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The Intrinsic Apoptosis Pathway Mediates the Pro-Longevity Response to Mitochondrial ROS in *C. elegans*

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SUMMARY

The increased longevity of the *C. elegans* electron transport chain mutants *isp-1* and *nuo-6* is mediated by mitochondrial ROS (mtROS) signaling. Here we show that the mtROS signal is relayed by the conserved, mitochondria-associated, intrinsic apoptosis signaling pathway (CED-9/Bcl2, CED-4/Apaf1, and CED-3/Casp9) triggered by CED-13, an alternative BH3-only protein. Activation of the pathway by an elevation of mtROS does not affect apoptosis but protects from the consequences of mitochondrial dysfunction by triggering a unique pattern of gene expression that modulates stress sensitivity and promotes survival. In vertebrates, mtROS induce apoptosis through the intrinsic pathway to protect from severely damaged cells. Our observations in nematodes demonstrate that sensing of mtROS by the apoptotic pathway can, independently of apoptosis, elicit protective mechanisms that keep the organism alive under stressful conditions. This results in extended longevity when mtROS generation is inappropriately elevated. These findings clarify the relationships between mitochondria, ROS, apoptosis, and aging.

INTRODUCTION

The observed association of the aging process with the biology of reactive oxygen species (ROS), in particular ROS originating from mitochondria (mtROS), has led to the formulation of the oxidative stress theory of aging. Recently, however, more nuanced interpretations have been proposed to explain the basic observations that led to the formulation of the theory (Lapointe and Hekimi, 2010; Sena and Chandel, 2012). One possibility is that ROS damage is not causally involved in the aging process but that ROS levels are correlated with the aged phenotype because they modulate signal transduction pathways that respond to cellular stresses brought about by aging (Hekimi et al., 2011). In other words, ROS generation may be enhanced by the aging process because, in their role as signaling molecules, ROS help to alleviate the cellular stresses caused by aging. This hypothesis is supported by findings in a variety of organisms, in particular in *C. elegans* where changes in ROS generation or detoxification can be uncoupled from any effect on lifespan (Doonan et al., 2008; Van Raamsdonk and Hekimi, 2009, 2010; Yang et al., 2007). Most strikingly, moderate mitochondrial dysfunction (Felkai et al., 1999; Feng et al., 2001; Yang and Hekimi, 2010b), severe loss of mtROS detoxification (Van Raamsdonk and Hekimi, 2009), and elevated mtROS generation (Yang and Hekimi, 2010a), as well as treatments with pro-oxidants (Heidler et al., 2010; Lee et al., 2010; Van Raamsdonk and Hekimi, 2012; Yang and Hekimi, 2010a), can all lengthen rather than shorten lifespan. In addition, the pro-longevity effects of both dietary restriction (Schulz et al., 2007), and reduced insulin signaling in *C. elegans* (Zarse et al., 2012), appear to involve an increase in ROS levels. Such observations are not limited to *C. elegans*. For example, mtROS signaling can act to extend chronological lifespan of the yeast *S. cerevisiae* (Pan et al., 2011).

The longevity phenotype of *isp-1(qm150)* (Feng et al., 2001) and *nuo-6(qm200)* (Yang and Hekimi, 2010b) mutants is most unequivocally connected to mtROS generation (Yang and Hekimi, 2010a). *isp-1* encodes the "Rieske" iron sulfur protein, one of the major catalytic subunits of mitochondrial complex III, and *nuo-6* encodes the mitochondrial complex I subunit NDUFB4. The *qm150* and *qm200* mutations are missense mutations that do not lead to a full loss of protein function. Mitochondria isolated from both mutants show elevated superoxide generation, as measured by fluorescence sorting of purified mitochondria incubated with the dye MitoSox (Yang and Hekimi, 2010a). This is a very specific phenotype that is not accompanied by an increase in overall mitochondrial oxidative stress, nor by a measurable increase in overall oxidative damage. The long-lived phenotype can also be phenocopied by treatment of the wild-type with a very low level (0.1 mM) of the superoxide generator paraquat (PQ). In contrast, treatment of the mitochondrial mutants with PQ has no effect, suggesting that treatment with PQ extends lifespan by the same mechanisms as the mitochondrial mutations (Yang and Hekimi, 2010a).

Increased longevity can also result from induction of the mitochondrial unfolded protein stress response (mtUPR), which can be triggered by RNA interference knockdown of mitochondrial components (Dillin et al., 2002; Durieux et al., 2011; Lee et al., 2003). This response is however distinct from the response to

elevated mtROS as the lifespan increases produced by the elevated mtROS in the mutants and by the activated mtUPR are fully additive (Yang and Hekimi, 2010b).

How might elevated mtROS promote longevity? ROS are well known to act as modulators in signal transduction pathways, and it is as such that they might be enhancing longevity. One candidate signaling pathway that could include potential mtROS sensors as well as a mechanism of downstream signaling is the intrinsic apoptosis pathway. Apoptosis is a highly controlled process that in mammals is sensitive to mitochondrial function, including mtROS, via the intrinsic apoptosis signaling pathway (Wang and Youle, 2009). In *C. elegans* the intrinsic apoptotic machinery consists of the BH3-only protein EGL-1, CED-9 (Bcl2-like), CED-4 (Apaf1-like), and CED-3 (Casp9-like). CED-9 is tethered to the outer mitochondrial membrane and binds CED-4. However, in contrast to vertebrates, there is no evidence for any role for mtROS in regulating apoptosis in *C. elegans*.

Interestingly, the individual proteins or pairs of interacting proteins of the apoptotic signaling machinery appear to be able to carry out apoptosis-independent functions. For example, EGL-1 and CED-9 affect mitochondrial dynamics (Lu et al., 2011), CED-4 and CED-3 promote neuronal regeneration (Pinan-Lucarre et al., 2012), CED-4 appears to be involved in hypoxic preconditioning (Dasgupta et al., 2007) and S-phase checkpoint regulation (Zermati et al., 2007). These and similar findings in other organisms (Galluzzi et al., 2008) suggest that the proteins of the intrinsic apoptotic pathway have bona fide signal transduction activities in other processes. However, in no case to date has the full pathway, from a BH3-only protein to a caspase, been found to be involved in a process distinct from apoptosis.

Here, we show that the *isp-1* and *nuo-6* mutations and 0.1 mM PQ treatment induce a unique pattern of changes in gene expression. Strikingly, we found that mutations in the conserved intrinsic apoptosis signaling pathway (*ced-9*, *ced-4*, and *ced-3*) suppress the longevity of *isp-1* and *nuo-6* mutants, independently of inhibition of apoptosis. Moreover, unlike apoptosis, which requires the BH3-only protein EGL-1, the suppression of *isp-1* and *nuo-6* requires the BH3-only protein CED-13, which is not required for apoptosis. Treatment with PQ can bypass the need for CED-13, suggesting that mitochondrial ROS acts directly on activation of the pathway, possibly by acting on CED-9 and CED-4, which are associated with mitochondria. Loss of apoptotic signaling also suppresses most of the other phenotypes of *isp-1* and *nuo-6*, such as slow development, slow behaviors, altered gene expression, and sensitivity to heat, but not the primary defects of low oxygen consumption and low ATP levels. The finding that the hypometabolic and hypersensitive phenotypes can be suppressed at the same time as longevity, without suppression of the low oxygen consumption and ATP concentration, indicates that these phenotypes are actively induced by mtROS to protect from mitochondrial dysfunction.

RESULTS

Pro-Longevity mtROS Signaling Induces a Unique Pattern of Gene Expression

The effects of *isp-1(qm150)* and *nuo-6(qm200)* mutations on longevity are not additive, and a low dose of PQ (0.1 mM) increases the lifespan of the wild-type, but not of either of the mutants, (Yang and Hekimi, 2010a, b). These observations suggest that a common mechanism underlies the increased longevity in all three conditions. To test this further, we used Affymetrix *C. elegans* microarrays to characterize patterns of gene expression in the two mutants and in wild-type PQ-treated worms. The lists of genes with significantly altered expression overlap considerably (Table S1 available online); 621 genes were upregulated and 1,161 were downregulated in all three conditions (Figure 1A). This confirms that PQ treatment and the two mutations induce common changes in worm physiology. However, the only feature revealed by Gene Ontology (GO)-term analysis was a downregulation of large families of kinases and phosphatases whose expression is linked to the production of sperm (Reinke et al., 2000). GO-term analysis did not reveal any obvious pattern pointing to a specific biological process that could be responsible for the slow aging phenotype (Table S2). In particular, the list of genes upregulated in all three conditions is not significantly enriched in ROS-detoxifying activities or ROS-damage repair activities (Table S3). This strongly suggests that the longevity increase under the three conditions is not the result of an over-compensatory increase in oxidative defenses in response to increased levels of mtROS.

We compared the lists of gene with similar lists obtained by genome-wide expression studies of other mutants and treatments related to the aging process (Table S4). The overlaps were from 1% to 20% for upregulated genes and from 0% to 33% for downregulated genes. The highest overlap was with the short-lived *gas-1(fc21)* mutant. Although *gas-1* is short-lived, it encodes a subunit of a mitochondrial complex I, which is likely the source of the similarities. However, the comparison did not identify a particular process by GO-term analysis (Table S2). Finally, in contrast to a recent study in yeast where mitochondrial ROS signaling ultimately resulted in the specific silencing at subtelomeric loci (Schroeder et al., 2013), we found a uniform distribution of the downregulated loci across all chromosomes (Figure S1). Taken together our findings suggest that the pattern of gene expression induced by elevated mtROS is unique, which is consistent with the observation that PQ treatment is fully or partially additive to the pro-longevity effects of mutations in *daf-2*, *eat-2*, and *clk-1*, and is not fully suppressed by mutations in *daf-16*, *aak-2*, *wwp-1*, *hif-1*, *skn-1*, or *hsf-1* (Yang and Hekimi, 2010a).

The Changes in Gene Expression Are Necessary for the Pro-Longevity Effect of mtROS

To provide a proof-of-principle demonstration of the relevance for lifespan of the observed changes in gene expression, we focused on a small group of genes. The possibility that genome instability is important in determining lifespan remains a strong hypothesis in biogerontology. Furthermore, there are strong links between DNA damage and general stress responses (Ermolaeva et al., 2013). We tested 16 upregulated genes belonging to this group (Figures 1B, S2 and S3). All numerical values and statistics for survival data presented in the paper are provided in Table S5. The knockdown of 3 of the genes in *isp-1* mutants only had a very small effect on lifespan. The knockdown of the 13 other genes

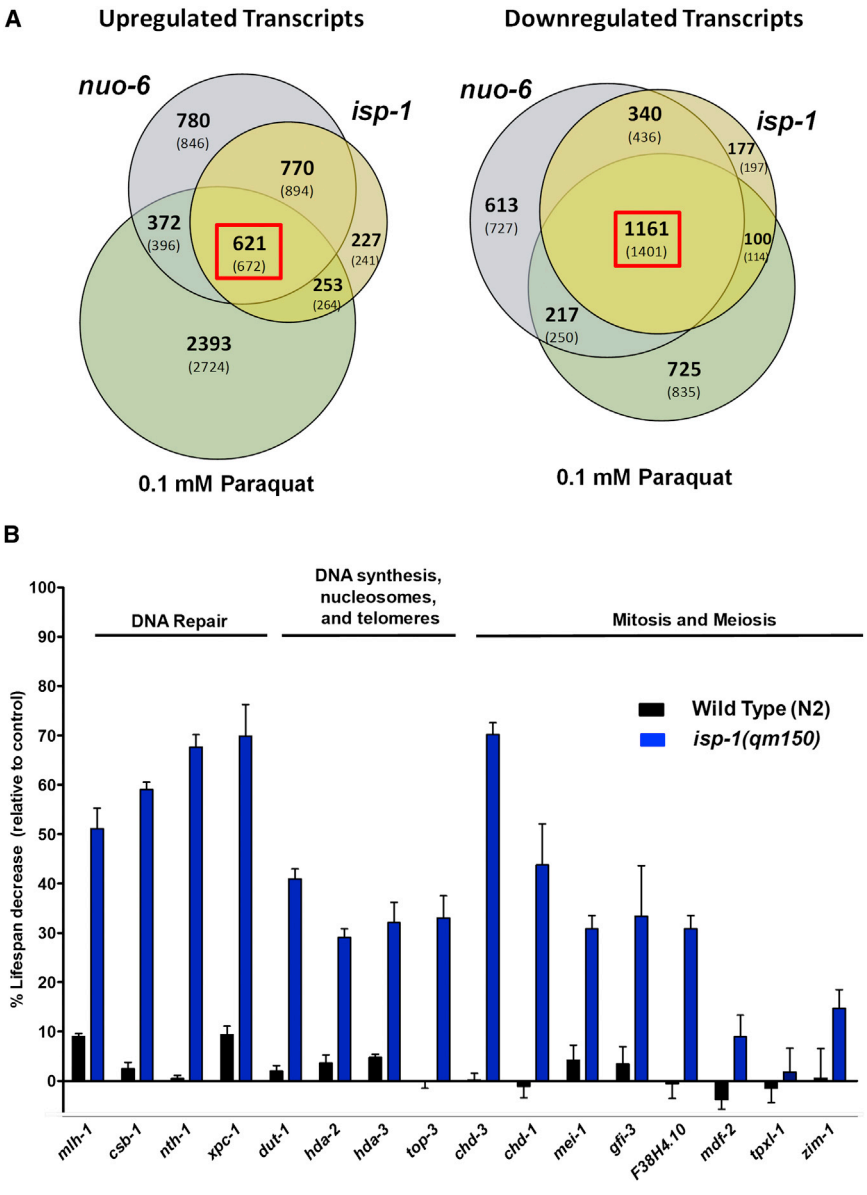

Figure 1. Whole-Genome Expression Profiling of *isp-1* and *nuo-6* Mutants, and the Wild-Type Treated with 0.1 mM Paraquat

(A) Venn diagrams illustrating the number of significantly upregulated or downregulated transcripts found in each condition tested when compared to untreated wild-type. Bolded numbers represent the actual number of probes whose expression was significantly changed relative to wild-type expression, while numbers in brackets represent the maximum number of different transcripts that could be detected as a result of high homology.

(B) Lifespan changes resulting from treatment of wild-type and *isp-1* mutants with RNAi against genes that are upregulated in all three conditions and whose activities are expected to be involved in genome stability. The majority of genes had large effects on the mutant but no, or very little, effect on the wild-type.

Bars represent the degree of lifespan shortening relative to control and error bars represent SEM. See also Figures S1, S2, and S3; Tables S1, S2, S3, S4, and S6. Numerical values and statistical analyses for all lifespan experiments are presented in Table S5.

had a much larger effect on the mutant than on the wild-type and the knockdown of 7 of these had virtually no effect on the wild-type but strongly suppressed the longevity of the mutants (Figures 1B, S2 and S3). The fact that the knockdown of at least some of the upregulated genes limits the longevity of *isp-1* without affecting the wild-type strongly suggest that at least some and probably many of the changes in gene expression we observed are necessary for the longevity resulting from mtROS signaling.

The Longevity Response of *isp-1* and *nuo-6*, but Not that of Other Longevity Mutants, Requires the Conserved Intrinsic Apoptotic Signaling Pathway

The intrinsic pathway of apoptosis, which uses conserved signaling proteins, is physically associated with mitochondria, and in vertebrates is sensitive to mitochondrial oxidative stress. We therefore tested whether this pathway was involved in the pro-longevity signal in worms by scoring the lifespan of double mutants of *isp-1* and *nuo-6* with *ced-9gf*, *ced-4*, and *ced-3* mutations (Figures 2A–2B, S4A–S4D). The mutations in all three *ced* genes significantly suppressed the longevity of both *isp-1* and *nuo-6*, without having any significant effects on lifespan by themselves. The suppression by *ced-4(n1162)* was consistently the most robust. The suppression by *ced-9(n1950)* was somewhat less effective, possibly because it is a gain-of-function allele and might therefore not be fully equivalent to a loss of *ced-4(n1162)*. The somewhat lesser suppression by *ced-3(n717)* suggests that CED-4 recruits other effectors as well.

We tested possible effects of *ced-4* on other lifespan mutants that had previously been shown to be genetically distinct from *isp-1*/*nuo-6*, including *eat-2*, *clk-1*, *daf-2*, and *glp-1*. For this, we tested lifespan in double-mutant combination with *ced-4*, but no effects on the lifespan of these mutants were detected (Figures S4E–S4H). In addition, previous findings had suggested that RNAi against subunits of the ETC prolong lifespan by a mechanism that is distinct from that of the genomic mutants *isp-1* and *nuo-6* (Yang and Hekimi, 2010b). We therefore tested RNAi against *isp-1* and *nuo-6* on *ced-4* mutants and, as predicted, *ced-4* did not suppress the longevity induced by the RNAi treatments (Figures S4I and S4J). We conclude that

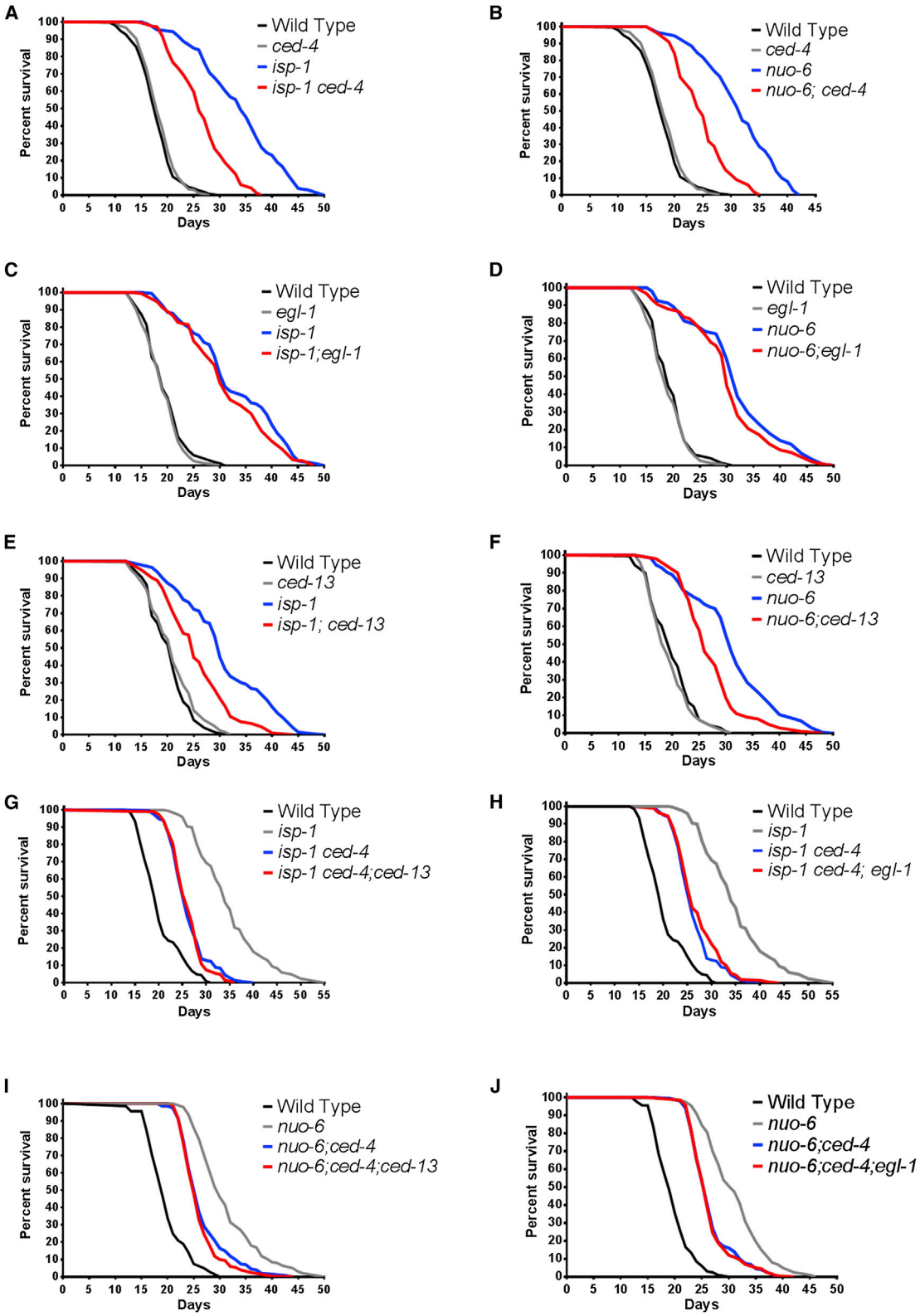

Figure 2. Genetic Interactions between Longevity and Cell Death Genes

(A and B) Effect of *ced-4(n1162)* on the survival of *isp-1(qm150)* and *nuo-6(qm200)*.

(C and D) Effect of *egl-1(n1084n3082)* on the survival of *isp-1* and *nuo-6*.

(legend continued on next page)

the intrinsic apoptotic signaling machinery uniquely mediates the longevity of *isp-1* and *nuo-6*.

The Longevity Response Is Independent of Apoptosis Per Se

As *ced-9gf*, *ced-4*, and *ced-3* affect apoptosis, we scored embryonic and pharyngeal apoptosis in *isp-1* and *nuo-6* mutants as well as in *ced-4*, *isp-1 ced-4*, and *nuo-6;ced-4* double mutants (Table S6). The pattern of apoptosis in the mitochondrial mutants was indistinguishable from the wild-type, and the pattern of apoptosis in the double mutants with *ced-4* was indistinguishable from that produced by the *ced-4* mutation alone, that is, most cell deaths were eliminated. These findings indicated that *isp-1* and *nuo-6* do not affect normal or mutant apoptosis, but they cannot establish whether the normal pattern of apoptosis is necessary for the mutants' increased longevity. For this we turned to the BH3-only protein EGL-1, which is required for all apoptosis in *C. elegans*. We scored both apoptosis and lifespan in *egl-1* mutants as well as in *egl-1;isp-1* and *egl-1;nuo-6* double mutants. As expected, the *egl-1* mutation, like the *ced-4* mutation, abolished apoptosis in all three genotypes (Table S6). However, in contrast to *ced-4*, *ced-9*, and *ced-3*, *egl-1* had no effect at all on lifespan (Figures 2C and 2D). Thus it is not the absence of apoptosis in the intrinsic pathway mutants that suppresses the lifespan of the mitochondrial mutants.

The Activity of the Intrinsic Apoptotic Signaling Pathway on Longevity Requires CED-13, an Alternative BH3-Only Protein

For canonical apoptotic signaling, the intrinsic pathway requires stimulation by a BH3-only protein. CED-13 is the only other protein in *C. elegans* to possess a BH3 domain (Schumacher et al., 2005). CED-13 has been shown to be able to have some effect on somatic apoptosis when overexpressed and is also able to interact with CED-9 in vitro in a way that is similar to that of EGL-1 (Fairlie et al., 2006). However, loss of CED-13 has very limited effects and only on DNA damage-induced germline apoptosis. We found however that the *ced-13(sv32)* mutation suppressed the longevity of *isp-1* and *nuo-6* mutants as efficiently as the mutations in the genes of the core pathway (Figures 2E and 2F). We verified whether CED-13 acted indeed in the same pathway as the other CED proteins by testing whether the effects of *ced-13(sv32)* were additive to those of *ced-4(n1162)* for suppression of the lifespan of *isp-1*. We found that the lifespan of the triple mutants *isp-1 ced-4;ced-13* and *nuo-6;ced-4*;*ced-13* were indistinguishable from those of the double mutants *isp-1 ced-4* and *nuo-6;ced-4*, respectively (Figures 2G and 2I), indicating that *ced-13* acts in the same pathway as *ced-4*. As expected, *egl-1* had no effect either in triple combinations (Figures 2H and 2J). Thus, rather than EGL-1, CED-13 is the BH3-only protein that is required for pro-longevity signaling through the intrinsic pathway.

mtROS Act Downstream of CED-13 for Longevity

We first determined if treatment with 0.1 mM PQ (which does lengthen wild-type lifespan) or with 0.5 mM PQ (which is too toxic to lengthen wild-type lifespan) had any effect on apoptosis (Table S6). No effect was found at either concentration, which is consistent with mtROS being capable of regulating the CED-13-dependent activation of the pathway and not apoptosis. We then treated mutants of all four genes (*ced-13*, *ced-9gf*, *ced-4*, and *ced-3*) with 0.1 mM PQ. The effect of PQ on lifespan was almost completely suppressed by *ced-4* and *ced-9*, partially by *ced-3* but not at all by *ced-13* and *egl-1* (Figure 3). This suggested that PQ (and thus mtROS) act downstream of CED-13. As the *ced-13* mutation is capable of suppressing the lifespan of *isp-1* and *nuo-6* mutants, its inability to suppress the longevity induced by PQ suggests that the level of mtROS is insufficient in the *isp-1* and *nuo-6* mutants to trigger the pathway in the absence of stimulation by CED-13 but that the level of mtROS induced by PQ treatment is sufficient to directly activate the mitochondria-associated CED-9 and/or CED-4. Although *ced-4* does not suppress the longevity induced by RNAi against ETC subunits the position of CED-13 upstream of CED-4 and of mtROS could in principle allow it to regulate RNAi-dependent longevity through a parallel pathway. However, no suppression of *isp-1(RNAi)* by *ced-13* or *egl-1* was observed (Figure S5). All further analyses of the pathway described below were conducted with *ced-4* for part of the pathway downstream of ROS activity, with *ced-13* for the part of the pathway upstream of ROS activity, and with *egl-1* as control for apoptosis per se.

Loss of the Intrinsic Pathway Signaling Does Not Suppress Low Oxygen Consumption and ATP Levels

isp-1 and *nuo-6* encode subunits of mitochondrial respiratory complexes and the mutations lead to reduced oxygen consumption (Figure S6A and Table S5). This is likely a primary phenotype directly resulting from altered function of the electron transport chain. Lower electron transport chain function is expected to lead to ATP depletion. We found that ATP levels were low in both mutants and particularly severely in *isp-1* mutants (Figure S6B and Table S5). Neither oxygen consumption nor ATP levels were affected in *ced-4*, *ced-13*, or *egl-1*. To test whether suppression by the *ced* mutations was achieved by restoration of electron transport or ATP levels, we measured oxygen consumption and ATP levels in suppressed double mutants (Figures S6C and S6D and Table S5). No effect on oxygen consumption or ATP levels was observed, indicating that this is not the mechanism by which phenotypic suppression is achieved.

(E and F) Effect of *ced-13(sv32)* on the survival of *isp-1* and *nuo-6*.
(G) Effects of *ced-4* and *ced-13* on *isp-1* survival in the triple mutant combination.
(H) Effects of *ced-4* and *egl-1* on *isp-1* survival in the triple mutant combination.
(I) Effects of *ced-4* and *ced-13* on *nuo-6* survival in the triple mutant combination.
(J) Effects of *egl-1* and *ced-4* on *nuo-6* survival in the triple mutant combination.
See also Table S7; Figures S4 and S5. Numerical values and statistical analyses for all lifespan experiments are presented in Table S5.

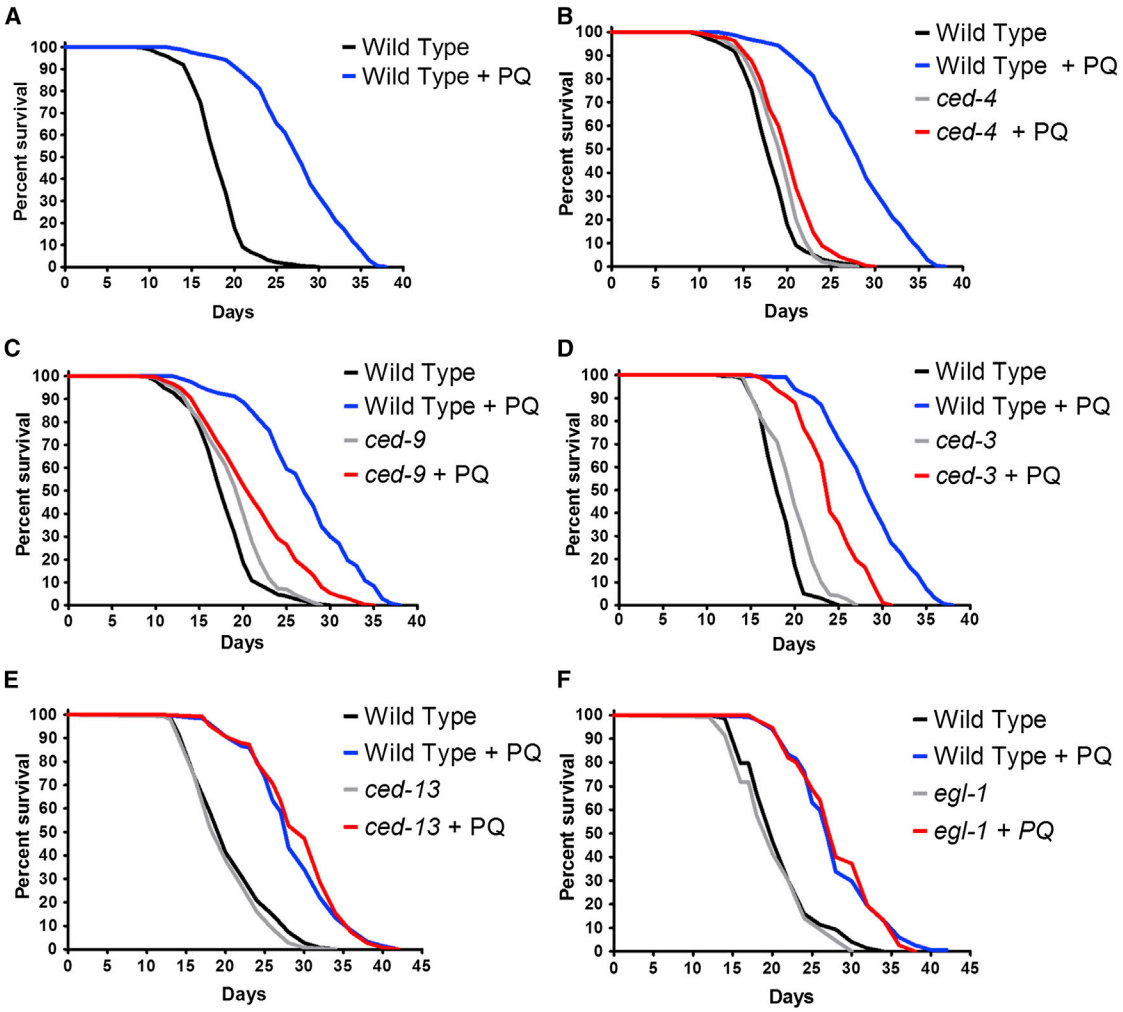

Figure 3. Lifespan Extension by 0.1 mM PQ Requires the Intrinsic Apoptosis Pathway

(A) Effect of 0.1 mM PQ treatment on the wild-type.

(B–F) Effects of 0.1 mM PQ treatment on: (B) *ced-4(n1162)*, (C) *ced-9(n1950 gf)*, (D) *ced-3(n717)*, (E) *ced-13(sv32)*, and (F) *egl-1(n1084n3082)*. See also Table S5. Numerical values and statistical analyses for all lifespan experiments are presented in Table S5.

Loss of the Intrinsic Pathway Suppresses the Hypometabolic and Gene Expression Phenotypes of *isp-1* and *nuo-6* Mutants

The *isp-1* and *nuo-6* mutations induce other phenotypes in addition to an increase in lifespan, including slow embryonic and postembryonic development, as well as slow behaviors such as pumping, defecation, and thrashing. Mutations in *ced-13* and *ced-4*, but not *egl-1*, partially suppressed all these phenotypes of both *isp-1* and *nuo-6*, (Figure 4 and Table S5). The fact that the *egl-1* mutation, which abolishes cell death but has no effect on lifespan, had no effect on any of the phenotypes implies, as for longevity, that these phenotypes do not depend on changes in apoptosis. One phenotype that is not rescued and is in fact worsened by *ced-4* is brood size (Figure 4D). This suggests that the germline phenotype due to mitochondrial dysfunction does not involve the longevity pathway we have uncovered. Recent findings suggest that apoptosis in the germline is necessary for oocyte quality (Andux and Ellis, 2008), which might be the cause of the reduction in brood size.

As described above, the *isp-1* and *nuo-6* mutations result in many changes in gene expression relative to wild-type. We determined whether *ced-4(n1162)*, which suppresses the increased lifespan of the mutants as well as most other phenotypes also suppressed the gene expression changes. Using Affymetrix *C. elegans* microarrays as before, we compared the changes in gene expression in *isp-1 ced-4* and *nuo-6; ced-4* double mutants relative to wild-type to those in the single mutants relative to wild-type (Table S1). The *ced-4* mutation partially suppressed both upregulated and downregulated changes in both *isp-1* and *nuo-6*. 57% of the genes upregulated in *nuo-6* were back to wild-type levels in *nuo-6;ced-4*, and 36% of the genes upregulated in *isp-1* were back to wild-type level in *isp-1 ced-4*. Similarly *ced-4* suppressed the

downregulation of 62% of the genes in the case of *nuo-6* but only 18% in the case of *isp-1*. GO-term analysis of the list of genes affected by *ced-4* in both mutants showed a meaningful enrichment only in the kinases and phosphatases linked to sperm production that were downregulated in the mutants (Table S2). However, as the low brood size of the mutants was not suppressed by *ced-4* the significance of this observation is unclear.

Constitutive Activation of the CED Pathway Leads to Heat-Stress Hypersensitivity

To further investigate the hypothesis that the CED pathway is a stress pathway that responds to mitochondrial dysfunction, we examined the effects of a severe heat stress. To establish the level of stress on mitochondrial function produced by this treatment we measured ATP levels after the animals had experienced 37°C for 1.5 hr. The stress led to a severe ATP depletion in all genotypes (Figure 5A and Table S5). However, the depletion was substantially more severe for the two mitochondrial mutants. While the wild-type, *ced-13*, *ced-4*, and *egl-1* experienced an ~30% drop, *isp-1* and *nuo-6* lost >50% of their already low ATP levels. Surprisingly, but consistent with our other findings, *ced-13* and *ced-4*, but not *egl-1*, suppressed the severity of this effect (Figure 5B and 5C, and Table S5). To explore this further, we treated young adult for 4 hr at 37°C and scored survival (Treinin et al., 2003). Treatment of all genotypes with this longer heat stress decreased survival, but much more severely in the mitochondrial mutants. Treatment of the wild-type, *ced-13*, *ced-4*, and *egl-1* resulted in ~80% survival but the treatment killed virtually all *isp-1* or *nuo-6* mutants (Figure 5D and Table S5). Again, *ced-13* or *ced-4*, but not *egl-1*, suppressed the mitochondrial mutants such that the double mutants had much higher survival rates (40%–50%). Taken together these observations suggest that resources required for acute survival are not available in animals in which the CED pathway is strongly and constitutively activated by mitochondrial dysfunction because they have been diverted to processes involved in long-term survival.

CED-13 Acts Upstream of mtROS for All Phenotypes

We focused on *isp-1* to explore further the epistatic relationships in the *ced-13*-dependent pathway. Previous observations indicated that the longevity effect of PQ is not additive to *isp-1* (Yang and Hekimi, 2010a), which we have confirmed (Table S5). On the other hand, the observation that *ced-13* does not suppress the longevity induced by PQ treatment (Figure 3), places its action upstream of that of mtROS. This suggests that PQ should suppress the suppressed longevity of *isp-1;ced-13* double mutants, which is what we observed (Figure 6A). Similarly, the slow defecation, pumping, and thrashing of *isp-1* mutants are partially suppressed by *ced-13* and by *ced-4* (Figures 4A–4C). If mtROS act downstream of *ced-13* but upstream of *ced-4*, PQ treatment should suppress the suppressive effect of *ced-13* but not that of *ced-4*, which is what we observed (Figures 6B–6D). Finally, *ced-13* and *ced-4* partially suppress the lethality induced by heat treatment (Figure 5D). Thus treatment with PQ should partially suppress the lethality suppression of *ced-13* but not that of *ced-4*, which is what we observed (Figure 6E).

SOD-3 Is Involved in Generating the Pro-Longevity mtROS Signal

Treatment with PQ and altered ETC function in the mitochondrial mutants are believed to generate superoxide (Yang and Hekimi, 2010a). However, only peroxide is believed to cross membranes readily, which might be necessary to affect the CED pathway proteins, which are associated with the outer mitochondrial membrane. The main mitochondrial superoxide dismutase SOD-2 is not required to generate the pro-longevity ROS signal, as PQ treatment can further lengthen the already long lifespan of *sod-2* mutants (Van Raamsdonk and Hekimi, 2009), which we have confirmed (Table S5). *sod-3*, which encodes a minor, inducible, mitochondrial superoxide dismutase very similar to SOD-2 in structure, was the only ROS handling enzyme whose expression was found to be increased by microarray analysis (Table S3). Interestingly, we found that *sod-3* was absolutely required for the pro-longevity signal induced by PQ treatment as this treatment was without effect on longevity in the *sod-3(tm760)* knockout background. (Figure 6F). This suggests that peroxide is the necessary intermediate for pro-longevity signaling. The specificity of the action of SOD-3 could be achieved by specific submitochondrial localization in relation to the outer membrane localization of CED-4/CED-9 complexes. Interestingly, both SODs have recently been found to be closely associated with the ETC (Suthammarak et al., 2013).

DISCUSSION

A Model for Lifespan Determination by Mitochondrial Dysfunction and mtROS Signaling

Previous studies have suggested that the mechanism of lifespan extension operating in the long-lived *isp-1* and *nuo-6* mutants is based on increased mtROS generation due to mitochondrial dysfunction. Here, we provide further evidence for this by showing that PQ treatment and the mutations induce a common pattern of changes in gene expression which is at least in part required for longevity. Most importantly, we show that the mtROS signal requires the activity of the intrinsic apoptosis signaling pathway (including CED-9, CED-4, and CED-3), activated by a dedicated BH3-only protein, CED-13 (Figure 7). However, the recruitment of this pathway by mtROS and the consequences on longevity are fully independent of apoptosis per se. The known association of CED-9 and CED-4 with mitochondria; their involvement in sensing mtROS in vertebrates; and our findings from epistasis analysis that PQ, and therefore ROS, acts immediately downstream of CED-13 suggest that this pathway is the most immediately affected by mtROS and likely functions upstream of other pathways that might also be engaged (Lee et al., 2010; Walter et al., 2011). We found that loss of CED-3 suppresses less efficiently than loss of CED-4, suggesting that CED-4 could have other effectors in addition to CED-3, which is consistent with the existence of CED-3-independent activities of CED-4. ROS-independent activation by CED-13 provides the opportunity for input from upstream signals to modulate the sensitivity of the pathway to mtROS. For example, *ced-13* expression appears to be regulated by *cep-1*, the *C. elegans* homolog of p53 (Schumacher et al., 2005). Furthermore, *cep-1* appears to affect lifespan modulation by

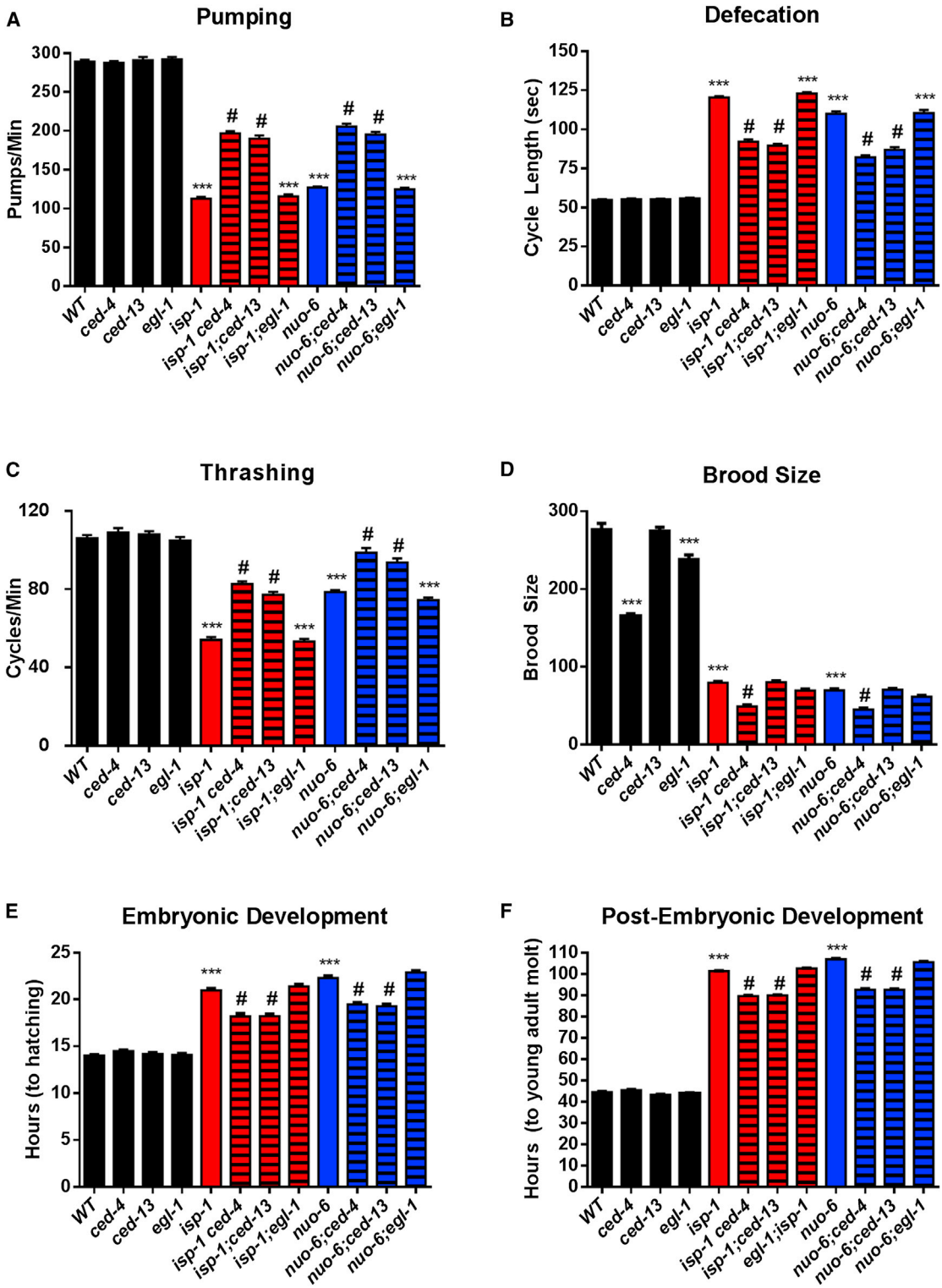

Figure 4. The Behavioral and Growth Defects of *isp-1(qm150)* and *nuo-6(qm200)* Mutants Are Partially Suppressed by *ced-4(n1162)* and *ced-13(sv32)* but Not *egl-1(n1084n3082)*

(A) Pharyngeal pumping rate. *isp-1* and *nuo-6* pump at a significantly slower rate than the wild-type. Loss of *ced-4* or *ced-13* but not *egl-1* partially rescues the slow pumping rates of *isp-1* and *nuo-6*. None of the cell death genes affects the pumping rate of the wild-type.

(B) Defecation cycle length. *isp-1* and *nuo-6* mutants have a significantly lengthened defecation cycle length. Loss of *ced-4* or *ced-13* but not *egl-1* partially rescues the slow defecation phenotype of *isp-1* and *nuo-6*. None of the cell death genes affect the defecation cycle length of the wild-type.

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mitochondrial dysfunction in a complex manner (Baruah et al., 2014; Ventura et al., 2009). Thus the action of CED-13 might have affinities with that of BH3-only proteins such as PUMA and NOXA, which are regulated by p53 (Nakano and Vousden, 2001; Oda et al., 2000).

Loss of the CED pathway cannot rescue the low oxygen consumption and the low ATP levels. This is expected as *isp-1* and *nuo-6* mutations are point mutations in subunits of the mitochondrial respiratory chain and the ATP and oxygen phenotypes are likely primary defects that cannot be fixed. However, the loss of the CED pathway abolishes a large part of the increased longevity and several other phenotypes of the mutants, such as slow growth and behavior, a large subset of the changes in gene expression, and the hypersensitivity to heat stress. Taken together this suggests that mtROS acts in the mutants through the CED pathway to trigger phenotypic changes that alleviate the consequences of the primary defects, including protective changes that ultimately result in increased lifespan. Interestingly, the slow growth and behavioral phenotypes are the type of effects expected from mitochondrial dysfunction and the resulting low ATP production. Even slow aging can be postulated to result from low energy production, based on the observation that cold temperature and low metabolic rates are associated with longer lifespans. Thus it appears that the mtROS/CED pathway amplifies phenotypes that would be produced to a lesser extent by the immediate effects of mitochondrial dysfunction on energy metabolism alone. This could be a protective mechanism that, in the wild-type, allows the mitochondria to recover their function when the dysfunction is only transient, sparing ATP and rerouting its use to protective mechanisms (Figure 7). In this model, longevity in the mutants is the result of both a slowing down of ATP-dependent processes that limit lifespan and by an abnormally intense activation of the protective pathway induced by elevated mtROS acting through the CED cascade. This model is consistent with the finding that, in contrast to the mutants that are only partially suppressed by *ced-4* and *ced-9gf*, the longevity induced by PQ, which does not affect oxygen consumption nor ATP levels (Yang and Hekimi, 2010a), can be almost completely suppressed by *ced-4* and *ced-9gf* (Figure 3).

Activation of the Apoptotic Pathway by mtROS

In vertebrates mtROS are involved in the regulation of apoptosis by the intrinsic mitochondrial pathway. However, no clear role for mtROS in *C. elegans* apoptosis has yet been discovered. We confirmed this by showing that neither mtROS-generating mitochondrial mutations nor PQ treatment affect the extent of somatic apoptosis (Table S5). Two biological roles for apoptosis have been proposed: a role in shaping the development of multicellular organisms by eliminating cells that are not needed, and a protective role by eliminating cells that are damaged. In the somatic lineage of worms, apoptosis appears to have a developmental role but in the germline it might have a protective role for fertility by eliminating damaged gamete precursors (Gartner et al., 2008) and reallocating resources to produce high-quality gametes (Andux and Ellis, 2008). In vertebrates, the mtROS-sensitive intrinsic pathway is part of a protective program and participates in the elimination of defective cells, including cells with defective mitochondria. Our findings suggest that in *C. elegans*, the intrinsic apoptotic machinery, including CED-9, CED-4, and CED-3, is also sensitive to mtROS when stimulated by the BH3-only protein CED-13. Stimulation by CED-13 leads to the activation of a protective program but not to apoptosis. How stimulation of the same pathway by CED-13 and EGL-1 results in different outcomes is unknown at the present time but likely involves cell type-specific differences. A program of protective apoptosis similar to that in vertebrates is probably not possible in *C. elegans* because of its very small number of postmitotic cells. Losing damaged cells is not an option without losing important functions and bodily integrity. However, stimulating protective and repair mechanisms in the face of injury remains useful. Thus it appears that what is conserved from nematode to vertebrates is the use of the proteins of the intrinsic pathway to transduce a mtROS signal that stimulates a protective response to mitochondrial dysfunction. It is interesting to speculate whether a nonapoptotic protective function of the intrinsic pathway is also acting in vertebrate postmitotic cells such as neurons and could have a role in protecting from neurodegeneration.

EXPERIMENTAL PROCEDURES

Strains and Genetics

All strains were maintained by standard methods, at 20oC, on solid agar (NGM plates), fed E. coli OP50, and grown continuously. The following genotypes were used: Bristol N2 (wild-type); LGI: *nuo-6(qm200)*, *sod-2(ok1030)*; LGII: *eat-2(ad1116)*; LGIII: *daf-2(e1370)*, *clk-1(qm30)*, *ced-4(n1162)*, *ced-9(n1950); glp-1 (e2141ts);* LGIV: *isp-1(qm150)*, *ced-3(n717)*; LGV: *egl-1(n1084n3082)*; LGX: *ced-13(sv32)*, *sod-3(tm783).*

(C) Thrashing rate. *isp-1* and *nuo-6* mutants have a significantly decreased rate of thrashing. Loss of *ced-4* or *ced-13* but not *egl-1* partially rescues the slow thrashing phenotype of *isp-1* and *nuo-6*. None of the cell death genes affect the thrashing rate of the wild-type.

(D) Brood size (the number of progeny produce by self-fertilization of a single hermaphrodite). Both *isp-1* and *nuo-6* have significantly reduced brood sizes. The reduction in brood size was enhanced by loss of *ced-4* but not *ced-13* or *egl-1*. Loss of *ced-4*, and to a lesser degree *egl-1*, also significantly reduced brood size the wild-type background.

(E) Length of embryonic development. The time taken for a 2-cell stage embryo to reach hatching is significantly increased in *isp-1* and *nuo-6* mutants. Loss of *ced-4* and *ced-13* but not *egl-1* partially rescues this phenotype of *isp-1* and *nuo-6*. None of the cell death genes affect the rate of embryonic development of the wild-type.

(F) Length of postembryonic development. The time taken for freshly hatched L1-stage larva to reach the young adult stage is significantly increased in *isp-1* and *nuo-6* mutants. Loss of *ced-4* and *ced-13* but not *egl-1* partially rescues this phenotype of *isp-1* and *nuo-6*. None of the cell death genes affect the rate of postembryonic development of the wild-type. Bars represent the mean value of 25 animals. Error bars represent standard error of the mean. Significance was determined using a Student's t test (*** denotes $p < 0.0001$ as compared to the wild-type; # denotes $p < 0.0001$ as compared to either *isp-1* or *nuo-6* single mutants).

Complete numerical values and statistics are provided in Table S5.

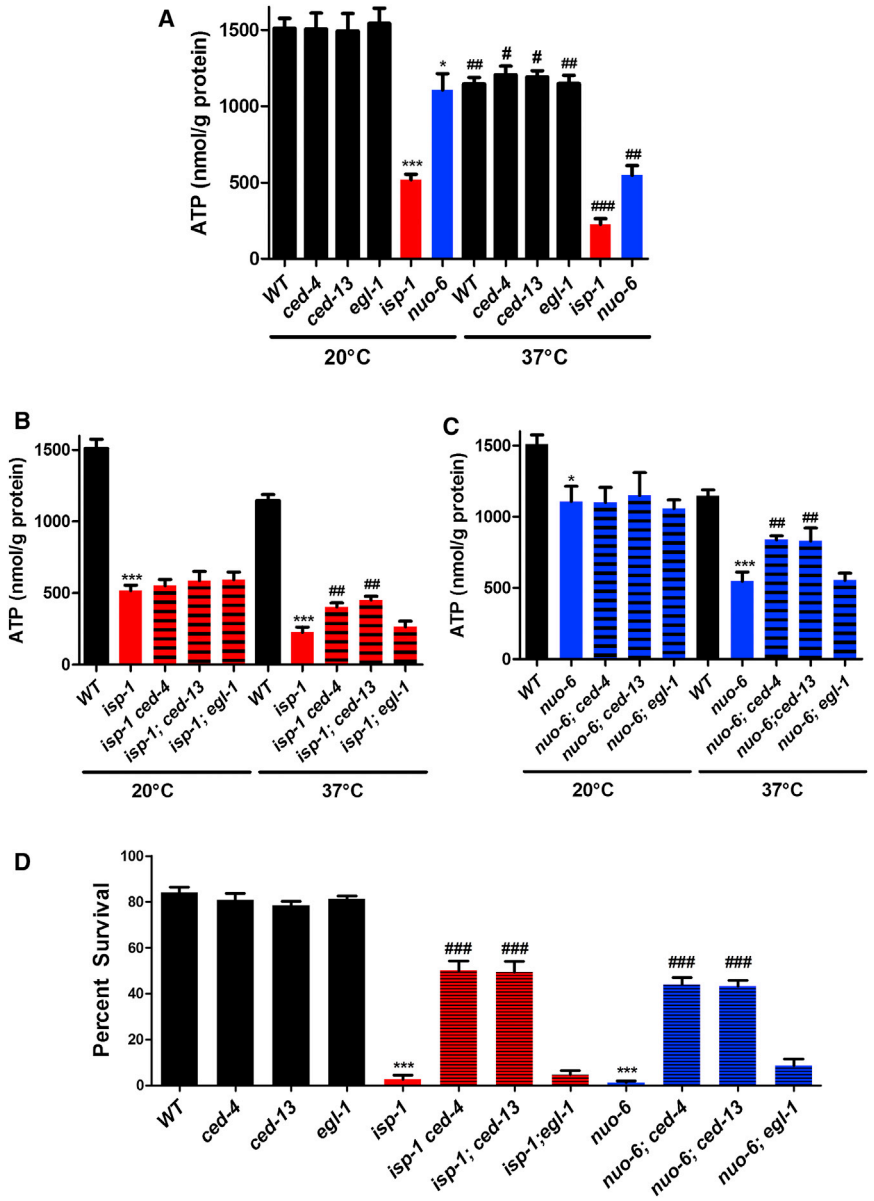

Figure 5. Effects of *isp-1*, *nuo-6* and Cell Death Genes on ATP Levels and Survival under Heat Stress

(A) *isp-1* and *nuo-6* mutants, but not *ced-4*, *ced-13* or *egl-1* mutants exhibit reduced ATP levels when grown under standard conditions (20°C). Acute exposure (1.5 hr) to heat (37°C) reduces the ATP levels of all genotypes.

(B) Loss of *ced-4*, *ced-13* or *egl-1* does not affect ATP levels in *isp-1* mutants at 20°C. However, the reduction in ATP levels after heat stress is significantly reduced in *ced-4 isp-1* and *isp-1;ced-13*, but not *egl-1;isp-1* double mutants compared to *isp-1(qm150)*.

(C) Mutations in *ced-4*, *ced-13* and *egl-1* do not affect ATP levels in *nuo-6* mutants at 20°C. However, the reduction in ATP levels after heat stress is significantly less in *ced-4;nuo-6* and *nuo-6;ced-13*, but not *egl-1;isp-1* double mutants compared to *nuo-6(qm200)*.

(D) Exposure to heat stress for 4 hr significantly decreases the survival of all genotypes, but much more severely for *isp-1(qm150)* and *nuo-6(qm200)* mutants. However, loss of *ced-4* or *ced-13* but not *egl-1* strongly rescues the survival of *isp-1* and *nuo-6* mutants.

Significance was determined using a Student's t test (A) * denotes $p < 0.05$, *** denotes $p < 0.0001$ as compared to the wild-type. # denotes $p < 0.05$ compared to the control at 20°C, ## denotes $p < 0.05$ compared to the wild-type at 37°C. ### denotes $p < 0.005$ compared to the wild-type at 37°C. (B) *** denotes $p < 0.0005$ relative to the wild-type control, ## denotes $p < 0.05$ relative to *isp-1(qm150)* at 37°C. (C) * denotes $p < 0.05$ as compared to the wild-type control at 20°C, *** denotes $p < 0.001$ as compared to the wild-type control at 37°C, ## denotes $p < 0.005$ as compared to the *nuo-6(qm200)* at 37°C. Error bars represent standard error of the mean. Complete numerical values and statistics are provided in Table S5.

Lifespan Analysis

All lifespan measurements were performed at 20°C and set up using a 4 hr limited lay. An experimental pool of 50 animals was used for each genotype in any given experiment, and lost or animals that died prematurely were replaced from a backup pool. Statistical analysis was performed using GraphPad Prism (v5.0) and Student's t tests in Microsoft Excel.

Paraquat Treatment

Paraquat (Sigma-Aldrich, St. Louis, USA) was added to NGM plates at a final concentration of 0.1 mM, 0.15 mM or 0.5 mM. OP50 grown on regular NGM plates was transferred onto NGM-PQ plates using a platinum pick instead of seeding directly onto the NGM-PQ plates. Control NGM plates containing no PQ were treated in a similar fashion.

Gene Expression Studies

A total of 2,000 synchronized young adults grown at 20°C on NGM plates were collected, frozen in liquid nitrogen, and total RNA was extracted using a QIAGEN RNeasy Tissue Microarray Mini kit. Total RNA samples were analyzed for concentration and dissolution spectrophotometrically using a Nanodrop ND-100 Spectrophotometer. RNA samples were processed by Génome Québec (Montreal) and hybridized onto Affymetrix *C. elegans* GeneChips. Raw expression data were analyzed using FlexArray v1.6.1 (Génome Québec) and normalized using the GC-RMA method. Comparisons of each genotype were compared to the wild-type using the Empirical Base (Wright & Simon) algorithm and fold changes were represented on a $\log_2$ scale. A threshold of $p < 0.05$ and a fold change of 1.3 ($\log_2$) was set to determine differentially expressed targets.

Comparisons of Gene Expression Patterns

Comparisons made to other published data sets were done using raw Affymetrix data sets wherever possible (obtained from NCBI Gene Expression Omnibus [GEO] (http://www.ncbi.nlm.nih.gov/geo/). Raw data were imported to FlexArray and handled identically to the data that was generated in this study. For studies that did not deposit their data to GEO or used technologies other than Affymetrix, comparisons of gene lists (upregulated and downregulated transcripts) were conducted using Microsoft Excel.

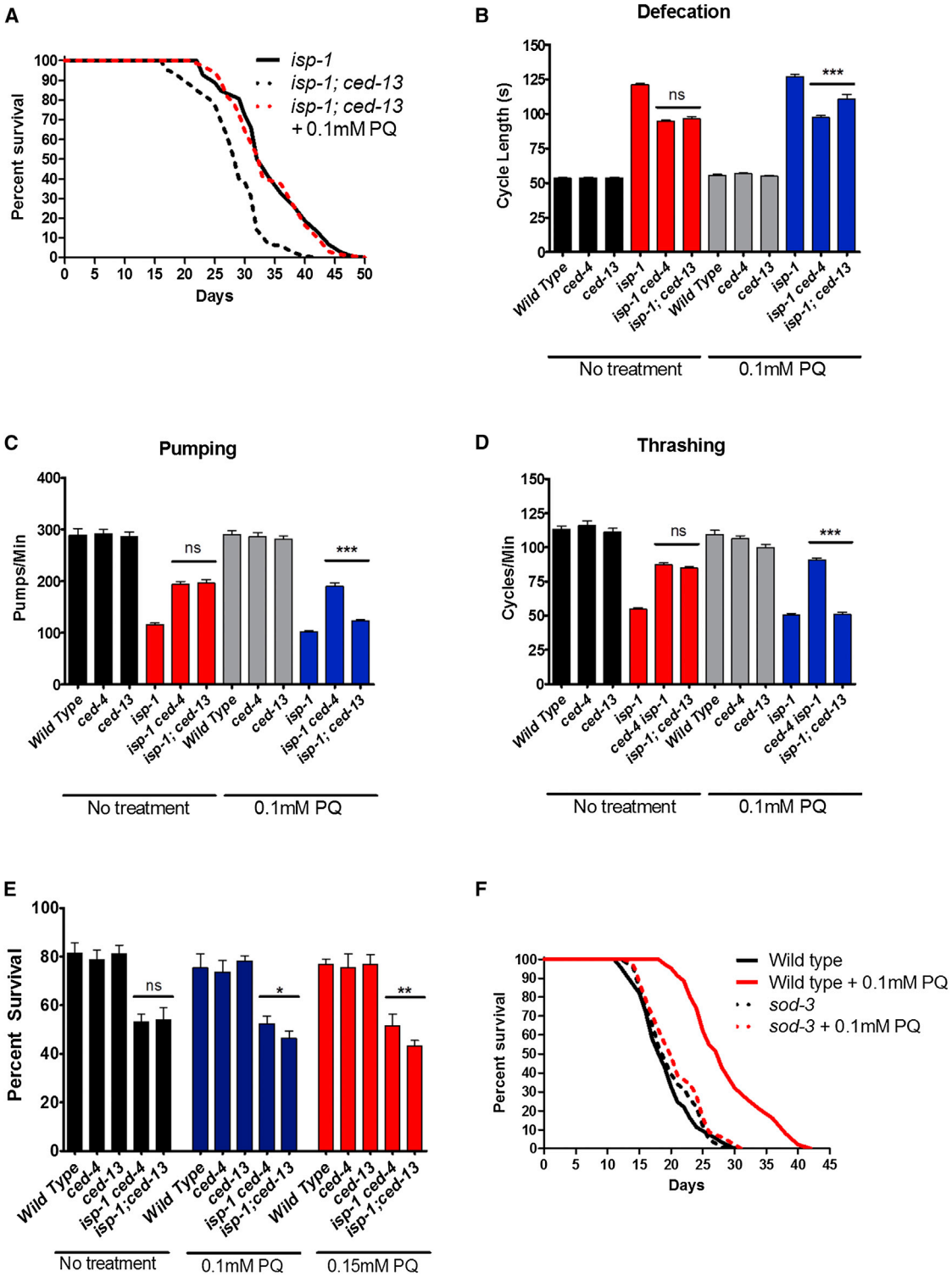

Figure 6. Epistatic Relationships between Genotypes and Treatments

(A) Treatment of *isp-1;ced-13* with 0.1 mM PQ rescues lifespan to the *isp-1* level (n > 50, p < 0.0001 for the difference between treated and untreated double mutants).

(B) Treatment with 0.1 mM PQ does not affect the defecation of *isp-1 ced-4* but partially restores the defecation of *isp-1;ced-13* toward the *isp-1* level (n = 25).

(C) Treatment with 0.1 mM PQ does not affect the pumping rate of *isp-1 ced-4* but partially restores *isp-1;ced-13* pumping toward the *isp-1* level (n = 10).

(D) Treatment with 0.1 mM PQ does not affect the trashing rate of *isp-1 ced-4* but partially restores *isp-1;ced-13* thrashing toward the *isp-1* level (n = 15).

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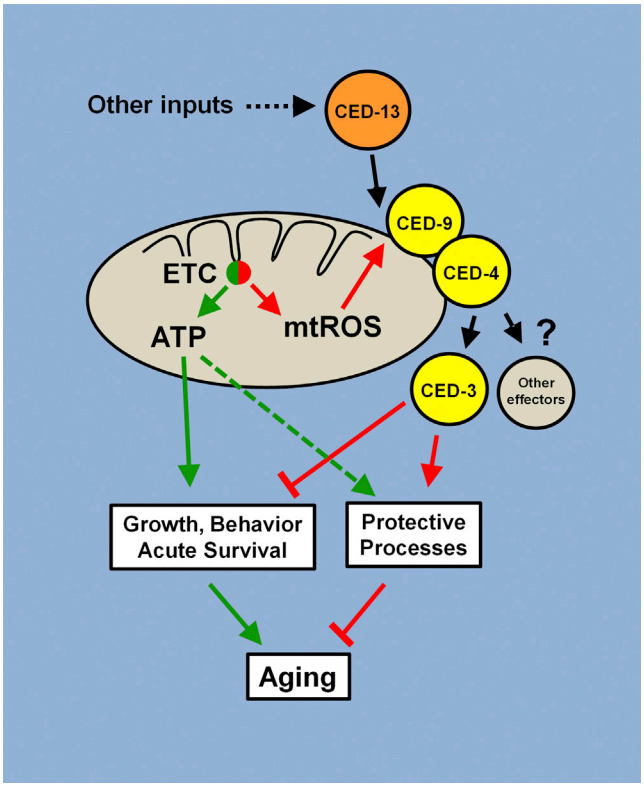

Figure 7. A Model for the Regulation of Lifespan by mtROS Signaling through the Intrinsic Apoptosis Pathway

The intrinsic apoptosis pathway (composed of CED-9, CED-4, and CED-3) is sensitive to mtROS from the ETC when it is activated by the alternative BH3-only protein CED-13. Mitochondrial dysfunction leads to an increase in mtROS which activates the CED signaling pathway to reduce ATP usage and redistribute it to protective rather than active functions. We propose that the mitochondrial dysfunction in *isp-1(qm150)* and *nuo-6(qm200)* mutants induces the mutant phenotypes, including longevity, both by directly lowering ATP generation and by stimulating mtROS signaling to alter ATP usage. In the wild-type this mechanism could provide a protective role in case of transient mitochondrial dysfunction or nutrient shortage. In the mutants its continuous action leads to the mutant phenotypes, including longevity.

Gene Ontology Term Analysis

GO-term analysis was performed using Cytoscape (v2.8.3) and the BiNGO plugin (v2.44). A hypergeometric test using the Benjamini & Hochberg false discovery rate (FDR) correction was implemented at a significance level of 0.05.

Measurement of Apoptosis

Quantification of corpses or cells was performed as previously described (Lu et al., 2009; Schwartz, 2007).

Whole-Worm Phenotypes

All phenotypes were measured as before (Yang and Hekimi, 2010b).

Oxygen Consumption

Mixed populations of worms were collected and washed 3× in M9 to a final volume of 50 μL of packed worms. A total of 25 μL of worms were then resuspended to a final volume of 50 μL using M9 buffer and loaded into a chamber of an Oroboros Oxygraph-2K. The remaining 25 μL of worms were freeze-thawed 3× in liquid nitrogen and resuspended in lysis buffer for immediate determination of protein concentration by a BCA Protein Assay kit (Thermo Scientific, Rockford, USA).

ATP Measurements

Young adult populations were collected using a 4 hr limited lay. Worms were picked and washed three times in M9. Worm pellets were subjected to 3 cycles of freeze-thaw using liquid nitrogen and subsequently spun down for 15 min at top speed. The resulting supernatant was assayed using an ATP Determination kit (Life Technologies, Carlsbad, USA). Protein concentrations were determined as described above.

Heat Stress Assays

Young adults were picked onto NGM plates that were preheated to 37°C and incubated for 4 hr at 37°C. Animals were allowed to recover for 30 min and scored for viability. For ATP measurements after heat stress, mixed populations were transferred onto preheated NGM plates and incubated for 1.5 hr at 37°C. Animals were then collected and washed three times with M9 and flash frozen and stored in liquid nitrogen. ATP measurements were performed as described above. For experiments performed using paraquat, paraquat plates were made as described and worms were grown on paraquat for one generation. Young animals that were grown on paraquat were subsequently assayed on preheated paraquat plates.

ACCESSION NUMBERS

The GEO ascension number for all gene array data in this paper is GSE54024.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.02.055.

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(E) Treatment with 0.1 mM and 0.15 mM PQ decreases the acute survival of *isp-1;ced-13* worms but not of *isp-1 ced-4* at 37°C (for 4 hr). Significance for all experiments was determined using the Student's t test (* denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.
(F) Treatment with 0.1 mM PQ increases wild-type lifespan but not *sod-3(tm783)* lifespan ($n = 150$, $p < 0.0001$ for the difference between the wild-type and *sod-3* treated with PQ). Error bars represent mean + SEM. See also Figure S6. Complete numerical values and statistics are provided in Table S5.

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