Increased mitochondrial fusion allows the survival of older animals in diverse *C. elegans* longevity pathways

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Mitochondria are dynamic organelles that undergo fusion and fission events. Mitochondrial dynamics are required for mitochondrial viability and for responses to changes in bioenergetic status. Here we describe an insulin-signaling and SCF LIN-23-regulated pathway that controls mitochondrial fusion in *Caenorhabditis elegans* by repressing the expression of the mitochondrial proteases SPG-7 and PPGN-1. This pathway is required for mitochondrial fusion in response to physical exertion, and for the associated extension in lifespan. We show that diverse longevity pathways exhibit increased levels of elongated mitochondria. The increased mitochondrial fusion is essential for longevity in the diverse longevity pathways, as inhibiting mitochondrial fusion reduces their lifespans to wild-type levels. Our results suggest that increased mitochondrial fusion is not a major driver of longevity, but rather is essential to allow the survival of older animals beyond their normal lifespan in diverse longevity pathways.
Mitochondria play diverse roles in signaling, physiology, and metabolism\(^1\). Mitochondrial dynamics regulate the morphology, number, and function of mitochondria to allow adaptation to cellular needs\(^1\). Mitochondrial fission is required for: mitophagy; the mitotic segregation of mitochondria to daughter cells; and the distribution of mitochondria to subcellular locations, such as neuronal axons\(^2\). Mitochondrial fusion is required for maintaining mitochondrial membrane potential and respiratory capacity, and to protect against apoptosis in mammalian cells\(^3\).

Cells adjust mitochondrial morphologies to coordinate between the cellular demand for energy and the availability of resources\(^3\). Elongated morphology is associated with increased efficiency of ATP production and reduced generation of reactive oxygen species (ROS), while fragmented morphology is linked to reduced ATP production and mitochondrial uncoupling.

Mitochondrial fusion and fission events are tightly regulated and require the activity of evolutionarily conserved GTPases\(^4\).

Mitochondrial fission in yeast, invertebrates, and mammals requires the dynamin-related protein DNML1 (Dnm1 in budding yeast and DRP-1 in \textit{C. elegans}). Mitochondrial fission requires mitofusins for outer membrane fusion (MFN1 and MFN2 in mammals, Fzo1 in budding yeast, and FZO-1 in \textit{C. elegans}), and inner membrane fusion (OPA1 in mammals, Mgm1 in budding yeast, and EAT-3 in \textit{C. elegans})\(^4-6\). In mammalian cells, overexpression of the outer membrane fusion proteins MFN1 and MFN2 can lead to either clustering of spherical mitochondria\(^7\) or elongated mitochondria\(^8\). It is likely that the different outcomes result from differences in the level of expression, as it was shown that modest overexpression of the inner membrane fusion protein OPA1 induces mitochondrial fission, while higher levels of expression induce fragmentation\(^9\). In \textit{C. elegans}, the overexpression of either the outer membrane mitofusin FZO-1 or the inner membrane fusion EAT-3 was reported to induce mitochondrial fragmentation\(^6\), however, in light of the

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**Fig. 1** CAND-1/SCFLIN-23 regulates mitochondrial shape via \textit{spg-7} and \textit{ppgn-1}. \textbf{a} Real-time PCR quantification of \textit{spg-7} mRNA levels in adults of the indicated genotypes, presented in arbitrary units (a.u.) normalized to control \textit{rpl-19} (ribosomal protein L19) mRNA. \textit{Error bars} denote s.e.m. from four biological replicates, each with at least two technical replicates. \textit{P} values were determined by Student’s \textit{t}-test. \textbf{b} Images of tubular, elongated, and fragmented mitochondria visualized by mitochondria-targeted GFP expressed in body-wall muscle cells. \textit{Scale bar}, 10 μm. \textbf{c} The percentages of muscle cells with tubular, elongated, and fragmented mitochondria for the indicated genotypes and RNAi treatments. All animals were fed either gene-specific RNAi or control RNAi so that the feeding conditions were matched. \textit{P} values were determined by \textit{χ}²-test. Sample size (\(n\)) of muscle cells from left to right are: 729; 126; 210; 124; 210; 201; 234; 274; 209; 297; 318; 457. Mitochondrial morphology was scored blinded. \textbf{d} Real-time PCR quantification of \textit{ppgn-1} mRNA levels normalized to control \textit{rpl-19} mRNA in adults of the indicated genotypes. \textit{Error bars} denote s.e.m. from three biological replicates, each with at least two technical replicates. \textit{P} values determined by Student’s \textit{t}-test. For all panels, asterisks above \textit{bars} denote \textit{P} value comparisons to wild-type/controls; asterisks above \textit{lines} denote comparisons under the lines: *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\); ns = not significant.
mammalian results, the failure to generate elongated mitochondria could have resulted from excessive expression levels.

Mitochondrial dysfunction, impaired energy homeostasis, and increased production of ROS are associated with aging in both invertebrates and vertebrates. In large-scale *C. elegans* RNA interference (RNAi) screens for genes regulating lifespan, one of the largest classes of genes were those encoding mitochondrial proteins affecting the electron transport chain (ETC). The RNAi depletion of which can either shorten or extend lifespan. Lifespan extension in response to ETC impairment occurs, at least in part, from the activation of the mitochondrial unfolded protein response (UPR(m))14. In this study, we present evidence that increased mitochondrial elongation, which occurs independently of UPR(m), allows the survival of older animals in diverse longevity pathways.

Insulin/IGF-1 signaling (IIS) is an evolutionarily conserved pathway that controls lifespan. In *C. elegans*, IIS reduces lifespan predominantly by inhibiting the activity of the FOXO transcription factor DAF-16 via inhibitory phosphorylation that blocks its nuclear localization. In the absence of IIS activity, DAF-16 enters the nucleus and regulates gene expression to extend lifespan.

In this study, we describe a pathway for the control of mitochondrial fusion in *C. elegans* that is regulated by IIS and a cullin-RING ubiquitin ligase (CRL). CRLs are multisubunit ubiquitin ligases (E3s) that ubiquitylate substrate proteins to induce proteasome-mediated degradation or post-translational regulation. CRLs include a cullin protein as a scaffold, a RING finger protein, which binds the ubiquitin-conjugating enzyme, a substrate receptor that binds the substrate, and (generally) an adaptor that links the substrate receptor to the core complex. Substrate receptors are variable components, and core CRL complexes function with multiple substrate receptors. The binding of a different substrate receptor to the core CRL complex changes the substrates that are targeted and the cellular function. The most widely studied class of CRLs contains the cullin CUL1 and is designated SCF for its core components: the Skp1 adaptor; CUL1; and F-box protein substrate receptors.

The CAND1 protein functions as an exchange factor for CRL substrate receptors. CAND1 affects the steady-state levels of different substrate receptors with the core CRL components. In diverse organisms, the loss of CAND1 selectively affects a subset of CRLs, suggesting that certain substrate receptors are particularly reliant on CAND1 activity.

Here, we describe a *C. elegans* mitochondrial fusion pathway that is regulated by CAND-1, the E3 SCF-CUL1, and IIS. We show that this pathway is responsible for an increase in elongated mitochondria in response to physical exercise. Additionally, we show that increased levels of elongated mitochondria are associated with diverse lifespan extension pathways, and that the increase in mitochondrial elongation is required to allow the survival of older animals during extended lifespans.

**Results**

The mitochondrial protease *spg-7* is a *cand-1* suppressor. Inactivation of *C. elegans cand-1* results in developmental and morphological defects, including impenetrant embryonic and larval arrest, developmental delays, altered cell divisions, and morphological defects. To identify CAND-1 molecular pathways, we isolated a genetic suppressor mutation, *ek25*, that suppresses multiple *cand-1* (tm1683) loss-of-function phenotypes (Supplementary Table 1). *ek25* was identified as an insertion mutation in an intron of the *spg-7* gene and the 3′ untranslated repeat (UTR) of the *Y47G6A.15* gene (Supplementary Figs. 1 and 2). *Y47G6A.15* is not conserved even in closely related *Caenorhabditis* species. *spg-7* is the ortholog of the mammalian mitochondrial m-AAA protease AFG3L2. RNAi depletion of *spg-7* in *cand-1* mutants enhanced *cand-1* mutant phenotypes, and aborted suppression in *cand-1*; *spg-7(ek25)* animals (Supplementary Table 1). In contrast, RNAi inactivation of *Y47G6A.15* had no obvious effects (Supplementary Table 1).

CAND-1 positively regulates *spg-7* mRNA levels. *cand-1* mutants have lower levels of *spg-7* mRNA than wild type, and the *spg-7(ek25)* mutant allele rescues the decrease in *spg-7* expression in *cand-1* mutants (Fig. 1a and Supplementary Fig. 3). These results suggest that *spg-7(ek25)* acts as a gain-of-function mutation that suppresses *cand-1* mutant phenotypes by restoring *spg-7* expression.

**CAND-1 and LIN-23 regulate mitochondrial morphology.** Since the *spg-7(ek25)* suppressor regulates the expression of a mitochondrial m-AAA protease, we wanted to determine whether *cand-1* mutants have a mitochondrial phenotype. To characterize mitochondrial morphology, we used a transgenic strain expressing mitochondria-targeted green fluorescent protein (GFP) in muscle cells. Morphological morphology was scored blinded, and the scoring correlates with a quantitative assessment of mitochondrial area (Supplementary Fig. 4). The majority of wild-type hermaphrodite body-wall muscle cells have longitudinally arrayed tubular mitochondria (Fig. 1b, c). Smaller percentages of muscle cells exhibit elongated mitochondria in an interconnected mesh-like network, or fragmented mitochondria (Fig. 1b, c). Inactivation of *cand-1* significantly increases the percentage of cells exhibiting elongated mitochondria; moreover, this phenotype is rescued by the *spg-7(ek25)* suppressor mutation in *cand-1*, *spg-7(ek25)* animals (Fig. 1c and Supplementary Fig. 5). Inactivation of the COP9/Signalosome component CSN-3, which, like CAND-1, is required for CRL function, also increased the proportion of elongated mitochondria (Fig. 1c and Supplementary Fig. 5). This suggests that the increased mitochondrial elongation phenotype in *cand-1* mutants arises from loss of CRL activity. The *E3 SCF-CUL1*, which contains the substrate receptor LIN-23, is particularly reliant on CAND-1 for activity. We found that inactivation of *lin-23* similarly reduces *spg-7* expression and has increased levels of elongated mitochondria (Fig. 1a, c and Supplementary Fig. 5).

**CAND-1 and SCF-CUL1 regulate mitochondria through DAF-16.** The *spg-7(ad2249)* loss-of-function and *spg-7(tm2312)* deletion (Δ) mutants exhibit mitochondrial elongation, but the proportion of mitochondria with elongated morphology is not as large as that observed in *cand-1(RNAi)* or *lin-23(RNAi)* animals (Fig. 1c and Supplementary Fig. 5). Mammalian m-AAA proteases form hexameric complexes in the inner mitochondrial membrane that exist as hetero-oligomeric complexes of AFG3L2 and SPG7, or homo-oligomeric complexes of AFG3L2. We considered whether the partial mitochondrial fusion phenotype of *spg-7* AFG3L2 mutants could arise from functional redundancy between *SPG-7* and PPPG-1, the *C. elegans* ortholog of mammalian SPG7.

We found that inactivation of *ppg-1* alone increased mitochondrial fusion, while inactivating both *ppg-1* and *spg-7* further increased the level of elongated mitochondria, implying that both m-AAA proteases negatively regulate mitochondrial elongation (Fig. 1c). CAND-1 and LIN-23 also promote *ppg-1* expression (Fig. 1d), suggesting that the increase in mitochondrial elongation in *cand-1* and *lin-23* mutants arises from the failure to adequately express both *spg-7* and *ppg-1*.

To determine how the *spg-7(ek25)* mutation increases *spg-7* mRNA levels, we looked for transcription factor-binding sites...
Fig. 2 CAND-1/SCF^LIN-23^ activates AKT-1 to inhibit DAF-16 and reduce EAT-3 levels. a DAF-16::GFP nuclear localization in body-wall muscle cells for animals with the indicated RNAi treatments. White arrows indicate nuclei in the control RNAi image. Scale bar, 10 μm. b Quantification of the mean level of nuclear and cytoplasmic DAF-16::GFP intensity in body-wall muscle cells. Error bars denote s.e.m. P values were determined by Student’s t-test. Sample size (n) of body-wall muscle cells from left to right are: 44; 61; 102; 87. c Western blot with anti-GFP of AKT-1::GFP from whole-animal lysate of L4/young-adult-stage animals treated with the indicated RNAi showing the altered mobility of AKT-1::GFP on SDS-PAGE; anti-histone H4 staining is used as a loading control. Similar results were obtained in two to five biological replicates. d Western blot showing staining for an antibody that recognizes AKT phospho-substrates in animals expressing AKT-1::GFP and subjected to the indicated RNAi treatments. Significant decreases in AKT phospho-substrates were observed for cand-1, cul-1, and lin-23 RNAi conditions in three to five biological replicates, with pdk-1 RNAi shown as a control that is known to reduce AKT-1 activity. The identities of the AKT-1 phospho-substrate proteins observed in the western blot are not known. e Graph showing the levels of AKT phospho-substrate signal relative to control protein bands (anti-tubulin or anti-histone H4) for three to five biological replicates. The control RNAi is set to 100 in arbitrary units. Error bars denote s.e.m. P values were determined by Student’s t-test. f Western blot showing EAT-3 protein levels for the indicated genotypes and RNAi treatments. The expected molecular weights of the L-isoform and S-isoform of EAT-3 are 110.1 and 85.5–86.4 kDa, respectively. g Quantification of EAT-3 levels normalized to α-tubulin. Error bars denote s.e.m. from two to four biological replicates. For all panels, asterisks above bars denote P value comparisons to wild type/controls; asterisks above lines denote comparisons under the lines: *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant.
that are affected by the spg-7(ek25) mutation. The spg-7(ek25) mutation disrupts a consensus DAF-16-binding element (DBE) within a region confirmed for DAF-16 binding by chromatin immunoprecipitation sequencing (ChIP-seq) (Supplementary Fig. 2). DAF-16 was also shown to bind to the pgpn-1 regulatory region. A meta-analysis of DAF-16-responsive genes suggests that DAF-16 can repress gene expression through DBE sites (Methods). daf-16(mu86) deletion mutants have elevated levels of spg-7 and pgpn-1 mRNA, suggesting that DAF-16 is a transcriptional repressor of both spg-7 and pgpn-1 (Fig. 1a, d). Unexpectedly, the reduction of pgpn-1 mRNA levels observed in cand-1 mutants was also rescued by the spg-7(ek25) suppressor allele. The mechanism for this effect is not known but could involve feedback loop(s) or the co-regulation of spg-7 and pgpn-1 expression.

We wanted to determine whether CAND-1 and SCF LIN-23 promote spg-7 and pgpn-1 expression by inhibiting DAF-16 activity. One of the primary mechanisms to control DAF-16 activity is by regulating its nuclear localization. We observed that inactivation of cand-1, cul-1, and lin-23 significantly increased DAF-16::GFP nuclear localization, suggesting that CAND-1 and SCF LIN-23 normally act to inhibit DAF-16 nuclear localization (Fig. 2a, b). To determine whether the CAND-1-mediated inhibition of mitochondrial fusion is dependent on DAF-16 activity, we combined daf-16 and cand-1 loss-of-function mutations. The addition of the daf-16 mutation rescued the cand-1-elongated mitochondria phenotype, suggesting that CAND-1 inhibits mitochondrial elongation by negatively regulating DAF-16 activity (Fig. 1c).

To determine how SCF LIN-23 inhibits DAF-16 nuclear localization, we analyzed the kinase AKT-1, which phosphorylates DAF-16 to prevent its nuclear localization. Inactivating cand-1 or the SCF LIN-23 components cul-1 or lin-23 leads to the accumulation of a faster-migrating form of AKT-1::GFP without affecting its overall levels (Fig. 2c and Supplementary Fig. 6). AKT-1 is activated by PDK-1 phosphorylation. RNAi depletion of pdk-1, or the inhibition of the IIS pathway upstream of PDK-1 and AKT-1, results in the accumulation of a faster-migrating form of AKT-1::GFP that is consistent with the unphosphorylated, inactive form (Fig. 2c). RNAi does not produce a further shift in the migration of AKT-1::GFP when combined with lin-23 RNAi (Fig. 2c). This suggests that the lower molecular weight form of AKT-1::GFP in lin-23(RNAi) animals reflects the loss of PDK-1-dependent activating phosphorylation.
experiment (Fig. 2c). These results suggest that SCF \textsuperscript{LIN-23} increases AKT-1 activity by promoting its activating phosphorylation.

**SPG-7 and PPGN-1 regulate mitochondrial fusion protein EAT-3.** In yeast and mammals, the inner mitochondrial fusion protein, Mgm1 or OPA1, respectively, is cleaved into long (L) and short (S) isoforms, both of which are required for mitochondrial fusion\textsuperscript{29, 30}. In mammals, there are two cleavage sites, S1 and S2. S1 is cleaved by the protease OMA1, and S2 is cleaved by the protease YME1L1\textsuperscript{29}. \textit{C. elegans} lacks an OMA1 homolog, and the corresponding S1 cleavage site is not conserved in EAT-3/OPA1. In contrast, \textit{C. elegans} has a YME1L1 ortholog, \textit{ymel-1}, and its target S2 site is conserved in EAT-3. The expected sizes of EAT-3 are consistent with an L-isoform (after removal of the mitochondrial targeting sequence) and an S-isoform cleaved at the conserved S2 site (Fig. 2f).

Mammalian m-AAA components AFG3L2 and SPG7 can cleave OPA1 when overexpressed or expressed ectopically in yeast\textsuperscript{31, 32}. We tested whether inactivating \textit{C. elegans} spg-7 and ppgn-1 affects EAT-3 levels. Co-inactivation of spg-7 and ppgn-1 significantly increased the overall level of EAT-3, indicating that both m-AAA proteases negatively regulate EAT-3 levels (Fig. 2f, g). cand-1 mutants, which have reduced expression of spg-7 and ppgn-1, have higher levels of EAT-3 protein compared to wild type (Fig. 2f, g). Significantly, the cand-1; spg-7(ek25) strain, which restores expression of spg-7 and ppgn-1 mRNA, does not have elevated EAT-3 levels (Fig. 2f, g). The levels of eat-3 mRNA remain unchanged in the above conditions, indicating that the negative regulation of EAT-3 protein is post transcriptional (Supplementary Fig. 7). It is not known whether the negative regulation of EAT-3 protein is direct or indirect. EAT-3 appears to function downstream of CAND-1, LIN-23, SPG-7, and PPGN-1, as the eat-3(RNAi) mitochondrial fragmentation phenotype is epistatic to the mitochondrial fusion associated with inactivation of these genes (Fig. 3a, b).

The levels of FZO-1::GFP (outer mitochondrial membrane fusion protein) and DRP-1::GFP (inner mitochondrial fission protein) were not affected by inactivation of the mitochondrial

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**Fig. 4** Model for regulation of mitochondrial fusion. **a** Proposed linear pathway for CAND-1 and SCF\textsuperscript{LIN-23} regulation of mitochondrial fusion; see text for description. **b, c** Schematic of the proposed intracellular pathway regulating mitochondrial fusion and lifespan extension in the presence **b** or absence **c** of CAND-1.
Regulation of mitochondrial fusion in physical exertion. Acute physical exertion in mice induces mitochondrial fusion in skeletal muscle cells, presumably to increase the efficiency of ATP production. We wanted to determine whether physical exertion similarly increases mitochondrial fusion in C. elegans, and whether the fusion is DAF-16-dependent. To induce physical exertion, we utilized swimming behavior. Animals were kept in a state of constant swimming by gently rocking them in M9 buffer solution that contained OP50 bacteria. Swimming, which manifests as an intense thrashing motion (Supplementary Movies 1 and 2), appears to be vigorous exercise based on the observation that ATP levels decrease during swimming (Fig. 5a). Wild-type animals and daf-16(mu86) mutants expressing mitochondria-targeted GFP swam vigorously at an equivalent swimming rate (Fig. 5b and Supplementary Movies 1–3). Wild-type animals showed an increase in elongated mitochondria that reached an elevated plateau at 60 min that persisted through the remainder of the 5-h test period (Fig. 5c and Supplementary Fig. 9a). Significantly, daf-16 mutants did not exhibit increased levels of elongated mitochondria, implying that DAF-16 is required for this physiological response (Fig. 5c and Supplementary Fig. 9b). Consistently, the nuclear localization of DAF-16::GFP increased in response to swimming in wild-type animals (Fig. 5d and Supplementary Fig. 10). Notably, cand-1; spg-7(ek25) mutants, which have stabilized spg-7 and ppgn-1 expression, exhibited a lack of mitochondrial fusion in response to physical exertion despite a slightly faster swim rate (Fig. 5b, c and Supplementary Fig. 9c). Similarly, we did not observe an increase in mitochondrial fusion in spg-7(ek25) mutants, which also swam at a rate equivalent to wild-type animals (Supplementary Fig. 11). This suggests that the DAF-16-mediated fusion pathway genes (Supplementary Fig. 8a, b). We also did not observe a major change in the levels of mitochondria-targeted GFP upon RNAi depletions of the mitochondrial fusion pathway genes (Supplementary Fig. 8c). This suggests that the mitochondrial fusion pathway does not significantly affect the level of mitophagy, as changes in mitophagy affect the level of mitochondria-targeted GFP.

Our data support the model shown in Fig. 4, in which CAND-1 activates SCF\(^\text{LIN}^{23}\), and SCF\(^\text{LIN}^{23}\) activates AKT-1, thereby reducing DAF-16-mediated transcriptional repression of spg-7 and ppgn-1. SPG-7 and PPGN-1 inhibit mitochondrial fusion, at least in part, by negatively regulating the level of EAT-3.

Fig. 5 DAF-16 is required for physical exertion-induced mitochondrial fusion. a ATP levels decrease during swimming. Graph of ATP levels (normalized to whole-animal protein levels) at the indicated times of continuous swimming. Error bars denote s.e.m. from two biological replicates, each with three technical replicates. P values were determined by Student’s t-test. b Average swim strokes per minute of 12 animals each for the indicated genotypes upon induction of swimming behavior. Error bars denote s.e.m. P values determined by Student’s t-test. c The percentages of muscle cells with elongated mitochondria in wild type, daf-16(mu86), and cand-1(tm1683); spg-7(ek25) animals for the indicated times post induction of swimming behavior. Full distributions of mitochondrial morphology and sample size (n) are shown in Supplementary Fig. 9. d DAF-16::GFP nuclear localization in body-wall muscle cells for animals at 0 and 300 min post induction of swimming behavior. White arrows indicate nuclei in the 0 min image. Scale bar, 10 µm. For all panels, asterisks above bars denote P value comparisons to wild type/controls; asterisks above lines denote comparisons under the lines: *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant.
**Fig. 6** Increased mitochondrial elongation extends lifespans. **a–e** Survival curves for adults of the indicated RNAi treatments. The wild-type survival curves for **a–c** were analyzed at the same time and are shown in each panel for comparison. RNAi depletions of *lin-23 a, cand-1 a, ppgn-1 e, and spg-7 + ppgn-1 d* significantly increased mean lifespan. **b** cand-1 mutants and spg-7(tm2312) mutants **d** had increased the mean lifespan relative to wild type, while cand-1; spg-7(ek25) **e** animals had lifespan comparable to wild type. **e, f** eat-3 RNAi **f** depletions significantly decreased the mean lifespan of *daf-2 (e1370)* mutants. All lifespan experiments were performed with four biological replicates. See Supplementary Table 2 for statistics. **g** The percentages of muscle cells with predominantly tubular, elongated, or fragmented mitochondria in adult hermaphrodites of the indicated genotypes/RNAi treatments visualized by mitochondria-targeted GFP expression in body-wall muscle cells. *P* values were determined by χ²-test. Sample size (n) of muscle cells from left to right are: 278; 102; 179; 207; 136; 218. Mitochondrial morphology was scored blinded. The wild-type control from Fig. 1c was analyzed at the same time and is shown here for comparison. Asterisks above bars denote *P* value comparisons to wild type/control; asterisks above lines denote comparisons under the lines: *P* < 0.05; **P** < 0.01; ***P** < 0.001; ns = not significant.
suggesting that the extended lifespan of cand-1 increase in elongated mitochondria, had normal lifespan, cand-1
Supplementary Table 2 for statistics on lifespan data). Interestingly, the cand-1(tm1683); spg-7(ek25) performed with four biological replicates. See Supplementary Table 3 for statistics
– listed days: 30 min on days 1 c was 30 min of swimming on day 1, followed by a reduction in swim time of 2 min per day on subsequent days. a to swim regimen A (30 min of swimming per day).

Fig. 7 Exercise extends lifespan that is dependent on the mitochondrial fusion pathway. a–d Survival curves for adults that were kept continuously on agar plates or removed from the plates for brief periods for the described swim regimens. a Comparison of wild-type kept on agar plates continuously or subject to swim regimen A (30 min of swimming per day). b Test of swim regimens B and C. Swim regimen B was the following swimming times per day on the listed days: 30 min on days 1–5; 25 min on days 6 and 7; 20 min on days 8 and 9; 15 min on day 10; 10 min on day 11; and 5 min on day 12. Swim regimen C was 30 min of swimming on day 1, followed by a reduction in swim time of 2 min per day on subsequent days. c, d Test of swim regimen C with wild type, cand-1(tm1683); spg-7(ek25) c, or daf-16(mu86) d. The wild-type control and wild-type swim regimen C survival curves were analyzed at the same time and are shown in c, d for comparison. All swimming regimens significantly increased the mean lifespan of wild type a–d. Swim regimen C did not increase the mean lifespan of cand-1(tm1683); spg-7(ek25) c or daf-16(mu86) mutants d relative to the control, non-swimming condition. All lifespan experiments were performed with four biological replicates. See Supplementary Table 3 for statistics

Mitochondrial fusion is required for IIS lifespan extension. DAF-16 is required for the extended lifespan of IIS pathway mutants27. Because CAND-1 and SCF LIN-23 inhibit DAF-16 nuclear localization, we hypothesized that inactivation of these regulators would extend lifespan. As expected, RNAi depletion of cand-1 and lin-23 (begun in the late-L4 larval stage to ensure normal larval development) extended lifespan (Fig. 6a; see Supplementary Table 2 for statistics on lifespan data). Interestingly, the cand-1; spg-7(ek25) strain, which rescues the cand-1 increase in elongated mitochondria, had normal lifespan, suggesting that the extended lifespan of cand-1 mutants requires increased levels of elongated mitochondria (Fig. 6b).

Notably, we observed that increasing mitochondrial fusion independently of DAF-16 produces a modest extension of lifespan. ppgn-1(RNAi), spg-7(Δ), and spg-7 + ppgn-1 double RNAi animals, all of which exhibit increases in elongated mitochondria (Fig. 1c), had extended lifespan (Fig. 6c, d). In contrast to spg-7(Δ) mutants, spg-7(RNAi) animals do not exhibit increased levels of elongated mitochondria (Fig. 3b), potentially because of the impenetrant effects of RNAi, and consistently had normal lifespan (Fig. 6c).

The mitochondrial fusion pathway includes activating AKT-1 to repress the DAF-16 transcription factor. The IIS pathway has the same downstream pathway, and so mutation of the IIS receptor DAF-2 would also be expected to activate DAF-16-mediated repression of spg-7 and ppgn-1 to activate mitochondrial fusion. Consistent with this, daf-2 mutants exhibit increased levels of elongated mitochondria (Fig. 6g). To determine the extent to which increased levels of elongated mitochondria contribute to the extended daf-2 mutant lifespan, we inactivated the mitochondrial fusion gene eat-3 in daf-2 mutants. Strikingly, eat-3 RNAi reduced the extended lifespan of daf-2 mutants to wild-type levels (Fig. 6e). To test an alternate method to reduce elongated mitochondria in daf-2 mutants, we RNAi-depleted the outer membrane fusion protein FZO-1; moreover, this also significantly reduced the extended lifespan of daf-2 mutants (Fig. 6f). These results suggest that increased levels of mitochondrial elongation are required for lifespan extension in daf-2 mutants.

Elongated mitochondria are known to produce ATP more efficiently3. We analyzed ATP levels in several key mutants, and found a correlation between increased levels of elongated mitochondria and increased ATP/protein ratios (Supplementary Fig. 12). The daf-2 mutant is known to have significantly increased ATP levels35, and had the highest ATP levels in our assay. Additionally, statistically significant increases in ATP levels were observed in lin-23(RNAi), cand-1(Δf), and ppgn-1(RNAi) animals, all of which have increased levels of elongated mitochondria. spg-7(RNAi) animals, which do not exhibit increased levels of elongated mitochondria (Fig. 3b), did not have significantly increased ATP levels. The observation that cand-1(Δf); spg-7(ek25) mutants had significantly lower ATP levels than cand-1(Δf) mutants suggests that a significant portion of the increase in ATP levels in cand-1(Δf) mutants can be attributed to the reduction of spg-7 expression. That ATP levels in cand-1(Δf); spg-7(ek25) remained higher than in wild type may reflect the impact of the loss of CAND-1 on other CRL-regulated processes.

Physical exertion extends lifespan. We observed that during swimming, DAF-16 nuclear localization increases, which is associated with lifespan extension. We wanted to determine whether exercise would increase the median lifespan. Making animals swim initially for 30 min per day, followed by consecutive reductions in swim time of 1 min per day, produced significant lifespan extension. However, this swimming regimen (A) caused a rapid drop-off in the viability of older animals (Fig. 7a, see Supplementary Table 3 for statistics). To limit the stress on older animals, we tested two different swimming regimens (B and C)
that further reduced the extent of swimming as the animals aged: an initial rate of 30 min swimming/day was tapered via arbitrary reductions (regimen B, Fig. 7 legend), or reductions of two min per day (regimen C). These age-moderated swim regimens produced further lifespan extensions relative to regimen A (Fig. 7b).

Notably, cand-1; spg-7(ek25) and daf-16 mutants, which failed to exhibit increased mitochondrial fusion in response to swimming, did not exhibit lifespan extension in response to swimming (Fig. 7c, d). This suggests that the induction of mitochondrial fusion during exercise contributes to the observed lifespan extension.

Because of how the swim experiments were carried out, the lifespans of the control animals are not directly comparable to the lifespans of control animals in other (non-swim) experiments. Control animals in the swim experiments had shorter lifespans than the control animals in non-swim experiments. Presumably, this difference arose because all animals in the swim experiments (including controls) were kept at room temperature during the swim periods (rather than the lower temperature of 20 °C) and were transferred daily to new plates. Notably, the swim regimens B and C produced a significant extension of lifespan even when compared to control animals from the non-swim lifespan experiments (Supplementary Table 3).

Mitochondrial fusion is broadly required for longevity.

To determine whether mitochondrial fusion is more broadly correlated with longevity, we analyzed the mitochondrial morphologies of animals that exhibit longevity from five distinct mechanisms: age-1(RNAi) and pdk-1(RNAi) animals have extended lifespan from loss of IIS15; eat-1(RNAi) and eat-6(RNAi) animals have extended lifespan linked to caloric/dietary restriction36; clk-1(RNAi) extends lifespan because of mitochondrial ETC dysfunction; glp-1(RNAi) extends lifespan as a result of the loss of the germline37; and vhl-1, the von Hippel Lindau tumor suppressor ortholog, mutants/RNAi animals have lifespan extension due to deregulation of the hypoxia transcriptional program38.

We analyzed the distribution of mitochondrial morphologies in the seven long-lived strains. Strikingly, six of the seven long-lived mutants/RNAi animals had increased levels of elongated mitochondria in response to swimming, with vhl-1(RNAi) animals the lone exception, with predominantly tubular mitochondria (Fig. 8a, b and Supplementary Fig. 5). Caloric restricted mutants, such as eat-6, have lifespan extension independent of DAF-1636. We found that the increase in elongated mitochondria in eat-6 mutants was not affected by daf-16 RNAi, suggesting that mitochondrial morphology in these mutants is regulated through a DAF-16-independent pathway (Fig. 8a). In other organisms, nutrient limitation increases mitochondrial fusion3, and it is possible that a similar mechanism operates in C. elegans.

Strikingly, the increase in elongated mitochondria in these diverse mutants is essential for their longevity. RNAi depletion of the mitochondrial fusion gene eat-3 abolished the extended lifespans of diverse longevity mutants/RNAi: glp-1(e2141ts); eat-2(ad1116) and eat-6(ad467); and clk-1(RNAi) (Fig. 9a–d). Significantly, eat-3 RNAi depletion in a wild-type background or in vhl-1(ok161) mutants, neither of which have significantly elevated elongated mitochondria, did not affect their lifespans (Fig. 9e). This suggests that eat-3 RNAi does not have a negative
impact on lifespan directly, but rather acts indirectly by reducing the beneficial effects of increased levels of elongated mitochondria. Elongated mitochondria have decreased production of ROS. We measured mitochondrial ROS levels using the dye MitoSOX Red in daf-2, glp-1, and eat-6 mutants, all of which have elevated levels of elongated mitochondria. Each of these long-lived mutants had reduced ROS levels, and ROS levels increased significantly upon treatment with eat-3 RNAi to block mitochondrial fusion (Supplementary Fig. 13 and Supplementary Table 4). This suggests that increased levels of elongated mitochondria contribute to reduced ROS levels.

Fig. 9 Mitochondrial elongation is required for longevity in diverse mutants. a–i Survival curves for adults of the indicated genotypes, overexpressions (oe), and RNAi treatments. The wild-type and eat-3(RNAi) survival curves are shown in graphs for comparison. eat-3 RNAi significantly reduced the extended lifespans relative of all tested genotypes, overexpression, and RNAi treatments except for vhl-1 mutants and wild-type control e. All lifespan experiments were performed with four biological replicates. See Supplementary Table 2 for statistics.
We considered the possibility that the effect on lifespan by the mitochondrial fusion pathway was primarily due to changes in energy levels that were a secondary consequence of the changes in mitochondrial morphology. We analyzed three energy sensors that have an impact on lifespan: LET-363/TOR, SIR-2.1, and AAK-2/AMPK. LET-363/TOR responds to metabolic inputs to regulate lifespan through inhibition of the transcription factors DAF-16, SKN-1, and PHA-4. SIR-2.1 is a NAD+-dependent histone deacetylase whose overexpression increases lifespan by activating DAF-16, UPRmt, and autophagy. The AMP-activated protein kinase AAK-2 extends lifespan in response to ROS signals and impaired glycolysis, such as inactivation of GPI-1, glucose phosphate isomerase, and PHA-4. Overexpression of SIR-2.1 or AAK-2, and RNAi depletion of let-363/TOR or gpi-1, all exhibited increased mitochondrial elongation (Figs. 8b, c and 9a, i and Supplementary Fig. 5). cat-3 RNAi reduced the extended lifespan of these animals, suggesting that the elongated mitochondrial morphology contributes to their lifespan extension (Fig. 9f-i and Supplementary Fig. 5).

The UPRmt links mitochondrial ETC dysfunction during larval stages with lifespan extension in adults. To determine whether the IIS/SCF\[^{\text{LIN-23}}\]-regulated mitochondrial fusion pathway activates the UPRmt, we analyzed the induction of the UPRmt reporters Phsp-6::GFP. RNAi depletion of spg-7 is known to induce UPRmt, and, consistently, we observed increased expression of Phsp-6::GFP in spg-7(RNAi) animals (Fig. 10a, b). Notably, ppgn-1 RNAi, which induces substantially more mitochondrial fusion than spg-7 RNAi (Figs. 1c and 3b), did not induce Phsp-6::GFP expression (Fig. 10a, b). Additionally, cand-1, lin-23, and cul-1 RNAi depletions did not induce Phsp-6::GFP, indicating that the mitochondrial fusion pathway does not induce UPRmt.

The induction of UPRmt is mediated by the transcription factor ATFS-1, which is required for the expression of hsp-6 and other UPRmt-regulated genes. Counterintuitively, a gain-of-function mutation of ATFS-1, atfs-1(et17gf), with constitutively activated UPRmt, exhibits reduced lifespan, not lifespan extension. We used the atfs-1(et17) gain-of-function mutant to ask whether activation of the mitochondrial fusion pathway reduces lifespan further, as would be expected if the pathway further activated the UPRmt. We found that RNAi depletion of cand-1, lin-23, spg-7, and ppgn-1 increased the lifespan of atfs-1(et17) mutants, suggesting that the lifespan extension operates independently of the UPRmt (Fig. 10c, d). Induction of UPRmt by loss of spg-7 is abolished upon the loss of atfs-1. However, we did not observe significant differences in mitochondrial morphology between spg-7(Δ) mutants and spg-7(Δ); atfs-1(RNAi) mutants (Fig. 10e). The UPRmt is
also not induced upon swimming, unlike the mitochondrial fusion pathway (Fig. 1D). These results suggest that the increase in mitochondrial fusion associated with inactivation of the IIS/SCF<sup>LIN-23</sup>-regulated pathway occurs independently of UPR<sup>M</sup>.

**Discussion**

Our study has revealed a pathway by which the ubiquitin ligase SCF<sup>LIN-23</sup> and IIS control mitochondrial fusion in *C. elegans* (Fig. 4a). We found that the CRL regulator CAND-1 and SCF<sup>LIN-23</sup> are required to activate AKT-1. Active AKT-1 inhibits the nuclear localization of DAF-16, which inhibits the expression of the mitochondrial m-AAA proteases SPG-7 and PPGN-1. Decreased expression of SPG-7 and PPGN-1 is associated with increased levels of the mitochondrial fusion protein EAT-3, whose activity is essential for the increased mitochondrial fusion that is observed upon inactivating the pathway components CAND-1, LIN-23, and PPGN-1. Our analysis does not indicate whether SPG-7 and PPGN-1 directly reduce EAT-3 levels or whether the impact on EAT-3 is indirect. Given the additional functions of the yeast and mammalian orthologs of *spg-7* and *ppgn-1*, it is likely that reducing their activity affects multiple mitochondrial pathways<sup>29</sup>. Additionally, SCF E3s are known to target multiple substrates<sup>16</sup>, <sup>17</sup> and we cannot rule out the possibility that CAND-1 and SCF<sup>LIN-23</sup> have an impact on mitochondria through other pathways.

Our work identifies the SCF<sup>LIN-23</sup>-mediated activation of AKT-1 as a new regulatory mechanism for IIS, for which AKT-1 is a critical component. Currently, it is unclear how SCF<sup>LIN-23</sup> promotes AKT-1 phosphorylation. Ubiquitination has been linked to AKT activation in mammalian cells, wherein K63-linked poly-ubiquitination of AKT is required for its translocation to the plasma membrane, where it is activated<sup>49</sup>, <sup>50</sup>. In mammalian cells, the K63 linkage is only detectable with anti-K63 ubiquitin antibodies immediately after stimulation of the relevant signaling pathways, but not in unstimulated cells<sup>49</sup>, <sup>50</sup>. We have been unable to detect K63 poly-ubiquitin on immunoprecipitated AKT-1::GFP. However, the inability to rapidly induce signaling in intact animals limits the implications of this negative result.

In mouse skeletal muscle cells, physical exercise increases mitochondrial fusion<sup>34</sup>. We have shown a similar result in *C. elegans* using swimming as a source of exercise. *C. elegans* swimming is associated with a reduction in the level of ATP, indicating that the “thrashing” swim strokes involve a greater expenditure of energy than the normal sinusoidal movement on plates. Swimming induces an increase in the level of elongated mitochondria, and this increase appears to be controlled by the IIS/SCF<sup>LIN-23</sup>-regulated pathway, as the increase in elongated mitochondria is blocked in *daf-16* mutants as well as *cand-1; spg-7(ek25)* mutants, which are no longer responsive to *daf-16*-mediated inhibition of *spg-7* expression.

In rodents, modest physical exercise correlates with increases in mean lifespan and healthspan<sup>31</sup>, and we have found that exercise regimens increase *C. elegans* lifespan. The lifespan extension in response to exercise is not observed in *daf-16* mutants or *cand-1; spg-7(ek25)* mutants, which are unable to activate the IIS/SCF<sup>LIN-23</sup> pathway to increase mitochondrial fusion, suggesting that increased mitochondrial elongation positively contributes to exercise-induced longevity.

We observed an increase in elongated mitochondria in diverse longevity pathways, including: IIS inactivation; caloric restriction; germline depletion; ETC dysfunction leading to UPR<sup>M</sup>; TOR inactivation; Sir2p overexpression; AMPK overexpression; impaired glycosylation; and exercise. RNAi depletions of six genes that cause ETC dysfunction leading to extended lifespan were previously shown to increase mitochondrial fusion<sup>52</sup>. These observations suggest that increased levels of elongated mitochondria are broadly associated with longevity.

Notably, the extended lifespans for the longevity pathway mutants with increased mitochondrial elongation were significantly reduced by *eat-3* RNAi, which reduces mitochondrial fusion. *eat-3* RNAi does not affect the lifespans of wild-type animals or long-lived *vhl-1* mutants, neither of which exhibit increased levels of elongated mitochondria. This suggests that inactivation of the mitochondrial fusion protein EAT-3 does not directly reduce lifespan, but rather indirectly reduces lifespan by countering the beneficial effects of elongated mitochondria. RNAi depletion of the outer membrane mitochondrial fusion protein EFO-1 also reduces *daf-2* mutant-extended lifespan.

*ppgn-1* mutants have a substantial increase in elongated mitochondria, yet have only modestly longer lifespans. It has been reported that blocking fission by inactivating DRP-1 reduces<sup>33</sup> or has no effect on the lifespan of wild-type animals<sup>34</sup>, but increases the extended lifespans of IIS pathway mutants<sup>34</sup>. These observations suggest that increasing mitochondrial elongation, by itself, does not greatly extend lifespan. Rather, increased levels of elongated mitochondria contribute to older animal survival, the precise level of the increase would not be expected to be predictive of the extent of longevity.

Elongated mitochondria have increased mitochondrial efficiency<sup>32</sup>; in addition, we have observed increased ATP levels in mitochondrial fusion pathway mutants that have increased mitochondrial elongation. This leads to the hypothesis that increased mitochondrial efficiency resulting in sufficient ATP production is required in diverse longevity pathways to allow the survival of long-lived adults.

We observed that *vhl-1* mutants do not have increased levels of elongated mitochondria and their extended lifespan is not reduced by *eat-3* RNAi. Interestingly, a large-scale Cas9 inactivation screen in human cells found that inactivation of VHL allowed a bypass of the complete block on mitochondrial respiration<sup>55</sup>. VHL functions as a substrate receptor for the CRL2VHL ubiquitin ligase, which degrades the hypoxia-inducible factor HIF1A. During hypoxia, the HIF1A transcriptional response is associated with a major reorganization of energy pathways to allow energy generation in the absence of oxidative mitochondrial metabolism<sup>35</sup>. Thus, unregulated HIF1A transcriptional activity can functionally replace the requirement for mitochondrial respiration in human cells<sup>35</sup>.

*C. elegans* VHL-1 similarly targets the degradation of the HIF1A ortholog HIF-1<sup>56</sup>. HIF-1 is required for the lifespan extension of *vhl-1* mutants<sup>38</sup>. If the role of HIF-1 in providing alternate energy pathways under hypoxic conditions is conserved, then *vhl-1* mutants would have increased energy capacity via HIF-1-mediated induction of energy metabolism. It is possible that increased energy capacity is a requirement for survival beyond the normal lifespan, and that most longevity mutants increase energy capacity via mitochondrial elongation, while *vhl-1* mutants utilize HIF-1-mediated transcriptional activity. It will be interesting to determine whether increased mitochondrial fusion and/or energy metabolism contributes to the survival and healthspan of older animals in other species.

**Methods**

**C. elegans strains.** The following *C. elegans* strains were used, with strain designations in parentheses: wild type Bristol (N2), wild type Hawaiian (CB4856), *cand-1(tm1683)* (ET327), *cand-1(tm1683)* outcrossed 8x into Hawaiian strain CB4856 (ET335), *cand-1(1m1863)*; spg-7(ek25) (ET329), lin-23(e1883)/mln-1 (ET351), *daf-16(msu86)* (CFI038), *spg-7(ad2249)* (DA2249)<sup>37</sup>, *spg-7(tm2312)*
RNA interference. Feeding RNAi constructs (expressed in HT115 bacteria) were obtained from the Arrhinger library60. RNAi-feeding bacteria were induced in 1 μM IPTG in liquid culture plus 10% media plus 100 μg/ml carbenicillin/ml. Double RNAi treatments were performed by combining RNAi bacteria at a 1:1 ratio (unless otherwise indicated) using OD600 optical densities to quantify the bacteria prior to seeding plates. Unless otherwise indicated, eggs were placed on the RNAi plates and adults from the next generation were analyzed.

The isolation of the ek25 cand-1 suppressor mutation. cand-1(tm1683) mutants were synchronized as L1-stage larvae by isolating eggs by hypochlorite treatment61 and allowing the eggs to hatch overnight in M9 buffer supplemented with 0.5 mM N-nitroso-N-ethylurea (ENU) for 4 h. The plates with OP50 bacteria until the L4/young-adult stage, when they were grown in a 500 ml liquid culture supplemented with OP50 bacteria for 11 days, adults and synchronized as L1 larvae. In all, 10,000 F2 mutant L1 larvae were isolated from the gravid adults and synchronized as L1 larvae. The F1 mutant animals were cultured on NGM plates with OP50 bacteria. One of these cloned animals (cand-1(tm1683); ek25) had 100% healthy progeny without visible cand-1 mutant phenotypes.

SNP mapping. The cand-1(tm1683); ek25 strain was outcrossed 10 times into the Hawaiianized cand-1(tm1683) strain ET352. The resulting Hawaiianized cand-1; ek25 strain appears to be largely Hawaiian throughout the genome with the exception of the +7.5 to +11.5 region of chromosome V, which contains the csn-3 gene and remains N2 Bristol. The cand-1(tm1683) and ek25 strains were Hawaiianized (using Dra I site) in order to distinguish Hawaiian from N2 Bristol genomic DNA. We placed on a rocking platform at room temperature to prevent the animals from rolling off the plates with OP50 bacteria until the L4/young-adult stage, when they were grown in a 500 ml liquid culture supplemented with OP50 bacteria for 11 days, adults and synchronized as L1 larvae. The F1 mutant animals were cultured on NGM plates with OP50 bacteria. One of these cloned animals (cand-1(tm1683); ek25) had 100% healthy progeny without visible cand-1 mutant phenotypes.

Lifespan analysis. Survival assays67 were performed at 20°C. Eggs were isolated from gravid adults by hypochlorite treatment and allowed to hatch on RNAi-feeding bacteria plates, with the exception of cand-1, cul-1, lin-3, clk-1, and let-36 RNAi, where wild-type bacterial RNAi-silenced clones were transferred to RNAi-silenced C. elegans bacteria plates as late-L4 stage larvae to ensure that prior larval development was normal. Animals were transferred to fresh plates every alternate day throughout the lifespan study. For all lifespan studies, animals in the L4 larval stage were picked on day 0. Lifespan analysis of wild-type adult animals subjected to a swimming regimen was performed by adding 10,000 F2 mutant L1 larvae to plates placed on a rocking platform at room temperature to prevent the animals from settling down. The daily swim regimes are described in the main text. After
swimming, animals were transferred to fresh agar plates with OP50 bacteria. The non-swim control animals were placed at room temperature for the duration of the swim period, and also transferred to new OP50-seeded NGM plates at the same minute for each genotype. The authors declare that all data supporting the findings of this study are available within this article, its Supplementary Information files, or are available from the corresponding author upon reasonable request.

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Author contributions

S.N.C. and E.T.K. designed the study, performed the experiments, analyzed the results, and wrote the manuscript.

Additional information

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