PHENOTYPIC EFFECTS OF UBIQUINONE DEFICIENCIES AND METHODS OF SCREENING THEREOF

Background of the invention

(a) Field of the Invention

This invention relates to the phenotypic effects of ubiquinone deficiencies and methods of screening thereof.

(b) Description of Prior Art

Ubiquinone (UQ), and its reduced form ubiquinol, is a prenylated benzoquinone/ol lipid and is the major site of production of reactive oxygen species (ROS). It is a co-factor in the mitochondrial respiratory chain where it becomes reduced by the activity of Complex I and Complex II, and oxidized by the activity of Complex III. During these processes, ubisemiquinone species are formed, which are unstable and generate superoxide. Furthermore, ubiquinone/ubiquinol is a redox-active cofactor of other enzyme systems that produce ROS, for example the plasma membrane NAD(P)H oxidoreductases, as well as the lysosomal and peroxisomal electron transport chains. In all these locations ROS can be produced during redox reactions involving ubiquinone/ubiquinol.

In addition, ubiquinone is a ubiquitous natural anti-oxidant, whose presence in biological membranes helps to detoxify ROS produced by endogenous processes or by toxicants or radiations. Unfortunately, dietary ubiquinone has very poor penetration into cells, in particular into sub-cellular organelles.

Reactive oxygen species have been implicated in numerous human diseases, including, but not exclusively, diabetes (Nishikawa et al., (2000). *Nature*, **404**, 787-790; Brownlee (2001). *Nature* **414**, 813-820), hypoxia/reoxygenation injury (Li et al., (2002). *Am J Physiol Cell Physiol* **282**, C227-C241; Lesnefsy et al., (2001). *J. Mol Cell Cardiol* **33**, 1065-1089; Cuzzocrea et al., (2001). *Pharmacological Reviews* **53**, 1, 135-159), Parkinson's (Betarbet et al., (2002). *Bioessays* **24**, 308-318),

atherosclerosis atherosclerosis (Lusis, (2000). *Nature*, **407**, 233-241), and Alzheimer's disease (Butterfield et al.,(2001). *Trends in Molecular Medicine*, **7**, 12, 548-554; Tabner et al., (2002) *Free Radical Biology & Medicine*, 32, 11, 1076-1083, 2002).

The gene *clk-1* of the nematode *Caenorhabditis elegans* affects many physiological rates, including embryonic and post-embryonic development, rhythmic behaviors, reproduction and life span. *clk-1* encodes a 187 amino acid protein that localizes to mitochondria, and that is homologous to the yeast protein Coq7p, which has been shown to be required for UQ biosynthesis. *clk-1* has also been shown to be necessary for UQ biosynthesis (Jonassen, T. et al.,(2001). *Proc Natl Acad Sci U S A* **98**, 421-6.; Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6), as UQ_9 is entirely absent from mitochondria purified from *clk-1* mutants (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6) (the subscript refers to the length of the isoprenoid side chain). Instead, these mitochondria accumulate demethoxyubiquinone (DMQ_9), which is an intermediate in the synthesis of UQ_9 (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6). Recent evidence suggests that *clk-1* encodes a DMQ hydroxylase (Stenmark, P. et al., (2001). *J Biol Chem* **2**, 2). In *E. coli*, DMQ_8 is able to sustain respiration in isolated membranes although at a lower rate than UQ_8. Similarly, DMQ_9 is capable to convey electron transport in eukaryotic mitochondria, as the function of purified mitochondria (Feikai, S. et al., (1999). *Embo J* **18**, 1783-92) and of mitochondrial enzymes (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6) from *clk-1* mutants appear to be almost intact compared to the wild type. Furthermore, synthetic DMQ_2 can function as a co-factor for electron transport from complex I and, more poorly, from complex II (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6). Interestingly, only DMQ_9 is present in all three *clk-1* alleles irrespective of the severity of their effect on physiological rates, which suggests that the lack of UQ cannot solely account for the Clk-1 phenotype (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6).

Recently, it has been found that *clk-1* mutants are unable to grow on a UQ-deficient bacterial strain in spite of the presence and the activity of DMQ_9 (Jonassen, T. et al.,(2001). *Proc Natl Acad Sci U S A* **98**, 421-6).

Although, dietary UQ is generally not capable to reach mitochondria, this has been interpreted to suggest that DMQ_9 is insufficient for normal mitochondrial function, and that dietary bacterial UQ_8 can reach the mitochondria and function there in trace amounts (Jonassen, T. et al.,(2001). *Proc Natl Acad Sci U S A* 98, 421-6).

It would be highly desirable to be provided with characterization of phenotypic effects of UQ deficiencies and screening methods for compounds that can affect the activity of *clk-1* and/or relieve UQ deficiencies in multicellular organisms.

Summary of the invention

In accordance with the present invention there is provided a method of screening for a compound allowing survival of *clk1* homozygous mutant vertebrate embryos, which comprises the step of breeding heterozygous *clk1* subjects to obtain *clk1* homozygous mutant embryos and determining viability of *clk1* homozygous embryos; wherein at least one of the heterozygous subject is treated with the compound prior to the breeding; and wherein viable embryos are indicative of a compound allowing survival of *clk1* homozygous embryos.

The method in accordance with a preferred embodiment of the present invention, wherein the subject is a mouse.

The method in accordance with a preferred embodiment of the present invention, wherein the compound is suitable for partial or complete functional replacement of endogenous ubiquinone.

The method in accordance with a preferred embodiment of the present invention, wherein the compound is administered by at least one route selected from the group consisting of oral, intra-muscular, intravenous, intraperitoneal, subcutaneous, topical, intradermal, and transdermal route.

In accordance with the present invention, there is provided a method of screening for a compound suitable for rescue of mutant phenotype of *mclk1* homozygous cell line, which comprises the step of determining a

mutant phenotype in a *mclk1* knockout cell line, wherein cell line is treated with the compound prior to the determining, and wherein the level of the phenotype is indicative of a compound suitable for rescue.

In accordance with the present invention, there is provided a method of screening for a compound suitable for partial or complete functional replacement of endogenous ubiquinone, which comprises the step of determining a mutant phenotype in a *mclk1* knock-out homozygous ES cell line; wherein the cell line is treated with the compound prior to the determining; and wherein level of the phenotype is indicative a compound suitable for partial or complete functional replacement of ubiquinone.

The method in accordance with a preferred embodiment of the present invention, wherein the phenotype is cellular respiration and/or growth rate.

In accordance with the present invention, there is provided a method of screening for a compound suitable for partial or complete functional replacement of ubiquinone in a subject, which comprises the step of assessing at least one phenotype selected from the group consisting of viability, fertility, and total or partial absence of a mutant phenotype of a *coq-3* homozygous mutant worm; wherein the worm is treated with the compound prior to the assessing; and wherein at least one phenotype selected from the group consisting of the viability, fertility and total or partial absence of the mutant phenotype is indicative of a compound suitable for partial or complete functional replacement of ubiquinone in the subject.

The method in accordance with a preferred embodiment of the present invention, wherein the compound is capable of reaching mitochondria in the subject.

In accordance with the present invention, there is provided a method for screening for a compound suitable for partial or complete functional replacement of ubiquinone in a subject, which comprises the step of assessing at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of a Clk-1 phenotype of a *clk-1* homozygous mutant worm grown on ubiquinone-depleted substrate;

wherein the worm is treated with the compound prior to the assessing; and wherein at least one phenotype selected from the group consisting of the viability, fertility and total or partial absence of said Clk-1 phenotype is indicative of a compound suitable for partial or complete functional replacement of ubiquinone in the subject.

The method in accordance with a preferred embodiment of the present invention, wherein the ubiquinone-depleted substrate is a non-ubiquinone producer bacteria.

The method in accordance with another embodiment of the present invention, wherein the ubiquinone-depleted substrate is a bacteria producing ubiquinone having side-chains shorter than 8 isoprene units.

The method in accordance with another embodiment of the present invention, wherein the compound is capable of reaching at least non-mitochondrial sites of ubiquinone requirement in the subject.

The method in accordance with a preferred embodiment of the present invention, wherein the bacteria is selected from the group consisting of RKP1452, AN66, IS-16, DM123, GD1, DC349, JC349, JC7623, JF496, KO229(pSN18), KO229(Y37A/Y38A), KO229(R321V), and KO229(Y37A/R321V).

The method in accordance with a preferred embodiment of the present invention, wherein the bacteria has a mutation in at least one of genes selected from the group consisting of *ubiCA, ubiD, ubiX, ubiB, ubiG, ubiH, ubiE, ubiF,* and *ispB.*

The method in accordance with a preferred embodiment of the present invention, wherein the bacteria carries at least one of the plasmids selected from the group consisting of pSN18, Y37A/Y38A, R321V, Y37A/R321V.

The method in accordance with a preferred embodiment of the present invention, wherein the functional replacement of ubiquinone is for a function of ubiquinone as co-factor of CLK-1.

In accordance with the present invention, there is provided a method for screening a compound capable of inhibiting activity of *clk-1* and/or other processes required to make ubiquinone from demethoxyubiquinone in a subject, which comprises the step of determining at least one phenotype selected from the group consisting of growth, fertility and total or partial absence of a Clk-1 phenotypes of a wild-type worm on a ubiquinone-depleted substrate; wherein the worm is treated with the compound prior to the determining; and wherein at least one phenotype selected from the group consisting of total or partial absence of growth, absence of fertility and total or partial absence of said Clk-1 phenotypes is indicative of a compound capable of inhibiting activity of *clk-1* and/or other processes required to make ubiquinone from demethoxyubiquinone in a subject.

In accordance with the present invention, there is provided a method of screening for a compound suitable for complete or partial functional ubiquinone replacement, which comprises the step of determining a mutant phenotype of a subject in which *mclk1* and/or a known ubiquinone biosynthetic enzyme gene is deleted and/or any other gene which when altered leads to absence or reduction of ubiquinone; wherein the subject is treated with the compound prior to the determining; and wherein level of the phenotype is indicative of a compound suitable for complete or partial functional ubiquinone replacement.

The method in accordance with a preferred embodiment of the present invention, wherein the subject is a mouse, ES cell line, or any cell line in which *mclk1* is deleted or any gene coding for a known ubiquinone biosynthetic enzyme gene is deleted and/or any other gene which when altered leads to absence or reduction of ubiquinone.

In accordance with the present invention, there is provided a mouse which is incapable of producing ubiquinone and comprising a gene knock-out of *mclk1*; wherein the mouse expresses the phenotype related to an absence of ubiquinone and the presence of demethoxyubiquinone.

In accordance with the present invention, there is provided a DNA construct, which comprises an alteration of *mclk1*; wherein the DNA

construct is instrumental in producing a mouse *mclk1* knockout strain of the present invention.

In accordance with the present invention, there is provided an ES cell line which is incapable of producing ubiquinone and comprising a gene knock-out of *mclk1*; wherein the ES cell line expresses the phenotype related to an absence of ubiquinone and the presence of demethoxyubiquinone.

In accordance with the present invention, there is provided a *coq-3* mutant subject which is incapable of producing ubiquinone; wherein mutation is a deletion of *coq-3* or a deletion of a ubiquinone biosynthetic enzyme and/or any other gene which when altered leads to absence or reduction of ubiquinone.

The mutant in accordance with a preferred embodiment of the present invention, wherein the subject is a worm.

The mutant in accordance with a preferred embodiment of the present invention, wherein the mutant is selected from the group of worm identified using PCR primers selected from the group consisting of SHP172, SHP1773, SHP1774, SHP1775, SHP1840 and SHP1865.

In accordance with the present invention, there is provided a method of screening for a compound suitable for complete or partial functional ubiquinone or demethoxyubiquinone replacement, which comprises the step of determining a mutant phenotype in a subject in which a ubiquinone biosynthetic enzyme gene and/or any gene whose alteration leads to an absence or reduction of ubiquinone or demethoxyubiquinone is altered; wherein the subject is treated with the compound prior to the determining; and wherein level of phenotype is indicative of a compound suitable for complete or partial functional ubiquinone or demethoxyubiquinone replacement.

In accordance with the present invention, there is provided a method for reducing and/or increasing ubiquinone level in a multicellular subject, which comprises the step of targeting *coq-3* in the subject.

In accordance with the present invention, there is provided a method of screening for a genetic suppressor of *clk-1*, which comprises the step of determining at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of a Clk-1 mutant phenotype of *clk-1* mutant worms grown on ubiquinone-depleted bacteria; wherein the worm carries the genetic suppressor prior to the determining; and wherein at least one phenotype selected from the group consisting of the viability, fertility and total or partial absence of said Clk-1 mutant phenotype is indicative of a genetic suppressor of *clk-1*.

In accordance with the present invention, there is provided a method of screening for a genetic suppressor of *coq-3*, which comprises the step of determining at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of a mutant phenotype of *coq-3* mutant worm; wherein the worm carries the genetic suppressor prior to the determining; and wherein the at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of said mutant phenotype is indicative of a genetic suppressor of *coq-3*.

In accordance with the present invention, there is provided a method of screening for a compound suitable for complete or partial functional ubiquinone replacement, which comprises the step of determining a mutant phenotype of a subject in which *mclk1* is deleted only in a subset of cells and/or periods of the life cycle, wherein the subject is treated with the compound prior to the determining; and wherein level of the phenotype is indicative of a compound suitable for complete or partial functional ubiquinone replacement.

The method in accordance with a preferred embodiment of the present invention, wherein the compounds are useful in treating a disease selected from the group consisting of reactive oxygen species (ROS) mediated disease, diabetes, hypoxia/reoxygenation injury, Parkinson's disease, artherosclerosis and Alzheimer's disease.

In the present application, the term "ubiquinone-depleted substrate" is intended to mean a substrate being not producing ubiquinone or being producing ubiquinone with side-chains too short to be effective. An

example of what will be considered ubiquinone with side-chains too short to be effective would be ubiquinone with side-chains shorter than 8 isoprene units.

Brief description of the drawings

Fig. 1 illustrates the *coq-3* gene and its deletion in *coq-3(qm188);*

Figs. 2A-E illustrate the targeted disruption of the mouse *mclk1* gene;

Fig. 3 illustrates the severe developmental delay in *mclk1* mutant embryos;

Figs. 4A-C illustrate the generation of the *mclk1*^{flox} allele. Analysis by Southern blot on neomycin resistant clones;

Fig. 5 illustrates the comparison of COQ-3 proteins from different species (SEQ ID NOS: 3-6);

Figs. 6 A-E illustrate the *Mus musculus* genomic sequence of *mclk-1* (Exons are in bold) (SEQ ID NO: 15);

Figs. 7 A-E illustrate the *Mus musculus* genomic sequence in mutant knock-out allele of *mclk-1* (Exons are in bold, neomycin cassette is in lowercase) (SEQ ID NO: 16); and

Figs. 8 A-E illustrates the sequence of *mclk1*^{flox} allele. (Exons in bold, loxp sequence in italic, DNA fragment inserted underlined.) (SEQ ID NO: 21)

Detailed description of the invention

In accordance with the present invention, there is provided characterization of phenotypic effects of ubiquinone deficiencies in multicellular organisms.

Ubiquinone is necessary for *C. elegans* development and fertility

clk-1 mutants are incapable of completing development when fed on an *ubiG E. coli* mutant strain (Jonassen, T. et al.,(2001). *Proc Natl Acad Sci U S A* **98**, 421-6), which produces no ubiquinone (UQ). The *ubiG* gene product is required at two steps of the UQ biosynthesis pathway, and *ubiG* mutants do not produce any UQ. Tests were performed to verify whether

this growth phenotype resulted from a specific toxicity of the *ubiG* strain (GD1) for *clk-1* mutants, or from the absence of UQ. For this purpose, a systematic analysis of the growth of *clk-1* mutant worms on a variety of *E. coli* mutants that are defective for UQ biosynthesis (*ubi* mutants) was conducted. Nine *E. coli* enzymes have been described as participating in UQ biosynthesis. They are all membrane-bound, except the first one, *ubiC*, which is a soluble chorismate lyase. The next enzyme in the pathway is the prenyltransferase *ubiA* that attaches the isoprenoid side chain to the quinone ring (8 subunits in *E. coli*). The other enzymes are grouped in three categories: decarboxylases (*ubiD, ubiX*), monooxygenases (*ubiB, ubiH, ubiF*), and methyltransferases (*ubiG, ubiE*). Standard procedures were used for bacterial and worm cultures, except that the NGM plates contained 0.5 % glucose, to minimize the reversion of UQ-deficient strains. To evaluate the development of worms on various bacterial strains, adult hermaphrodites were picked and bleached on a plate containing the test bacteria, following standard methods. This step ensures that no OP50 bacteria contamination is present on the test plate. L1 larvae that hatched from the bleached eggs were transferred to a fresh plate, and the growth of the worms was examined. The genotypes of the bacterial strains used are described in Table 1. The growth of the three *clk-1* mutant strains on strains of bacteria mutant for each of these genes was examined (Table 1). Three *clk-1* mutant alleles have been identified: *qm30* and *qm51*, which are putative nulls, and *e2519*, which carries a point mutation in the *clk-1* gene and displays a relatively milder phenotype.

Table 1

***E. coli* strains used**

Strain	Genotype
OP50	*ura*
RKP1452	Km^R, Δ*ubiC*A::Km^R
AN66	*thr-1 leuB6 ubiD*410
IS-16	*ubiX*, derived from the THU strain
DM123	RM1734 *yigR*::Kan
GD1	*ubiG*::Kan
DC349	FadR mel adhC81 acdA1
AN70	Hfr metB StrR ubiE-401
JC7623Δ4-1	JC7623, *ubiE*::KanR
JF496	*ubiF411 asnB50*::Tn5

It was found that on all the bacterial *ubi* - (mutant) strains tested, L1 larvae from the wild-type strain N2 are capable of completing development to adulthood and these adults have a brood-size of approximately 320, which is similar to their brood size on *ubi* + bacteria (OP50) (Table 2). This indicates that endogenously synthesized UQ is sufficient to maintain a wild-type phenotype, without a requirement for dietary UQ. A number of worm mutants that are not known to be involved in UQ synthesis (*dpy-9, eat-2, mau-2*), including long-lived mutants (*daf-2* and a number of strains that show a Clk-1-like phenotype that have not been fully characterized) were examined. In no case was the growth of the mutants impaired on *ubi* - bacteria. In contrast, all three *clk-1* mutants behave identically on most *ubi* - bacterial strains tested: they develop very slowly, or not at all, and produce no progeny (Table 2). However, the *clk-1* mutants can develop and produce some progeny on *ubiD, ubiX* and *ubiH* mutant strains, which are point mutants producing residual amounts of ubiquinone (around 15 % of the wild type). Thus, the relatively low levels of bacterial UQ_8 are sufficient to allow for the growth of *clk-1* mutants.

Table 2

Growth and brood-size analysis of wild-type and *clk* worms on *ubi* + and *ubi* - bacteria

		N2 (Wild type)		*clk-1* mutants	
Strain	Genotype	Growth	Progeny	Growth	Progeny
OP50	*ubi* +	+	323 ± 16	+	*qm30*: 94 ± 12 *qm51*: 83 ± 10 *e2519*: 177 ± 4
RKP1452	*ubiCA* KO	+	331 ± 37	–	0
AN66	*ubiD*	+	313 ± 16	+	*qm30*: 82 ± 5 *qm51*: 93 ± 6 *e2519*: 182 ± 26
IS-16	*ubiX*	+	336 ± 8	+	*qm30*: 96 ± 11 *qm51*: 83 ± 10 *e2519*: 164 ± 8
DM123	*ubiB* KO	+	312 ± 25	–	0
GD1	*ubiG* KO	+	315 ± 15	–	0
DC349	*ubiH*	+	329 ± 16	+	*qm30*: 105 ± 6 *qm51*: 90 ± 3 *e2519*: 168 ± 11
JC7623	*ubiE* KO	+	313 ± 4	–	0
JF496	*ubiF*	+	330 ± 4	–	0

***C. elegans* is sensitive to ubiquinone side-chain length**

Ubiquinone (UQ) is composed of a quinone ring and an isoprenoid chain, whose length is species-specific. There are 9 isoprene repeats in *C. elegans*, 8 in *E. coli*, and 6 in *S. cerevisiae*. In mammals, both UQ_9 and UQ_{10} are detected (the subscript refers to the length of the isoprenoid side chain). UQ_{10} is the major UQ species present in humans, while UQ_9 is predominant in mice and rats (Dallner, G. and Sindelar, P. J. (2000). *Free Radic Biol Med* **29**, 285-94). The differential tissue distribution of UQ_9 and UQ_{10} is presented in Table 3 (Dallner, G. and Sindelar, P. J. (2000). *Free Radic Biol Med* **29**, 285-94).

Table 3

Ubiquinone tissue distribution in rat and human

	Rat			Human		
	UQ_9 µg/g tissue	UQ_{10} µg/g tissue	UQ_{10}/UQ_9 (%)	UQ_9 µg/g tissue	UQ_{10} µg/g tissue	UQ_9/UQ_{10} (%)
Heart	202	17	8	3	114	2.5
Liver	131	21	14	2	55	3.5
Kidney	n.d	n.d	-	3	67	4.5
Brain	37	19	34	1	13	7
Spleen	23	9	28	1	25	4
Lung	17	2	10.5	1	8	11
Intestine	51	19	27	n.d	n.d	-

The length of the UQ side-chain is controlled by polyprenyl-diphosphate synthases. These enzymes are encoded by essential genes, and have been cloned in many organisms, including *S. cerevisiae* (*coq1*: hexaprenyl-diphosphate synthase), *E. coli* (*ispB*: octaprenyl-diphosphate synthase), and *Rhodobacter capsulatus* (*sdsA*: solanesyl-diphosphate synthase).

To evaluate the importance of UQ side-chain length, using *C. elegans*. The exogenous UQ fed to the worms was manipulated by exposing the worms

to an *E. coli* mutant strain where the original *ispB* gene is knocked-out, and replaced by different versions of *ispB* carried on rescuing plasmids. The *ispB* version dictates the side-chain length of the bacterially-manufactured UQ. N2 (Bristol) was used as wild-type strain, and analyzed *clk-1(qm30), clk-1(qm51), clk-1(e2519)* and *daf-2(e1370)* mutants strains. The genotypes of the bacterial strains used are described in Table 4. The plasmids encoding mutant versions of *ispB* are described in Table 5. Table 6 is providing the results obtained from brood size measurements. The entire progeny of 10 worms was counted and the experiment was performed twice.

Table 4

Genotypes of the bacterial strains used in the study of the effect of UQ side-chain length

Strain	Genotype	Reference
OP50	***ura***	Laboratory collection
KO229	***ispB***::Camr	Okada et al., 1997*

* Okada et al., (1997). *Journal of bacteriology*, **179**, 9, 3058-3060

Table 5

Plasmids encoding versions of *ispB*

Plasmid	Characteristics	Major UQ produced	Minor UQ produced	Reference
pSN18	***Amp^r***, encodes ***Rhodobacter capsulatus ispB*** homolog (***sdsA***)	UQ_9	-	Okada et al., 1997*
Y37A/ Y38A	***Amp^r***, encodes a mutant version of ***E. coli ispB*** gene	UQ_7	UQ_8, UQ_6	Kainou et al., 2001**
R321V	***Amp^r***, encodes a mutant version of ***E. coli ispB*** gene	UQ_6	UQ_7, UQ_5	Kainou et al., 2001
Y37A/ R321V	***Amp^r***, encodes a mutant version of ***E. coli ispB*** gene	UQ_6	UQ_7, UQ_5	Kainou et al., 2001

* Okada et al., (1997). *Journal of bacteriology*, **179**, 9, 3058-3060

** Kainou et al., (2001). *The Journal of Biological Chemistry* **276**, 11, 7876-7883

Table 6

Brood-size analysis

	N2	*clk-1 (qm30)*	*clk-1 (qm51)*	*clk-1 (e2519)*	*daf-2 (e1370)*
OP50 (UQ_8)	240, 282	94, 108	112, 121	158, 181	254, 228
pSN18 (UQ_9)	266, 246	107, 102	94, 117	170, 193	249, 264
Y37A/Y38A (UQ_7)	255, 291	0, 0	0, 0	149, 178	266, 237
R321V (UQ_6)	236, 258	0, 0	0, 0	82, 93	218, 259
Y37A/R321V (UQ_6)	247, 273	0, 0	0, 0	95, 101	241, 229

Growth rate on various bacterial strains

Post-embryonic growth of the worms on the various bacterial strains was qualitatively evaluated. It was observed that N2 and *daf-2* mutants grow at similar rates on all bacterial strains. However, *clk-1(qm30)* mutants had a similar growth rate on OP50 and KO229(pSN18), but were delayed by 3-5 days on the other strains. Also, the post-embryonic development of *clk-1(e2519)* mutants was delayed by ~1 day on KO229(R321) mutants, as compared to OP50. Their growth on KO229(Y37A/Y38A) is less severely affected. Finally, the onset of egg laying by *clk-1(e2519)* was delayed by 1 day on KO229(Y37A/Y38A) and by 3 days on KO229(R321A) and KO229(Y37A/R321A).

Thus, these experiments revealed a process in *C. elegans* that is sensitive to ubiquinone side-chain length as indicated by the behaviour of *clk-1* mutants on bacterial strains that produce short chain ubiquinones. An inappropriate chain length severely alters development and fertility in *qm30* and mildly or not at all in *e2519*.

The observation that *clk-1(e2519)* mutants are almost unaffected in spite of the fact that they are known to produce no detectable ubiquinone, indicates that CLK-1 participates in processes that are different from ubiquinone synthesis. One can also infer that the *e2519* mutation does not greatly affect this additional function or functions of CLK-1. However, these processes are ubiquinone-dependent as *clk-1(e2519)* mutants cannot develop in the total absence of ubiquinone. For example, ubiquinone could act as a redox co-factor in these processes.

Endogenous ubiquinone is necessary for *C. elegans* development and fertility

To test whether dietary UQ is sufficient for *C. elegans* development, a knockout mutation of the worm gene *coq-3* was produced (SEQ ID NO:1). *coq-3* encodes a methyltransferase (SEQ ID NO:2) whose homologues (Coq3p and *UbiG*) have been extensively characterized in the yeast *S. cerevisiae* and in *E. coli*, respectively. The enzyme acts at two different steps of Q synthesis and neither UQ nor DMQ is produced in the yeast

and bacterial mutants. The worm COQ-3 protein is 29% identical to *S. cerevisiae* Coq3p and 28% to *E. coli UbiG* (Fig. 5 and SEQ ID NOS:3-6). A method of random mutagenesis and PCR-based screening was used to identify a deletion in *coq-3* adapted from a standard protocol. The *coq-3* gene is located on chromosome 4 of *C. elegans*, and as shown in Fig. 1, is part of an operon, comprising the *gdi-1* gene and the NADH-ubiquinone oxidoreductase gene. *coq-3* contains five predicted exons. The deletion in *coq-3(qm188)* removes 2456 bp (SEQ ID NO:7), and thus eliminates exons 3 and 4 (SEQ ID NOS: 1 and 8), and prevents any functional protein to be produced. To verify the genotype of *coq-3*, PCR analysis was performed, and used sets of primers whose priming regions are either outside of the *coq-3* gene, or inside the region corresponding to the deletion obtained in the *qm188* mutation. To check the presence of a deletion in the *coq-3* gene, PCR analyses were carried out using genomic DNA from single worms. Each DNA preparation was simultaneously tested with primers recognizing sequences either outside the *coq-3* gene (SHP 1772 (5'-CTGATTTCTTCCAGAGCTCTCTTGCCGCAC3') (SEQ ID NO: 9), SHP 1773 (5'-AGCATTCCCGAGATGATGCACTCCTTGAGG-3') (SEQ ID NO: 10), SHP 1774 (5'-TAGCGACTCTCAGCGACAAGCTTAACC-3') (SEQ ID NO: 11) and SHP 1775 (5'-GAGGCCGGTTCCGAGACGATGGCATCG-3') (SEQ ID NO: 12)), or inside the obtained deletion (SHP 1840 (5'-CCTCCTCGCGCACTACACACCATC-3') (SEQ ID NO: 13) and SHP 1865 (5'-CGAAGCGACGACTGCATCGTAGGC-3') (SEQ ID NO: 14)). Fig. 1 displays the primers' localization. When using primers amplifying the whole *coq-3* gene, a band of 4.3 kb was obtained with a wild-type worm. In contrast, a mutant band was amplified at 1.8 kb from a *coq-3/coq-3* worm. When using primers annealing in the deletion region, both wild-type and heterozygote worms gave a PCR product of 1.1 kb, while no band was detected from a *coq-3/coq-3* homozygote worm, which confirmed the homozygote nature of *coq-3/coq-3* mutants.

Self-fertilizing *coq-3(qm188)/+* hermaphrodites produce ¼ of homozygous *coq-3(qm188)/coq-3(qm188)* progeny, as verified by PCR. These *coq-3* homozygotes develop slowly and appear substantially smaller than wild-type worms. Most are sterile, but approximately 25% (n = 31) produce

some progeny (5-10 eggs) that arrests at the L1 stage and die quickly thereafter. For brood-size measurements, the entire progeny of 20 worms was counted. These observations indicate a partial maternal rescue effect of *coq-3* homozygotes by the heterozygous mothers, as the phenotype of the first homozygous generation (slow development to adulthood) is less severe than that of the second homozygous generation (arrest at the L1 stage). UQ provided to the embryo by the mother or to maternal deposits of *coq-3* mRNA or protein can provide the maternal effect.

It is also observed that the brood size of heterozygous *coq-3/dpy-4* worms was much reduced (185 ± 64; n=20) suggesting that the level of *coq-3* expression might be limiting for UQ biosynthesis and that the worm's reproductive capacity is very sensitive to reduced level of endogenous UQ biosynthesis.

To ascertain that the observed phenotypes are solely due to the mutation in the *coq-3* gene, the genomic fragment corresponding to the wild-type *coq-3* gene was introduced into *coq-3/+* heterozygotes using the *rol-6* transformation marker by germline transformation. The micro-injection procedure was followed to generate standard extrachromosomal arrays. A PCR fragment (50 ng/µL) comprising the *coq-3* genomic sequence was injected to assay for rescue. pRF4 plasmid (120 ng/µL) was used as a co-injection marker to screen for transgenic worms. *coq3/dpy-4* worms were utilized for injection since *coq-3* homozygotes are lethal. The homozygous rescued lines were selected by checking the absence of the Dpy phenotype in their progeny, and the genotype was confirmed by PCR analysis.

Homozygous *coq-3* transgenic animals (displaying the marker phenotype, Rol) develop normally and are fertile, indicating that the phenotype observed is indeed due to the *coq-3* deletion. However, the extrachromosomal array carrying the *coq-3* and *rol-6* sequences is incapable of producing a strong maternal effect. Indeed, homozygous animals without the array (phenotypically non-Rol) issued directly from mothers carrying the array (phenotypically Rol) did not develop beyond the L2 stage. The expression of genes from extrachromosomal arrays is

sometimes silenced and is poor in the *C. elegans* germline. The observation of a maternal effect indicates that the mother deposits an essential product in the oocytes (UQ and/or *coq-3* mRNA). In either case, proper expression of *coq-3* in the germline is necessary for the effect.

The lethal phenotype of *coq-3* mutants indicates that dietary UQ is not sufficient for the growth and development of worms. This is consistent with findings in other systems that indicate that dietary UQ cannot reach the mitochondrial compartment, or only in extremely small amounts. The possibility that dietary UQ could be sufficient for worms was proposed to account for the viable phenotype of *clk-1* mutants grown on *ubi* + bacteria, and their lethal phenotype when grown on *ubi* - mutant bacteria. However, the phenotype of *coq-3* mutants clearly indicates that even in the presence of dietary bacterial UQ_8, a total absence of endogenous UQ_9 and DMQ_9 (in *coq-3* mutants) is not equivalent to the replacement of endogenous UQ9 by endogenous DMQ_9 (in *clk-1* mutants).

In this context, it is of particular interest that *clk-1* mutants cannot thrive by feeding on *ubiF* mutants. Indeed, UQ biosynthesis in *ubiF* mutants is blocked at the same level as in *clk-1* mutants, and *ubiF* bacteria thus produce DMQ_8. As DMQ_9 performs efficiently in the mitochondrial respiratory chain (Miyadera et al., 2001), our findings demonstrate that neither endogenous nor dietary DMQ can replace UQ at non-mitochondrial sites of UQ requirement.

Ubiquinone is necessary at mitochondrial and non-mitochondrial sites

The results presented here demonstrate that UQ is necessary for *C. elegans* growth and development at different subcellular locations. First, in the mitochondria, endogenous DMQ_9 can functionally replace endogenous UQ_9. Indeed, *clk-1* mutant mitochondria do not contain UQ_9 but are functionally competent (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6), and the phenotype of *coq-3* mutants, which produce neither UQ_9 nor DMQ_9, is much more severe than that of *clk-1* mutants. Second, at non-mitochondrial sites, endogenous DMQ_9 or dietary DMQ_8 or dietary UQ with a side-chain length shorter than 8 isoprene units cannot functionally

replace endogenous UQ_9, while dietary UQ_8 can. In fact, *clk-1* mutants, which have functional mitochondria and make DMQ_9, cannot develop and grow without dietary UQ_8, even in the presence of dietary DMQ_8 from *ubiF* bacteria or dietary UQ with a short side-chain.

This is consistent with the findings by numerous studies on UQ uptake and metabolism in other systems, such as rodents (Dallner, G. and Sindelar, P. J. (2000). *Free Radic Biol Med* **29**, 285-94). Dietary UQ in these experiments appears to be taken up only poorly (2-3% of the initially ingested ubiquinone) and the majority is then distributed to the plasma membrane, the lysosomes and the golgi, with only minute quantities, if at all, appearing in the mitochondria. Given that every cell endogenously produces UQ, no active uptake system has been identified to assimilate this rather complex lipid.

These studies clarify the roles of endogenous and dietary UQ in the worm's biology. Also, for the first time it demonstrated the functional importance of UQ at non-mitochondrial locations for an organism's viability or fertility. Action of dietary UQ at non-mitochondrial sites could underly the beneficial effects of dietary UQ for patients with mitochondrial diseases (Dallner, G. and Sindelar, P. J. (2000). *Free Radic Biol Med* **29**, 285-94). For example, UQ has been found to participate in reactions that regulate the redox state of the cell at the plasma membrane. Disease states which arise from deficient mitochondria are often found to increase cellular oxidative stress and dietary UQ could stimulate a protective function at the plasma membrane. In addition, in bacteria, quinones have been found to act as the primary signal of the redox state of the cell. In *E. coli*, UQ negatively modulates the phosphorylation status and function of ArcB, an important global regulator of gene expression.

The *coq-3* and *clk-1* mutant strains provide genetic systems to identify compounds that selectively replace ubiquinone at the mitochondria and/or at non-mitochondrial sites. Screens for such compounds can be based on their ability to rescue selectively the phenotypes of *coq-3* or *clk-1* mutants grown on UQ deficient bacteria or not. For example, compounds that can reach the mitochondria, should rescue the phenotype of *coq-3* mutants.

On the other hand, compounds selective for sites outside the mitochondria should rescue the phenotype of *clk-1* worms grown on UQ-deficient bacteria, but should not rescue the lethal phenotype of *coq-3* animals grown on wild-type bacteria. The development of such bio-available ubiquinone mimetics is of great medical interest.

Study of the phenotypic consequences of a disruption in the gene *mclk-1* of *Mus musculus*

The *mclk1* locus was disrupted in murine embryonic stem (ES) cell by homologous recombination and produced heterozygous and homozygous mice using standard methods. An IFIX II genomic library from mouse strain 129/SvJ DNA (Stratagene) was screened with a genomic *mclk1* fragment, and six overlapping genomic clones were obtained. Genomic DNA fragments from two clones were subcloned into Bluescript SK and characterized in detail. A 7 kb *NotI-BamHI* fragment containing part of the *mclk1* promoter and exons I, II and III was subcloned into Bluescript SK (pL5). A 1.6 kb fragment containing part of the exon II and the exon III was removed from pL5 by *StuI/BamHI* digestion and replaced with a neomycin cassette consisting of a 1.1 kb *XhoI* blunted-*BamHI* fragment from pMC1Neo polyA to produce pL5+Neo. A 2.8 kb *PstI-SacI* genomic fragment containing introns IV and V and 500bp from 5'UTR region was subcloned in Bluescript (pL15). A 2.5kb *EcoRV-XhoI* fragment from pL15 was inserted into the *SmaI-XhoI* sites of pL5+Neo to produce the final replacement targeting vector pL17. A *KpnI* fragment from the targeting vector was isolated and electroporated into R1 embryonic stem (ES). Successfully targeted clones were identified by Southern blot analysis. Genomic DNA was digested with *BglII*, and then hybridized with a 3'external probe flanking the 3' region of the targeting vector (*SacI-XhoI* fragment). A neomycin probe was used to detect random integrations in the genome. ES clones were injected into CD-1 mouse blastocysts and germline transmission was obtained. Out of 2000 G418-resistant clones analyzed, 4 were homologous recombinants. Two independently targeted ES cell clones with the correct karyotype were used to generate homozygous (-/-) *mclk1* mice. Figs. 2 A, C and D display the maps of the wild-type *mclk1* locus and of the targeting vector, where black boxes

represent exons. The targeting vector consists of the replacement of a part of exon II and the exons III and IV by the neomycin gene, indicated as a white box in Fig. 2. The restriction enzymes sites indicated are: *BamH*I; B, *Bgl*II; E, *EcoR*I; K, *Kpn*I; R, *EcoR*V; S, *Sac*I; X, *Xho*I. The genomic sequence of the *Mus musculus* wild-type *mclk-1* locus and mutant knock-out allele of *mclk-1* is given in Figs. 6A-E (SEQ ID NO: 15) and 7A-E (SEQ ID NO: 16) respectively.

For genotype determinations, DNA was prepared from tails of adult mice or yolk sacs of embryos. Southern blot analysis was done as described above. PCR was done for 30 cycles (95°C, 30 sec; 58°C, 30 sec; 72°C, 30 sec). The primers used to detect wild-type mclk1 allele were as follows: forward (KO5) 5'- ggt gaa gtc ttt tgg gtt tga gca t-3' (SEQ ID NO: 17); reverse (KO6) 5'-tgt cta agg tca tcc ccg aac tgt g-3' (SEQ ID NO: 18). They amplify a band of 302 bp. The targeted mclk1 allele was detected with the primers KO7 (5'-gcc agc gat atg act cag tgg gta a-3') (SEQ ID NO: 19) and KO8 (5'-cac ctt aat atg cga agt gga cct g-3') (SEQ ID No: 20), which give a product of 397 bp. Fig. 2 E shows the PCR analyses.

Heterozygous (+/-) mice are viable and fertile. They show no obvious anatomical or behavioral defects. However, after crossing heterozygous male and female mice, no new born (-/-) mice were observed in more than 81 offspring (Table 7), indicating that homozygous disruption of *mclk1* results in embryonic lethality. To determine the nature of the lethality, embryos from heterozygous intercrosses were analyzed at different days of gestation (Table 7). *mclk1* (-/-) embryos were present at expected mendelian frequencies at E8.5. By E13.5, however, all *mclk1* (-/-) embryos detected were in the process of being resorbed. The homozygous embryos also showed a developmental delay that is clearly evident by day 9.5 post coitum (E9.5) (Fig. 3). The mutant is dramatically smaller compared to the wild-type littermate.

Table 7

Genotype distribution from *mclk1* heterozygous crosses

Stage	Total	+/+#	+/-	-/-	n.d.
E 8.5	74	18 (24 %)	40 (54 %)	14 (19 %)	2
E 9.5	85	23 (27 %)	48 (56 %)	12 (14 %)	2
E 10.5	181	50 (28 %)	114 (63 %)	16 (9 %)	3
E 11.5	137	35 (26 %)	84+2* (63 %)	12+1* (9 %)	2+1*
E 12.5	66	8 (12 %)	41+2* (65 %)	2+2* (6 %)	1+10*
Newborn	81	26 (32%)	55 (68 %)	0	-

n.d.: not determined. *Embryos being resorbed. #The genotype of embryos was determined by PCR analysis and that of pups by southern blotting, as described in Methods.

Northern blot analysis of total E11.5 embryo RNA showed that the amount of *mclk1* mRNA was reduced by approximately 50% in heterozygous embryos when compared to normal embryo and could not be detected in *mclk1* (-/-) embryos. Fig. 2B shows Northern blot analyses of total RNA levels in tissues from *mclk1* +/+ and +/- mice and from E 11.5 *mclk1* +/+, +/- and -/- littermates. The expression level of *cox1*, a mitochondrially encoded subunit of cytochrome oxidase (complex IV), is shown as one of the controls. The expression level of *cox1* gives a good measure of the capacity for oxidative phosphorylation in a given tissue. Northern blots were performed using the full length mouse *mclk1* cDNA as a probe. The decreased level observed in homozygous embryos is likely to be due to the beginning of the resorption process. An approximately 50% decrease of *mclk1* transcript was observed in liver, heart, kidney, muscle, stomach and cerebellum of 44-day old *mclk1* (+/-) mouse as compared to wild-type littermates. Immunoblotting with a polyclonal antibody revealed a band of ~ 21 kDa in liver and heart extracts from (+/+) and (+/-) mice. This signal was reduced by 50% in (+/-) mice as compared to the (+/+) mice (Fig. 2D). The results confirmed that the *mclk1* mutation is a null mutation and

demonstrated a gene-dosage effect of reduced protein levels in (+/-) mice. Total protein extracts from liver and heart of two day-old mice were probed with antibodies against mCLK1 and against the controls COX1 and Porin. Porin is a protein of the outer mitochondrial membrane encoded in the nucleus. Western blots were performed using monoclonal antibodies against cytochrome oxidase subunits I (1D6-E1-A8) and IV (20E8-C12) from Molecular Probes, and a monoclonal antibody against human porin 31HL was from Calbiochem.

The amounts of ubiquinone-9 (UQ_9) and -10 (UQ_{10}) in homogenates of *mclk1* (+/+), (+/-) and (+/-) embryos were determined by HPLC. Cell-free extracts for quinone analysis and enzyme activity measurements were prepared as follows. The samples were homogenized in 50 mM potassium phosphate buffer (pH 7.4), and centrifuged at 1,000 x g for 5 min at 4°C. The supernatants were used for the determination of quinone content and the measurements of enzyme activity. Protein concentration was determined with bovine serum albumin as the standard. Quinones were extracted as described (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6), with slight modifications. Briefly, the quinones extracted in n-hexane/EtOH were dried under nitrogen gas, dissolved in acetone, and left at -80°C. After 30 minutes, the samples were centrifuged at 17,000xg, 15 min, 4 °C, and the supernatant was dried under nitrogen gas. The residue was dissolved in EtOH, vortexed for 2 min, and applied to an HPLC (Model 100A, Beckman) equipped with a guard column, and an analytical column (CSC 80 å, ODS2, C-18, 5 µm, 4.6 x 250 mm). The mobile phase was methanol/ethanol (70/30, v/v) with a flow rate of 2 ml/min. The elution was monitored by a wavelength detector (165 variable wavelength detector, Beckman) at 275 nm. The concentration of quinones was determined spectrophotometrically as described (Miyadera, H. et al., (2001). *J Biol Chem* **276**, 7713-6).

For *mclk1* +/+ embryos, a major peak elutes at 11.9 minutes and is identical to standard UQ for elution time. A smaller peak around 17.3 minutes corresponds to UQ_{10}. The quinone profile of heterozygous *mclk1* (+/-) embryos is identical to that of the wild type. The amount of UQ_9 and UQ_{10} were similar in wild-type and heterozygous embryos (Table 8).

However, the presence of neither UQ_9 nor UQ_{10} was observed in *mclk1* (-/-) embryos (Table 8). These mutant embryos instead exhibited a major peak eluting 0.46 minutes earlier than UQ_9 , which fits the criteria for being DMQ_9.

Table 8

Quinone content of ES cells and embryos

		Quinone type		
Sample	Genotype	DMQ_9 (ng/mg protein)	UQ_9 (ng/mg protein)	UQ_{10} (ng/mg protein)
Embryos				
	+/+	ND	126.7	13.6
	+/-	ND	125.8	14.5
	-/-	37.1	ND	ND
ES cells				
	ES1 (+/+)	ND	265	16.8
	ES2 (+/-)	ND	89.5	4.2
	ES7 (-/-)	38.4	ND	ND

N.D.: not detected.

mclk1 (+/+), (+/-) and (-/-) ES cell lines were derived from E3.5 blastocysts obtained from heterozygous matings as per standard procedures. The quinone profiles observed in these lines follow the same pattern as those obtained from the equivalent mutant embryos, including concentration (Table 4). In particular, only DMQ_9 was detected in the *mclk1* (-/-) ES cell line (ES 7).

As in the case of the *clk-1* mutants in *C. elegans*, the DMQ produced in *mclk1* mutants appears to be sufficient for the maintenance of a relatively

high level of oxygen consumption (62% of the wild type). It is surprising that such levels of mitochondrial function are insufficient to carry out embryogenesis. However, a number of elements could participate in the severity of the phenotype. Again, UQ is found in almost all biological membranes and is known to be a co-factor of the uncoupling proteins (UCP) in the mitochondria, to regulate the permeability transition pore, and to function in plasma membrane and lysosomal oxido-reductase systems. Although DMQ can partially replace UQ in the respiratory chain, it is possible that DMQ is less efficient as a UQ analogue for some of the other functions of UQ, whose resulting impairement participates in the severity of the phenotype. Finally, it has recently been discovered that, in bacteria, quinones are the primary signal for the regulation of growth in response to oxygen availability. Given the conservation between prokaryotes and eukaryotes of crucial molecular mechanisms that sense environmental signals (e.g. the PAS domain proteins), the full UQ deficiency of *mclk1* mutants directly affects the regulation of embryonic growth.

Studies of tissue-specific and temporally controlled knockout of the *mclk1* gene

In addition, studies of tissue-specific and temporally controlled knockout of *mclk1* gene have been initiated in *Mus musculus*. *mclk1*^{flox} allele was created and chimeric mouse was generated as follows. In order to investigate the functional role of mCLK1 protein in specific cells, the technique of conditional gene inactivation was used with Cre-loxP mediated recombination. To produce an *mclk1* allele that can be modified by Cre-recombination, a targeting vector containing approximately 7.5 kb of *mclk1* genomic DNA was constructed in which a selection cassette flanked by loxP sites was introduced downstream of exon 4 with a third loxP site upstream of exon 2 (see Figs. 4A-C and Figs. 8A-E (SEQ ID NO:21)). In Figs. 4A-C, a horizontal line represents *clk1* genomic DNA. Exons are represented by unfilled boxes. The gray box represents a neo-TK expression cassette, with the direction of neo and TK transcription indicated by arrows. The black head arrows represent loxP sites. The restriction sites are : *Bgl*II (B), *Bspe*I (P), *EcoR*I (E), *Hind*III (H), *Sac*I (S), *Swa*I (W), *Xho*I (X). Following transfection of ES cells, homologous

recombinants were identified by Southern blot analysis. Genomic DNA was digested with *Bgl*II, and then hybridized with the 3'external probe flanking 3'region of the targeting vector (*Sac*I-*Xho*I fragment). After analysing the promising neomycin-resistant clones by extensive southern blot, three clones (30, 48 and 84) showed the correct homologous recombination (Fig. 4). Fig. 4A displays a schematic representation of *mclk1* locus and the targeting vector. The different probes used for southern blot are drawn. Fig. 4B gives the expected fragment sizes upon digestion with the different enzymes. Fig. 4C displays the southern blot were performed on *Bgl*II or *EcoR*I digested DNA using different probes. A 9 kb band obtained if there is insertion of the selection cassette flanked by loxP sites downstream of exon 4 without insertion of the third loxP site upstream of exon 2, and is indicated by a * in Fig. 4C.

A detailed description of the generation of the *mclk1*^{flox} allele follows. *mclk1* genomic DNA was isolated from a strain 129/SvJ mouse library (Stratagene) and a *Hind*III-*Xho*I fragment of approximately 7.5 kb containing exons 2, 3, 4, 5 and 6 was subcloned into pBluescript. A primer containing a loxP site (3'-CCG GAG CTA GCG AGC TCG GAA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT GGC GAA TT-5') (SEQ ID NO: 11) was introduced into a BsepI site upstream the exon 2. A cassette containing the neor and HSV-tk genes flanked by two loxP sites was inserted into the *Swa*I site in intron 4 to yield the targeting replacement vector pL75. This cassette, a 4.3 kb *Xho*I/*Not* I fragment, was isolated from the plasmid CDLNTKL (SEQ ID No: 12) and the recessed 3' termini were filled with Klenow enzyme.

To generate homologous recombinants, R1 ES cells derived from 129/Sv mice (at passage 12) were electroporated with *Hind*III-*Xho*I targeting vector fragment. Homologous recombinants were identified by Southern blot hybridization. Genomic DNA was digested with *Bgl*II, and then hybridized with the 3'external probe flanking the 3'region of the targeting vector (*Sac*I-*Xho*I fragment). Other probes were used to detect random insertions in the genome. Hybridizations were performed for 16 hours at 65°C in 6 x SSC, 5 x Denhart, 0.5 % SDS. Blots were then washed for 20 min each, twice 3 x SSC, 0.1 % SDS, then twice with 1 x SSC, 0.1 % SDS.

To generate type I and type II deletions, 5 x 106 homologous recombinant cells were electroporated with 25 μg pBS185 containing the cre-recombinase gene, plated and selected 48 h later with 2 μM gancyclovir. Surviving clones were analyzed by Southern blot. Genomic DNA was digested with *Sac*II, and then hybridized with the 3'external probe flanking 3'region of the targeting vector (*Sac*I-*Xho*I fragment).

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of screening for a compound allowing survival of *clk1* homozygous mutant vertebrate embryos, which comprises the step of breeding heterozygous *clk1* subjects to obtain *clk1* homozygous mutant embryos and determining viability of *clk1* homozygous embryos; wherein at least one of said heterozygous subject is treated with said compound prior to said breeding; and wherein viable embryos are indicative of a compound allowing survival of *clk1* homozygous embryos.

2. The method of claim 1, wherein said embryo is a mouse.

3. The method of claim 1, wherein said compound is suitable for partial or complete functional replacement of endogenous ubiquinone.

4. The method of claim 1, wherein said compound is administered by at least one route selected from the group consisting of oral, intra-muscular, intravenous, intraperitoneal, subcutaneous, topical, intradermal, and transdermal route.

5. A method of screening for a compound suitable for rescue of mutant phenotype of *mclk1* homozygous cell line, which comprises the step of determining a mutant phenotype in a *mclk1* knockout cell line, wherein cell line is treated with said compound prior to said determining, and wherein the level of said phenotype is indicative of a compound suitable for rescue.

6. A method of screening for a compound suitable for partial or complete functional replacement of endogenous ubiquinone, which comprises the step of determining a mutant phenotype in a *mclk1* knock-out homozygous ES cell line; wherein said cell line is treated with said compound prior to said determining; and wherein level of said phenotype is indicative of a compound suitable for partial or complete functional replacement of ubiquinone.

7. The method of claim 6, wherein said phenotype is cellular respiration and/or growth rate.

8. A method of screening for a compound suitable for partial or complete functional replacement of ubiquinone in a subject, which comprises the step of assessing at least one phenotype selected from the group consisting of viability, fertility, and total or partial absence of a mutant phenotype of a *coq-3* homozygous mutant worm; wherein said worm is treated with said compound prior to said assessing; and wherein said at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of said mutant phenotype is indicative of a compound suitable for partial or complete functional replacement of ubiquinone in said subject.

9. The method of claim 8, wherein said compound is capable of reaching mitochondria in said subject.

10. A method for screening for a compound suitable for partial or complete functional replacement of ubiquinone in a subject, which comprises the step of assessing at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of a Clk-1 phenotype of a *clk-1* homozygous mutant worm grown on ubiquinone-depleted substrate; wherein said worm is treated with said compound prior to said assessing; and wherein said at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of said Clk-1 phenotype is indicative of a compound suitable for partial or complete functional replacement of ubiquinone in said subject.

11. The method of claim 10, wherein said ubiquinone-depleted substrate is a non-ubiquinone producer bacteria.

12. The method of claim 10, wherein said ubiquinone-depleted substrate is a bacteria producing ubiquinone having side-chains shorter than 8 isoprene units.

13. The method of claim 10, wherein said compound is capable of reaching at least non-mitochondrial sites of ubiquinone requirement in said subject.

14. The method of any one of claims 10 and 13, wherein said bacteria is selected from the group consisting of RKP1452, AN66, IS-16, DM123, GD1, DC349, JC349, JC7623, JF496, KO229(pSN18), KO229(Y37A/Y38A), KO229(R321V), and KO229(Y37A/R321V).

15. The method of any one of claims 10, 13 and 14, wherein said bacteria has a mutation in at least one of genes selected from the group consisting of *ubiCA, ubiD, ubiX, ubiB, ubiG, ubiH, ubiE, ubiF*, and *ispB*.

16. The method of any one of claims 10, 13-15, wherein said bacteria carries at least one of the plasmids selected from the group consisting of pSN18, Y37A/Y38A, R321V, Y37A/R321V.

17. The method of any one of claims 10-16, wherein said functional replacement of ubiquinone is for a function of ubiquinone as co-factor of CLK-1.

18. A method for screening a compound capable of inhibiting activity of *clk-1* and/or other processes required to make ubiquinone from demethoxyubiquinone in a subject, which comprises the step of determining at least one phenotype selected from the group consisting of growth, fertility and total or partial absence of a Clk-1 phenotypes of a wild-type worm on a ubiquinone-depleted substrate; wherein said worm is treated with said compound prior to said determining; and wherein at least one phenotype selected from the group consisting of total or partial absence of growth, absence of fertility and total or partial absence of said Clk-1 phenotypes is indicative of a compound capable of inhibiting activity of *clk-1* and/or other processes required to make ubiquinone from demethoxyubiquinone in a subject.

19. The method of claim 18, wherein said ubiquinone-depleted substrate is a non-ubiquinone producer bacteria.

20. The method of claim 18, wherein said ubiquinone-depleted substrate is a bacteria producing ubiquinone having side-chains shorter than 8 isoprene units.

21. The method of claim 18, wherein said bacteria is selected from the group consisting of RKP1452, AN66, IS-16, DM123, GD1, DC349, JC349, JC7623, JF496.

22. The method of any one of claims 18, wherein said bacteria has a mutation in at least one of gene selected from the group consisting of *ubiCA, ubiD, ubiX, ubiB, ubiG, ubiH, ubiE* and *ubiF*.

23. The method of any one of claims 18 and 22, wherein said bacteria carries at least one of the plasmids selected from the group consisting of pSN18, Y37A/Y38A, R321V, Y37A/R321V.

24. A method of screening for a compound suitable for complete or partial functional ubiquinone replacement, which comprises the step of determining a mutant phenotype of a subject in which *mclk1* and/or a known ubiquinone biosynthetic enzyme gene is deleted and/or any other gene which when altered leads to absence or reduction of ubiquinone; wherein said subject is treated with said compound prior to said determining; and wherein level of said phenotype is indicative of a compound suitable for complete or partial functional ubiquinone replacement.

25. The method of claim 24, wherein said subject is a mouse, ES cell line, or any cell line in which *mclk1* is deleted or any gene coding for a known ubiquinone biosynthetic enzyme gene is deleted and/or any other gene which when altered leads to absence or reduction of ubiquinone.

26. A mouse which is incapable of producing ubiquinone and comprising a gene knock-out of *mclk1*; wherein said mouse expresses the phenotype related to an absence of ubiquinone and the presence of demethoxyubiquinone.

27. A DNA construct, which comprises an alteration of *mclk1*; wherein said DNA construct is instrumental in producing a mouse *mclk1* knockout strain of claim 26.

28. A ES cell line which is incapable of producing ubiquinone and comprising a gene knock-out of *mclk1*; wherein said ES cell line expresses

the phenotype related to an absence of ubiquinone and the presence of demethoxyubiquinone.

29. A *coq-3* mutant subject which is incapable of producing ubiquinone; wherein mutation is a deletion of *coq-3* or a deletion of a ubiquinone biosynthetic enzyme and/or any other gene which when altered leads to absence or reduction of ubiquinone.

30. The mutant of claim 29, wherein said subject is a worm.

31. The mutant of claim 30, wherein said mutant is selected from the group of worm identified using PCR primers selected from the group consisting of SHP172, SHP1773, SHP1774, SHP1775, SHP1840 and SHP1865.

32. A method of screening for a compound suitable for complete or partial functional ubiquinone or demethoxyubiquinone replacement, which comprises the step of determining a mutant phenotype in a subject in which a ubiquinone biosynthetic enzyme gene and/or any gene whose alteration leads to an absence or reduction of ubiquinone or demethoxyubiquinone is altered; wherein said subject is treated with said compound prior to said determining; and wherein level of phenotype is indicative of a compound suitable for complete or partial functional ubiquinone or demethoxyubiquinone replacement.

33. The method of claim 32, wherein said subject is a worm.

34. A method for reducing and/or increasing ubiquinone level in a multicellular subject, which comprises the step of targeting *coq-3* in said subject.

35. A method of screening for a genetic suppressor of *clk-1*, which comprises the step of determining at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of a Clk-1 mutant phenotype of *clk-1* mutant worm grown on ubiquinone-depleted substrate; wherein said worm carries said genetic suppressor prior to said determining; and wherein said at least one phenotype selected from the group consisting of viability, fertility and total or partial

absence of said Clk-1 mutant phenotype is indicative of a genetic suppressor of *clk-1*.

36. The method of claim 35, wherein said bacteria is selected from the group consisting of RKP1452, AN66, IS-16, DM123, GD1, DC349, JC349, JC7623, JF496, KO229(pSN18), KO229(Y37A/Y38A), KO229(R321V), and KO229(Y37A/R321V).

37. The method of any one of claims 35, wherein said bacteria has a mutation in at least one of genes selected from the group consisting of *ubiCA, ubiD, ubiX, ubiB, ubiG, ubiH, ubiE, ubiF* and *ispB*.

38. The method of any one of claims 35, wherein said bacteria carries at least one of the plasmids selected from the group consisting of pSN18, Y37A/Y38A, R321V, and Y37A/R321V.

39. A method of screening for a genetic suppressor of *coq-3*, which comprises the step of determining at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of a mutant phenotype of *coq-3* mutant worms; wherein said worm carries said genetic suppressor prior to said determining; and wherein said at least one phenotype selected from the group consisting of viability, fertility and total or partial absence of said mutant phenotype is indicative of a genetic suppressor of *coq-3*.

40. A method of screening for a compound suitable for complete or partial functional ubiquinone replacement, which comprises the step of determining a mutant phenotype of a subject in which *mclk1* is deleted only in a subset of cells and/or periods of the life cycle, wherein said subject is treated with said compound prior to said determining; and wherein level of said phenotype is indicative of a compound suitable for complete or partial functional ubiquinone replacement.

41. The method of any one of claims 1-25, 32 and 35-40, wherein said compounds are useful in treating a disease selected from the group consisting of reactive oxygen species (ROS) mediated disease, diabetes,

hypoxia/reoxygenation injury, Parkinson's disease, artherosclerosis and Alzheimer's disease.

SEQUENCE LISTING

<110> MCGILL UNIVERSITY

HEKIMI, Siegfried

HIHI, Abdelmadjid

LEVAVASSEUR, Frangoise

SHOUBRIDGE, Eric

GAO, Yuan

PAQUET, Michel

BÉNARD, Claire

<120> PHENOTYPIC EFFECTS OF UBIQUINONE DEFICIENCIES AND METHODS OF SCREENING THEREOF

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35 40 45

Pro Phe His Ala Leu His Ser Leu Asn Arg Ile Arg Val Pro Trp Ile
50 55 60

Val Asp Asn Val Arg Lys Ser Asp Gln Lys Ala Pro Pro Arg Leu Val
65 70 75 80

Asp Val Gly Ser Gly Gly Gly Leu Leu Ser Ile Pro Leu Ala Arg Ser
85 90 95

Gly Phe Asp Val Thr Gly Ile Asp Ala Thr Lys Gln Ala Val Glu Ala
100 105 110

Ala Asn Gln Ser Leu Thr Ala Lys Pro Leu Gln Ile Ala Gly Ile Ser
115 120 125

Lys Arg Leu Arg Phe Glu His Thr Ser Val Glu Asp Phe Cys Gln Lys
130 135 140

Pro His Asn Lys Ser Ala Tyr Asp Ala Val Val Ala Ser Glu Ile Val
145 150 155 160

Glu His Val Ala Asp Leu Pro Gly Phe Ile Gly Cys Leu Ala Glu Leu
165 170 175

Ala Arg Pro Gly Ala Pro Leu Phe Ile Thr Thr Ile Asn Arg Thr Trp
180 185 190

Leu Ser Lys Leu Ala Ala Ile Trp Leu Ala Glu Asn Val Leu Lys Ile
195 200 205

Val Pro Pro Gly Val His Asp Trp Glu Lys Phe Ile Thr Pro Ala Glu
210 215 220

Leu Thr Ser His Leu Glu Lys Ala Gly Cys Arg Val Thr Ala Val His

225 230 235 240

Gly Leu Met Phe His Pro Val Gly Asn His Trp Thr Trp Ile Glu Ser

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Val Glu Lys Phe Gly Asp Leu Ser Ala Glu Trp Ala Asp Glu Leu Gly

35 40 45

Pro Phe His Ala Leu His Ser Leu Asn Arg Ile Arg Val Pro Trp Ile

50 55 60

Val Asp Asn Val Arg Lys Ser Asp Gln Lys Ala Pro Pro Leu Val Asp

65 70 75 80

Val Gly Ser Gly Gly Gly Leu Leu Ser Ile Pro Leu Ala Arg Ser Gly
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Phe Asp Val Thr Gly Ile Asp Ala Thr Lys Gln Ala Val Glu Ala Ala
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Asn Gln Ser Leu Thr Ala Lys Pro Leu Gln Ile Ala Gly Ile Ser Lys
115 120 125

Arg Leu Arg Phe Glu His Thr Ser Val Glu Asp Phe Cys Gln Lys Pro
130 135 140

His Asn Lys Ser Ala Tyr Asp Ala Val Val Ala Ser Glu Ile Val Glu
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His Val Ala Asp Leu Pro Gly Phe Ile Gly Cys Leu Ala Glu Leu Ala
165 170 175

Arg Pro Gly Ala Pro Leu Phe Ile Thr Thr Ile Asn Arg Thr Trp Leu
180 185 190

Ser Lys Leu Ala Ala Ile Trp Leu Ala Glu Asn Val Leu Lys Ile Val
195 200 205

Pro Pro Gly Val His Asp Trp Glu Lys Phe Ile Thr Pro Ala Glu Leu
210 215 220

Thr Ser His Leu Glu Lys Ala Gly Cys Arg Val Thr Ala Val His Gly
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Thr Arg Cys Lys Ser Thr Asp Ala Ser Glu Asp Glu Val Lys His Phe
35 40 45

Gln Glu Leu Ala Pro Thr Trp Trp Asp Thr Asp Gly Ser Gln Arg Ile
50 55 60

Leu His Lys Met Asn Leu Thr Arg Leu Asp Phe Val Gln Arg Thr Val
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Arg Asn Gln Val Lys Ile Gln Asn Pro Glu Ile Phe Val Pro Gly Phe
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Asn Tyr Lys Glu Phe Leu Pro Glu Tyr Val Cys Asp Asn Ile Gln Arg
100 105 110

Glu Met Gln Glu Ser Ile Glu Thr Asn Leu Asp Lys Arg Pro Glu Val
115 120 125

Ser Val Leu Asp Val Gly Cys Gly Gly Gly Ile Leu Ser Glu Ser Leu
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Ala Arg Leu Lys Trp Val Lys Asn Val Gln Gly Ile Asp Leu Thr Arg
145 150 155 160

Asp Cys Ile Met Val Ala Lys Glu His Ala Lys Lys Asp Pro Met Leu
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Glu Gly Lys Ile Asn Tyr Glu Cys Lys Ala Leu Glu Asp Val Thr Gly
180 185 190

Gln Phe Asp Ile Ile Thr Cys Met Glu Met Leu Glu His Val Asp Met
195 200 205

Pro Ser Glu Ile Leu Arg His Cys Trp Ser Arg Leu Asn Pro Glu Lys
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Gly Ile Leu Phe Leu Ser Thr Ile Asn Arg Asp Leu Ile Ser Trp Phe
225 230 235 240

Thr Thr Ile Phe Met Gly Glu Asn Val Leu Lys Ile Val Pro Lys Gly
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Thr His His Leu Ser Lys Tyr Ile Asn Ser Lys Glu Ile Leu Ala Trp
260 265 270

Phe Asn Asp Asn Tyr Ser Gly Gln Phe Arg Leu Leu Asp Leu Lys Gly
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35 40 45

Ala Glu Arg Ala Gly Gly Leu Phe Gly Lys Lys Val Leu Asp Val Gly
50 55 60

Cys Gly Gly Gly Ile Leu Ala Glu Ser Met Ala Arg Glu Gly Ala Thr
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Val Thr Gly Leu Asp Met Gly Phe Glu Pro Leu Gln Val Ala Lys Leu
85 90 95

His Ala Leu Glu Ser Gly Ile Gln Val Asp Tyr Val Gln Glu Thr Val
100 105 110

Glu Glu His Ala Ala Lys His Ala Gly Gln Tyr Asp Val Val Thr Cys
115 120 125

Met Glu Met Leu Glu His Val Pro Asp Pro Gln Ser Val Val Arg Ala
130 135 140

Cys Ala Gln Leu Val Lys Pro Gly Gly Asp Val Phe Phe Ser Thr Leu
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Asn Arg Asn Gly Lys Ser Trp Leu Met Ala Val Val Gly Ala Glu Tyr
165 170 175

Ile Leu Arg Met Val Pro Lys Gly Thr His Asp Val Lys Lys Phe Ile
180 185 190

Lys Pro Ala Glu Leu Leu Gly Trp Val Asp Gln Thr Ser Leu Lys Glu
195 200 205

Arg His Ile Thr Gly Leu His Tyr Asn Pro Ile Thr Asn Thr Phe Lys
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Val Gly Cys Gly Gly Gly Leu Leu Thr Glu Pro Leu Gly Arg Leu Gly

35 40 45

Ala Ser Val Ile Gly Ile Asp Pro Val Asp Glu Asn Ile Lys Thr Ala

50 55 60

Gln Cys His Lys Ser Phe Asp Pro Val Leu Asp Lys Arg Ile Glu Tyr

65 70 75 80

Arg Val Cys Ser Leu Glu Glu Ile Val Glu Glu Thr Ala Glu Thr Phe

85 90 95

Asp Ala Val Val Ala Ser Glu Val Val Glu His Val Ile Asp Leu Glu

100 105 110

Thr Phe Leu Gln Cys Cys Cys Gln Val Leu Lys Pro Gly Gly Ser Leu

115 120 125

Phe Ile Thr Thr Ile Asn Lys Thr Gln Leu Ser Tyr Ala Leu Gly Ile

130 135 140

Val Phe Ser Glu Gln Ile Ala Ser Ile Val Pro Lys Gly Thr His Thr

145 150 155 160

Trp Glu Lys Phe Val Ser Pro Glu Thr Leu Glu Ser Ile Leu Glu Ser

165 170 175

Asn Gly Leu Ser Val Gln Thr Val Val Gly Met Leu Tyr Asn Pro Phe

180 185 190

Ser Gly Tyr Trp His Trp Ser Glu Asn Thr Ser Leu Asn Tyr Ala Ala

195 200 205

Tyr Ala Val Lys Ser Arg Val Gln Glu His Pro Ala Ser Ala Glu Phe
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ctcttttctt ttttttttgg aaataaattt gaaaattttc agaatgtact caaaatcgtg 480

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gaaaaagcgg gttgccgggt gacggcggtg catggattaa tgtttcatcc ggttggaaat 600

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