shown). *exp-1::gfp* expression could also be restored when a *dsc-1*-rescuing construct was introduced into the *dsc-1* mutant lines (data not shown). In addition, we observed that *dsc-1(+)* was not required for the expression of *exp-1* in the PDA neuron, a neuron that is known to express *exp-1* but is not known to have a role in defecation. Taken together, these results indicate that *dsc-1(+)* is required for the expression of *exp-1* in an enteric muscle-specific manner.

As a control, we also examined the expression of *unc-49* in *dsc-1* mutants (Figure 5.6C,D). *unc-49*, encodes a typical anion-gated GABA channel (Bamber et al., 1999), which is expressed in the sphincter, and should promote relaxation of this muscle in response to GABA. However, the exact role of the sphincter in hermaphrodite defecation is less clear than for the other two muscles. Given that *unc-49* mutants are not expulsion defective (McIntire et al., 1993a), the sphincter probably plays only a minor role in the expulsion step in hermaphrodites. However, in males there is a remodeling of the system in adulthood such that the expulsion step is achieved by relaxation of the sphincter, rather than by contraction of the IM and AD (Reiner and Thomas, 1995). The expression of *unc-49* does not appear to be in any way altered in *dsc-1* mutants, which is consistent with the expression pattern of *dsc-1*. Moreover, we found that *dsc-1* mutant males do not have the Con phenotype that is

associated with the Exp phenotype (Figure 5.2F). This further supports a requirement for *dsc-1* only in the IM and AD muscles, and only for the expression of genes like *exp-1*, that act in the IM and AD. As has previously been reported (Reiner and Thomas, 1995), we found that *exp-1* males are also not Con, whereas *unc-25* males are severely Con. This is consistent with a requirement for GABA for the expulsion in males, not by acting on EXP-1 to promote contraction of the IM and AD, but rather by acting on UNC-49 to promote relaxation of the sphincter.

The *dsc-1* expulsion defect is due to the absence of EXP-1

In order to test whether the lack of *exp-1* expression was responsible for the Exp defect of *dsc-1*, we used the Nde-box to drive *exp-1* expression in the IM and AD muscles (Fig 5.6E). We found that this construct was able to fully rescue the Exp defect of *exp-1* mutants. We also found that this fusion was also able to rescue the Exp defect of *dsc-1*, which indicates that the expulsion defect of the *dsc-1* mutants is indeed due to the lack of *exp-1* expression. However, the rescue achieved with this construct was somewhat weaker in *dsc-1* than in *exp-1* and also weaker than the rescued achieved with *dsc-1(+)* expression (Figure 5.5C). This suggests that *dsc-1* may also be required for the expression of some positive regulators of *exp-1* or some other genes that contribute, but are not essential, for enteric muscle function.

Consistent with the finding that the GABA-gated EXP-1 channel is responsible for the *dsc-1* phenotype, neither *exp-1* nor *dsc-1* mutants can be rescued by exogenous GABA (data not shown).

dsc-1 regulates the defecation cycle length

We have described two defecation defects for the *dsc-1* mutant: an expulsion defect and a cycle length defect, both of which result from a lack of *dsc-1(+)* expression in the IM and AD. One possibility is that the shortened defecation cycle length is in fact a secondary effect of the Exp defect. It has been shown for other Exp mutants, like the *unc-43(gf)*

mutant, that a lack of enteric muscle contractions leads to a progressive shortening of the cycle as the intestine fills with bacterial debris. This eventually leads to a forceful expulsion of the gut contents, which is generally followed by one or two longer cycles (Reiner et al., 1999). We also observed a similar oscillation in cycle length with *dsc-1*, albeit over a very small range (see legend of Figure 5.7), suggesting that the Exp phenotype could contribute to the shortened cycle length of *dsc-1*, but only in a minor way. Consistent with this, we found that the *Nde-box::exp-1* construct, which rescues the Exp defect of *exp-1* and *dsc-1* fully rescues the cycle length defect of *exp-1*, but only partially that of *dsc-1* (Figure 5.6E).

Although the cycle length of *dsc-1* mutants is affected by the absence of the expulsion step, several observations suggest that *dsc-1* has a role in cycle length regulation that is independent of its effect on the expulsion step of the cycle. The first observation relates to the fact that *dsc-1(qm133)* was identified in a screen for suppressors of the slow defecation cycle phenotype of *clk-1* mutants, in which we did not recover any other Exp mutant, although the screen may have begun to reach saturation (Branicky et al., 2001).

Secondly, we examined the defecation cycle lengths of other Exp mutants in both *clk-1(+)* and *clk-1(qm30)* backgrounds (Figure 5.7). Our survey included *unc-25*, a mutant of GAD (Jin et al., 1999), *hlh-8*, a mutant that affects the development of the enteric muscles (Corsi et al., 2000), and *exp-1*, which, as described above, affects the enteric muscle contraction in response to GABA (Beg and Jorgensen, 2003). All mutants showed the oscillation in cycle length characteristic of Exp mutants (see legend of Figure 5.7). We found that only *exp-1,* like *dsc-1,* significantly shortened the average cycle length in a wild-type background. The others (*unc-25* and *hlh-8*) significantly increased the average cycle length (Figure 5.7A), which suggests that an expulsion defect per se does not necessarily lead to an average shortening of the defecation cycle length.

Moreover, we found that, in contrast to what is observed with *dsc-1* mutants, none of the Exp mutants, including *exp-1,* could suppress the lengthened defecation cycle of *clk-1* mutants (Figure 5.7B).

Thirdly, we observed that overexpression of *dsc-1* can significantly increase the defecation cycle length. The transgenic lines that express *dsc-1,* either under the control of the *dsc-1* promoter or the Nde-box, which presumably leads to overexpression, have significantly longer defecation cycle lengths than the wild type (Figs 5.5C, 5.1A). As a loss of *dsc-1* activity results in a shortened defecation cycle length, this suggests that the level of *dsc-1(+)* expression is somehow involved in setting the defecation rate. A similar observation has been made with the *itr-1* gene, except that the effect is reversed (loss-of-function mutations slow down, whereas overexpression speeds up, the cycle (Dal Santo et al., 1999).

Fourthly, we found that *dsc-1* can also significantly shorten the lengthened defecation cycle of the *itr-1(sa73)* mutant (data not shown). This again supports a role for *dsc-1* in cycle length regulation, and suggests that *dsc-1* affects the cycle length by a mechanism that is independent of the regulation that depends on calcium signaling in the gut. However, given that *itr-1(sa73)* is not a null allele, potential interactions between *dsc-1* and *itr-1* need to be further investigated.

Discussion

The developmental roles of Paired-like homeobox genes

The Paired class of homeobox genes, which are related to the Drosophila gene *paired* (*prd*), can be divided into three sub-classes according to the residue at position 50 of the HD. The Pax or Prd-type genes are characterized by a Serine at position 50 and also contain a second DNA binding domain, the so-called Paired domain (PD). The other two Prd-like sub-classes lack the PD and have either a Lysine (K_{50}) or a Glutamine (Q_{50}) at position 50 (reviewed in (Galliot et al., 1999)). A number of

orthologous gene families have been identified within each sub-class. For example, more than 15 orthologous families of K50 or Q50 Prd-like genes have been identified. In *C. elegans*, there are 13 Prd-like genes that fall into only 8 of these families.

C. elegans Prd-like genes have mostly been studied for their roles in the nervous system, and with one exception, have all been shown to have roles in specifying neuron sub-type identity, that is, particular aspects of neuronal function such as choice of neurotransmitter synthesis, connectivity or sensory abilities. Briefly, *unc-30*, the worm Rx family ortholog, specifies the identities of 19 GABAergic neurons (Eastman et al., 1999; Jin et al., 1999). The worm Otx family orthologs *ttx-1, ceh-36* and *ceh-37*, specify the identities of the ASH, the AWB and AWC, and the ASE sensory neurons, respectively (Lanjuin et al., 2003; Satterlee et al., 2001). *ceh-10*, specifies the identity of the AIY interneuron (Altun-Gultekin et al., 2001; Svendsen and McGhee, 1995). *unc-4* specifies the identities of the VA motor neurons (Miller et al., 1992; White et al., 1992). *ceh-17*, a worm Prx family member, also functions in a subset of neurons, but appears to be required for axonal outgrowth rather than subtype identity (Pujol et al., 2000).

Remarkably, the function of some of these genes is precisely conserved in vertebrates. For example, vertebrate orthologues of *unc-30*, *ceh-36* and *ceh-37*, and *ceh-17* (Pitx2, Otx1 and Phox2b, respectively) are all expressed in cell types similar to those in which they are expressed in the worm, and can complement the mutant worm genes (Lanjuin et al., 2003; Pujol et al., 2000; Westmoreland et al., 2001). However, this does not mean that they are not involved in other processes as well. For example, Pitx2 is also involved in the development of the pituitary gland, craniofacial region, eyes, heart, abdominal viscera, and limbs (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Liu et al., 2001).

dsc-1 encodes a Q50 Prd-like protein, which does not appear to have any clear orthologue in any species but *C. briggsae*. It is expressed

in a few sensory neurons (Figure 5.4), as well as in the non-striated enteric muscles. We have determined that *dsc-1* is required in three of these muscles for the expression of *exp-1*, a GABA-gated cation channel. Our analysis suggests that as in other systems, Prd-like genes in *C. elegans* may play key developmental roles in non-neuronal tissues. We speculate that other Prd-like genes in *C. elegans* might also be involved in specifying neurotransmitter sensitivity. Indeed *unc-42*, another Prd-like gene in *C. elegans* that is without a clear vertebrate orthologue, appears to be required for regulating glutamate receptor expression in the nervous system (Baran et al., 1999).

Enteric muscle development and function in *C. elegans*

Muscles in worms are of two broad types. The main class of muscles are the striated muscles, the so-called "body-wall muscles," which attach to the outer body wall and contract longitudinally for locomotion. The second class are the non-striated muscles, which are a diverse group of single-sarcomere muscles. These muscles include the pharyngeal muscles, which are used for feeding, the uterine and vulval muscles, which are used for egg-laying, the male-tail associated muscles, which are used for mating, and the enteric muscles we have discussed here (the AD, IM and sphincter), which are required for the expulsion part of the defecation behavior (www.wormatlas.org).

Only very few genes required for the development of the enteric muscles have been identified so far. The most extensively characterized is *hlh-8*, the *C. elegans* homolog of Twist, a basic helix-loop-helix (bHLH) transcription factor that has an evolutionarily conserved role in mesodermal patterning (Corsi et al., 2000; Harfe et al., 1998). *hlh-8* mutants have defects in the patterning of the post-embryonic mesodermal lineages, and in the development of the IM and AD muscles, which develop embryonically. One downstream target of *hlh-8* in the AD muscle is the NK-2 class homeobox gene *ceh-24*, whose expression is dependant on *hlh-8(+)* activity. However, *ceh-24* mutants do not appear to have

expulsion defects (Harfe and Fire, 1998), suggesting that the activity of *ceh-24* is not necessary for the main aspects of muscle function. *hlh-8* is not required for the development of the sphincter muscle (Corsi et al., 2000). In fact, although a number of genes have been reported to be expressed in the sphincter (www.wormbase.org Release WS144) none have been shown to be required for its development.

Here we have shown that *dsc-1* is required for an aspect of terminal differentiation of the AD and IM muscles, that is, the specification of their sensitivity to GABA. Like *hlh-8*, *dsc-1* does not appear to have a role in the sphincter, although it does contain some elements in its promoter that can drive its expression there. Indeed, the transcriptional *dsc-1::gfp* reporter is expressed exclusively in the sphincter (Figure 5.4C); conversely, the translational reporter is expressed only in the AD and the IM (Figure 5.4F). This suggests that there are elements in the *dsc-1* promoter that direct expression to the sphincter, while there are elements in the intronic and/or exonic regions that prevent expression in the sphincter but instead promote expression in the AD and IM. We speculate that *dsc-1* participates in a combinatorial code of expression that identifies all muscles that are involved in defecation as a group, but also ensures that *exp-1* is only expressed in the muscles that need to contract during defecation.

The roles of *dsc-1* in defecation

Two types of defecation mutants have been identified in *C. elegans*: those that affect the aBoc and/or Exp step, and those that affect periodicity. In general, these two classes of mutants are distinct, although a few mutants fall into both classes (e.g. the *flr* mutants and the *unc-43(gf)* mutant; (Reiner et al., 1999; Take-Uchi et al., 1998; Take-Uchi et al., 2005). Mutants that affect both the aBoc and Exp steps (defining the *aex* genes) have general synaptic transmission defects (Doi and Iwasaki, 2002; Iwasaki et al., 1997). Similarly, there is a class of dominant Exp mutants that are also egg-laying defective that affect another general property,

which is muscle excitability (Reiner et al., 1995). Most of the recessive Exp mutants reported are defective in either the function of the GABAergic DVB neuron (Hobert et al., 1999), the synthesis or transport of GABA (McIntire et al., 1993a; McIntire et al., 1993b), or the response of the enteric muscles to GABA (Bamber et al., 1999; Beg and Jorgensen, 2003). Consistently, we find that *dsc-1* is required for an aspect of GABA neurotransmission, that is, the expression of the appropriate GABA receptor in the IM and AD.

As described in the Introduction, a number of genes affecting the periodicity of the defecation cycle have been identified, in particular, *itr-1* (*dec-4/lef-1*), *elo-2*, *dsc-4*, *flr-1* and *flr-4*. Although these genes appear to affect diverse processes, all affect defecation by acting in the gut, and presumably altering some of its properties (Dal Santo et al., 1999; Kniazeva et al., 2003; Shibata et al., 2003; Take-Uchi et al., 1998; Take-Uchi et al., 2005). In contrast, we have found that *dsc-1* modulates the defecation cycle length by acting in the enteric muscles. As the gut appears to control the rhythm of muscle contraction in this system, we speculate that *dsc-1* is required for a property of these muscles that allows them to act in a feedback mechanism from the muscles to the gut. One type of feedback mechanism that is operating in this system works through gut distention. Indeed, a lack of enteric muscle contractions leads a progressive shortening of the defecation cycle until the contents of the gut are forcefully expulsed by pressure alone, and is then followed by a few longer cycles. We speculate that *dsc-1* is necessary for a feedback from the muscles that may involve a humoral signal that acts to coordinate the calcium signal generated in the gut with the timed contractions of the various muscles types. In this context, it is interesting to note that the *flr-1* and *flr-4* mutants, which have a shortened defecation cycle length, are also Exp. Furthermore, expression of both genes in the intestine alone is sufficient to rescue both the cycle length and the Exp defects. Possibly

these genes could somehow be involved in transducing the putative humoral signal from the muscle to the gut.

Interestingly, we have found that *dsc-1* suppresses the lengthened defecation cycle of *clk-1(qm30)* mutants. Although *exp-1* is a target of *dsc-1* in the IM and AD muscle for the expulsion step of the defecation behavior, *dsc-1* likely acts through different targets for cycle length regulation. Indeed mutation of *exp-1* cannot suppress the lengthened defecation cycle of *clk-1*. The identification of the downstream effectors of *dsc-1* would likely give new insight into the slow defecation phenotype of *clk-1* mutants, and more generally, into mechanisms that regulate the defecation cycle length in *C. elegans*.

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Figure 5.1: The defecation phenotypes of *dsc-1* mutants.
(A) *dsc-1* mutants have a shortened defecation cycle length. The bars represent the mean of the mean cycle lengths of n animals that had each been scored for 5 consecutive defecation cycles; the error bars represent the standard deviation of the animal means (n=20-30 animals). **(B)** *dsc-1* mutants have an expulsion defective (Exp) phenotype. The bars represent the mean number of expulsions per 6 pBocs; the error bars represent the range (of number of expulsions/6 pBocs) observed (n=20 animals). For both (A) and (B) the asterisks indicate that the data are significantly different from that of N2. All significant differences detected were at a level of $P<0.0001$.

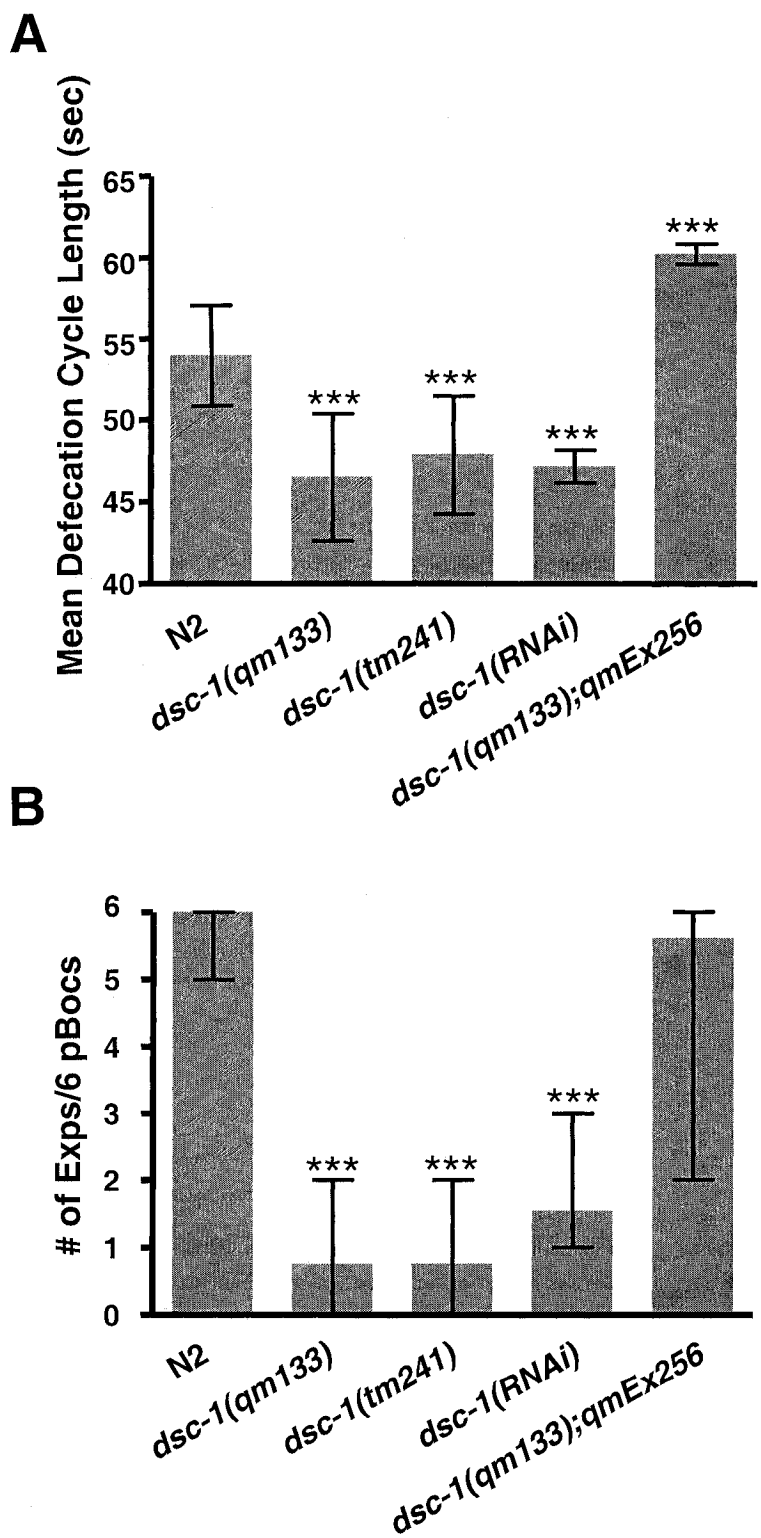

A
Mean Defecation Cycle Length (sec)
65
60
55
50
45
40
***
***
***
***
N2
dsc-1(qm133)
dsc-1(tm241)
dsc-1(RNAi)
dsc-1(qm133);qmEx256
B
# of Exps/6 pBocs
6
5
4
3
2
1
0
***
***
***
N2
dsc-1(qm133)
dsc-1(tm241)
dsc-1(RNAi)
dsc-1(qm133);qmEx256

Figure 5.2: The Con phenotype of Exp mutants.

Nomarski images of the tail region of wild-type and Exp mutant hermaphrodites **(A – D)** and their male counterparts **(E – H)**. Arrows indicate the width of the intestinal lumen. N2 hermaphrodites and males are neither expulsion defective (Exp) nor Constipated (Con). *dsc-1(qm133)* **(B)** *exp-1(sa6)* **(C)** and *unc-25(e156)* **(D)** hermaphrodites are Exp and, as a result, exhibit the Con phenotype, as the gut lumen fills with bacterial debris and becomes distended. Neither *dsc-1* **(F)** nor *exp-1* **(G)** males are Con, indicating that they are not expulsion defective. In contrast, *unc-25(e156)* males are Exp, and as a result are severely Con **(H)**. The bar represents 10μM.

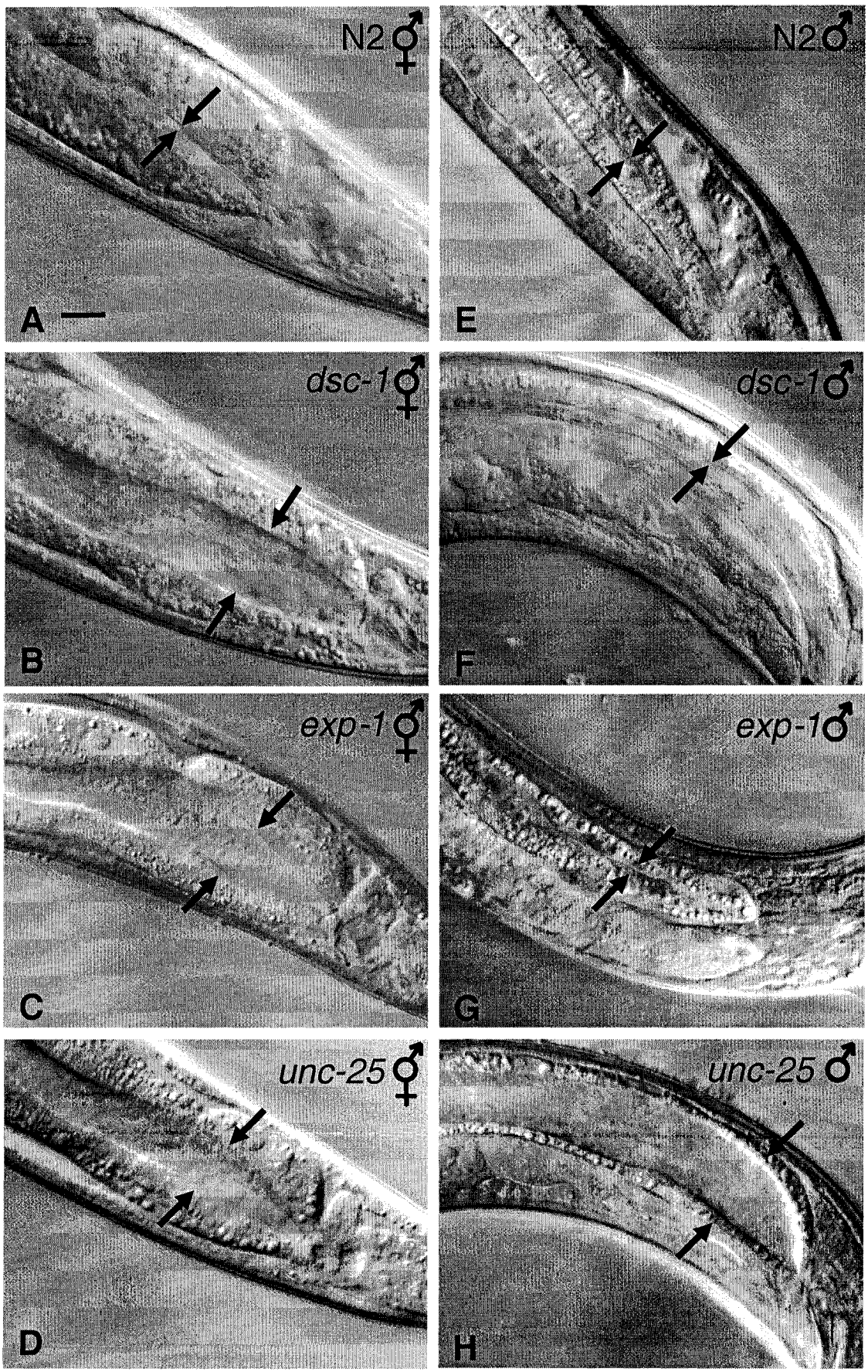
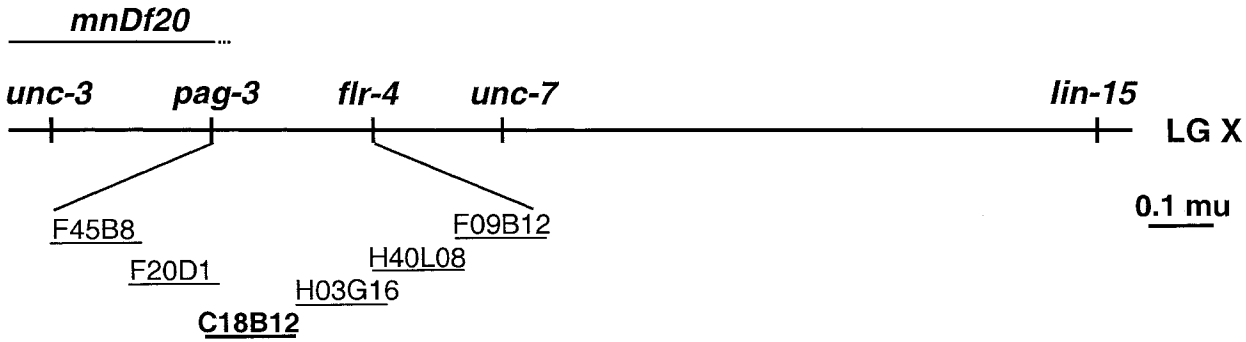

N2 ♀
A
N2 ♂
E
dsc-1 ♀
B
dsc-1 ♂
F
exp-1 ♀
C
exp-1 ♂
G
unc-25 ♀
D
unc-25 ♂
H

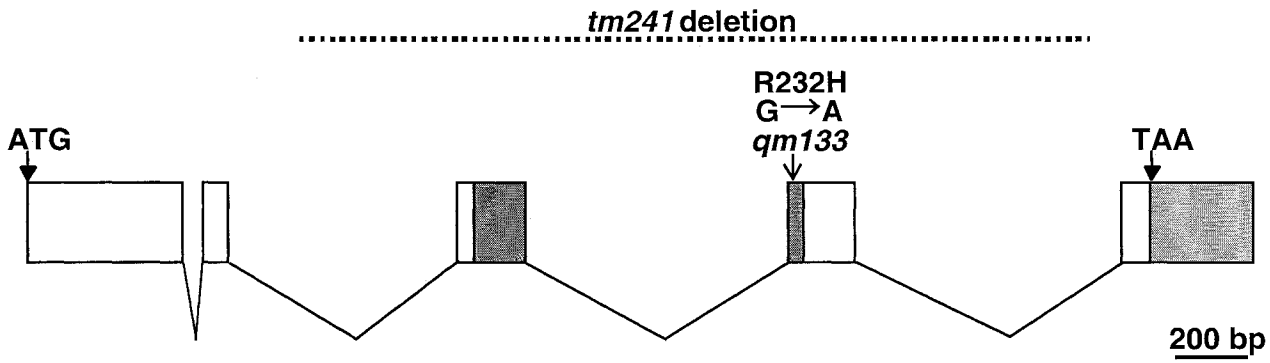
Figure 5.3: Positional Cloning of *dsc-1*.

(A) Mutations, deficiencies and cosmids used for the positional cloning of *dsc-1*. (**B**) Genomic structure of *dsc-1* and the lesions of the two *dsc-1* alleles. The dark grey box indicates the homeobox and the light grey box indicates the 3'UTR. **(C)** Comparison between the homeodomain of DSC-1 with the consensus Q_{50} Paired-like homeodomain (Prd HD) (from (Galliot et al., 1999), and the homeodomains of the six other Q50 Paired-like homeodomain proteins in *C. elegans*. The residues shaded in grey indicate the six residues that are diagnostic of the Paired class; the residues in bold indicate invariant or highly conserved residues that are not specific to the Paired class; the box indicates the Q_{50}. The *qm133* mutation results in an R→H substitution in an absolutely conserved residue at position 53 of the homeodomain (indicated by an asterisk).

A



B



C

Prd HD	QRRNRTTFTS	EQLEE**L**EKA**F**	QKTHYPDIFM	REELALRIDL	TEARVQVW**F**Q	**N**RR**A**K**W**RKQE
dsc-1	RRRFRTNFTE	LQSTF**L**EDS**F**	KESHYPDHKA	KKYMADFLKI	PEDRITVW**F**Q	**N**RR**A**K**W**RRKE
ceh-10	KRRHRTIFTQ	YQIDE**L**EKA**F**	QDSHYPDIYA	REVLAGKTEL	QEDRIQVW**F**Q	**N**RR**A**K**W**RKTE
unc-4	RRRTRTNFSG	WQLEE**L**ESA**F**	EASHYPDVFM	REALAMRLDL	LESRVQVW**F**Q	**N**RR**A**K**W**RKRE
ceh-8	QRRNRTTFTT	FQLHA**L**EAA**F**	DKTHYPDVYA	RETLAAKVQL	PEVRVQVW**F**Q	**N**RR**A**KFRRQE
ceh-17	QRRIRTTFTS	GQLKE**L**ERS**F**	CETHYPDIYT	REEIAMRIDL	TEARVQVW**F**Q	**N**RR**A**KYRKQE
unc-42	ERRHRTTFTQ	EQLQE**L**DAA**F**	QKSHYPDIYV	REELARITKL	NEARIQVW**F**Q	**N**RR**A**KHRKHE
R08B4.2	QRRYRTTFSA	FQLDE**L**EKV**F**	ARTHYPDVFT	REELATRVQL	TEARVQVW**F**Q	**N**RR**A**KYRKQE

* R232H

Figure 5.4: The expression patterns observed for *dsc-1* transcriptional and translational reporter *gfp* fusions.

(A – C) The pattern of expression observed with a transcriptional *gfp* reporter. *dsc-1* is expressed in 4 pairs of sensory neurons in the head **(A)**, the PVD neuron **(B)**, and the sphincter muscle **(C)**. **(D – F)** The pattern of expression observed with a fully functional translational *gfp* reporter. Expression of a translational fusion of *dsc-1* can be detected in the nuclei of the same sensory neurons that express the transcription fusion **(D, E)**, however, in contrast to the transcriptional fusion, the translational fusion is expressed in the intestinal (IM) and anal depressor (AD) muscles, but not in the sphincter (Sph) **(F)**. The bar represents 10μM. All animals were adults, albeit of different ages and sizes.

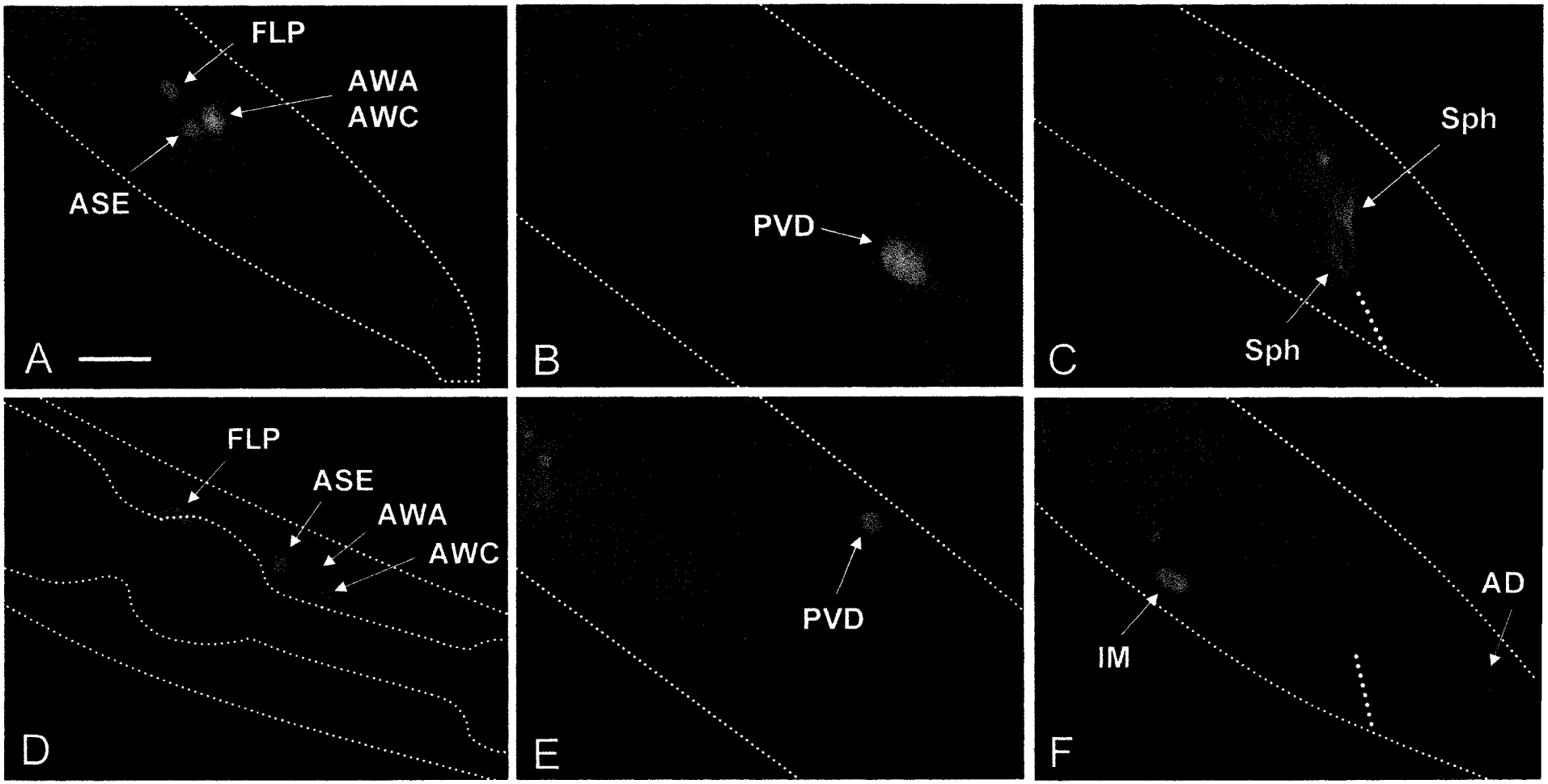

FLP
AWA
AWC
ASE
A
PVD
B
Sph
Sph
C
FLP
ASE
AWA
AWC
D
PVD
E
AD
IM
F

Figure 5.5: Expression of *dsc-1(+)* in two enteric muscles is sufficient to rescue the defecation defects of *dsc-1* mutants.

(A) Schematic of construct used to express *dsc-1* specifically in the anal depressor (AD) and intestinal muscles (IM). **(B)** Expression of the construct depicted in (A) in *dsc-1(qm133)* animals. The dotted line represents the anus. The bar represents 10μM. **(C)** Comparison of the degree of rescue of *dsc-1(qm133)* mutants obtained by expression of the *dsc-1* cDNA specifically in the IM and AD under the NdE-box promoter in comparison to the expression of the *dsc-1* genomic region under its own promoter, which expresses *dsc-1* in sensory neurons as well as in the two enteric muscles (shown in Figure 5.3). Dark grey bars represent transgenic worms and light grey bars represent the non-transgenic siblings from the transgenic lines. For the left panel, the bars represent the average number of expulsions per 6 pBocs observed for 3 independent transgenic lines and the error bars represent the standard deviations of the means obtained for each transgenic line. For the right panel, the bars represent that average defecation cycle length observed for 3 independent transgenic lines and the error bars represent the standard deviations of the means obtained for each transgenic line. 12 transgenic and 12 non-transgenic animals were scored per line.

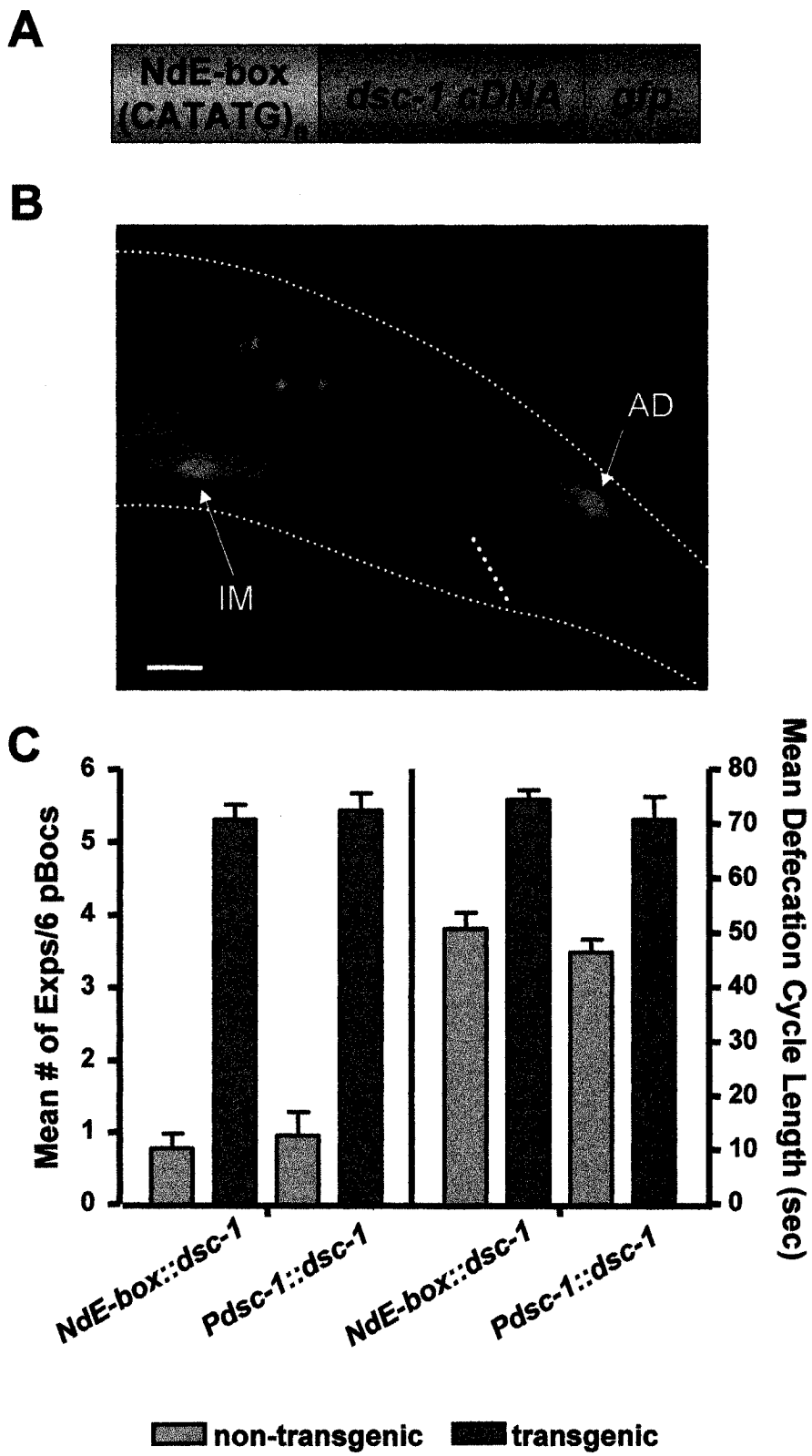

A
NdE-box
(CATATG)n
dsc-1 cDNA
gfp
B
AD
IM
C
Mean # of Exps/6 pBocs
Mean Defecation Cycle Length (sec)
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
NdE-box::dsc-1
Pdsc-1::dsc-1
NdE-box::dsc-1
Pdsc-1::dsc-1
non-transgenic
transgenic

Figure 5.6: The Exp defect of *dsc-1* is due to the lack of *exp-1* expression.

(A,B) Expression of an *exp-1::gfp* transcriptional reporter clone (pAB04 from (Beg and Jorgensen, 2003)) in wild-type and *dsc-1* mutant backgrounds. (The same extra-chromosomal array was expressed in both backgrounds; the same results were obtained with >4 independent extra-chromosomal arrays). **(C,D)** Expression of an integrated *unc-49B::gfp* translational reporter clone in wild-type and *dsc-1* mutant backgrounds. The same integrated array (*oxIs22* from (Bamber et al., 1999)) was expressed in both backgrounds. The bar represents 10µM. AD, anal depressor; IM, intestinal muscle; Sph, sphincter; NC, nerve chord. **(E)** Expression of an *Nde-box::exp-1* fusion in *exp-1(sa6)* and *dsc-1(qm133)* backgrounds. For the left panel, the bars represent the average number of expulsions per 6 pBocs observed for 3 independent transgenic lines and the error bars represent the standard deviations of the means obtained for each transgenic line. For the right panel, the bars represent that average defecation cycle length observed for 3 independent transgenic lines and the error bars represent the standard deviations of the means obtained for each transgenic line. 12 transgenic and 10 non-transgenic animals were scored per line.

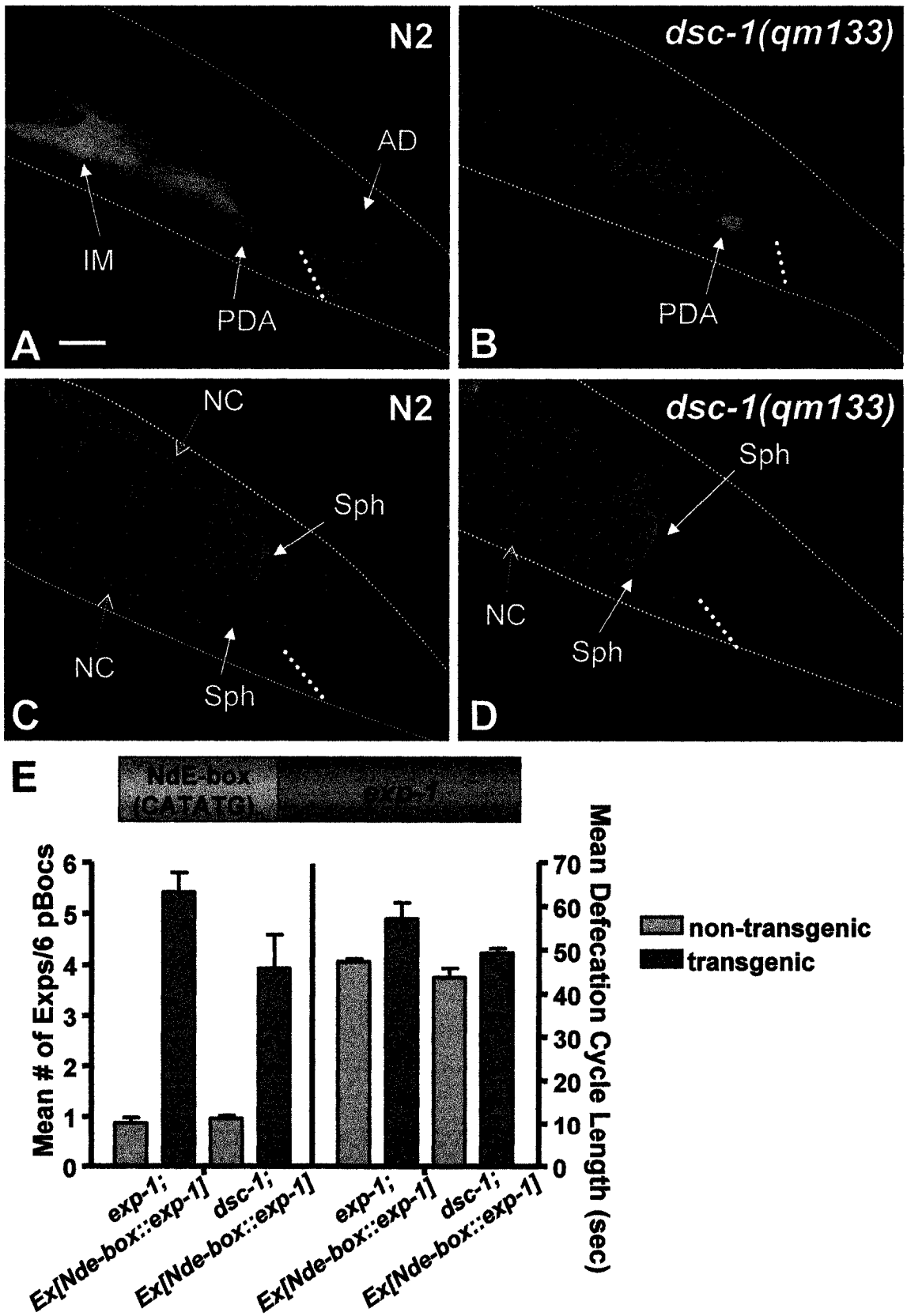
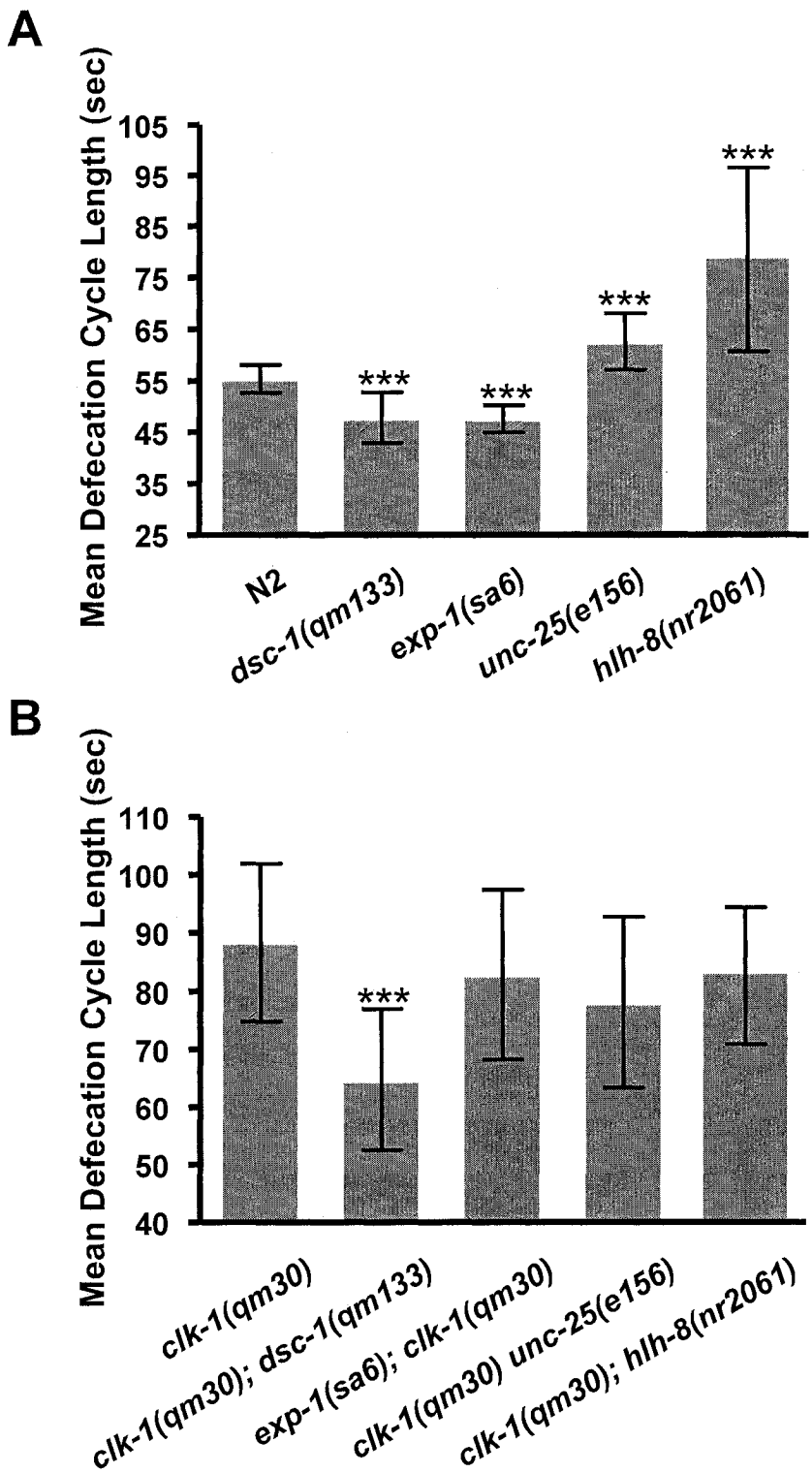

N2
AD
IM
PDA
A
dsc-1(qm133)
PDA
B
NC
N2
Sph
NC
Sph
C
dsc-1(qm133)
Sph
NC
Sph
D
E
Nde-box (CATATG)
exp-1
Mean # of Exps/6 pBocs
Mean Defecation Cycle Length (sec)
non-transgenic
transgenic
exp-1; Ex[Nde-box::exp-1]
dsc-1; Ex[Nde-box::exp-1]
exp-1; Ex[Nde-box::exp-1]
dsc-1; Ex[Nde-box::exp-1]

Figure 5.7: The effect of Exp mutations on the defecation cycle length in wild-type and *clk-1* mutant backgrounds.

The bars represent the mean of the mean cycle lengths of n animals that had each been scored for 5 consecutive defecation cycles; the error bars represent the standard deviation of the animal means (n>10 animals) **(A)** In a wild-type (N2) background, both *dsc-1(qm133)* and *exp-1(sa6)* significantly shorten the defecation cycle; *unc-25(e156)* and *hlh-8(nr2061)* significantly lengthen the cycle. All the Exp mutants show an oscillation in cycle length, with the cycles getting progressively shorter as the worm becomes increasingly constipated (Con), which eventually produces a forceful expulsion, and is then followed by 1 or 2 longer cycles. This phenotype is revealed by the variability of the cycle length in individual animals scored for 5 cycles. This can be expressed as a per animal standard deviation. The average per animal standard deviations are as follows: N2: 0.89, *dsc-1:* 1.90, *exp-1:* 3.43, *unc-25:* 3.92, and *hlh-8*: 11.68. **(B)** Of the 4 Exp mutants surveyed, only the *dsc-1(qm133)* mutation can significantly suppress the lengthened defecation cycle of *clk-1(qm30)* mutants. The asterisks indicate the data that are significantly different from N2 (A) or *clk-1* (B). All significant differences detected were at a level of $P<0.0001$.


A
Mean Defecation Cycle Length (sec)
105
95
85
75
65
55
45
35
25
***
***
***
***
N2
dsc-1(qm133)
exp-1(sa6)
unc-25(e156)
hlh-8(nr2061)
B
Mean Defecation Cycle Length (sec)
110
100
90
80
70
60
50
40
***
clk-1(qm30)
clk-1(qm30); dsc-1(qm133)
exp-1(sa6); clk-1(qm30)
clk-1(qm30) unc-25(e156)
clk-1(qm30); hlh-8(nr2061)

Connecting Statement: Bridging Chapters 5 and 6

In the previous chapters, I reported on the isolation and characterization of mutations that suppress the defecation phenotypes of *clk-1* mutants. These "*dsc*" mutants were isolated in screens carried out using the *clk-1(qm30)* null allele, but as we showed in Chapter 2, they also suppress the missense *clk-1(e2519)* allele. In the following chapter, I report on the isolation and characterization of 9 *clk-1(e2519)*-specific mutations that were isolated in screens for suppressors of the slow growth phenotype of *clk-1* as well as of their requirement for dietary ubiquinone.

Chapter 6:

Growth Suppressors of *clk-1*

Uncoupling the pleiotropic phenotypes of *clk-1* with tRNA missense suppressors in *Caenorhabditis elegans*

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Manuscript submitted to Molecular and Cellular Biology.

Abstract

clk-1 encodes a demethoxyubiquinone (DMQ) hydroxylase that is necessary for ubiquinone biosynthesis. When *C. elegans clk-1* mutants are grown on bacteria that synthesize ubiquinone (UQ), they are viable, but have a pleiotropic phenotype that includes slowed development, behaviors and aging. However, when grown on UQ-deficient bacteria, the mutants arrest development transiently before growing up to become sterile adults. We identified 9 suppressors of the missense mutant *clk-1(e2519)*, which harbors a Glu to Lys substitution. All suppress the mutant phenotypes on both UQ-replete and UQ-deficient bacteria. However, each mutant suppresses a different subset of phenotypes, indicating that most phenotypes can be uncoupled from each other. In addition, all suppressors restore the ability to synthesize exceedingly small amounts of UQ, although they still accumulate the precursor DMQ, suggesting that the presence of DMQ is not responsible for the Clk-1 phenotypes. We cloned 6 of the suppressors and all encode $tRNA^{Glu}$ genes whose anticodons are altered to read the substituted Lys codon of *clk-1(e2519)*. To our knowledge, these suppressors represent the first missense suppressors identified in any metazoan. The pattern of suppression we observe suggests that the individual members of the $tRNA^{Glu}$ family are expressed in different tissues and at different levels.

Introduction

Suppressor analysis has been routinely used in *Caenorhabditis elegans* for the study of gene function and the identification of genetic pathway components. Suppressor analysis of nonsense alleles has also led to the discovery of informational suppressors encoding components of the translational machinery, including suppressor tRNAs (for example (1, 21, 22, 40)). In fact, *C. elegans* is the only metazoan from which nonsense tRNA suppressors have been recovered in classic forward genetic screens.

We have used a suppressor approach to understand the pleiotropic phenotype of the *C. elegans clk-1* mutants. Mutations in *clk-1* are highly pleiotropic, affecting the rates of many physiological processes, over a wide range of timescales (41). These mutations result in an average lengthening of the cell cycle of early embryos, of embryonic and post-embryonic development, as well as of the defecation, swimming, and pharyngeal pumping cycles of adults. *clk-1* mutations also affect reproductive features like the rates of germline development and egg production (35, 41), and lead to an increased lifespan (23).

clk-1 encodes a highly conserved hydroxylase (9, 18, 29, 36) that is required for the hydroxylation of 5-demethoxyubiquinone to 5-hydroxyubiquinone, which is converted by another enzyme (COQ-3) into ubiquinone (UQ, also called coenzyme Q, CoQ). In the absence of CLK-1, worms are devoid of UQ_9 (the subscript refers to the number of isoprene units in the side chain, and is a species-specific trait) (16, 30), and instead accumulate the precursor demethoxyubiquinone (DMQ_9) (30). Several observations suggest that DMQ can partially substitute for UQ. In particular, mitochondrial respiration is only slightly affected in *clk-1* mutants (2, 3, 10, 30). This is in contrast to the *coq-3* mutants, which are also defective in UQ biosynthesis but do not make DMQ, and are inviable (12). Although DMQ may not be functional in yeast (33), it has been shown in both isolated membranes and in vitro studies that DMQ can function as an electron transporter in the mitochondrial electron transport

chain (30, 39). Furthermore, mouse *mclk1* -/- ES cells which do not contain measurable amounts of UQ are still capable of high levels of mitochondrial respiration (25). Despite the fact that DMQ appears capable to function as an electron transporter, it cannot entirely substitute for UQ in worms as *clk-1* mutants require UQ from their bacterial food source in order to proceed through development and become fertile adults (12, 16). When the mutants are fed on *E. coli* mutants that are defective in the biosynthesis of UQ_8 (*ubi* mutants), they arrest development transiently, but eventually grow up to become sterile adults (5).

Several observations indicate a complex relationship between the Clk-1 phenotypes and UQ. Firstly, *clk-1* mutants exhibit a profound mutant phenotype when grown on UQ-replete bacteria, although their mitochondria contain bacterially-derived UQ_8 (17). In addition, there is no correlation between the absence of endogenous UQ_9 and the severity of the overall phenotype. Indeed, no UQ can be detected in any of the three *clk-1* mutants (*e2519*, *qm30* and *qm51*) and all three accumulate the same amount of DMQ (30), yet for most, if not all the phenotypes, the *qm30* and *qm51* null mutants, which produce no CLK-1 protein, are more severely affected than the missense mutant, *e2519,* which produces wild-type amounts of mutant CLK-1 protein (13). These observations suggest several possibilities: (1) Some of the mutant phenotypes could be due to the presence of DMQ rather than to the lack of UQ. Indeed DMQ is generally not present in the wild type and likely has different redox properties than UQ (31). (2) UQ_8 might not be functionally equivalent to UQ_9. (3) Endogenously synthesized UQ may not be functionally equivalent to exogenously supplied UQ, regardless of the chain length. (4) CLK-1 could have other functions, in addition to the biosynthesis of ubiquinone.

Here we describe our analysis of mutants that were isolated in screens for growth suppressors of *clk-1*. We identified 9 suppressors that are specific to the missense mutant *clk-1(e2519)* (Glu148Lys). All suppressors are dominant and suppress some, or, in some cases, all the

Clk phenotypes on both UQ-replete and UQ-deficient bacteria. The suppressors still accumulate a large amount of DMQ_9 but are able to synthesize a very small amount of UQ_9. However, the *clk-1* mutants cannot be suppressed when this same amount of UQ_9 is obtained exogenously (from bacteria). This suggests that viability can be supported by exogenous UQ but that wild-type rates of development and behavior require endogenous UQ biosynthesis. In addition, the large amount of DMQ_9 retained in the suppressor mutants indicates that DMQ_9 is unlikely to contribute to the Clk phenotypes.

We have cloned 6 of the suppressors and all encode $tRNA^{Glu}$ genes whose anticodons are altered to read the substituted Lys codon of *clk-1(e2519)*. To our knowledge, these suppressors represent the first missense suppressors identified in any metazoan. The pattern of suppressed phenotypes we observe among the suppressor mutants suggests that the individual members of the $tRNA^{Glu}$ family might be expressed in different tissues, at different times during development and at different levels. It also indicates that most Clk phenotypes can be uncoupled from each other, an observation that is particularly relevant to understanding the long lifespan phenotype.

Materials and Methods

General Culture Methods and Strains

C. elegans were cultured under standard conditions at 20°C (37). For the most part, worms were fed on either the standard *E. coli* strain OP50, which produces UQ_8, or on GD1 (14) or DM123 (28) (*ubiG*::Kan and *yigR*::Kan, respectively), which are UQ_8-deficient *E. coli* strains. These strains were cultured in 2YT growth medium supplemented with 0.5% glucose and 50 µg/mL kanamycin then on NGM plates supplemented with 50 µg/mL kanamycin, to prevent contamination from other bacterial strains. For some experiments, worms were fed on *ispB* mutant bacteria (*ispB::Cm*^{r}) engineered to produce UQ_8 or UQ_9 (KO229 pSN18 and

KO229 pKA3, respectively) (32). These bacteria were grown in 2YT and then on NGM plates containing 50 µg/mL of spectinomycin and ampicillin, respectively.

A two-step procedure was used to transfer worms from OP50 to the various test bacteria. First the worms were transferred without bacteria to an intermediate plate seeded with the test bacteria. The worms were left to crawl around on these plates for ~2 hours to rid them of any trace amount of OP50. The worms were then transferred from the intermediate plates to the experiment plates containing the same test bacteria.

The Bristol strain N2 was used as the wild type. The *clk-1* alleles used in this study were *e2519* and *qm30*. The mutants used for linkage and mapping are as follows: LGI: *unc-11(e47), dpy-5(e61), unc-75(e950), unc-101(m1), unc-54(e190),* LGII: *lin-31(n301), unc-85(e1414), clr-1(e1745), dpy-10(e128), unc-4(e120), unc-53(e404),* LGIII: *dpy-17(e164),* LGIV: *unc-24(e138),* LGV: *dpy-11(e224),* LGX: *unc-1(e719), lon-2(e678), unc-6(e78), dpy-6(e14), unc-115(mn481), unc-9(e101), unc-84(e1410), dsc-1(qm133), unc-7(e5).*

Isolation of Suppressor Mutations

clk-1(e2519) worms were mutagenized with EMS, essentially as described (37). Briefly, L4 stage worms were incubated with 50 mM EMS for 4 hours at 20 °C, with agitation every 30 min. The mutagenized P0's were transferred to 90 mM plates, 20 worms/plate and left to produce progeny. The resulting F1's were bleached as gravid adults (using a solution of 20:3:2 of H20: 10% NaOCl: 5M KOH), and the resulting F2's were left to hatch in M9 buffer for ~48 hrs, under which condition they will arrest as L1-stage larvae. For the growth rate suppressor screen, the L1's were spotted onto 90 mM plates seeded with OP50 and were screened 2-3 days later for the presence of fast growing worms. For the growth arrest/sterility suppressor screen, the L1's were spotted onto 90 mM plates seeded with DM123 and were screened 3-4 days later for the presence of non-arrested worms. The plates were also re-screened a few days later for

the presence of F3 eggs laid by a fertile F2 worm that was missed in the initial screening, but no such eggs were found.

The number of haploid genomes screened was estimated from the number of F2 worms scored and the number of F1 worms from which they originated (assuming that each F1 worm carries 2 independently mutagenized haploid genomes, and that 4 F2 worms need to be scored from each F1 to be able to score the 2 haploid genomes). Based on this, we estimate that we scored 200,000 haploid genomes in the growth rate suppressor screen (from which we isolated 2 suppressors: *qm194* and *qm195*) and 100,000 haploid genomes in the growth arrest/sterility suppressor screen (from which we isolated 7 suppressors: *qm196*, *qm197, qm198, qm199, qm210, qm211*, and *qm213*), which suggests that the growth arrest/sterility suppressor screen is a much more sensitive screen. It should also be noted that because all suppressors recovered were dominant, the actual numbers of haploid genomes screend are likely greater than our estimates. We also screened approximately 100,000 haploid genomes in the *clk-1(qm30)* background for both screens, from which we did not recover any suppressors.

The mutants described here in detail (*qm199, qm210, qm211* and *qm213*) have been backcrossed to the *clk-1(e2519)* strain at least 3 times.

Mapping and Cloning of Suppressors

All linkage and mapping was carried out using strains that were homozygous for *clk-1(e2519)*. For linkage analyses homozygous suppressor mutants were crossed with mutants homozygous for a recessive visible mutation. F2 animals homozygous for the visible mutation were transferred to DM123 plates as young adults. The entire F3 brood was scored as either suppressed (when 75 or 100% of the F3's were suppressed) or not suppressed (when 0% were suppressed). Linkage is indicated by <75% of the F3 plates being suppressed. The recombination distance (p) was calculated by considering the recombination frequency (f; the number of suppressed plates/total number

of plates) to be equal to 2p/(1+p). The f was also used to calculate the 95% confidence interval from the Binomial Distribution.

For 3-point mapping experiments, hermaphrodites homozygous for 2 closely linked markers were crossed with homozygous suppressor males. Recombinant F2's were singled as adults. To assay for the presence of the suppressor mutation, 4 F3's with the recombinant phenotype (either homozygous or heterozygous for the recombinant chromosome) from each recombinant F2 were singled as adults onto DM123 plates. Their entire F4 brood was scored as either suppressed (75% or 100% suppressed) or not suppressed (0% suppressed). If any of the F4 plates were suppressed, the recombinant F2 was considered to have carried the suppressor mutation. The 95% confidence intervals were calculated from the Binomial Distribution.

The key mapping data for *qm199*, *qm210*, *qm211* and *qm213* have been included in Table 1. The approximate map positions of *qm195* and *qm197* were determined by linkage analyses and are also given in Table 1. Based on the map positions of these 6 mutants, we identified their corresponding genes using a candidate approach. Genomic DNA was extracted from the mutants and the candidate genes were sequenced. Primer sequences and PCR conditions are available upon request. *qm195* was found to contain the same mutation in the same gene as *qm199,* although they were isolated independently in different screens. *qm194*, *qm196,* and *qm198* have all been linked to various regions of LGX, however we have not determined to which genes they correspond.

Phenotypic Analyses

Brood Size: The total broods of individual animals were counted. For brood size measurements on DM123 and GD1, the P0's were transferred to the UQ- bacteria as young adults, and the broods produced by their F1 progeny were counted.

Embryonic Development: 2- to 4-cell embryos were dissected out of gravid hermaphrodites and monitored every hour until hatching.

Post-embryonic Development: Eggs were picked to plates and examined 3 hours later. Larvae that had hatched during this time were considered to be 1.5hours old at the end of the interval. Larvae were examined every hour until they molted into adults.

Lifespan: On Day 0, eggs were picked to plates and left to hatch for 6 hours. Eggs that had hatched during this interval were used for the experiment. The worms were monitored daily until death. Worms deemed to die from either internal hatching or gut extrusion were not included in the study and were replaced by other worms.

Defecation: Worms were scored for 3 to 5 consecutive defecation cycles as described (4), and the mean cycle length was calculated for each worm. For experiments on KO229 pKA3 and KO229 pSN18, N2 worms were grown on the test bacteria for 3 generations and the *clk-1* mutant strains for 4 generations.

Pharyngeal Pumping: Worms were scored for 5 individual minutes, and the mean number of pumps per minute was calculated for each worm.

Statistical analyses: The Student's t-test was used for all phenotypes except the lifespan phenotype, for which the Log rank test was used. All tests were 1-tailed. Differences between the samples were considered to be significant when $p<0.05$. In the text, we consider a significant difference from *clk-1(e2519)* but not N2 to indicate full suppression, significant differences from both N2 and *clk-1(e2519)* to indicate partial suppression, and a significant difference from N2 but not *clk-1(e2519)* to indicate no suppression.

HPLC Analyses

Worms were collected from plates nearing starvation, and were washed several times with M9 buffer. The worms grown on KO229 pSN18 were cleaned by sucrose flotation. ~400 μL of worms were used for each run. The extraction was performed essentially as described (34). Briefly, worm pellets were completed to 1 mL with water and homogenized in the presence of 50µL of 2,6-di-tert-butyl-4-methyl-phenol (BHT; 10 mg/mL in

EtOH; from Sigma). 100µL of homogenate was taken at this step to measure protein concentration, using the Bio-Rad protein assay. 1 mL of 0.1 M SDS was added and the samples were further homogenized. 2 mL of EtOH was added and the samples were vortexed. 2 mL of hexane was added and the samples were vortexed, and then centrifuged at 1000 x *g* for 5 min at 4°C. The upper organic layer was collected and the hexane extraction was repeated. The samples were evaporated using a cooled speed vacuum, and were kept at –80°C. The Beckman Coulter System Gold Hardware and the 32 Karat Software were used to analyze the samples. Shortly after reconstitution with 750 µl mobile phase (70% methanol and 30% ethanol), the samples were loaded on a reverse phase column (Inertsil ODS-3 C-18, 5 µM, 4.6 X 250 mM, GL Science) and the elution was monitored by a UV detector at 275 nm.

For peak identification, a "standard" containing commercial UQ_9 (Sigma) mixed with lipid extract from *clk-1(qm30)* worms grown on OP50 (for DMQ_9 and UQ_8) was prepared. For quantification, the amount of UQ_9 was determined by comparison to UQ_9 standards of known concentration, which were also used to estimate the amount of DMQ_9 and UQ_8. The recovery rate was estimated by determining how much UQ_6 (Sigma) was present in the sample after a known amount had been added to the samples before the lipid extraction was performed. The amount of quinone was normalized to the amount of protein.

Results

Identification of suppressors of the growth phenotypes of *clk-1(e2519)*

clk-1 mutants exhibit a highly pleiotropic phenotype: when grown on UQ-replete bacteria the mutants grow slowly, have slowed behaviors, delayed reproduction and an increased lifespan (23, 35, 41); when grown on UQ-deficient bacteria the mutants transiently arrest development at the second larval (L2) stage, eventually growing up to become sterile adults (5, 12). We carried out suppressor screens in an effort to better understand the

bases of the growth phenotypes and also to further explore the mutants' requirement for dietary UQ. The first screen was aimed at identifying mutants that could suppress the slow growth rate of *clk-1* mutants on the standard UQ-replete bacterial strain OP50. We screened for suppressors in both the *clk-1(qm30)* and *clk-1(e2519)* backgrounds. We were not able to find any suppressors in the *qm30* background, but isolated 2 suppressors (*qm194* and *qm195*) in the *e2519* background. The second screen was aimed at finding suppressors of the growth arrest and/or sterility on the UQ-deficient DM123 bacteria. Again we screened in both the *qm30* and *e2519* backgrounds, and only found suppressors in the *e2519* background. This second screen was a much more efficient, or sensitive, screen, as we were able to isolate 7 suppressors (*qm196, qm197, qm198, qm199, qm210, qm211*, and *qm213),* although we screened only half as many haploid genomes as had been screened in the first screen (see Materials and Methods). All the suppressors isolated in this screen suppressed both the growth arrest and sterility on the UQ-deficient bacteria.

As mentioned above, we were unable to find suppressors in the *qm30* background, which suggested that the suppressors may be *e2519*-specific. To test this directly, we transferred a number of the suppressors into the *qm30* background and found that none could suppress any phenotypes on UQ+ or UQ- bacteria (data not shown), indicating that we had in fact isolated 9 *e2519*-specific suppressors. Given the number of haploid genomes screened, it is likely that no single gene can be mutated so as to suppress the growth phenotypes of *qm30* mutants.

Growth suppressors suppress both UQ- and UQ+ phenotypes

In order to determine whether the mutants isolated in the two screens represent two different classes of suppressors, we tested both groups of suppressors for suppression of the Clk-1 phenotypes on UQ- and UQ+ bacteria. We found that all 9 suppressors are able to suppress the growth arrest and sterility on UQ- bacteria (Figure 6.1 and data not shown).

Indeed all the suppressors are fully fertile on the UQ- bacteria. In some cases the suppressors even have wild-type brood sizes.

We then tested whether the suppressors are able to suppress the growth and behavioral Clk phenotypes observed on UQ+ bacteria (Figure 6.2). For these analyses we focused on 4 of the mutants that we had repeatedly backcrossed and which we believe to be representative of the 9 mutants isolated. We found that all mutants are able to partially suppress the slow rate of embryonic development of *clk-1(e2519)*, with *qm211* having the weakest effect (Figure 6.2A). All mutants are also able to partially suppress the slow post-embryonic development, with the exception of *qm211*, which did not suppress this phenotype at all (Figure 6.2B). Similarly, we found that, with the exception of *qm211*, the suppressors are able to fully suppress the increased lifespan of *clk-1(e2519)* (Figure 6.2C). In contrast, we found that all the suppressors could fully suppress the slow pharyngeal pumping rate of *clk-1(e2519)* (Figure 6.2D). For the slow defecation rate of *clk-1(e2519)*, we found that *qm213* suppresses fully and *qm211* partially, while *qm199* and *qm210* do not suppress this phenotype at all. This complex pattern of suppression is likely due to differences in the level, timing and/or tissue-specificity of expression of each suppressor (see Discussion).

Suppressors restore UQ biosynthesis

HPLC analysis revealed that all the suppressors restore UQ biosynthesis (Figure 6.3E-H and data not shown), although not to wild-type levels. As can be seen from a quantification of the levels of the quinones present in the various strains (Figure 6.4), the predominant Q species in the wild-type is UQ_9, whereas in *clk-1(e2519),* as well as in the suppressors, the predominant Q species is DMQ_9, with only a very small amount of UQ_9 detectable in the suppressors. In fact, the suppressor that makes the most UQ_9 (*qm213*) makes 20 times less than the wild-type and the suppressor that makes the least UQ_9 (*qm210*) makes 63 times less than the wild-type, yet this appears to be sufficient for growth and fertility on UQ-deficient

bacteria, and even for almost wild-type rates of growth and for some behaviors.

In order to test whether this small amount of UQ_9 could really be responsible for the observed suppression, we chose to examine the defecation rate of the wild-type, *clk-1(e2519)* and *qm213; clk-1(e2519)* on a transgenic *E. coli* strain that has been engineered to produce UQ_9 (Figure 6.5). We chose the defecation rate because it is one of the phenotypes that can be fully rescued by some of the suppressors. The KO229 strain lacks the octaprenyl diphosphate synthase encoded by the *ispB* gene, which is required for the synthesis of the UQ side chain. The pKA3 plasmid contains a wild-type *ispB* gene from *E. coli* and restores UQ_8 biosynthesis, whereas the pSN18 plasmid contains a *Synechocystis* sp. strain PCC6803 *ispB* homolog, which results in the synthesis of UQ_9 as well as some UQ_8 (32).

We found that both *clk-1(e2519)* and *qm213; clk-1(e2519)* mutants have significantly slower defecation on both KO229-based strains than on OP50. Possibly, the mutants are sensitive to the lack of menaquinone (MK) that is a feature of the KO229-based strains (15), or some other difference unrelated to UQ. Alternatively, the *clk-1(e2519)* mutants could be sensitive to the level of UQ in their food source. Indeed, under our growth conditions, both the pKA3- and pSN18-containing strains produce less total UQ than OP50, ~3 and ~9 times less than OP50, respectively (data not shown).

We also observed that the slow defecation of *clk-1(e2519)* is not suppressed when fed on the KO229 pSN18 strain, although this strain makes UQ_9. On the other hand, the suppressed *qm213; clk-1(e2519)* strain is much less sensitive to the level of UQ, as it is faster than *clk-1(e2519)* on all food sources. Thus, the defecation rate of *qm213; clk-1(e2519)* on KO229 pKA3 (which makes only UQ_8) is significantly faster than that of *clk-1(e2519)* on KO229 pSN18 (which makes UQ_9). This suggests that exogenously obtained UQ_9 is not equivalent to

endogenously synthesized UQ_9. Although *clk-1(e2519)* mutants grown on KO229 pSN18 assimilate more UQ_9 than the suppressors produce (Figure 6.4), it is possible that this UQ_9 does not reach the required cells or subcellular location. However, it has previously been shown that, at least for UQ_8, most of the UQ obtained exogenously can reach the mitochondria (17) (see Discussion).

Suppressors encode $tRNA^{Glu}$ genes

Using linkage analyses we determined that 7 of the suppressors map to LG X (*qm194* to *qm198, qm199, qm211*), 1 to LG I (*qm210*), and 1 to LG II (*qm213*) (data not shown). Using 2- and 3-point mapping strategies we further refined the genetic positions of 6 of the suppressors (the key data are summarized in Table 6.1). We determined that in each of the intervals to which a suppressor had been mapped there was a least one gene coding for a $tRNA^{Glu}$. Given that the *e2519* mutation results in the substitution of a Glu codon for a Lys codon (GAG to AAG) one possible mechanism of suppression could be the mutation of a $tRNA^{Glu}$. We sequenced the candidate tRNA genes in each of these 6 mutants, and in each case identified one tRNA gene that contained a C to T transition at position 36 of the gene. *qm195* and *qm199* contain the same mutation in the same $tRNA^{Glu}$ gene, although they were isolated independently in two different screens, whereas the other 4 contain the same mutation but in distinct $tRNA^{Glu}$ genes. These genes have been named *rte-1* to *rte-5* for RNA, Transfer, glutamic acid (E). The C36T mutation found in all the suppressors alters the anticodon from CTC to CTT and would allow for the decoding of an AAG Lys codon as a Glu codon. This suggests that the suppressors act by restoring the production of some CLK-1(+) protein, which in turn is capable of restoring a small amount of UQ biosynthesis. The other 3 suppressors have been linked to the X chromosome, which contains multiple $tRNA^{Glu}$ genes. Although the molecular identities of these 3 mutations have not been formally determined, we believe that they also

correspond to $tRNA^{Glu}$ genes, given that they are similar to the others suppressors in heritability and phenotype (data not shown).

Discussion

The pleiotropic phenotype and dietary requirements of *clk-1* mutants

We have identified 9 suppressors of the missense mutant *clk-1(e2519).* The 6 suppressors cloned all encode $tRNA^{Glu}$ genes, whose anticodons have been altered to read the altered Glu codon of the *e2519* mutant. The suppressors still predominantly accumulate the UQ intermediate DMQ, although they restore a very small amount of UQ biosynthesis. This small amount of UQ appears to be sufficient for continuous growth and fertility on UQ-deficient bacteria. The suppressors also suppress some phenotypes on UQ-replete bacteria, although different subsets of Clk-1 phenotypes are suppressed by different mutations. These observations allow us to resolve some questions about the *clk-1* mutants.

Firstly, the fact that different subsets of phenotypes are suppressed by the different mutants indicates that the phenotypes can be de-coupled from each other (Figure 6.2). From an analysis of the patterns of suppression, we can deduce that the increased lifespan of *clk-1* is not due, and does not require slow embryonic development or slow behaviors. In particular, it is interesting to note that a slow rate of pharyngeal pumping is not necessary for an increased lifespan as the *clk-1(e2519); qm211* mutants have a wild-type rate of pumping but a long lifespan that is indistinguishable from that of *clk-1(e2519).* This observation suggests that the mechanism of lifespan extension of *clk-1* mutants is not simply caloric restriction, although slow pumping can lead to caloric restriction in worms and increase lifespan in worms (24). On the other hand, the suppressor mutants cannot tell us whether slow post-embryonic development is required for the increased lifespan. However, previous results have suggested that this is not the case (5).

Secondly, the quinone contents of the mutants allows us to address some questions about DMQ_9. Previously we had speculated that the large amount of DMQ_9 present in *clk-1* mutants but not in the wild-type, could be contributing to the phenotype. Indeed, there is *in vitro* and *in vivo* evidence that DMQ can act in the electron transport chain (25, 30, 39). There has also been some *in vitro* evidence to suggest that DMQ could be a lesser prooxidant than UQ (31), which could explain the lowered levels of ROS observed in the *clk-1* mutants (19, 35). However, the biological role of DMQ is somewhat controversial given that DMQ does not seem to support respiration in yeast, although it appears to act as a prooxidant in this system (33). Our previous observations suggest that DMQ contributes to the viability of the *clk-1* mutants on UQ-replete bacteria, as mutants completely devoid of endogenous quinones are inviable, even on UQ-replete bacteria (12). Our present observations, however, suggest that the Clk-1 phenotypes are not due to the presence of DMQ_9, as the suppressors still accumulate a very large amount of DMQ_9, yet most of the Clk-1 phenotypes are suppressed. Thus, the presence of DMQ does not contribute to those phenotypes that are fully rescued by the suppressors (lifespan, defecation and pharyngeal pumping). However, the developmental rates are only partially suppressed, so it remains possible that the presence of DMQ affects these phenotypes. Alternatively, it could be that these phenotypes have a higher threshold, requiring more CLK-1(+) activity.

Thirdly, the suppressors allow us to address whether there is a specific requirement for endogenously synthesized UQ versus exogenous, dietary, UQ. *clk-1* mutants grown on the standard UQ_8-replete bacterial strain (OP50) assimilate UQ_8 and transport it all the way to their mitochondria (17). However, the mutants contain significantly less UQ_8 than there is UQ_9 in the wild type. Therefore, the Clk-1 phenotypes on UQ-replete bacteria could be due to (1) too little UQ, (2) a non-equivalence of UQ_8 and UQ_9, or (3) a subtle non-equivalence of endogenously-

synthesized versus exogenously-acquired UQ. The amount of UQ_9 synthesized by the suppressors is dramatically less than the amount of UQ_8 obtained from the bacteria, yet the phenotypes are suppressed, suggesting the Clk phenotypes are not due to too little UQ. *clk-1(e2519)* is not rescued by the UQ_9 producing bacterial strain indicating that even UQ_9 cannot rescue the mutants when it is obtained exogenously, so it is not the difference in chain length (UQ_8 vs UQ_9) that results in the Clk-1 phenotypes on OP50. In fact, this observation suggests that exogenously obtained UQ cannot entirely substitute for endogenously synthesized UQ. Therefore it appears as though viability can be supported by exogenous UQ but that wild-type rates of development and behavior require endogenous UQ biosynthesis. At this time we cannot distinguish whether there is any specific requirement for UQ_9 in *C. elegans*, or whether UQ_8 would also be sufficient if it were synthesized endogenously.

What the functionally important difference between exogenous and endogenous UQ might be is not immediately obvious. UQ is found in all cellular membranes, with the highest levels in the mitochondria, where it is involved in electron transport (reviewed in (6, 38)). In general, the majority of ubiquinone derived from the diet remains outside of the mitochondria (reviewed in (7)). However, it has been shown in worms and in cultured cells that exogenously acquired UQ can reach the inner mitochondrial membrane, and, at least in cells, can then participate in electron transport (11, 17, 27). Possibly, in worms, not enough of the UQ acquired from bacteria reaches the site of electron transport in the mitochondria to influence mitochondrial function, or the acquired UQ does not assimilate into all tissues and cell types, some of which may be crucial for the regulation of physiological rates. Alternatively, it could be that electron transport, or some other important mitochondrial function, requires that UQ be synthesized de novo in the inner mitochondrial membrane.

We are also considering another, altogether distinct, possibility to explain the high degree of rescue provided by the suppressors. Given that

the suppressors encode suppressor tRNAs, they act by restoring the production of some CLK-1(+) protein, which in turn restores a small amount of UQ biosynthesis. Thus, it remains possible that it is the presence of wild-type CLK-1(+) protein rather than the small amount of accumulated UQ_9 that confers suppression. It is possible that CLK-1 carries out some other function in addition to ubiquinone biosynthesis, and it is this other function that is important for the Clk-1 phenotypes. This would be consistent with the lack of rescue conferred by the Q_9-producing bacteria, and the observation that *clk-1(e2519)* mutants have a weaker phenotype than *clk-1(qm30)*, despite the fact that neither mutant produces any UQ. The *e2519* mutant produces a full-length protein, which could retain some activity for a function that is not related to UQ biosynthesis, thereby resulting in a milder phenotype. Unfortunately, at the present time we have no tool to disrupt UQ biosynthesis in the presence of CLK-1(+) function or vice versa.

C. elegans tRNA missense suppressors

All 6 suppressors that we have cloned, and likely all 9 that we have isolated, encode $tRNA^{Glu}$ genes whose anticodons are altered to read the substituted Lys codon of *clk-1(e2519)*. To our knowledge, these suppressors represent the first missense suppressors identified in *C. elegans*, and in any metazoan. In fact, *C. elegans* is the only metazoan in which any tRNA suppressors have been identified in forward genetic screens, and to date, all are nonsense amber suppressors. Amber mutations introduce TAG nonsense codons. All 8 of the amber suppressors cloned so far contain mutations in the anticodon of $tRNA^{Trp}$ genes (CCA to CTA) that can decode this nonsense codon as Trp.

Amber suppressors have several properties including that the strength of suppression is dose- and often temperature-dependent, that the suppressors are tissue-specific, that different suppressors exhibit different strengths of suppression, and that strong suppression is deleterious (reviewed in (20)). Our analysis suggests that the $tRNA^{Glu}$

suppressors are different in a number of ways. Most notably, we have not observed any deleterious effects with our suppressors. They can be maintained indefinitely in the homozygous state, and at any temperature, without exhibiting obvious growth defects or sterility. There might be a number of reasons for this. For example, producing elongated proteins, which could act as dominant negatives, may be more deleterious than replacing some lysines by glutamic acids. In addition, there are 23 genes that decode the Glu codon (GAG) versus only 10 for the Trp codon (TGG) (8), thus, mutation of any one $tRNA^{Glu}$ gene could have less of a negative effect than mutation of one of the $tRNA^{Trp}$ genes.

We also did not observe a very strong dosage effect of the suppressor mutations. For example, for the phenotypes on UQ-deficient bacteria, heterozygotes exhibit profound suppression. Similarly, the defecation phenotype can be almost completely suppressed by a suppressor in the heterozygous state. However, we do not know whether the suppressor mutations are fully dominant for every phenotype. Similarly, the suppressors described here cannot simply be classified as weak or strong. Indeed, different suppressors suppress different subsets of phenotypes and this cannot be clearly related to the amount of UQ biosynthesis they restore. Instead, the observation that they suppress different sets of phenotypes may suggest that some phenotypes have a stage- or tissue-specific focus of action and that the suppressors are stage- or tissue-specific as well, as are the amber suppressors (21, 22, 26). Similarly, differences in the levels of expression might also play a role (26). The *qm211* mutation has a weaker effect on the developmental phenotypes as well as on the lifespan phenotype, suggesting that the affected gene may be expressed at a lower level.

Finally, we can speculate on why missense suppressors have not been isolated before in *C. elegans*. The almost full suppression we observe, compared to the very small amount of UQ biosynthesis restored, suggests that in the case of *clk-1,* uniquely small amounts of wild-type

protein are sufficient for a near wild-type phenotype. It is likely that other genes may require a higher proportion of wild-type protein for a wild-type phenotype.

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Table 6.1: Genetic Positions and Molecular Identities of Suppressor Genes.

Suppressor allele	3 Point Mapping Used to Identify the Suppressor	Genetic Position (95% Confidence Interval)	Corresponding tRNA gene	Anticodon Sequence
+	-	-	$tRNA^{Glu}_{GAG}$	CTC
rte-1(qm199)	*unc-9* (6/23) *sup* (17/23) *dsc-1*	13.33 X (9.31 to 15.86)	C04C11.t1	CTT
rte-2(qm210)	*unc-75* (21/40) *sup* (19/40) *unc-101*	11.44 I (10.82 to 12.04)	Y53C10A.t2	CTT
rte-3(qm211)	*unc-6* (19/51) sup (32/51) *dpy-6*	-1.25 X (-1.52 to -0.96)	F27D9.t1	CTT
rte-4(qm213)	*lin-31* (50/52) sup (2/52) *clr-1*	-1.51 II (-1.95 to -1.35)	ZK622.t2	CTT
rte-5(qm197)	-	12.86 X[a] (8.67 to 18.89)	F02D10.t1	CTT
rte-1(qm195)	-	16.41 X[a] (4.3 to 23.26)	C04C11.t1	CTT

[a] Genetic positions are based on 2-point mapping experiments using *dpy-6* and *unc-84.*

Figure. 6.1: Brood sizes of N2, *clk-1* and *clk-1* suppressor strains grown on UQ- replete (OP50) and UQ-deficient (DM123 and GD1) bacteria.

The bars represents the mean brood size ± SD produced by 12-17 animals. Arrows indicate the complete sterility of *clk-1(e2519)* on DM123 and GD1 bacteria.

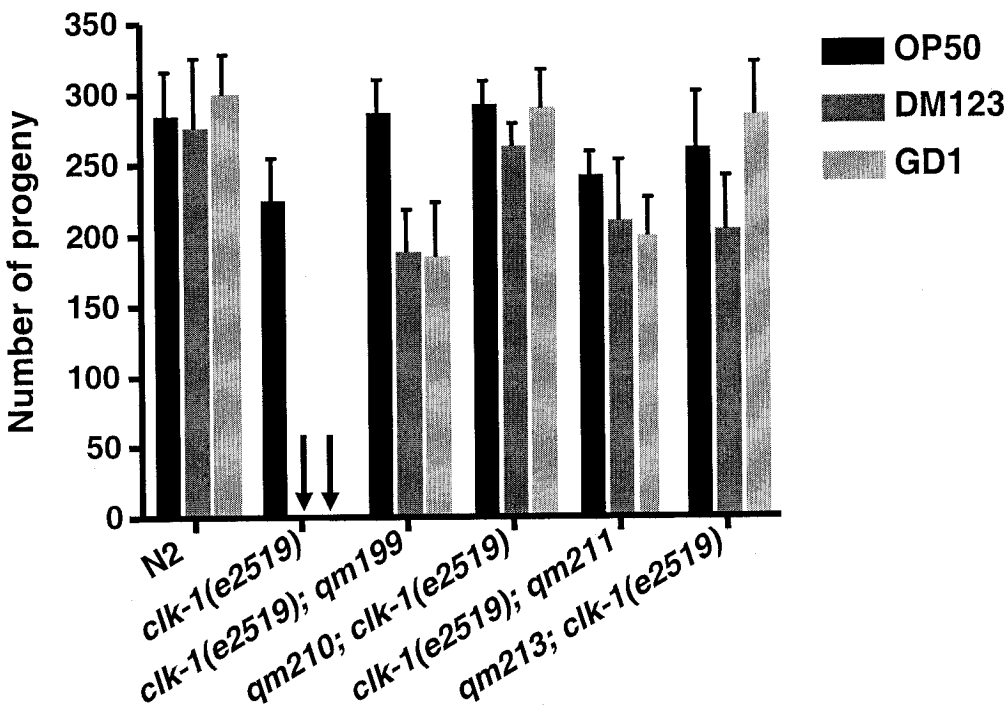
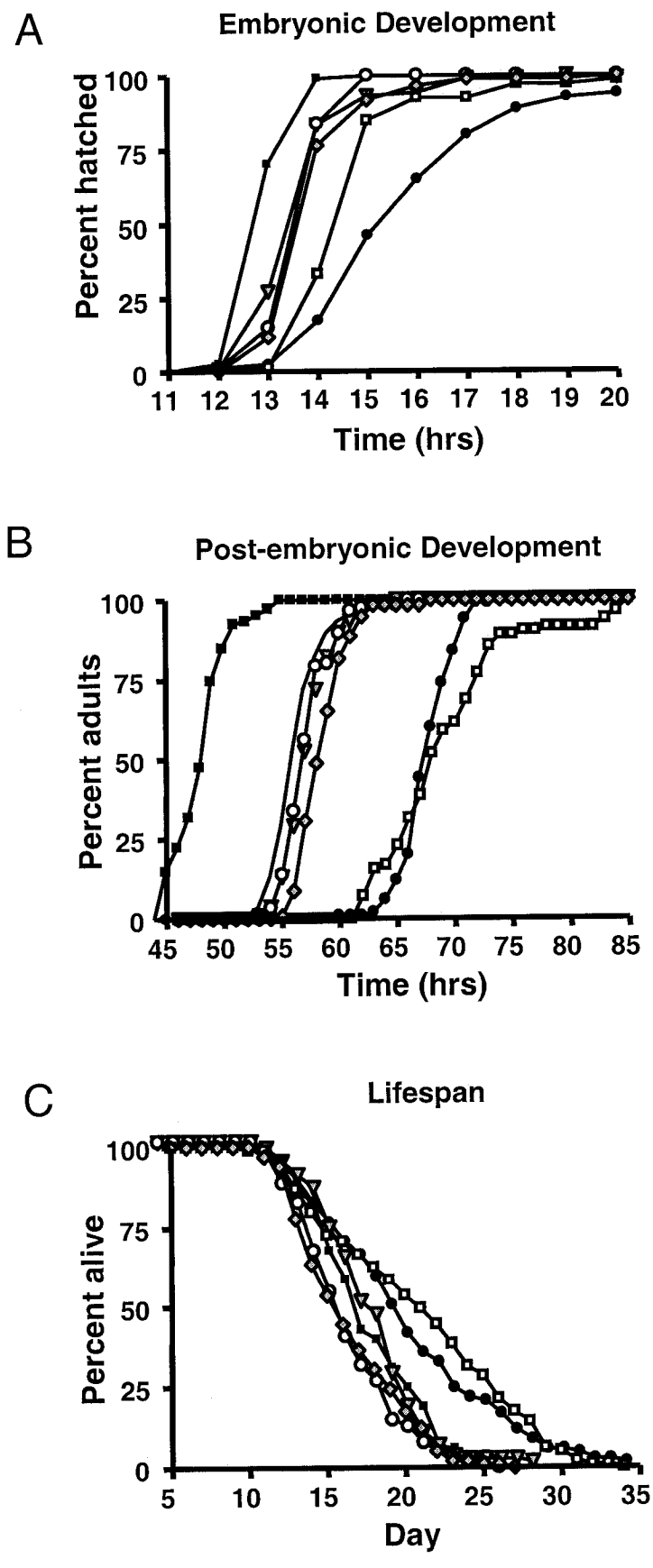

Number of progeny
350
300
250
200
150
100
50
0
OP50
DM123
GD1
N2
clk-1(e2519)
clk-1(e2519); qm199
qm210; clk-1(e2519)
clk-1(e2519); qm211
qm213; clk-1(e2519)

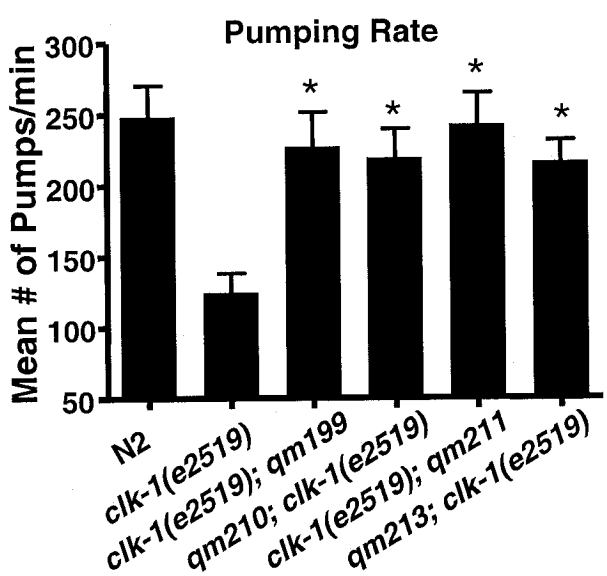
Figure 6.2: Developmental and behavioral phenotypes of N2, *clk-1* and *clk-1* suppressor strains grown on OP50 bacteria.

# indicates that the data is significantly different from the data for N2, and * from *clk-1(e2519)*; see Materials and Methods for further explanation. **(A-C)** The data given is for genotypes in the following order: N2, *clk-1(e2519)*, *clk-1(e2519);qm199*, *qm210;clk-1(e2519)*, *clk-1(e2519);qm211*, *qm213;clk-1(e2519)*. **(A)** The rate of embryonic development. Each point represents the percentage of embryos that had hatched by that time point. For each genotype n=66-111. The means ± SD are: 13.28±0.53 (*), 16.09±1.87 (#), 14.03±1.06 (#, *), 14.00±0.60 (#, *), 15.02±1.38 (#, *), 14.27±1.02 (#, *). **(B)** The rate of post-embryonic development. Each point represents the percentage of larvae that had molted to adults by that time point. For each genotype n=83-116. The means ± SD are: 49.10±5.27(*), 69.03±2.30 (#), 58.26±4.21(#, *), 57.66±2.68 (#, *), 69.92±5.16 (#), 59.31±3.64 (#, *). **(C)** Lifespan. Each point represents the percentage of worms alive at that time point. For each genotype n=100. The means ± SD are: 18.42±3.58(*), 20.81±5.49(#), 18.66±3.14(*), 17.13±3.12 (*), 21.04±5.99 (#), 16.62±3.53 (*). **(D)** The rate of pharyngeal pumping. The bars represent the mean pumping rate of 10 animals, with each animal's rate based on the mean of 5 measures; the error bars represent the standard deviation of the means. **(E)** The rate of defecation. The bars represent the mean defecation cycle length 12 animals, with each animal's cycle length based on the mean of 5 cycles; the error bars represent the standard deviation of the means.


A
Embryonic Development
Percent hatched
100
75
50
25
0
11 12 13 14 15 16 17 18 19 20
Time (hrs)
B
Post-embryonic Development
Percent adults
100
75
50
25
0
45 50 55 60 65 70 75 80 85
Time (hrs)
C
Lifespan
Percent alive
100
75
50
25
0
5 10 15 20 25 30 35
Day


N2
clk-1(e2519)
clk-1(e2519); qm199
qm210; clk-1(e2519)
clk-1(e2519); qm211
qm213; clk-1(e2519)

D


Pumping Rate
Mean # of Pumps/min
300
250
200
150
100
50
*
*
*
*
N2
clk-1(e2519)
clk-1(e2519); qm199
qm210; clk-1(e2519)
clk-1(e2519); qm211
qm213; clk-1(e2519)

E

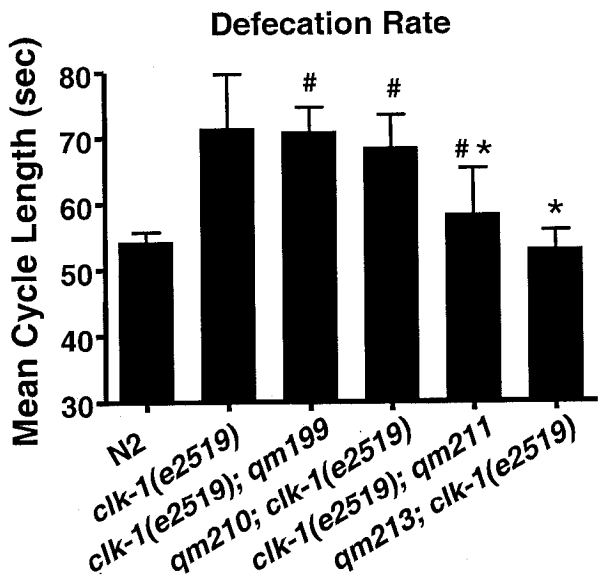

Defecation Rate
Mean Cycle Length (sec)
80
70
60
50
40
30
#
#
#*
*
N2
clk-1(e2519)
clk-1(e2519); qm199
qm210; clk-1(e2519)
clk-1(e2519); qm211
qm213; clk-1(e2519)

Figure 6.3: HPLC analysis of quinone content.

Representative chromatograms of quinones extracted from **(B)** N2 **(C)** *clk-1(e2519)* and suppressors **(E)** *clk-1(e2519); qm199* **(F)** *qm210; clk-1(e2519)* **(G)** *clk-1(e2519); qm211* and **(H)** *qm213; clk-1(e2519).* All worms were grown on OP50 bacteria. The traces are lined up with the standards **(A, D)** run on that same day. In **(E-H)** the arrows indicate the small amount of UQ_9 detected in the suppressors.

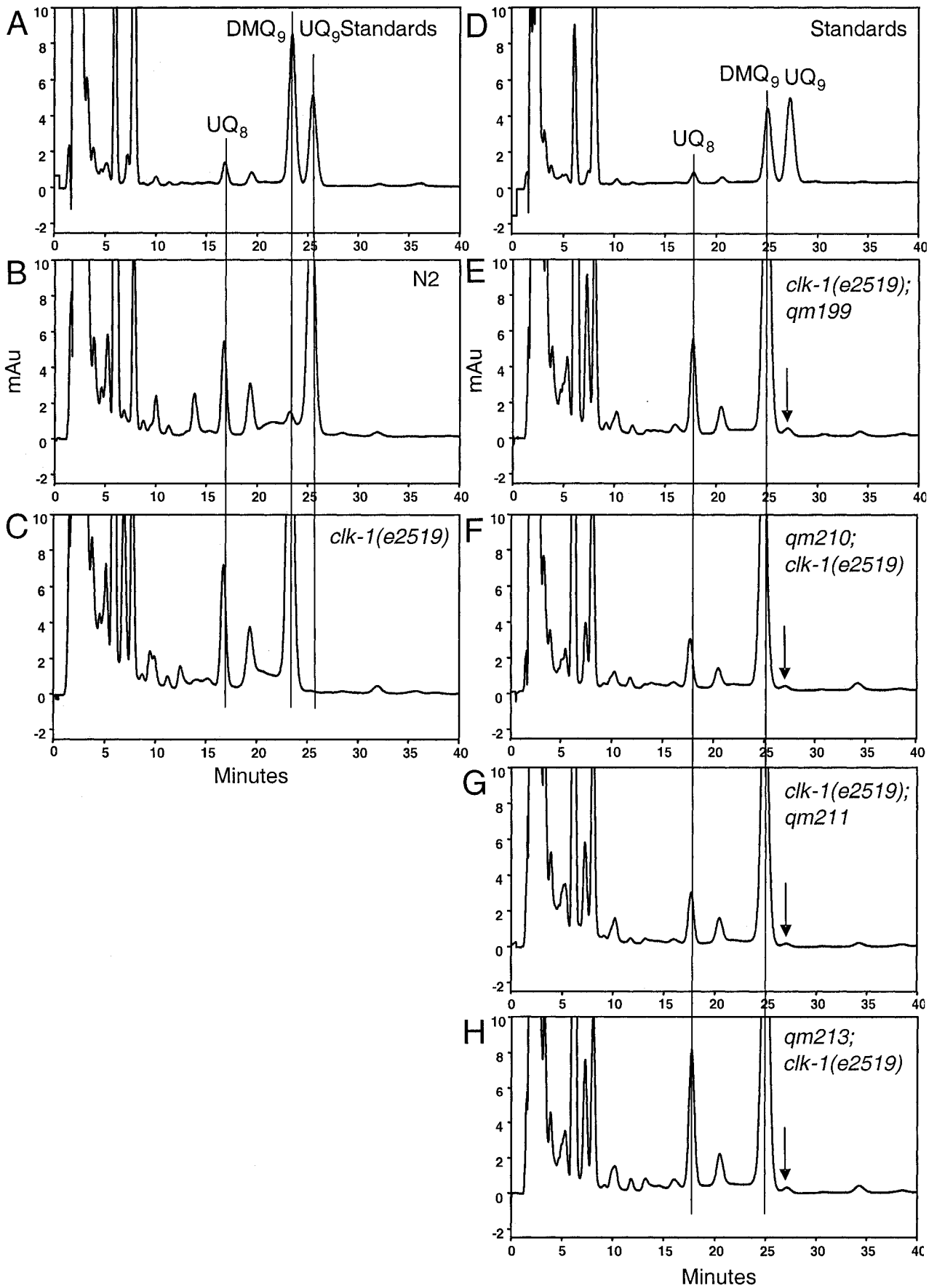
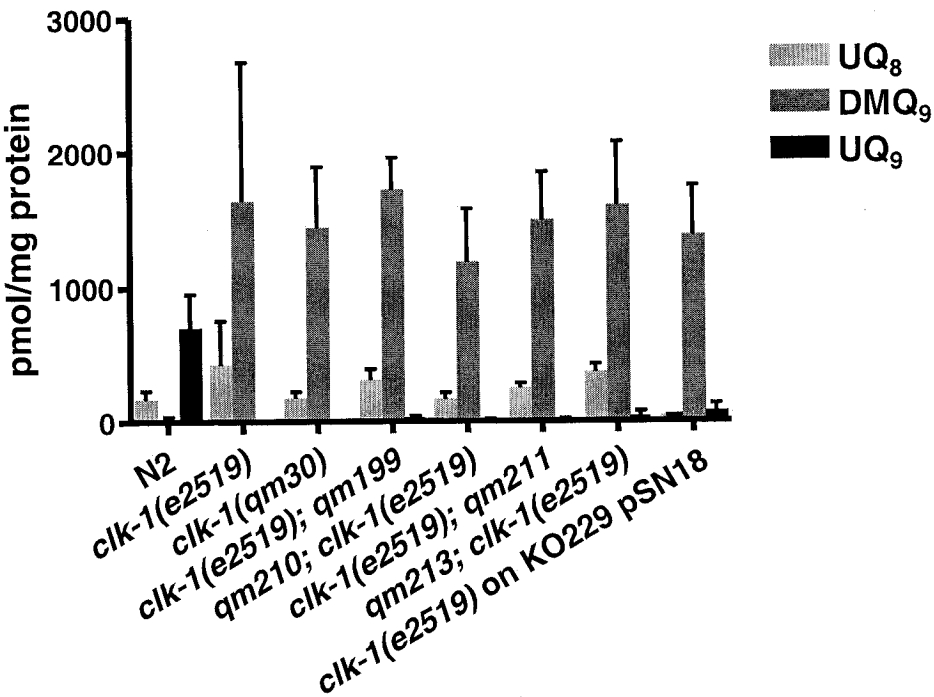

A
DMQ9
UQ9Standards
UQ8
B
N2
C
clk-1(e2519)
mAu
Minutes
D
Standards
DMQ9
UQ9
UQ8
E
clk-1(e2519);
qm199
F
qm210;
clk-1(e2519)
G
clk-1(e2519);
qm211
H
qm213;
clk-1(e2519)
mAu
Minutes

Figure 6.4: Quantification of quinone content.

(A) Quinone content of N2, *clk-1* and *clk-1* suppressor strains. Worms were grown on OP50 with the exception of the last bar, for which the worms were grown for >5 generations on the UQ_9 producing strain KO229 pSN18. Each bar indicates the mean ± SD from 3 independent extractions, with the exception of the last bar for which the data comes from 2 independent extractions. **(B)** The same results shown in (A) for UQ_9 at an expanded scale, which allows for the visualization of the small amount of UQ_9 present in the suppressors and *clk-1(e2159)* when grown on KO229 pSN18 . The arrows indicate that, even at this scale, there is no detectable amount of UQ_9 in *clk-1* mutants.

A


3000
2000
1000
0
pmol/mg protein
UQ8
DMQ9
UQ9
N2
clk-1(e2519)
clk-1(qm30)
clk-1(e2519); qm199
qm210; clk-1(e2519)
clk-1(e2519); qm211
qm213; clk-1(e2519)
clk-1(e2519) on KO229 pSN18

B

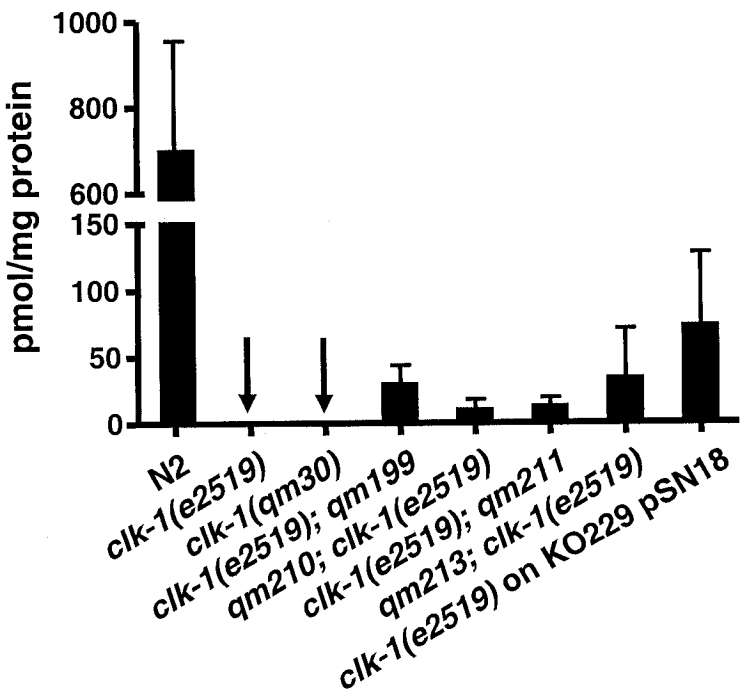
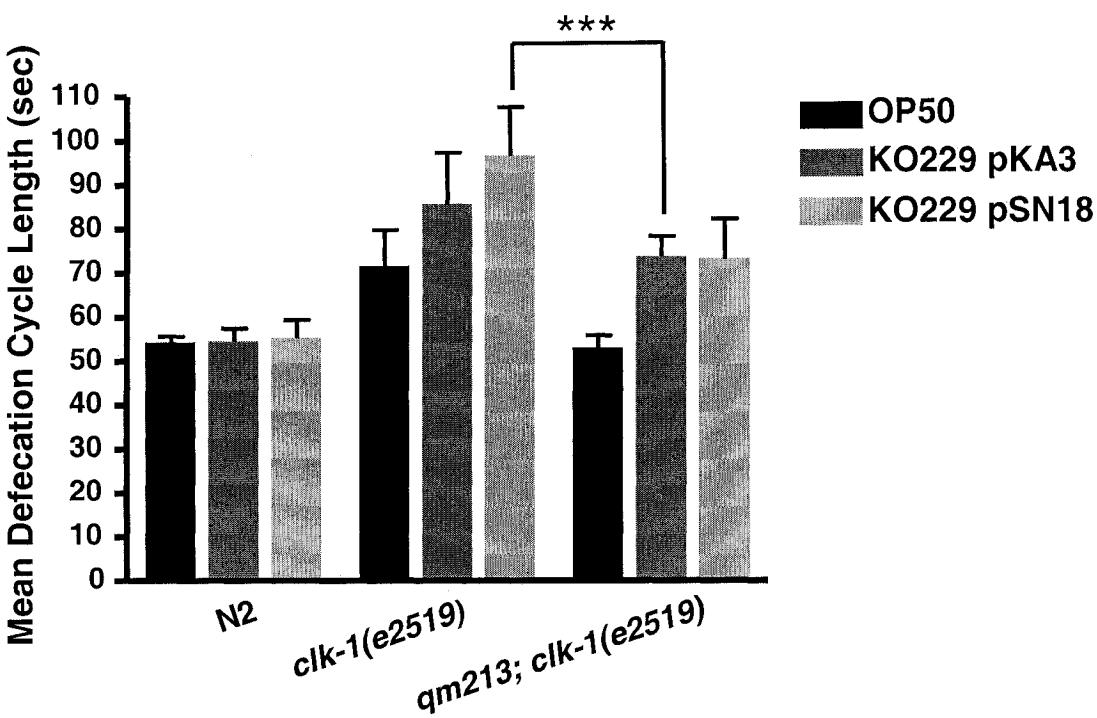

1000
800
600
150
100
50
0
pmol/mg protein
N2
clk-1(e2519)
clk-1(qm30)
clk-1(e2519); qm199
qm210; clk-1(e2519)
clk-1(e2519); qm211
qm213; clk-1(e2519)
clk-1(e2519) on KO229 pSN18

Figure 6.5: Defecation rate of N2, *clk-1* and a *clk-1* suppressor strain grown on UQ_8 and UQ_9 producing bacteria.

UQ_8 is the predominant UQ species in OP50 and KO229 pKA3, and UQ_9 in KO229 pSN18 ((32) and data not shown). The bars represent the mean defecation cycle length of 10 animals, with each animal's cycle length based on the mean of 3 cycles; the error bars represent the standard deviation of the means of the individual animals. The rate of *clk-1(e2519)* is significantly slower on KO229 pKA3 than on OP50 ($p<0.001$) and significantly slower on KO229 pSN18 than on KO229 pKA3 ($p<0.01$). *qm213; clk-1(e2519)* is also significantly slower on KO229 pKA3 and on KO229 pSN18 than on OP50 ($p<0.0001$), but there is no difference between KO229 pKA3 and pSN18. *qm213; clk-1(e2519)* is significantly faster than *clk-1(e2519)* on all bacteria: on OP50 ($p<0.0001$), KO229 pKA3 ($p<0.001$) and KO229 pSN18 ($p<0.0001$). *qm213; clk-1(e2519)* grown on KO229 pKA3 is significantly faster than *clk-1(e2519)* grown on KO229 pSN18 ($p<0.0001$) although they contain the same amount of Q_9 (see Figure 6.4), suggesting that Q_9 obtained exogenously is not equivalent to Q_9 synthesized endogenously.


***
Mean Defecation Cycle Length (sec)
110
100
90
80
70
60
50
40
30
20
10
0
N2
clk-1(e2519)
qm213; clk-1(e2519)
OP50
KO229 pKA3
KO229 pSN18

Chapter 7:

Summary and Conclusions

Summary of the thesis

The goal of the thesis was to investigate the function of CLK-1 and the phenotypic consequences of losing *clk-1(+)* activity using a suppressor analysis approach.

I report on the isolation of defecation suppressors of *clk-1*, which we call the *dsc* mutants. We isolated and genetically mapped eight mutations, which fall into five complementation groups. Four of these complementation groups define new genes, which we named *dsc-1* to *dsc-4*; the 5th corresponds to *dec-7*, a gene that had previously been identified. We demonstrate that the defecation cycle is not temperature compensated, but is actively altered in response to changes in temperature. We also show that *clk-1* mutants have two defecation defects. As had been previously described, they have an increased cycle length, and we show that they also cannot adjust their cycle length in response to temperature changes. The suppressors fall into two classes: Class I mutants suppress both the lengthened defecation cycle length and the inability to react properly to changes in temperature whereas the Class II mutants only suppress the lengthened cycle.

We have further characterized three *dsc* mutants, *dsc-4*, *dsc-3* (Class I mutants) and *dsc-1* (a Class II mutant). *dsc-4* encodes the worm homolog of the Microsomal Triglyceride Transfer Protein (MTP), a lipid transfer protein that is required for the formation of apoB-containing lipoproteins, such as the low density lipoprotein (LDL), in vertebrates. In addition to suppressing the defecation phenotypes of *clk-1*, *dsc-4* also suppresses the slow germline development of *clk-1.* We also show that, similar to *dsc-4* (MTP), reducing the function of *vit-5* (a worm apoB homolog) or *sod-1* (an extracellular superoxide dismutases) also suppress the slow germline development of *clk-1.* Based on these observations we propose that the rate of germline development in *C. elegans* is affected by the levels of a low density lipoprotein (LDL)-like lipoprotein particle. In addition, we suggest that, as in vertebrates, this LDL-like lipoprotein can

exist in both native and oxidized forms, with the native form being converted to the oxidized from through the action of ROS. We also show that *clk-1* suppresses the Multiple Vulvae (Muv) phenotype of activated *let-60/*Ras via lowering cytoplasmic ROS.

dsc-3 appears to be allelic to *tat-2*, which encodes a type IV P-type ATPase that is likely to be an aminophospholipid transporter. We show that the *dsc-3* mutants are hypersensitive to lowered levels of cholesterol in the media, and, in certain genetic backgrounds can be rescued by increased levels of cholesterol, which suggests that DSC-3 somehow regulates internal levels of cholesterol, possibly through an involvement in cholesterol absorption. Together with the identity of *dsc-4*, as well as interactions between *clk-1* and cholesterol, this demonstrates that *clk-1* affects some aspects of lipid metabolism in worms, likely by altering its level of oxidation.

The link between the Class I mutants and *clk-1* is less clear. *dsc-1* encodes a Paired-like homeodomain transcription factor (a class of transcription factors studied in *C. elegans* for their roles in neuronal specification), which is expressed and required in the enteric muscles for the proper regulation of the cycle length and also for the expulsion step of the defecation behavior. At present, only one neurotransmitter receptor, the GABA-gated cation channel EXP-1, is known to be required in these muscles for their function. We show that DSC-1(+) is required in these muscles for the expression of EXP-1, and that the defecation defects of the *dsc-1* mutants are due to the loss of EXP-1 expression. Thus, *dsc-1* encodes a Paired-like homeodomain transcription that is required for muscle specification in *C. elegans*. In addition, the *dsc-1* mutants reveal an unanticipated role for the enteric muscles in regulating the defecation cycle length.

Finally, I present our analysis of *clk-1* growth suppressors. These *clk-1(e2519)*-specific suppressor mutants were identified in screens for suppressors of the slow growth phenotype as well as of the requirement

for dietary ubiquinone (UQ). We show that the mutations suppress most Clk-1 phenotypes and re-establish a minute amount of UQ biosynthesis. This same amount of UQ is insufficient when obtained from the diet. Thus, wild-type rates of development and behavior can be sustained by vanishingly small amounts of UQ, but only if it is synthesized endogenously. We cloned 6 of the suppressors and all encode $tRNA^{Glu}$ genes whose anticodons are altered to read the substituted Lys codon of *clk-1(e2519)*. The pattern of suppressed phenotypes we observe among the suppressor mutants suggests that the individual members of the $tRNA^{Glu}$ family might be expressed in different tissues, and at different levels. It also indicates that most Clk phenotypes can be uncoupled from each other.

Conclusions

clk-1 mutants have a complex phenotype that includes an inability to react to changes in temperature

The Clk phenotype is characterized by an alteration in the timing of developmental and behavioral features. It has previously been shown that mutations in *clk-1* affect the length of cell cycles in the early embryo, the overall rate of embryogenesis, post-embryonic development, reproduction and aging. They also affect the rate of behaviors including swimming, pharyngeal pumping, and defecation (Branicky et al., 2000; Hekimi et al., 1995; Wong et al., 1995). In addition, it was shown for embryogenesis that the mutants cannot properly re-set their developmental rates in response to temperature shifts (Wong et al., 1995). This is not simply an inability to increase their rate of development, as there are conditions under which the mutant embryos develop faster than they should. We extend these studies and characterize in detail 2 new phenotypes. Firstly we show that the *clk-1* mutants cannot re-set their defecation rate after a shift to a new temperature. Together with the embryo studies, this indicates that reacting to changes in temperature must somehow be an active process as it requires *clk-1(+)* activity, and that an inability to react to changes in

temperature is part of the Clk-1 phenotype. We also characterize in detail the slow germline phenotype of the *clk-1* mutants. This is in fact a type of heterochronic phenotype, as the stage of development of the germline is not well synchronized with the developmental stage of the soma in the *clk-1* mutants. More specifically, when the mutants have reached the adult stage of somatic development, their stage of the germline is that of a late-stage larvae.

The Clk-1 phenotype is due to low UQ or low CLK-1 protein, not the presence of DMQ

As described in the Introduction, CLK-1 encodes a DMQ hydroxylase that is necessary for UQ biosynthesis. In the absence of CLK-1(+) activity, UQ cannot be produced, so instead, the mutants accumulate the precursor, DMQ. Given that DMQ is generally not present in the wild-type, and could have different redox properties than UQ (Miyadera et al., 2002), we have speculated that the large amount of DMQ present in the *clk-1* mutants, could be contributing to the mutant phenotype. The results obtained with our growth suppressor mutants suggest that the Clk-1 phenotypes are in fact not due to the presence of DMQ, as the suppressors still accumulate a very large amount of DMQ, yet most of the Clk-1 phenotypes are suppressed. Thus, the presence of DMQ does not at all contribute to those phenotypes that are fully rescued by the suppressors (lifespan, defecation and pharyngeal pumping). However, the developmental rates are only partially suppressed, so it remains possible that the presence of DMQ may be affecting these phenotypes.

Very little UQ or CLK-1 is sufficient for a near-WT phenotype

Our analysis of the growth suppressors revealed that all the suppressors restore UQ biosynthesis, although not nearly to wild-type levels.

The predominant quinone (Q) species in the wild-type is UQ, whereas in *clk-1(e2519),* as well as in the suppressors, the predominant Q species is DMQ, with only a very small amount of UQ detectable in the suppressors. In fact, the suppressor that makes the most UQ (*qm213*) makes 20 times

less than the wild-type and the suppressor that makes the least UQ (*qm210*) makes 63 times less than the wild-type, yet this appears to be sufficient for growth and fertility on UQ-deficient bacteria, and even for almost wild-type rates of growth and for some behaviors. However, given that the suppressors encode suppressor tRNAs, they act by restoring the production of some CLK-1(+) protein, which in turn restores a small amount of UQ biosynthesis. Thus, it remains possible that it is the presence of wild-type CLK-1(+) protein rather than the small amount of accumulated UQ that confers suppression. Unfortunately, at the present time we have no tool to disrupt UQ biosynthesis in the presence of CLK-1(+) function or vice versa. We also cannot measure the amount of CLK-1(+) protein restored by the suppressors as the *clk-1(e2519)* mutants already produce a wild-type level of full length, albeit mutant, protein (Hihi et al., 2003). However, given that there are 23 tRNAs genes that decode the Glu(GAG) codon (Duret, 2000), we expect that mutating only one of these genes should result in the production of only a minor amount of CLK-1(+) protein. Thus, only very small amounts of wild-type CLK-1 or UQ appear to be sufficient, at least under standard laboratory conditions. In fact, *clk-1* may be unique in just how little gene product is required, which could be one of the reasons that it is the only *C. elegans* gene for which missense tRNA suppressors have been found. Although we don't yet know of a function for CLK-1 other than UQ biosynthesis, we know that UQ has multiple cellular roles including for electron transport and as an important cellular antioxidant. Therefore, the much greater quantity of UQ produced by the wild type may in fact be needed when the animal is challenged with stressful conditions or if it is trying to meet a higher metabolic demand.

Some if not most Clk-1 phenotypes are separable and may be cell- or tissue-autonomous

Our analysis of the growth suppressors revealed that different subsets of Clk-1 phenotypes are suppressed by the different mutants. Their pattern of

suppression reveals that most, if not all phenotypes can be de-coupled from each other. For instance, the *qm211* mutant suppresses the slow embryonic development but not the slow post-embryonic development, indicating that these are separable development phenotypes. Moreover, as the *clk-1(e2519); qm211* mutants suppress the rate of defecation and pumping but still display a long lifespan that is indistinguishable from that of *clk-1(e2519),* we can deduce that the increased lifespan of *clk-1* is not due, and does not require slow embryonic development or slow behaviors. In particular, they reveal that a slow rate of pharyngeal pumping is not necessary for an increased lifespan. This observation suggests that the mechanism of lifespan extension of *clk-1* mutants is not simply caloric restriction, although slow pumping can lead to caloric restriction and in increase lifespan in worms (Lakowski and Hekimi, 1998). In fact the only phenotypes that the suppressors do not separate from each other are the slow post-embryonic development and the increased lifespan. However, we have previously shown that maternally-rescued *clk-1* mutants, which display wild-type rates of development and behavior early in life, still exhibit a long lifespan when in the background of the life-extending mutation *daf-2* (Burgess et al., 2003). This suggests that a slow rate of postembryonic development is not necessary for an increased lifespan.

Given that the suppressors encode tRNAs, this pattern of suppression is likely due to differences in the level and tissue-specificity of expression of each suppressor. Indeed it has been shown in *C. elegans*, at least for amber $tRNA^{Trp}$ suppressors, that different $tRNA^{Trp}$ genes are expressed at different levels as well as in different tissues (Kondo et al., 1988; Kondo et al., 1990). Although we have not examined the levels or patterns of expression, the *qm211* mutant, which overall appears to be a weaker suppressor, is likely to be expressed at a lower level. The other differences we observe among the suppressors are likely to be due to differences in tissue-specificity, which suggests that some phenotypes can have a tissue-specific focus of action. The clearest example of this is the

defecation cycle: it can be strongly suppressed by the weakest suppressor (*qm211*), but is not at all suppressed by a very strong suppressor (*qm199*), suggesting that *qm211* is expressed in the cells or tissue that requires *clk-1(+)* to regulate the defecation cycle length. This would be consistent with our observations with *dsc-1*. Indeed we found that *dsc-1* has a cell-specific focus of action (the enteric muscles) and can strongly suppress the slow defecation phenotype of *clk-1*.

clk-1 mutants have lowered levels of ROS, at least in some cellular compartments

We have made two key observations, which strongly suggest that the *clk-1* mutants have lowered levels of ROS, at least in particular cellular compartments. Firstly, we showed that, similar to *dsc-4* (MTP), reducing the function of the cytoplasmic superoxide dismutase, *sod-1*, can suppress the slow germline development of *clk-1*. The fact that we can suppress a Clk-1 phenotype by knocking down an enzyme that detoxifies ROS in the cytoplasm, which presumably acts by increasing the amount of ROS in the cytoplasm, strongly suggests that the mutants have low cytoplasmic ROS. Moreover, we found that *clk-1* mutations suppress the multiple vulva (Muv) phenotype conferred by gain-of-function *let-60*/Ras, which is part of a redox-sensitive pathway of signal transduction (Irani and Goldschmidt-Clermont, 1998; Irani et al., 1997). The suppression conferred by *clk-1* is suppressed by knocking down *sod-1* and to a lesser extent *sod-4* (which encodes an extracellular superoxide dismutase), which further shows that the mechanism of *clk-1* suppression of *let-60*/Ras is via lowering ROS in some cellular compartments. Moreover, another group has recently shown that *clk-1* mutants have lowered levels of oxidatively damaged proteins (Kayser et al., 2004).

At present, it is not clear how the absence of *clk-1* leads to a reduction of ROS, especially since it leads to the absence of UQ, a major cellular antioxidant. However, the mechanism appears to be conserved as loss of *mclk1* in embryonic stem cells also leads to a reduction in

oxidatively damaged lipids and DNA and a resistance to ROS-generating compounds (Liu et al., 2005).

The pleiotropic phenotype of *clk-1* mutants might be due to the multiple roles of ROS

It has been well established that ROS are toxic molecules that damage cellular constituents. In fact, it is becoming increasingly apparent that ROS play important roles in numerous human disease conditions, including atherosclerosis, diabetes, neurodegenerative diseases, carcinogenesis and ischemia/reperfusion injuries, as well as in the aging process (reviewed in (Balaban et al., 2005; Droge, 2002; Finkel, 2005)). However, it is also becoming appreciated that, like the well-studied redox signaling mediator nitric oxide (NO), at subtoxic levels, ROS such as superoxide, hydrogen peroxide, hydroxyl radicals and lipid hydroxyperoxides, play important cellular roles. For example, they have been shown to affect signal transduction by stimulating Ca^{2+} signaling, protein phosphorylation, and transcription factor activation, and may also serve as second messengers for receptor mediated signaling (reviewed in (Droge, 2002; Finkel, 2001; Suzuki et al., 1997)). Thus, it is possible that, at least part of the pleiotropic effects of *clk-1* mutations could be due to the multiple roles of ROS, particularly in signal transduction. Indeed, we have shown that some signal transduction pathways may be down-regulated in the mutants as the slow germline development can be suppressed by activating both the *let-60*/Ras and IP_3 signal transduction pathways.

clk-1 mutants have altered lipid metabolism

Two classes of *dsc* mutants were isolated in the *dsc* screen. A number of observations suggest that, for some phenotypes, the Class II *dsc* mutations actually compensate for the lack of *clk-1(+)* activity, and thus, can, in part, reveal that nature of the defects in the *clk-1* mutants. Most importantly, we found that the *dsc-3* and *dsc-4* mutants suppress both the lengthened defecation cycle and the inability of the mutants to re-set the cycle length in response to temperature changes, which essentially

restores the defecation behavior of the *clk-1* mutants to that of the wild-type. Moreover, closer examination of the *dsc-4* mutants showed that they in fact suppress other aspects of the phenotype including the slow germline development and the slow embryonic development (Y. Shibata and S. Hekimi, unpublished observations).

I present evidence that *dsc-4* and *dsc-3* affect defecation by a common mechanism. *dsc-4* encodes the worm homolog of MTP, which we show acts similarly to vertebrate MTP, in that it is required for the formation of an oxidizable lipoprotein particle. The exact function of DSC-3 is not presently clear, but its phenotype indicates that it is involved in some aspect of lipid metabolism, possibly lipid absorption. Thus, both *dsc-4* and *dsc-3* affect lipid metabolism in the worm, but they affect distinct aspects. That two specific suppressors of *clk-1* affect aspects of lipid metabolism strongly suggests that part of the Clk-1 phenotype is due to altered lipid metabolism. This is further supported by the finding that lowering media cholesterol can also suppress the slow germline development as well as the defecation phenotypes (Y. Shibata and S. Hekimi, unpublished observations) of *clk-1*. Although we do not presently know how CLK-1 affects lipid metabolism, given that one of the major roles of UQ is in the prevention of initiation and propagation of lipid peroxidation, we can speculate that it affects lipid metabolism by altering its level of oxidation.

Altered metabolism and/or altered ROS metabolism could be the basis of the Clk phenotype

To date, 11 *clk* mutants have been identified, four of which have been molecularly identified. The identities of *clk-1* (an enzyme required for UQ biosynthesis), *clk-2* (a gene involved in responding to damage and telomere length regulation), *gro-1* (a tRNA modifying enzyme) and *clk-8/tpk-1* (an enzyme required for the conversion of thiamine into its biologically active form) suggest that reducing very different gene activities can produce a very similar Clk phenotype. However, it is possible that all mutants affect a common process, which underlies the phenotype. One

candidate process is cellular metabolism. Indeed, *clk-1*, *clk-8*, and *gro-1* could all be expected to alter mitochondrial function. However, gross mitochondrial function, in terms of oxygen consumption and ATP production, do not appear to be affected in the mutants (Braeckman et al., 2002; Braeckman et al., 1999; de Jong et al., 2004). Another possibility is that, by subtly impairing mitochondrial function, all of these mutants somehow affect ROS metabolism. The phenotypes of *clk-2,* both in worms and in human cell lines are also consistent with an alteration of ROS metabolism (Benard and Hekimi, 2002; Benard et al., 2001; Jiang et al., 2003). Moreover, the *isp-1* mutants have essentially a strong Clk phenotype, but without the maternal-rescue effect. *isp-1* encodes an iron sulfur protein that is part of Complex III of the mitochondrial respiratory chain (Feng et al., 2001). Loss of *isp-1* dramatically slows down development, behavioral rates and aging and reduces oxygen consumption. As it decreases sensitivity to ROS and is not enhanced by mutations that increases resistance to ROS, it has been suggested to decrease the production of ROS. Thus, an alteration of some aspects of ROS metabolism may be the basis of the Clk phenotype.

***clk-1* can be suppressed by various mechanisms of suppression**

Suppressor analysis is routinely used in genetically tractable systems to identify unanticipated functional relationships between genes. In *C. elegans*, it has been particularly useful for identifying all the components of signal transduction pathways, such as of the Ras/Map Kinase pathway that affects vulval formation and of the insulin-like signaling pathway that affects dauer formation (Riddle and Albert, 1997; Sternberg and Han, 1998). Suppressors can act in several ways, for example, by restoring wild-type protein (which can be achieved by intragenic or informational suppressors), by altering the mutant pathway or process or by altering another compensatory pathway or process (reviewed in (Prelich, 1999)).

Our suppressor analysis of *clk-1* mutants seems to have yielded suppressors acting in all three of the ways described above. The growth

suppressors, which encode missense tRNA suppressor genes, are informational suppressors that act by restoring wild-type CLK-1 protein. The Class I *dsc* mutants (*dsc-3* and *dsc-4*) likely act independently of *clk-1*, but on a process that is affected by *clk-1*: lipid metabolism. The Class II *dsc* mutants (*dsc-1*, *dsc-2*, *dec-7*) seem to affect defecation in a variety of different ways, and do not seem to suppress other aspects of the Clk-1 phenotype, which suggests that they might act on defecation in ways distinct from that by which *clk-1* acts. In fact, they may affect the basic mechanisms of defecation, and by doing so, somehow are bypassing the need for *clk-1* activity. For example, the class II gene *dsc-1* is a transcription factor that is necessary for the proper differentiation of the enteric muscles, and thus, may simply be required for normal defecation *per se*. On the other hand, we have shown that *dsc-1* specifically affects the defecation cycle length, so its interaction with *clk-1* might actually be more specific than is suggested by its most obvious function in the enteric muscles.

Several classes of suppressors have yet to be identified. For example, we did not identify any suppressors that physically interact with CLK-1. Also, with the possible exception of *dsc-1*, we also did not find any truly *clk-1*-specific, feature-specific suppressors, that is, mutations that affect only one feature, such as growth or defecation, but are specifically downstream of *clk-1*. One would expect such mutations to be epistatic to *clk-1*, but no such suppressor mutation has yet been found.

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