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A nuclear role for the respiratory enzyme CLK-1/COQ7 in regulating mitochondrial stress responses and longevity

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The coordinated regulation of mitochondrial and nuclear activities is essential for cellular respiration and its disruption leads to mitochondrial dysfunction, a hallmark of ageing[1-3]. Mitochondria communicate with nuclei via retrograde signalling pathways that modulate nuclear gene expression in order to maintain mitochondrial homeostasis[4-6]. The monooxygenase CLK-1 was previously reported to be exclusively mitochondrial, with a role in respiration and longevity[7,8]. We have uncovered a distinct nuclear form of CLK-1 that independently regulates lifespan. Nuclear CLK-1 mediates a novel retrograde signalling pathway that is conserved from *Caenorhabditis elegans* to humans and is responsive to mitochondrial reactive oxygen species, thus acting as a barometer of oxidative metabolism. We show that, through modulation of gene expression, the pathway regulates both mitochondrial ROS metabolism and the mitochondrial unfolded protein response. Our results demonstrate a novel nuclear role for a mitochondrial respiratory enzyme that controls longevity.

Mitochondria function as cellular energy generators producing the fuel, predominantly in the form of adenosine triphosphate (ATP), required to drive biological processes. They act as a hub for many essential biochemical pathways, the metabolites of which are closely monitored by the cell[9]. The majority of the enzymes that are required for these pathways are encoded by the nuclear genome compared with a handful encoded directly by the mitochondrial genome. Therefore, coordinated regulation of nuclear and mitochondrial gene expression is essential[10]. Mitochondrial activity and dysfunction is monitored through a variety of mitochondrial readouts that activate or inhibit cytosolic signalling pathways, for example the rate of production of ATP or reactive oxygen species (ROS)[2,4,11]. These signalling pathways then ensure the appropriate changes in nuclear gene expression occur to maintain mitochondrial homeostasis.

The mitochondrial diiron containing monooxygenase CLK-1 catalyzes the hydroxylation of 5-demethoxyubiquinone, a critical step in the biosynthesis of ubiquinone, an essential cofactor of the electron transport chain[12,13]. However, *Caenorhabditis elegans clk-1* null mutants and heterozygous mice display altered mitochondrial metabolism and extended lifespans through a pathway that appears to be independent of ubiquinone biosynthesis[14-16] indicating a separate function for CLK-1 may exist.

CLK-1, and its human homologue COQ7, contain N-terminal mitochondrial targeting sequences (MTS)[17,18] and are assumed to reside exclusively within mitochondria. However, we have observed nuclear pools of CLK-1 and COQ7 in *C. elegans* and cultured human cells, respectively. Endogenous and exogenously expressed COQ7 display both mitochondrial and nuclear immunostaining in HeLa cells (Figs. 1a, b and Supplementary Fig. 1a), while adult transgenic worms expressing CLK-1 fused to green fluorescent protein (GFP) display fluorescence in both compartments (Fig. 1c). We identified a sequence in COQ7 required for nuclear localisation (NTS), adjacent to the MTS, but within the N-terminal region that is cleaved and degraded by the mitochondrial processing peptidase (MPP) following mitochondrial import[17,18] (Fig. 2b and Supplementary Fig. 1b). This suggested that a pool of COQ7, rather than being imported into mitochondria and cleaved, remains uncleaved and localises to the nucleus. This is supported by nuclear-specific immunostaining of endogenous COQ7 with antibodies that specifically recognise the N-terminal region (Fig. 1d and Supplementary Fig. 1c) and by the presence of both uncleaved and cleaved forms of COQ7 in cell lysates (Fig. 1g and Supplementary Figs. 1d-f). Taken together, these data establish that distinct mitochondrial and nuclear forms of CLK-1/COQ7 coexist within cells.

To address the functional significance of these two pools of CLK-1/COQ7, we explored whether its localisation could be regulated by mitochondrial activity. It has been proposed that the

regulation of mitochondrial protein import in response to changes in reactive oxygen species (ROS) production and peptide efflux can act as a gauge of mitochondrial activity and dysfunction[5,19]. We found that exposure of cells to an antioxidant (N-acetyl-L-cysteine; NAC) decreased the amount of nuclear COQ7, while the same treatment in worms reduced CLK-1-GFP nuclear localisation (Figs. 1e, f). Conversely, an increase in cellular or mitochondrial ROS enhanced nuclear COQ7 levels (Fig. 1g and Supplementary Fig. 2a). Interestingly, a mutant COQ7 with impaired mitochondrial targeting (COQ7 R11/14/16D) remained uncleaved and predominantly localised to nuclei, independently of ROS levels (Fig. 1h and Supplementary Figs. 2b, c). These data are consistent with a model in which a distinct pool of CLK-1/COQ7 is targeted to the nucleus following inhibition of mitochondrial import through a ROS-dependent pathway.

To further characterise the nuclear targeting of COQ7 we performed site-directed mutagenesis of conserved residues within the N-terminal NTS and identified a point mutant (R28A) that, compared to wild type COQ7, displayed significantly reduced nuclear localisation (Fig. 2a and Supplementary Figs. 3a, b). Furthermore, by inserting an epitope-tag (OLLAS) immediately C-terminal to the NTS but prior to the MPP cleavage site, we could demonstrate that uncleaved wild type COQ7 is present in nuclei but we could not detect any uncleaved R28A mutant (Fig. 2b, c). This is supported by immunoblots of cell lysates showing decreased amounts of uncleaved R28A mutant protein (Supplementary Fig. 2c). These data suggest that a pool of wild type COQ7 can be redirected to the nucleus before mitochondrial import but the R28A mutant cannot be and is therefore predominantly targeted to mitochondria where it is cleaved.

Since the loss of COQ7 expression would affect both its mitochondrial and nuclear functions, we created a system to specifically determine the effect of depleting nuclear COQ7. Cell lines were established with endogenous COQ7 expression replaced by ectopic expression of either wild type

COQ7 or the non-nuclear R28A mutant (Fig. 2d and Supplementary Fig. 3d). As expected, the uncleaved wild type COQ7 resided in the nuclear fraction but this form was absent in the R28A expressing cells (Fig. 2e). Importantly, both cell lines had normal levels of ubiquinone (Fig. 2f and Supplementary Fig. 3e). However, loss of nuclear COQ7 resulted in a decreased cell count that, in the absence of an increase in cell death, was indicative of impaired proliferation (Fig. 2g and Supplementary Fig. 3f). These data demonstrate that COQ7 has a biologically relevant nuclear role that is independent of its characterised mitochondrial biosynthetic function.

The N-termini of *C. elegans* CLK-1 and human COQ7 are not well conserved compared to the diiron-binding domain C-terminal to their predicted MPP cleavage sites (Supplementary Fig. 4a). Indeed, the N-terminal residues we identified as being critical for nuclear localisation of COQ7 do not appear to be conserved in CLK-1. However, both homologues localise to the nucleus in a ROS-dependent manner (Fig. 1) suggesting that their nuclear role is conserved but the precise molecular mechanism by which they are targeted to the nucleus is not. To address the non-mitochondrial role of worm CLK-1, we generated a GFP-tagged CLK-1 construct in which the predicted thirteen residue MTS, prior to the MPP site, was deleted. Transgenic worms expressing this protein showed it localised predominantly to the nucleus demonstrating that, similar to COQ7, when mitochondrial targeting is impaired nuclear localisation is prevalent (Fig. 3a). We next crossed transgenic worms expressing either full length CLK-1 (*clk-1*^{wt}) or the truncated nuclear-only form of CLK-1 (*clk-1*$^{nuc(+)}$) with the *clk-1* null worm *qm30* (*clk-1(-)*). As expected, full length CLK-1 was able to rescue ubiquinone biosynthesis in these worms, however, CLK-1$^{nuc(+)}$ could not. This infers that, similar to nuclear COQ7 in human cells, nuclear CLK-1 does not contribute to the mitochondrial biosynthetic role of CLK-1 in worms (Supplementary Fig. 4b).

We next analysed our two complementary models, nuclear only CLK-1 in worms and mitochondrial only COQ7 in human cells, for changes in ROS responses, a phenotype of *clk-1* null worms[20]. Expression of CLK-1$^{nuc(+)}$ in *clk-1* null worms partially rescued the increased ROS levels observed in these animals (Fig. 3b and Supplementary Fig. 4c). Importantly, we also observed increased basal and induced levels of ROS in human cells lacking nuclear COQ7 (Fig. 3d), suggesting nuclear CLK-1/COQ7 plays a conserved role in maintaining low levels of ROS. CLK-1$^{nuc(+)}$ was also able to significantly rescue the ROS-sensitivity phenotype of *clk-1* null worms when exposed to the mitochondrial respiratory chain inhibitor paraquat (Fig. 3c), whereas human cells expressing mitochondrial only COQ7 (R28A) were sensitive to ROS-induced cell death (Figs. 3e). These results indicate that there is a conserved role for nuclear CLK-1/COQ7 in the response of cells to ROS, potentially through regulation of ROS metabolism.

Mitochondrial glutamate metabolism is a key regulator of cellular redox balance and its inhibition is linked to increased ROS levels in cancer cells[21]. We examined whether the expression of the mitochondrial form of glutaminase (GLNA-1 in worms/GLS2 in humans) was regulated in a nuclear CLK-1/COQ7-dependent manner. In *clk-1* null worms, *glna-1* transcript levels were decreased compared to wild type animals, an effect that was rescued in the presence of CLK-1$^{nuc(+)}$ (Fig. 3f and Supplementary Fig. 4d). Loss of nuclear COQ7 in human cells also resulted in a decrease in expression of the *glna-1* homologue *GLS2* (Figs. 3h, j and Supplementary Fig. 4g), suggesting that nuclear CLK-1/COQ7 may regulate cellular ROS levels by modulating glutamate metabolism. Another regulator of ROS metabolism is the oxidoreductase WWOX, a hyperactivated form of which has been shown to increase cellular ROS levels in *Drosophila melanogaster*[22]. Furthermore, the transcript levels of one of the worm *WWOX* homologues are increased in *clk-1* null worms[23]. Intriguingly, we found that expression of CLK-1$^{nuc(+)}$ could rescue the increased expression of the closest *WWOX* homologue, *dhs-7,* in *clk-1(-)* worms, and that human *WWOX* expression was increased upon loss of nuclear COQ7 (Figs. 3g, h, j, and

Supplementary Figs. 4d, g). Taken together these data suggest nuclear CLK-1/COQ7 can potentially regulate metabolic pathways that alter cellular ROS production independently of ubiquinone.

Since cellular ROS levels were changed in a nuclear CLK-1/COQ7-dependent manner we sought to determine whether ROS-dependent gene expression was also altered. We found that transcripts of *sod-2*, encoding a ROS detoxification enzyme, were increased in *clk-1* null worms as previously reported[24], while transcripts of *skn-1*, encoding a transcription factor that is a central regulator of ROS homeostatic gene expression[25], were also increased (Supplementary Fig. 4e). The expression of nuclear CLK-1 in *clk-1* null worms abrogated the increased transcript levels of these genes (Supplementary Fig. 4e). Homologues of these genes, *SOD2* and *NRF2*, and the NRF2 target gene *HMOX1* were also increased in human cells lacking nuclear COQ7 (Fig. 3j and Supplementary Fig. 4f) suggesting that the altered ROS phenotype activates a ROS-defensive gene expression program. However, as the loss of nuclear CLK-1/COQ7 sensitises cells to oxidative stress (Fig. 3c and e) even in the presence of these increased ROS defenses, it suggests that survival pathways may also be altered. To address this we looked at the expression of the proapoptotic mitochondrial protease HTRA2, which is stimulated through a ROS-dependent retrograde pathway activated by disruption to mitochondrial proteostasis[26]. HTRA2 levels were significantly increased in cells lacking nuclear COQ7 (Figs. 3i, j and Supplementary Fig. 4g) and inhibition of HTRA2 activity rescued the ROS-sensitivity phenotype of these cells (Fig. 3k). These data further support a role for nuclear CLK-1/COQ7 in regulating retrograde ROS responses through modulation of gene expression.

In addition to changes in ROS metabolism, *C. elegans clk-1* null mutants and heterozygous mice have extended lifespans[7,16]. *C. elegans* lacking CLK-1 survive by obtaining ubiquinone from their diet while heterozygous *clk-1* mice display wild type ubiquinone levels, suggesting that their

increased longevity may be unrelated to CLK-1's function in ubiquinone biosynthesis[14,16]. Remarkably, expression of CLK-1$^{nuc(+)}$ in *clk-1* null worms caused a decrease in their enhanced longevity phenotype (Figs. 4a, b and Supplementary Table 1). This infers that nuclear CLK-1 can regulate longevity and that this is not related to the role of CLK-1 in mitochondrial ubiquinone biosynthesis. Interestingly, the expression of CLK-1$^{nuc(+)}$ did not rescue the delayed larval development observed in *clk-1* null worms[7] (Supplementary Fig. 5a), which is consistent with this phenotype being due to the loss of mitochondrial CLK-1.

The extended lifespans of *clk-1* mutants, and other mitochondrial longevity phenotypes, has been linked to activation of a distinct mitochondrial to nuclear retrograde pathway, the mitochondrial unfolded protein response (UPR^{mt})[5,6]. The UPR^{mt} responds to mitochondrial dysfunction by regulating nuclear gene expression[27] and there is an increase in the expression of some UPR^{mt}-responsive genes in *clk-1* null worms[5,23]. Thus, it is plausible that there could be crosstalk between CLK-1 nuclear signaling and the UPR^{mt}. Indeed, we found that the expression of an UPR^{mt}-responsive fluorescent reporter, which is activated in *clk-1* null worms, was significantly reduced in the presence of CLK-1$^{nuc(+)}$ (Fig. 4c), indicating that nuclear CLK-1 may potentially act as a suppressor of the UPR^{mt}. We subsequently monitored transcript levels for a range of UPR^{mt} genes[5,28] and identified a subset (*hsp-6*, *hsp-60*, *spg-7*) where increased expression in *clk-1* null worms was abrogated by expression of CLK-1$^{nuc(+)}$ (Fig. 4d and Supplementary Fig. 5b). Importantly, this regulation of the UPR^{mt} was conserved as the suppressed expression of homologues of these genes (*HSPD1*, *HSPA9*, *AFG3L2*) and of other UPR^{mt}-associated genes (*ATG16L1, CLPP*, *LONP1*, *SPG7*, *TIMM22*) was relieved upon loss of nuclear COQ7 in human cells (Figs. 4e, f and Supplementary Fig. 5c). The UPR^{mt} can be activated by an imbalance between mitochondrial and nuclear encoded mitochondrial proteins[6], but this was not observed in cells lacking nuclear COQ7 (Fig. 4f and Supplementary Fig. 5d; compare MTCO1 with COXIV). Furthermore, we noted that some of the UPR^{mt}-associated genes not regulated by nuclear CLK-

1/COQ7 (*DNAJA3*, *PMPCB*, *ymel-1/YME1L1*, *ENDOG*, *tim-17*) are downstream targets of a distinct UPR^{mt} instigated in the mitochondrial matrix[28] (Figs. 4d, e and Supplementary Figs. 5b, c). These data, therefore, indicate that nuclear CLK-1/COQ7 selectively suppresses a specific branch of the UPR^{mt}.

Since nuclear CLK-1/COQ7 appears to have a role in regulating gene expression, this could be through direct association with chromatin and components of the gene expression machinery or through an indirect intra-nuclear pathway. To address this we performed chromatin fractionation of cells expressing COQ7 or the non-nuclear R28A mutant, both featuring an OLLAS tag inserted in the N-terminus just prior to the MPP cleavage site to allow specific detection of the uncleaved protein (Fig. 2b). The uncleaved form of COQ7, but not the R28A mutant, was enriched in the chromatin fraction suggesting it does associate directly with DNA-protein complexes (Fig. 5a). We next performed chromatin immunoprecipitation (ChIP) for endogenous COQ7 followed by promoter microarray (ChIP-on-chip) analysis (Supplementary Fig. 5e). We identified a number of unique promoter sites enriched in COQ7 ChIPs compared with the IgG control (Supplementary Table 2). Interestingly, two genes whose expression is regulated by nuclear CLK-1/COQ7, *WWOX* and *TIMM22,* had sites that were enriched in the COQ7 ChIP (Fig. 5b). To confirm these were genuine COQ7 chromatin-bound sites, we performed ChIP followed by qPCR targeting these sites and demonstrated that wild type COQ7 expressing cells displayed an enhanced ChIP signal compared to cells expressing the non-nuclear R28A mutant (Fig. 5c). Importantly, we also showed that ChIP signals for endogenous COQ7 at the *WWOX* and *TIMM22* sites were decreased following treatment with the antioxidant NAC (Fig. 5d), which would correlate with the decrease in amount of nuclear COQ7 observed under such conditions (Fig. 1f). These data establish that the nuclear pool of COQ7 can associate directly with chromatin and that this may contribute to its ability to regulate gene expression.

Intriguingly, we did not detect COQ7 chromatin binding sites on the promoters of all the genes that we have found to be potentially regulated by nuclear COQ7 (although these could reside outside of the core promoter regions covered by the array) and, while we have demonstrated that nuclear COQ7/CLK-1 can suppress *WWOX* and *TIMM22* expression (Figs. 3g, h and 4e), we also have evidence that it positively regulates transcription (e.g. *GLS2/glna-1*; Figs. 3f and h). Therefore, while our current data point to COQ7 directly regulating transcription by associating with chromatin, it will be interesting in future studies to determine the precise mechanisms by which it positively or negatively impacts on gene expression.

In summary, we have discovered a conserved role for the respiratory enzyme CLK-1/COQ7 as a direct mediator of mitochondrial to nuclear retrograde signaling that responds to changes in cellular oxidative status and regulates mitochondrial ROS metabolism and proteostasis. The nuclear pool of CLK-1/COQ7 can associate with distinct regions of chromatin and affect gene expression. CLK-1 was previously assumed to modulate lifespan solely by acting in mitochondria, but we have now established that a nuclear form acts independently to inhibit longevity. CLK-1 null worms have defective oxidative phosphorylation[29] and, similar to the many other respiration mutants that are long-lived[30], this is likely to contribute to their increased lifespan. This suggests that the mitochondrial and nuclear forms of CLK-1 contribute to lifespan phenotypes through distinct mechanisms (Fig. 5e and Supplementary Fig. 6).

Previously, elevation of ROS signaling and activation of the UPR^{mt} were reported to promote longevity[6,20,31-33], thus the modulation of these pathways by nuclear CLK-1/COQ7 would be consistent with its role in limiting lifespan. Our data suggests that nuclear CLK-1/COQ7 functions as a rheostat to maintain ROS homeostasis and dampen stress-responsive pathways such as the UPR^{mt}. In such a model, basal levels of ROS, produced by mitochondria during normal functioning, direct a pool of CLK-1/COQ7 to the nucleus where it regulates gene expression.

Some CLK-1/COQ7-regulated genes are directly involved in mitochondrial ROS metabolism and therefore, the prolonged presence of CLK-1/COQ7 in the nucleus instigates a decrease in ROS production to suboptimal levels. Reduced ROS leads to CLK-1/COQ7 being predominantly localised to mitochondria, and not the nucleus, so its affects on gene expression are relieved, basal ROS production returns, and homeostasis is maintained. Under these conditions, nuclear CLK-1/COQ7 partially suppresses the UPR^{mt} suggesting that it acts to prevent activation of this pathway during non-stress conditions. Upon mitochondrial dysfunction that results in enhanced levels of ROS, the pool of nuclear CLK-1/COQ7 increases and protects against the ROS stress but limits hyperactivation of the UPR^{mt}. While the UPR^{mt} offers protection following the accumulation of unfolded or misfolded proteins in mitochondria, it is not clear whether it plays a significant role in the cellular response to oxidative stress. Indeed, it has previously been reported that activation of the UPR^{mt} does not confer resistance to worms subjected to high levels of a mitochondrial ROS stress[5].

This work represents the first example of a mitochondrial respiratory enzyme where two distinct forms from the same translation product are targeted to separate organelles, with the mitochondrial form participating in oxidative phosphorylation and the nuclear form acting as a sensor of metabolic activity through regulating gene expression. In worms, the transcription factor ATFS-1 also localises to both organelles but, unlike CLK-1, only translocates to the nucleus during times of mitochondrial stress when normal proteostasis is disrupted, usually being degraded following mitochondrial import[5]. It will be of interest to determine if redirection from mitochondria to nuclei represents a paradigm for other proteins in maintaining homeostasis during mitochondrial activity or dysfunction. This study contributes to our understanding of the fundamental signalling processes that mediate the response of cells and organisms to changes in respiratory activity that occur during ageing and in disease.

Methods

Plasmids and molecular cloning.

pcDNA3.1-COQ7, pcDNA3.1-COQ7(37-217), pcDNA3.1-COQ7-3xMyc (C-terminal 3xMyc tag), pEGFP-N1-COQ7 (C-terminal GFP tag), pEGFP-C2-COQ7 (N-terminal GFP tag), pEGFP-C2-COQ7(11-217) (N-terminal GFP tag), pEGFP-C2-COQ7(30-217) (N-terminal GFP tag), and pGEX6P1-COQ7 (N-terminal GST tag) were cloned by PCR amplification followed by restriction digest and ligation into the respective parent plasmid. pcDNA3.1-COQ7(R28A), pcDNA3.1-COQ7(S36A), pcDNA3.1-COQ7(R11/14/16D)-3xMyc (C-terminal 3xMyc tag), pEGFP-N1-COQ7(R21A) (C-terminal GFP tag), pEGFP-N1-COQ7(Y26F) (C-terminal GFP tag), pEGFP-N1-COQ7(R28A) (C-terminal GFP tag), pcDNA3.1(CM) (CMV minimal promoter -58 to +63), pcDNA3.1(CM)-COQ7-OLLAS (sequence encoding OLLAS tag (SGFANELGPRLMGKR) inserted between amino acids 33 and 34), pcDNA3.1(CM)-COQ7-OLLAS/FLAG (sequence encoding OLLAS tag inserted between amino acids 33 and 34 and C-terminal FLAG tag), pcDNA3.1(CM)-COQ7(R28A)-OLLAS/FLAG (sequence encoding OLLAS tag inserted between amino acids 33 and 34 and C-terminal FLAG tag), and pcDNA3.1(CM)-COQ7(R11/14/16D)-3xMyc (C-terminal 3xMyc tag) were generated with QuikChange Lightning site directed mutagenesis kit (Agilent Technologies). Primer sequences for cloning/mutagenesis available on request.

siRNA.

Knock down of endogenous *COQ7* transcripts was performed using a pool of siRNA against- 5'-UTR: 5'-AGCAACCACUUCGUUGAACUU-3' and 5'-ACUUCGUUGAACGGAACUGUU-3'; and 3'-UTR: 5'-ACCUGUUUCUCUGCAAAUGUU-3'.

Cell culture, transfections and stable cell lines.

HeLa, COS7, and HEK293 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 5 mM glutamine, 100 units/ml penicillin/streptomycin, and 10% fetal bovine serum (Life Technologies). Plasmid DNA transfections were performed with jetPEI (Polyplus) and siRNA transfections with Lipofectamine RNAiMAX (Life Technologies). HEK293 stable cell lines were selected with 1 mg/ml G418 (Sigma) following transfection with pcDNA3.1-COQ7 or pcDNA3.1-COQ7(R28A), before monoclonal isolation and expansion. Hydrogen peroxide (Sigma), tert-butyl hydroperoxide (Luperox TBH70X, Sigma), and cobalt (II) chloride hexahydrate (Sigma) were diluted in water before use; HTRA2 inhibitor, UCF-101 (Calbiochem) was re-suspended in DMSO.

Antibody generation and purification.

COQ7 antibodies were raised in rabbit using bacterially expressed GST-COQ7 as an immunogen. Rabbits were immunised and serum harvested off-site (Genscript, USA) and were purified against the N-terminus, essentially as described[34]. Briefly, bacterially expressed GST or GST-COQ7(1-37) bound to GSH-sepharose were washed twice in 0.2 M borate pH 8.6 before incubation in 20 mM dimethyl pimelimidate in 0.2 M triethanolamine pH 8.3 for 30 minutes to crosslink the proteins to the resin. Crosslinking was terminated by addition of 0.2 M ethanolamine pH 8.2 for 1 h before two washes in 0.1 M glycine pH 2.5 to remove non-covalently linked molecules before equilibration in TBS (15 mM Tris-HCl pH 7.4, 150 mM NaCl). Antibodies to be purified were adjusted to 1x TBS before incubation with GSH-GST cross-linked resin for 4 h and GST-specific antibodies removed by centrifugation. The supernatant was added to GSH-GST-COQ7(1-37) cross-linked resin, incubated for 18 h and washed. COQ7(1-37)-specific antibodies ($COQ7^{N\text{-}term2}$) were eluted with 0.1 M glycine pH 2.5 before pH adjustment to neutral with 2 M Tris. $COQ^{N\text{-}term}$ anti-COQ7(1-37)-specific antibodies were derived using the same protocol but starting with anti-COQ7 (Santa Cruz, sc-135040). For $COQ7^{2}$ antibodies GST cleared supernatants were incubated with GSH-GST-COQ7 (full length) before glycine elution.

Antibodies.

The following antibodies were used for protein identification: anti-c-Jun (JUN, Santa Cruz, sc-1694); anti-CLPP (Santa Cruz, sc-134496); anti-COQ7 (Proteintech, 15083-1-AP); anti-COXIV (Pierce, MA5-15078); anti-FLAG (M2, Sigma, F1804); anti-GLS2 (Abcam, ab113509); anti-GST (GE Healthcare, 27-4577-01); anti-HSPA9 (Grp75/mtHsp70, Cell Signaling, 2816S); anti-HSPD1 (HSP60, BD Transduction Laboratories, 611562); anti-HTRA2 (OMI, Biovision, 3497-100); anti-Lamin B1 (LMNB1, Santa Cruz, sc-6216); anti-MTCO1 (1D6E1A8, Life Technologies, 459600); anti-Myc (4A6, Millipore, 05-742); anti-NRF2 (NFE2L2, Santa Cruz, sc-722); anti-WWOX (Cell Signaling, 4045); anti-OLLAS (Novus Biologicals, NBP1-06713 and Genscript, A01658); anti-Tubulin β (TUBB, Abcam, ab6046); anti-VDAC (Cell Signalling, 4866).

Immunofluorescence, microscopy and image analysis.

Cells for microscopy were grown on coverslips, fixed in 4% paraformaldehyde (PFA, Sigma) for 10 minutes then permeabilized in 0.2% Triton X-100 for 10 minutes. All steps were performed in 1x PBS (phosphate buffered saline, Fisher). For immunofluorescence, cells were blocked in 3% bovine serum albumin (BSA) for 1 h, incubated in primary antibody in 3% BSA for 18 h, washed,

incubated in Alexa Fluor conjugated secondary antibody (Life Technologies) in 3% BSA for 1 h, and washed again. Coverslips were mounted in ProLong Gold with DAPI (Life Technologies). For MitoTracker (MitoTracker Red CMXRos; Life Technologies) staining, the dye was applied at 10 μM to media 30 minutes before cell fixation. For endogenous COQ7 immunofluorescence, cells were fixed in PFA for 5 minutes and permeabilized in methanol at 20°C for 2 minutes, then 0.2% Triton X-100 for 5 minutes. Images were collected on an Olympus BX51 upright microscope using a 60x objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue software (Molecular Devices). Images were processed and analysed using Adobe Photoshop and ImageJ. Images shown in Figures 1a, b, d, h, 2a, c, and Supplementary Figures 1b and c are representative of 3 independent experiments.

Western blotting.

Western blotting was performed essentially as described[35]. Briefly, cell lysates, unless for fractionations, were prepared in Triton lysis buffer (TLB: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 1% Triton X-100, 10% glycerol plus protease inhibitors). Samples were resolved by SDS-PAGE (10-13% gels) and transferred to Immobilon-P membranes (Millipore), which were then immunoblotted. Primary antibodies were detected with IRDye 800CW or IRDye 680LT secondary antibodies (LI-COR) using the Odyssey imaging system or HRP-conjugated secondary antibodies (Amersham Biosciences), followed by enhanced chemiluminescence (Pierce). Densitometry was performed using ImageJ.

Mitochondrial Isolation.

Mitochondrial isolation was performed essentially as described[36]. Briefly, cell pellets were re-suspended in hypotonic buffer (10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 250 mM sucrose) and left on ice to swell for 10 minutes. Cells were homogenized on ice with 50 strokes of a Dounce Homogenizer before centrifugation at 500 g for 5 minutes to pellet the nuclear fraction. The supernatant was transferred to a new tube and centrifuged again at 10, 000 g for 20 minutes to pellet mitochondria.

Cell survival, death and proliferation assays.

Cell survival was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay. 5 mg/ml MTT was added to cells 4 h before experimental end points after which cells were washed and intracellular formazan (reduced MTT) solvated with 0.4 M HCl in

99% propanol for 1 h. The amount of formazan was assayed using a microplate reader spectrophotometer at A570 nm. Cell death was measured by lactose dehydrogenase presence in media, released from apoptotic or necrotic cells, using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega), following the manufacturer's instructions. Proliferation was based on MTT activity in the absence of detectable cell death.

Cellular ROS measurements.

Intracellular ROS levels were determined by incubating cells with 10 μM 2',7'-dichlorofluorescein diacetate (DCFDA, Life Technologies) for 30 minutes before which cells were washed, lysed in TLB and centrifuged at 20, 000 g for 10 minutes. DCF fluorescence was measured in triplicate from supernatants using a microplate reader spectrophotometer at excitation 485 nm and emission 520 nm.

RNA isolation and quantitative reverse transcription PCR.

RNA was isolated from cells using the RNeasy system (Qiagen) following the manufacturer's instructions. Contaminating DNA was removed using the DNA-free Kit (Life Technologies). Quantitative reverse transcription PCR analysis was performed for both HEK293 cells and worm samples using Power SYBR Green RNA-to-CT 1-Step Kit (Life Technologies) following manufacturer's instructions, with 10 μl reactions. Each experimental repeat was run in triplicate. Primer sequences can be found in Supplementary Table 3. References for primer sequences available on request. Reference genes for quantification: human, *RPL19*; *C. elegans*, *act-1* and *tba-1*. Changes in gene expression were quantitated using the ΔΔCt method[37], normalizing for each gene to the reference gene for the same sample and then to one control sample, as indicated in figure legends. The heat map was generated using Multi Experiment Viewer software and represents the mean values of four independent experimental repeats.

Quinone extraction and reverse phase high performance liquid chromatography.

Quinone extraction and determination was essentially as describe[38]. Briefly, cell or worms pellets that had been frozen and thawed three times were re-suspended in lysis buffer (15 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS) and vortexed. Quinones were extracted by vortexing re-suspended pellets in ethanol/hexane (2/5, v/v) for 10 minutes followed by centrifugation at 20, 000 g for 5 minutes and the top hexane supernatant layer removed. This was repeated three times and supernatants combined. Hexane was removed by drying in a SpeedVac Concentrator and the residue re-suspended in ethanol. Re-suspended quinones were applied to a reverse-phase column

(Jupiter 4 μm Proteo 90 Å, C-12, 250 x 4.6 mm, Phenomenex) and eluted in isocratic condition (1 ml/minute, diisopropyl ether/methanol (1/4, v/v)) by high-performance liquid chromatography (Ettan LC, GE Healthcare). Eluted quinones were compared using their retention times and UV spectrophotometry (A275 nm). Ubiquinone-10 (Coenzyme Q10, Sigma) and ubiquinone-9 (Sigma) were used as standards for human and *C. elegans* endogenous ubiquinone, respectively. Clioquinol (Vetranal, Sigma) was used to inhibit COQ7 activity[39] leading to accumulation of its substrate demethoxyubiquinone-10. Chromatograms in Figures 2g, 3c and Supplementary Figures 2f, 3a are representative of 3 independent experiments.

Chromatin fractionation.

Isolation of chromatin/DNA-associated protein complexes from cells was performed as described[40]. Briefly, cells were washed then scraped in CSK buffer (10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM magnesium chloride, 1 mM EGTA, 0.5% Triton-X-100 plus protease inhibitors) and left at 4°C for 5 minutes before centrifugation at 5000 g for 3 minutes. The supernatant was removed as the triton-soluble fraction (TS). The pellet was resuspended in CSK buffer containing 30 U DNase I (New England Biolabs) and incubated at 37°C for 20 minutes followed by precipitation of chromatin by addition of 0.25 M ammonium sulphate and incubation at 4°C for 5 minutes. Samples were then centrifuged at 5000 g for 3 minutes and the supernatant taken as the DNase –soluble fraction (DS).

Chromatin immunoprecipitation.

Chromatin immunoprecipitations (ChIPs) for qPCR analysis of promoter regions or promoter microarray were carried out as follows[41]. Cells were washed then crosslinked in 0.85% formaldehyde for 10 minutes at 20°C before quenching with a final concentration of 0.125 M glycine for 5 minutes. Cells were then washed and lysed in FA buffer (50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors) for 10 minutes at 4°C followed by sonication using a 4°C water bath sonicator (Bioruptor Sonicator, Diagenode) set to medium power with cycles of 30 seconds on 30 seconds off for 5 minutes or until average DNA fragment length of 0.3-0.7 kb was obtained. Samples were centrifuged at 21000 g for 10 minutes before addition of ABRI buffer (50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, protease inhibitors) 3:1 to supernatants. 1/20 of each sample was taken as input and the rest used for IPs. 2 μg of each antibody ($COQ7^2$ or rabbit IgG control, Santa Cruz, sc-2027) was added before rotation for 18 hours at 4°C before addition of 15 μl prewashed protein A Dynabeads (Life Technologies) for a

further 1 hour. ChIPed complexes were washed once in ABRI buffer, twice in WB1 buffer (20 mM Tris pH 8, 2 mM EDTA, 150 mM NaCl, 1% Triton-X-100), twice in WB2 buffer (20 mM Tris pH 8, 2 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate), and once in TE buffer (25 mM Tris, 1 mM EDTA) before elution in 1% SDS and 0.1 M $NaCHO_3$ with rotation/shaking at 30°C for 30 minutes. Eluted complexes and inputs were then adjusted to 150 mM NaCl and crosslinks reversed by incubation at 65°C for 18 hours. Samples were then treated with proteinase K (Sigma) for 2 hours at 50°C. ChIPed and input DNA was then phenol/chloroform/IAA (Sigma) purified and washed before final solvation in TE buffer.

For qPCR analysis of promoter enrichment primers against regions (Supplementary Table 3) of interest were used to amplify DNA in both ChIPs and inputs and antibody enrichment calculated as a percent of input using the following formulae: 2^X = input dilution factor, total input Ct value = input Ct – X, sample % input = $2^{(\text{total input Ct- sample Ct})}$ x 100.

For promoter microarray analysis, DNA samples for IgG control or COQ7 ChIPs were amplified using random primer PCR amplification. DNA was subjected to two rounds of PCR, initially reactions of 40 ng DNA, 1x Sequenase buffer (US Biochemical) and 5 μM of the degenerate primer 5'-GTTTCCCAGTCACGATCNNNNNNNNN-3' were subjected to two cycles of primer extension: 98°C for 5 minutes, 8°C for 4 minutes, slow ramped (10%) from 8°C to 37°C before a further 8 minutes at 37°C. During the first 8°C hold the reaction mixture was adjusted to 0.25 mM dNTPs, 3 mM DTT and 0.05 mg/ml BSA; during the second 8°C hold 1.5 U/μl Sequenase (US Biochemical) was added. DNA was purified with a PCR Cleanup Kit (Qiagen) and all eluted DNA was then subjected to a second round of PCR with 1 U/μl Biotaq polymerase (Gentaur), 0.25 mM dNTPs, 1x Biotaq buffer and 1 μM of the amplification primer 5'-GTTTCCCAGTCACGATC-3' denatured at 97°C for 3 minutes before 25 cycles of: 97°C for 30 seconds, 40°C for 10 seconds, 50°C for 30 seconds and 72°C for 90 seconds; followed by a final extension of 72°C for 5 minutes. Amplified DNA was again purified as before and 10 μg of each sample sent for processing at the on-site genomic technologies facility. Enriched probe sets were analysed for two repeats of the experiment and regions significantly enriched in COQ7 ChIPs compared to IgG control are listed in Supplementary Table 2.

***C. elegans* strains.**

C. elegans were cultured at 20°C on nematode growth medium (NGM) agar plates spotted with OP50 strain *Escherichia Coli*[42]. Strains used were wild type Bristol N2, MQ130 *clk-1*

(qm30), SJ4100 (zcIs13 [*hsp-6::gfp*]) and EG6699 ttTi5605; *unc-119* (ed3); oxEx1578. Transgenic strains made in this study were OL0092 ukSi1[*pclk-1::clk-1::gfp, cb-unc-119(+)*], OL0119 ukSi2[*pclk-1::clk-1 ΔMTS (13-187)::gfp, cb-unc-119(+)*], OL0100 ukSi1[*pclk-1::clk-1::gfp, cb-unc-119(+)*]; *clk-1 (qm30)*, OL0177 ukSi1[*pclk-1::clk-1::gfp, cb-unc-119(+)*]; stIs10116[*phis-72::his-24::mcherry::let-858 3' UTR]*, OL0120 ukSi2[*pclk-1::clk-1 ΔMTS (13-187)::gfp, cb-unc-119(+)*]; *clk-1 (qm30)*, OL0121 *clk-1 (qm30); hsp-6::gfp* and OL0123 ukSi2[*pclk-1::clk-1 ΔMTS (13-187)::gfp, cb-unc-119(+)]; clk-1 (qm30); hsp-6::gfp.*

Generation of *C. elegans* transgenic strains.

Transgenic strains OL0092 and OL0119, were generated using MosI mediated single copy insertion (MosSCI) as described previously[43]. Briefly, a plasmid containing *pclk-1::clk-1::gfp* was generated using Gateway techniques (Invitrogen). pDONR clones were generated for the *clk-1* promoter and open reading frame using Gateway and gene-specific primers. The vector pJA256[44] was used as the pDONR clone for GFP. The final destination vector used was pCFJ150, which contains an *unc-119* rescue fragment. *pclk-1::clk-1 ΔMTS (13-187)::gfp* was generated by mutagenesis of the *pclk-1::clk-1::gfp* plasmid. Primer sequences are available on request. MosSCI was performed using the direct injection protocol. *unc-119* animals were injected with an injection mix consisting of: pCFJ150 containing *pclk-1::clk-1::gfp* or *pclk-1::clk-1 ΔMTS (13-187)::gfp* (50 ng/µl), pCFJ601 (50 ng/µl), pMA122 (10 ng/µl), pGH8 (10 ng/µl), pCFJ90 (2.5 ng/µl) and pCFJ104 (5 ng/µl). Selection was based on heat shock survival, wild type movement, no expression of mCherry markers and GFP expression. Insertion of the transgene was confirmed by sequencing.

Worm imaging.

MitoTracker staining was performed as follows, approximately 2-3 day old adult worms were washed off NGM agar plates using M9 buffer. Worms were washed twice in M9 buffer, then once in MR buffer (10 µM MitoTracker Red CMXRos (Invitrogen) in M9 buffer), before being incubated in MR buffer for 2 hours on a rotating mixer at room temperature in the dark. The worms were then pelleted before being washed once with M9 buffer and being allowed to recover on a standard NGM agar plate for 1 hour at 20^{o}C. They were then mounted on agarose pads and imaged at 100x magnification on a Leica DM5000B fluorescence microscope and images captured by Leica DFC340FX camera. Cells in the tail region were imaged. For NAC experiments, synchronised *clk-1::gfp; his-24::mcherry* worms were grown on untreated NGM agar plates or plates containing 10 mM NAC. Adult worms were imaged as described and the proportion of

nuclear CLK-1-GFP was determined by colocalisation with the HIS-24-mCherry nuclear marker. For *hsp-6::gfp* reporter assays between 40 and 60 young adult worms were mounted on agarose pads as described and imaged at 5x magnification. Fluorescence intensity was quantified using ImageJ. Images shown in Figures 1c and 3a are representative of 3 independent experiments.

Measurement of worm ROS levels.

ROS were measured using dihydroethidium (DHE). Briefly, synchronised adult worms were washed 3 times in PBS and then incubated in 3 μM DHE for 30 minutes. Following incubation worms were washed in PBS and then mounted on agarose pads and imaged at 63x magnification. The fluorescence intensity in the head of the worm was quantified using ImageJ.

Paraquat sensitivity assay.

Synchronised adult worms were treated with 40 mM Paraquat, dissolved in M9 buffer, for 6 hours in a 96 well plate. Approximately 20 worms were plated in each well, with each strain measured in triplicate. Survival was measured every 2 hours with worms being scored as dead if they failed to respond to gentle tapping of the plate.

Lifespan assays.

Lifespan assays were performed as described[45]. Briefly, 100 synchronized L1 stage worms were plated onto standard NGM agar plates spotted with OP50 bacteria. Upon reaching adulthood worms were transferred daily to fresh plates until egg laying ceased. Worms were scored daily, being judged as dead when they failed to respond to gentle prodding from a worm pick. Any worms that crawled off the plates during the assay were censored. Presented lifespan plots are representative of lifespan from the L1 larval stage from 3 independent experiments. The experiments were not randomised and the investigators were not blinded to allocation during experiments and outcome assessment. No statistical methods were used to predetermine sample size.

Development assays

Gravid adult worms were bleached and the embryos plated on NGM agar plates without food overnight. Synchronised L1 worms were transferred to spotted NGM plates and larval stage was determined every 24 hours until all of the worms reached adulthood. Approximately 50 worms per time point for each genotype were mounted on agarose pads and scored then discarded.

Worm RNA extraction.

Synchronized L4 stage worms were pelleted and vortexed in 250 µl Trizol (Life Technologies) for 2 minutes and left for 30 minutes to dissolve. 50 µl chloroform was added and tubes vortexed for 2 minutes before centrifugation at 20, 000 g for 5 minutes. The supernatant was removed to a new tube and 125 µl propanol was added for 30 minutes before centrifugation at 20, 000 g for 5 minutes. Supernatant was discarded and the pellet dissolved in RNase-free water. Contaminating DNA was removed using the DNA-free Kit (Life Technologies).

Statistical analysis.

Values are expressed as mean; error bars represent the standard error of the mean. Data were analysed, unless otherwise stated, using unpaired Student's t-test and confidence given by the p values indicated in figure legends. For analysis of the proportion of cells with nuclear staining (Supplementary Fig. 1a) a z-test was used. For lifespan assays, survival time was estimated with the Kaplan–Meier method and the log-rank method was applied to compare survival curves.

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Contributions

R.M.M. conceived and designed the study, performed the majority of the experiments, analysed the data and wrote the paper; R.G.B. generated worm strains, imaged worms and conducted lifespan experiments; K.F. generated worm strains; T.A. and N.R. screened non-nuclear COQ7 mutants; G.B.P. conceived and designed the study; A.J.W. conceived and designed the study, analysed the data and wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Figure Legends

Figure 1: CLK-1 and its human homologue COQ7 localise to both mitochondria and nuclei. (**a**) Endogenous COQ7 and COXIV (mitochondrial marker) immunostaining in HeLa cells. Nuclei stained with DAPI. (**b**) HeLa cells expressing COQ7 tagged at the C-terminus with Myc epitope (COQ7-myc) were stained with anti-Myc and anti-COXIV antibodies. Quantification of nuclear COQ-7-Myc staining is in Supplementary Fig. 1a. (**c**) Wild type CLK-1 ($CLK\text{-}1^{wt}$) fused at the C-terminus to GFP localises to both mitochondria and nuclei in adult *C. elegans*. Arrow marks nuclei. MT (MitoTracker, mitochondrial marker). (**d**) HeLa cells immunostained with an antibody specific to the N-terminus (amino acids 1-36) of COQ7 ($COQ7^{N\text{-}term}$). (**e**) Nuclear staining of endogenous COQ7 is decreased by antioxidant N-acetyl-L-cysteine (NAC, 10 mM, 24 h). 50 HeLa cells assessed in 3 independent experiments (error bars, s.e.m. $*P < 0.05$). **(f)** Percent of mCherry-positive nuclei that are also GFP-positive in *C. elegans* expressing CLK-1-GFP and the nuclear marker HIS-24-mCherry. 25 worms assessed in 3 independent experiments (error bars, s.e.m. $**P < 0.005$). **(g)** The ratio of nuclear (uncleaved) to mitochondrial (mito, cleaved) COQ7 in lysates from HEK293 was quantified from 3 independent immunoblotting experiments (error bars, s.e.m. $*P < 0.05$, $**P < 0.005$). Cells were treated with hydrogen peroxide (H_2O_2, 150 μM, 3h; cellular ROS) or rotenone (50 μM, 3 h; complex I inhibitor, mitochondrial ROS), with or without 10 mM NAC. A representative immunoblot is shown. **(h)** Blocking mitochondrial targeting of COQ7 enhances nuclear localisation of uncleaved COQ7. HeLa cells expressing the COQ7 (R11/14/16D) mutant, that disrupts mitochondrial targeting, fused at the C-terminus to the Myc epitope were immunostained with anti-Myc and anti-COXIV antibodies.

Figure 2: Nuclear COQ7 supports cell proliferation without affecting ubiquinone levels.
(a) Reduced nuclear localisation of the COQ7 (R28A) mutant. Fluorescence in COS7 cells expressing COQ7 or COQ7 (R28A) fused at the C-terminus to GFP. Nuclei stained with DAPI. **(b)** Schematic depicting location of the mitochondrial targeting sequence (MTS), the nuclear targeting sequence (NTS) and the mitochondrial processing peptidase cleavage site (MPP) on COQ7. The N-terminal region of COQ7 is degraded following cleavage by MPP in mitochondria. Also shown are positions of the OLLAS and FLAG epitope-tags. (**c**) HeLa cells expressing dual OLLAS and FLAG tagged COQ7 or COQ7 (R28A) immunostained with anti-FLAG and anti-OLLAS antibodies. The anti-FLAG antibodies recognise total COQ7 (uncleaved and cleaved) and

the anti-OLLAS antibody specifically recognises uncleaved nuclear COQ7. **(d)** Immunoblot of cell lysates from HEK293 cells stably expressing either wild type (WT) COQ7 or the R28A mutant in the presence of siRNA against untranslated regions of *COQ7*. R28A cells display a decrease in nuclear uncleaved COQ7. Quantification of the ratio of uncleaved (nuclear) to cleaved (Mito, mitochondrial) COQ7 from 3 independent experiments is presented (error bars, s.e.m. $^{**}P < 0.005$). **(e)** Immunoblot of cell lysates from the stable HEK293 cells expressing either wild type (WT) COQ7 or the R28A mutant separated into mitochondrial and nuclear pellet fractions. COXIV (mitochondrial marker), Lamin B1 (nuclear matrix marker). Uncropped images of blots are shown in Supplementary Fig. 7. **(f)** Ubiquinone (UQ10) levels are similar in COQ7 WT and R28A cells. Reverse-phase HPLC chromatograms of quinones purified from cells (UQ10 peak at 8.63 minutes). **(g)** Proliferation of COQ7 (R28A) expressing cells was reduced compared to COQ7 WT cells as measured by MTT assay (mean values from 4 wells of cells per condition in 3 independent experiments; error bars, s.e.m. $^{*}P < 0.05$). Cell survival was not altered under these conditions (see Supplementary Fig. 4f).

Figure 3: Nuclear CLK-1/COQ7 regulates cellular ROS metabolism.

(a) CLK-1 lacking the MTS (CLK-1$^{nuc(+)}$) is predominantly nuclear in adult worms. Arrows mark nuclei. MT (MitoTracker, mitochondrial marker). **(b)** Nuclear CLK-1 acts to lower cellular ROS levels. Expression the nuclear form of CLK-1 (*clk-1*$^{nuc(+)}$) partially rescued the increased ROS phenotype of *clk-1* null worms (*clk-1(-)*) as measured by the ROS-sensitive dye DHE. Wild-type CLK-1-GFP (*clk-1*^{wt}) completely rescues the phenotype (25 worms assessed in 3 independent experiments; error bars, s.e.m. $^{**}P < 0.005$, $^{***}P < 0.001$). **(c)** Expression of CLK-1$^{nuc(+)}$ in *clk-1* null worms significantly increases their survival in response to treatment with paraquat (40mM; respiration inhibitor). CLK-1^{wt} expression in *clk-1* null worms rescues survival to levels similar to *N2* worms (25 worms assessed in 3 independent experiments; error bars, s.e.m. $^{**}P < 0.01$). **(d)** Increased levels of ROS in untreated and oxidative stress treated (100 µM tert-butyl hydroperoxide, 1 h) HEK293 cells expressing non-nuclear COQ7 (R28A) compared to wild type (WT), monitored by DCF fluorescence (mean values from 4 wells of cells per condition in 3 independent experiments; error bars, s.e.m. $^{*}P < 0.05$, $^{**}P < 0.005$). **(e)** Nuclear COQ7 promotes resistance to ROS insults. Increased sensitivity of R28A cells to oxidative stress (1 mM $CoCl_2$, 4 h) measured by MTT assay (mean values from 4 wells of cells per condition in 3 independent experiments; error bars, s.e.m. $^{*}P < 0.05$). **(f, g)** Nuclear CLK-1 regulates genes involved in ROS metabolism. The altered transcript levels of *glna-1* and *dhs-7* in *clk-1* null worms are rescued by expression of CLK-1$^{nuc(+)}$ (mean values from 3 reactions per condition for 3 independent

experimental repeats; error bars, s.e.m. *$P < 0.05$). **(h)** The transcript levels of the *glna-1* and *dhs-7* homologues, *GLS2* and *WWOX*, are decreased or increased, respectively, upon loss of nuclear COQ7 (R28A compared to WT) (mean values from 3 reactions per condition for 4 independent experiments; error bars, s.e.m. *$P < 0.05$, **$P < 0.005$). **(i)** The transcript levels of *HTRA2*, encoding a proapoptotic mitochondrial protease, are increased in cells lacking nuclear COQ7 (R28A compared to WT) (mean values from 3 reactions per condition for 4 independent experiments; error bars, s.e.m. **$P < 0.005$). **(j)** Immunoblots of corresponding protein levels for gene transcripts analysed in Fig. 3h, i and Supplementary Fig. 4f (NRF2). Quantification from 3 repeats of the GLS2, WWOX and HTRA2 blots are shown in Supplementary Fig. 4g. Uncropped images of these blots are shown in Supplementary Fig. 7. **(k)** The ROS sensitivity phenotype observed upon loss of nuclear COQ7 is in part due to increased *HTRA2* expression. Inhibition of HTRA2 activity (10 mM UCF-101, 30 min) rescues the ROS sensitivity of COQ7 (R28A) cells following oxidative stress (1 mM $CoCl_2$, 4 h). Measured by MTT assay relative to DMSO treated cells (mean values from 4 wells of cells per condition in 3 independent experiments; error bars, s.e.m. *$P < 0.05$).

Figure 4: Nuclear CLK-1 regulates lifespan and suppresses UPR^{mt} gene expression.

(a, b) CLK-1$^{nuc(+)}$ expression partially rescues the increased lifespan observed in *clk-1*(-) worms while CLK-1^{wt} completely rescues the longevity phenotype. Lifespan plotted as percent survival and mean lifespans calculated. *N2* is the wild type strain. Lifespan data, including mean, maximum and 90th percentile lifespan with statistical analysis, for 3 independent experimental repeats is reported in Supplementary Table 1. **(c)** CLK-1$^{nuc(+)}$ expressing worms display decreased *hsp-6::gfp* reporter activity compared to *clk-1(-)* worms. Quantification of reporter fluorescence in CLK-1$^{nuc(+)}$ expressing worms (*clk-1(-); clk-1$^{nuc(+)}$*) relative to *clk-1(-)* worms (mean fluorescence of 50 worms per genotype pooled from 3 independent experiments; error bars, s.e.m. **$P < 0.005$). **(d)** qPCR measuring mRNA transcripts of UPR^{mt} genes in *clk-1(-)* or *clk-1(-); clk-1$^{nuc(+)}$* worms relative to wild type strain (*N2*) (mean values from 3 reactions per condition in 3 independent experiments; error bars, s.e.m. n.s., no significant difference, *$P < 0.05$). **(e)** Heat map depicting change in expression of UPR^{mt} genes (MT) and UPR^{ER} (ER) genes in R28A cells compared to WT COQ7 cells. Map generated from the qPCR data presented in Supplementary Figure 5c and is representative of 3 independent experiments. Scale represents mean fold change in expression. **(f)** Immunoblots of protein levels including the mitochondrial controls COXIV (nuclear-encoded) and MTCO1 (mitochondrial-encoded). Uncropped images of blots are shown in Supplementary Fig. 7.

Figure 5: COQ7 associates with chromatin.

(a) Chromatin fractionation of HEK293 cells expressing wild type or R28A (non-nuclear mutant) COQ7 tagged within the N-terminus with OLLAS, as shown in Fig. 2b, followed by anti-COQ7 immunoprecipitation and anti-OLLAS immunoblot. Each fraction was also immunoblotted for the markers Tubulin β (cytosolic), c-Jun (active chromatin), Lamin B1 (nuclear matrix). TS, triton-soluble fraction; DS, DNase-soluble fraction. **(b)** Schematic of the *WWOX* and *TIMM22* COQ7-associated promoter sites enriched in anti-COQ7 ChIPs compared to IgG control. Full data set and statistics of enriched sites are provided in Supplementary Table 2. **(c)** Anti-COQ7 ChIP was performed on HEK293 cells expressing wild type (WT) or the mitochondrial-only form (R28A) of COQ7 before qPCR analysis of *WWOX* and *TIMM22* promoter sites and two control intergenic sites (mean values from 3 reactions per condition in 3 independent experiments; error bars, s.e.m. n.s., no significant difference, $^{**}P < 0.005$). **(d)** Anti-COQ7 ChIP was performed on HEK293 cells treated with antioxidant (N-acetyl cysteine, NAC, 10 mM, 24 h) compared to untreated (mean values from 3 reactions per condition in 3 independent experiments; error bars, s.e.m. n.s., no significant difference, $^{*}P < 0.05$). (**e**) Model depicting the dual mitochondrial and nuclear role of CLK-1/COQ7 in regulating ROS homeostasis. Mitochondrial CLK-1/COQ7 regulates ubiquinone (UQ) biosynthesis and respiration via the electron transport chain (ETC), whilst nuclear CLK-1/COQ7, through modulation of gene expression, regulates ROS metabolism and suppresses the UPR^{mt}.

Supplementary information

Supplementary Figure 1: A distinct pool of COQ7 localises to the nucleus.

(a) HeLa cells expressing COQ7-Myc were scored for mitochondrial, nuclear, mitochondrial and nuclear, or disperse COQ7 immunostaining (100 cells assessed in each of 3 independent experiments; error bars, s.e.m. $^{**}P < 0.005$ compared to other localisations). Representative image of cells is shown in Fig. 1b. **(b)** The region of COQ7 required for nuclear localisation resides between amino acids 11 and 29. GFP fluorescence of COS7 cells expressing GFP-COQ7 and the deletion mutants GFP-COQ7(11-217), lacking amino acids 1-10, and GFP-COQ7(30-217), lacking amino acids 1-29. Orientating the GFP tag on the N-terminus of COQ7 abolished mitochondrial localisation and promoted nuclear localisation, probably due to disruption of the

interaction between the N-terminal MTS and the mitochondrial import machinery. Mitochondria are stained with MitoTracker (MT) and nuclei with DAPI. Schematic depicts the GFP-COQ7 deletion mutants used and summarises their localisation. **(c)** Endogenous uncleaved COQ7 is nuclear. HeLa cells immunostained with a second antibody specific to the N-terminus of COQ7 ($COQ7^{N\text{-}term2}$). **(d)** siRNA targeting *COQ7* transcripts decrease levels of both cleaved and uncleaved COQ7 protein. Immunoblots of lysates from HEK293 cells transfected with non-targeting (*CTRL*) or *COQ7* siRNA. Short and long exposures of the immunoblots are shown. **(e)** Immunoblot demonstrating that uncleaved wild type COQ7 (WT) migrates at the same position as COQ7 S36A (containing a point mutation in the predicted mitochondrial processing peptidase cleavage site) and that cleaved COQ7 migrates at the same position as COQ7(37-217) that lacks the N-terminal region cleaved by MPP. * denotes an aberrantly cleaved form of COQ7 S36A. **(f)** Uncleaved COQ7 is found in the nuclear fraction of cells. HEK293 cells treated with or without hydrogen peroxide (150 μM H_2O_2, 3 h) were fractionated into mitochondrial (mito) and a pellet fraction (containing nuclei and insoluble material). Fractions were immunoblotted for COQ7 and appropriate localisation markers; VDAC and COXIV for the mitochondrial fraction; c-Jun and Lamin B1 for the nuclear fraction. The presence of mitochondrial proteins, plus the cleaved mitochondrial form of COQ7, in the nuclear fraction indicates that the nuclear pellets contain some mitochondrial contamination.

Supplementary Figure 2: COQ7 nuclear localisation is dependent on ROS.

(a) Nuclear staining intensity of cells immunostained with anti-COQ7(1-36) ($COQ7^{N\text{-}term}$) following treatment with antioxidant (N-acetyl cysteine, NAC, 10 mM, 24 h) or low and high levels of exogenous ROS (hydrogen peroxide, 200 μM or 800 μM, respectively, 3 h) compared to untreated (0) control. 50 cells assessed in 3 independent experiments (error bars, s.e.m. $*P < 0.05$). **(b)** Immunoblots of lysates from HEK293 cells expressing COQ7-Myc or COQ7 (R11/14/16D)-Myc (mutant that disrupts mitochondrial targeting) treated with H_2O_2 (150 μM, 4 h) or NAC (10 mM, 6 h). NT = untreated cells. Uncropped images of blots are shown in Supplementary Fig. 7. **(c)** The intensity of nuclear anti-Myc immunostaining in HeLa cells expressing COQ7-Myc or COQ7-R11/14/16D-Myc was quantified (50 cells assessed in each of 3 independent experiments; error bars, s.e.m. n.s., no significant difference, $*P < 0.05$ relative to untreated).

Supplementary Figure 3: Identifying a non-nuclear mutant of COQ7.

(a) Alignment of mammalian COQ7 N-terminal protein sequences (amino acids 1 to 42) using Clone Manager; HS, *Homo sapiens*; CL, *Canis lupus familiaris*; MM, *Mus musculus*; RN, *Rattus norvegicus*. Conserved residues are in red and R28 is in blue. MPP marks the mitochondrial processing peptidase cleavage site. Residues mutated and used in the fluorescence studies in panel b are denoted with asterisks. **(b)** Mutations in the COQ7 N-terminus cause reduced nuclear localisation. GFP fluorescence in COS7 cells expressing COQ7 fused at the C-terminus to GFP and harboring the point mutations R21A, Y26F and R28A was analysed. Quantification of the percent of cells displaying nuclear staining is shown (40 cells assessed in each of 3 independent experiments; error bars, s.e.m. $^{**}P < 0.005$). The most significant loss of nuclear staining was observed with the R28A mutation. **(c)** COQ7 (R28A) mutant displays reduced levels of the uncleaved form. Lysates from HEK293 cells expressing OLLAS and FLAG tagged COQ7 ($COQ7^{O/F}$) or $COQ7(R28A)^{O/F}$ were immunoblotted with anti-COQ7 antibody. **(d)** Parent HEK293 cells (Ctrl) or cells stably expressing untagged (WT) or non-nuclear COQ7 (R28A) were transfected with *siCTRL* or *siCOQ7* that specifically targets endogenous *COQ7* mRNA. Transcript levels of endogenous *COQ7* mRNA (5'UTR amplicon) were analysed (mean values from 3 reactions per condition in 4 independent experiments; error bars, s.e.m. $^{**}P < 0.005$). **(e)** Reverse-phase HPLC chromatograms of quinones. Purified ubiquinone-10 (UQ10) was used as a standard. Levels of UQ10 and demethoxyubiquinone-10 (DMQ10) were measured in HEK293 cells treated with the COQ7 inhibitor clioquinol (CQ; 10 μM, 24 h), or from WT or R28A expressing HEK293 cells. CQ caused the appearance of DMQ10. UQ10 peak at 8.63 mins, DMQ10 peak at 8.39 mins. **(f)** Levels of lactate dehydrogenase (LDH) in media from cultured WT and R28A cells are similar, indicating that cell survival under basal conditions is not changed (mean values from 4 wells of cells per condition in 3 independent experiments; error bars, s.e.m. $^{**}P < 0.005$). Treatment with 0.1% (v/v) Triton X-100 (TX) for 30 minutes was used as a positive control.

Supplementary Figure 4: Nuclear COQ7/CLK-1 regulates ROS metabolic gene expression independently of ubiquinone biosynthesis.

(a) The mechanism of targeting of *C. elegans* CLK-1 and human COQ7 to nuclei is not conserved. Alignment of the N-termini and the start of the highly conserved diiron binding domains of CLK-1 and COQ7. The mitochondrial targeting sequences (MTS) are denoted by the black bars, conserved amino acids are highlighted in blue, the predicted mitochondrial processing peptidase (MPP) sites for both proteins is shown, the region in COQ7 containing determinants of nuclear localisation is denoted by the red bar and the residues required for nuclear-targeting are

highlighted in red. CE, *C. elegans*; HS, *Homo sapiens* **(b)** CLK-1$^{nuc(+)}$ expression does not rescue the loss of mitochondrial ubiquinone (UQ9) biosynthesis in *clk-1(-)* worms. Reverse-phase HPLC chromatograms of quinones extracted from the indicated strains (UQ9 peak at 8.78 minutes, DMQ9 peak at 8.42 minutes). CLK-1^{wt} but not CLK-1$^{nuc(+)}$ restores the UQ9 peak loss in *clk-1* (*clk-1(-)*) null worms. **(c)** Expression of the nuclear form of CLK-1 (*clk-1$^{nuc(+)}$*) in *clk-1* null worms (*clk-1(-)*) partially rescues the increased levels of ROS observed in these worms measured using the ROS-sensitive dye DHE. Quantification of staining is presented in Fig. 3b. **(d)** Nuclear CLK-1 regulates the expression of genes involved in ROS metabolism. qPCR analysis of transcripts from ROS-sensitive retrograde genes relative to *N2* (mean values from 3 reactions per condition in 3 independent experiments; error bars, s.e.m. *$P < 0.05$ for *clk-1(-)* compared to other strains). **(e)** CLK-1$^{nuc(+)}$ rescues the increased transcript levels of genes known to be responsive to ROS (*sod-2* and *skn-1*) in *clk-1* null worms (mean values from 3 reactions per condition in 3 independent experiments; error bars, s.e.m. *$P < 0.05$ for *clk-1(-)* compared to other strains). **(f)** Homologues of these genes, *SOD2* and *NRF2*, and the NRF2 target gene *HMOX1* are increased in cells that have lost nuclear COQ7 (R28A) (mean values from 3 reactions per condition for 4 independent experiments; error bars, s.e.m. *$P < 0.05$). (**g**) Quantification of immunoblots for GLS2, WWOX and HTRA2 proteins (mean values from 3 independent experiments; error bars, s.e.m. *P < 0.05, **P < 0.005). Representative blots are shown in Fig. 3j.

Supplementary Figure 5: Nuclear CLK-1 does not rescue the developmental phenotype of CLK-1 null worms but does regulate the expression of UPR^{mt} genes.

(a) Analysis of developmental timing. Worms were synchronised at L1 and larval stage was determined every 24 hours until all of the worms reached adulthood. Approximately 50 worms per genotype were monitored for each time point (mean values from 3 independent experiments; error bars, s.e.m.). **(b)** qPCR analysis of UPR^{mt} genes in CLK-1 transgenic worm strains relative to *N2* (mean values from 3 reactions per condition in 3 independent experiments; error bars, s.e.m. n.s., no significant difference, *$P < 0.05$). The increase in expression of *hsp-6*, *hsp-60* and *spg-7* in *clk-1(-)* worms was abrogated by expression of either CLK-1^{wt} or CLK-1$^{nuc(+)}$. mRNA levels of the endoplasmic reticulum UPR (UPR^{ER})-regulated gene *hsp-4* were not changed in any of the worm strains. **(c)** qPCR of analysis of transcripts of UPR^{mt} genes and UPR^{ER} genes in WT and R28A expressing HEK293 cells (mean values from 3 reactions per condition in 4 independent experiments; error bars, s.e.m. *$P < 0.05$. **$P < 0.005$). Heatmap of this data is shown in Fig. 4e. **(d)** The ratio of COXIV to MTCO1 protein levels in WT and R28A expressing cells quantified

from 3 independent immunoblots (error bars, s.e.m. n.s., no significant difference). See Fig. 4f for representative immunoblot.

Supplementary Figure 6: Model for the regulation of ROS metabolism, the UPR^{mt} and lifespan by nuclear CLK-1/COQ7.

(a) CLK-1/COQ7 regulates mitochondrial homeostasis. The majority of CLK-1/COQ7 localises to mitochondria by means of its mitochondrial targeting sequence (MTS), where it is required for ubiquinone (UQ) biosynthesis, an essential cofactor in the electron transport chain (ETC). However, basal levels of ROS, produced by the mitochondria, direct a pool of CLK-1/COQ7 to the nucleus where it regulates gene expression. Some CLK-1/COQ7-regulated genes are directly involved in mitochondrial ROS metabolism and therefore, the prolonged presence of CLK-1/COQ7 in the nucleus instigates a decrease in ROS production to suboptimal levels. Reduced ROS leads to CLK-1/COQ7 being predominantly localised to mitochondria, and not the nucleus, so its affects on gene expression are relieved, basal ROS production returns, and homeostasis is maintained. **(b)** Loss of nuclear COQ7 (R28A mutant) in human cells or loss of CLK-1 in worms (*clk-1(-)*), that scavenge UQ from their bacterial diet, alters ROS metabolism leading to increased ROS levels, augments the UPR^{mt}, and extends the lifespan of worms. **(c)** The augmented ROS levels, UPR^{mt} and extended lifespan in *clk-1(-)* worms is suppressed by expression of a nuclear-localised CLK-1 mutant (CLK-1$^{nuc(+)}$) that acts as a rheostat to try and maintain mitochondrial homeostasis.

Supplementary Figure 7: Uncropped western blots.

Supplementary Table 1: Complete lifespan analysis of *C. elegans* strains.

CLK-1$^{nuc(+)}$ expression partially rescues the increased lifespan observed in *clk-1*(-) worms while CLK-1^{wt} completely rescues the longevity phenotype. The presented lifespan data includes the mean, maximum and 90th percentile lifespan with statistical analysis for each worm strain from 3 independent experimental repeats.

Supplementary Table 2: ChIP array data

Supplementary Table 3: Primer sequences for qPCR experiments.