**Figure S1, Related to Figure 1;** *math-33* **inactivation reduces IIS-dependent** *Psod-3::GFP* **reporter induction without affecting stress response reporter activation**. (A) *Psod-3::GFP* reporter activity in wild type (N2) and *daf-2(e1370)* animals shifted to 25ºC. (B) *Phsp4::GFP* reporter activity in control and tunicamycin treated nematodes. (C) *Phsp16.2::GFP* reporter activity in control and heat stressed nematodes. (D) *Phsp6::GFP* reporter activity in control and paraquat exposed nematodes. Fluorescent (i.) and DIC (ii.) micrographs are presented. Animals were fed bacteria expressing *math-33* dsRNA or a control vector.

A
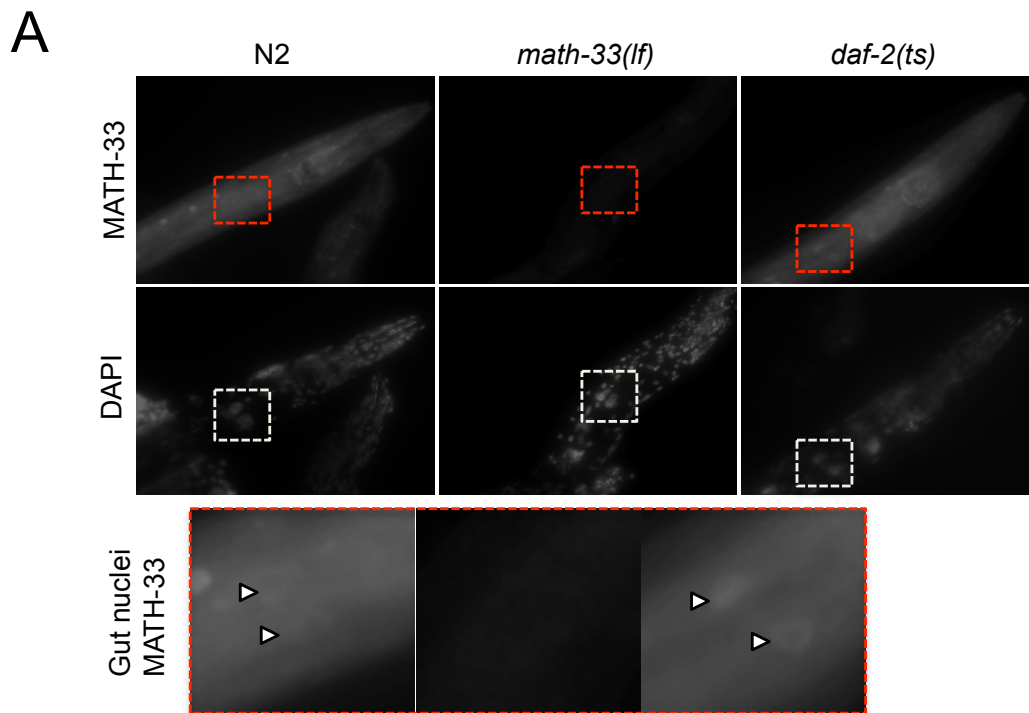
	N2	daf-2(ts)
% nuclear enrichment	4.1	19.1
number of gut nuclei	23	47
number of animals	8	16

Figure S2, Related to Figure 2; MATH-33 nuclear localization is enhanced in intestinal cells of *daf-2(1370)* mutants. (A) Immunostaining of MATH-33 using an affinity-purified anti-MATH-33 antibody. Nematodes were shifted to the restrictive temperature (25ºC) at the L4 stage and stained at day 1 of adulthood. DAPI staining indicates localization of nuclei. Magnified images of intestinal nuclei are presented (red boxes). The *daf-2(e1370)* temperature sensitive allele and the *math-33(tm3561)* loss-of-function alleles were used. (B) Quantification of nuclear to cytoplasmic ratio of MATH-33 signal intensity (see Supplemental Experimental Procedures).
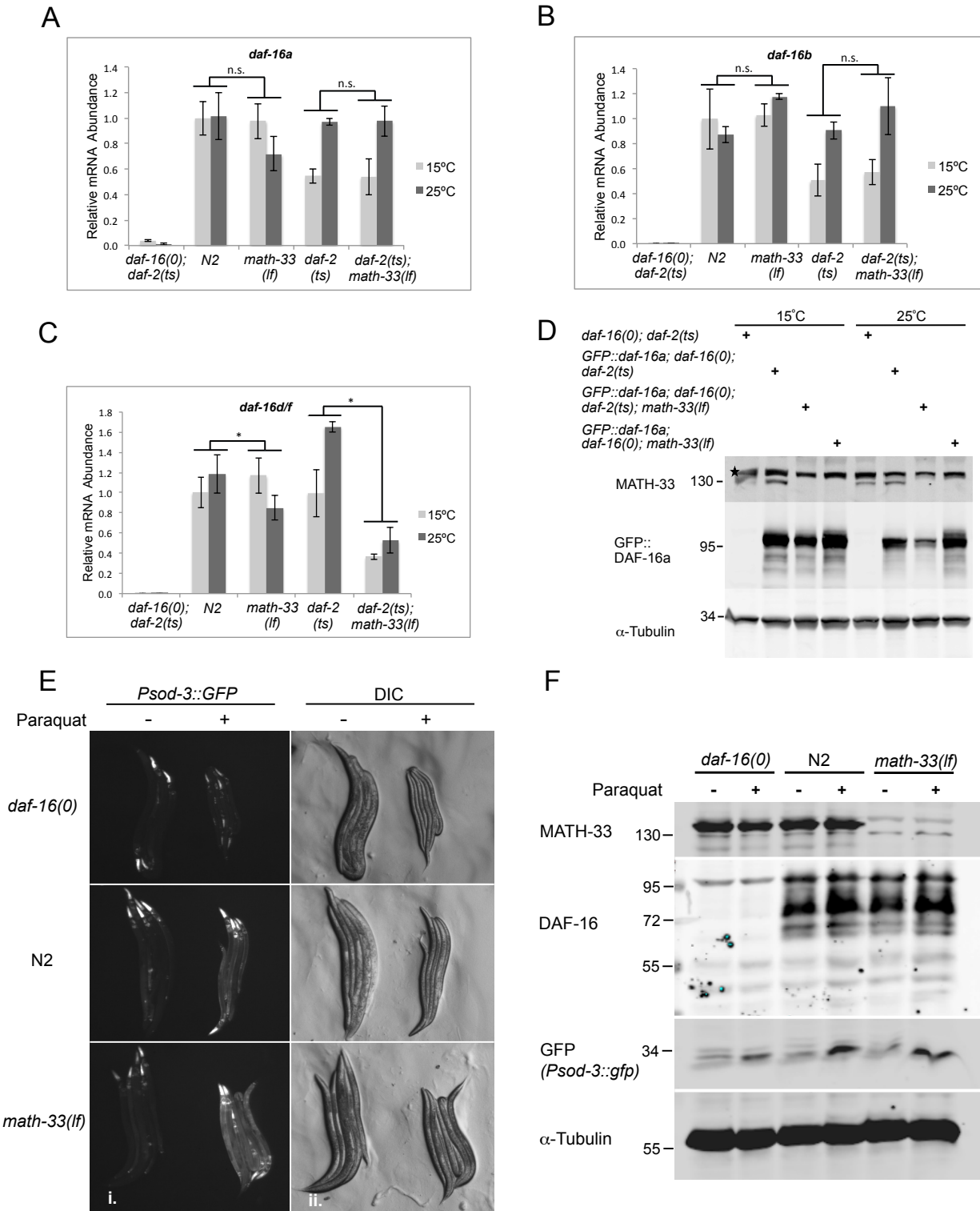
Figure S3, Related to Figure 3; Analysis of *daf-16* transcript and DAF-16a protein levels in IIS and oxidative stress analysis when *math-33* is inactivated. (A-C)

Quantification of endogenous *daf-16* isoform transcript levels using qRT-PCR when *math-33* is inactivated and IIS reduced. Fluctuations in *daf-16* isoform transcript levels between permissive and restrictive temperature in the *daf-2(e1370)* mutant background indicate a dynamic regulation for *daf-16* mRNA levels. RNA levels were normalized to three internal control genes (see methods). The mean normalized RNA level and STDEV of three biological repeats are shown. The normalized RNA level in N2 animals was set as 1. p-Values were calculated using Two-Way ANOVA. *: $p < 0.05$, n.s.: not significant. (D) Detection of DAF-16a isoform protein levels when *math-33* is inactivated and IIS diminished. GFP::DAF-16a levels detected by immunoblotting. Nematodes were raised at the semi-permissive temperature (15ºC) and shifted to the restrictive temperature (25ºC) for 24 hr or kept at 15ºC. Star denotes nonspecific bands. (E-F) Effect of *math-33* inactivation on DAF-16 activity and protein levels in wild type animals under oxidative stress conditions. (E) *Psod-3::GFP* reporter activity in control and paraquat exposed nematodes. Fluorescent (i.) and DIC (ii.) micrographs are presented. (F) Endogenous DAF-16 isoform levels and *Psod-3::GFP* reporter activity detected by immunoblotting. The *daf-2(e1370)* temperature sensitive allele, the *daf-16(mu86)* null allele and the *math-33(tm3561)* loss-of-function allele were used.
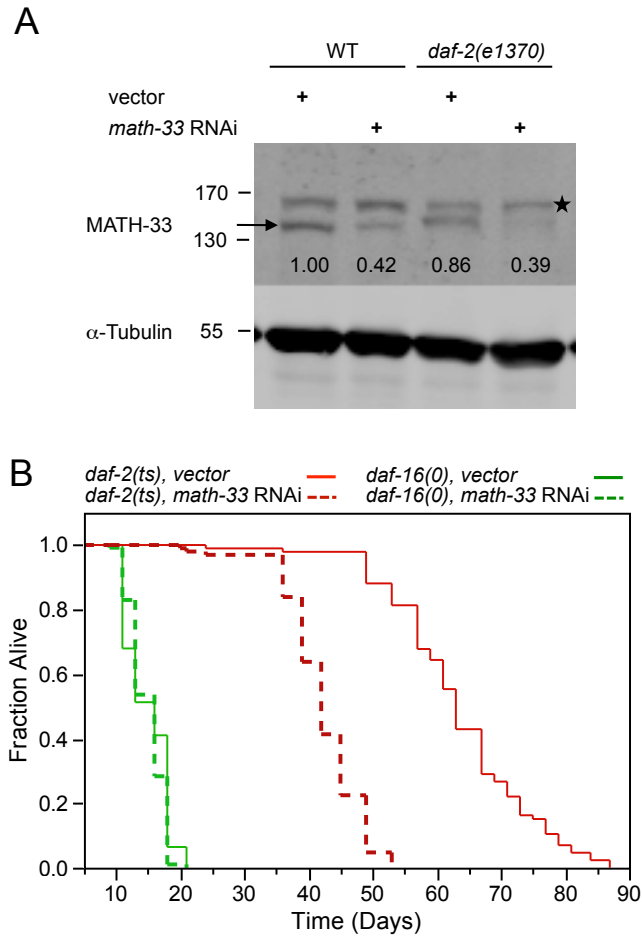
Figure S4, Related to Figure 4; *math-33* is required for increased longevity when IIS is reduced. (A) *math-33* RNAi-mediated knockdown reduces MATH-33 protein levels as indicated by immunoblotting using affinity-purified anti-MATH-33 antibody. Numbers display MATH-33 band densities calculated relative to tubulin signals. Star denotes nonspecific bands. (B) Effect of *math-33* RNAi-mediated knockdown on lifespan of *daf-2(e1370)* or *daf-16(mu86)* mutant animals when knockdown was initiated from hatching. Lifespan values are given in Supplementary information, Table S2.
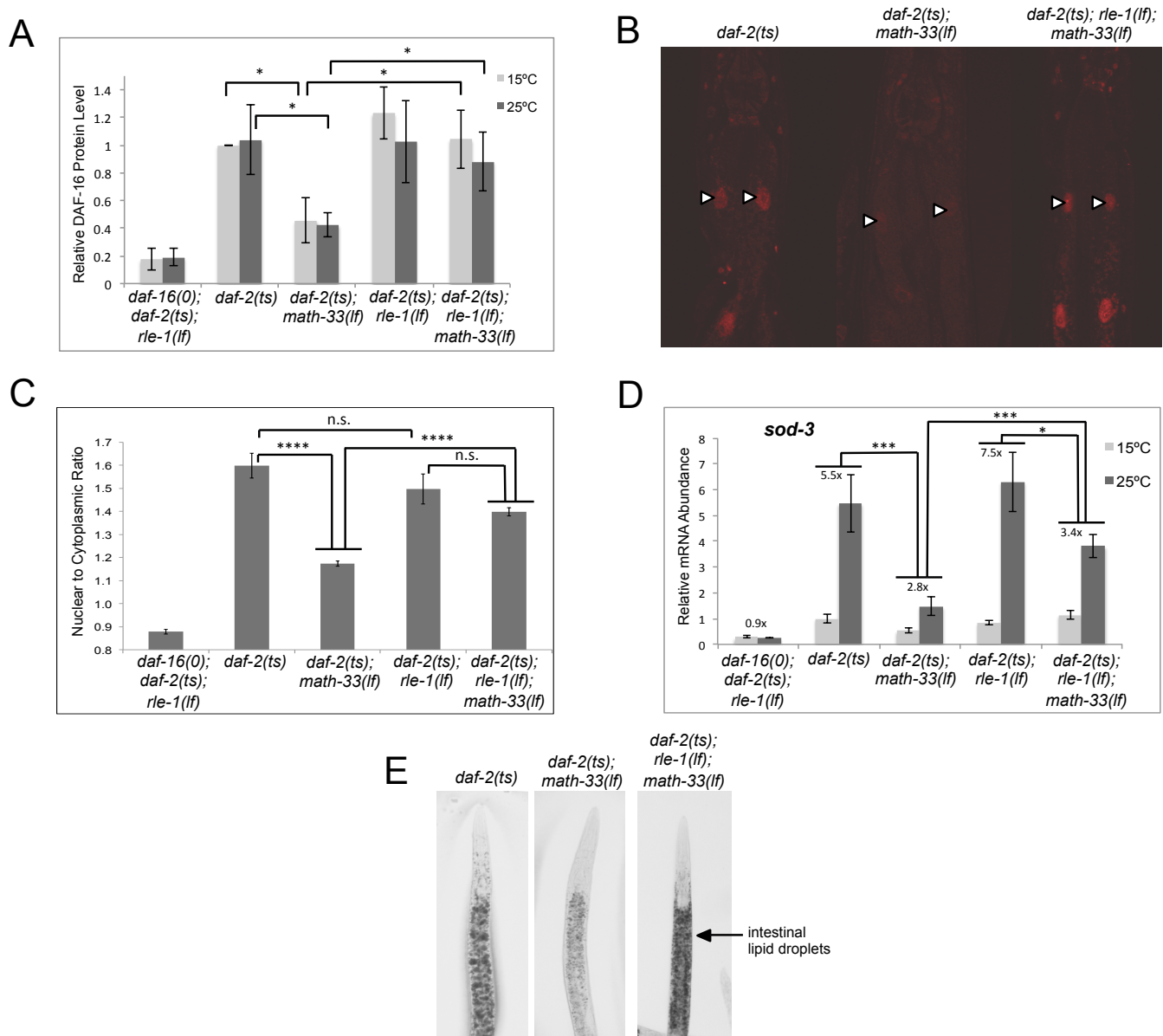
Figure S5, Related to Figure 5; *rle-1* is epistatic to *math-33* when IIS is downregulated. (A) Quantification of endogenous DAF-16 isoform levels from immunoblotting (Figure 5B). The normalized intensity level for DAF-16 isoforms in *daf-2(e1370)* animals on 15ºC was set as 1. DAF-16 isoforms of three independent experiments were quantified relative to the tubulin signal as a loading control. (B) Immunostaining of endogenous nuclear DAF-16 shown for anterior intestinal cells when IIS is reduced. Animals were shifted to the restrictive temperature (25ºC) at the

L4 stage for 24 hr. Arrowheads denote intestinal nuclei. Lipid droplet accumulation in *daf-2(e1370)* intestinal cells causes cytoplasmic background. (C) Quantification of nuclear to cytoplasmic ratio of DAF-16 levels detected by immunostaining (see Supplemental Experimental Procedures). (D) qRT-PCR of endogenous *sod-3* transcripts. RNA levels were normalized to three internal control genes (see methods). The mean normalized RNA level and STDEV of three biological repeats are shown. (E) Detection of fat storage using Oil Red O staining. Animals were shifted to the restrictive temperature (25ºC) after reaching the L2 larval stage to avoid dauer formation. (A-E) The *daf-16(mu86)* null allele, the *daf-2(e1370)* temperature sensitive allele, the *rle-1(cxTi510)* loss-of-function allele and the *math-33(tm3561)* loss-of-function allele were used. p-Values were calculated using two-tailed Student's t-test (A, C) and Two-Way ANOVA (D). ****: $p<0.0001$;***: $p<0.001$; *: $p<0.05$, n.s.: not significant.

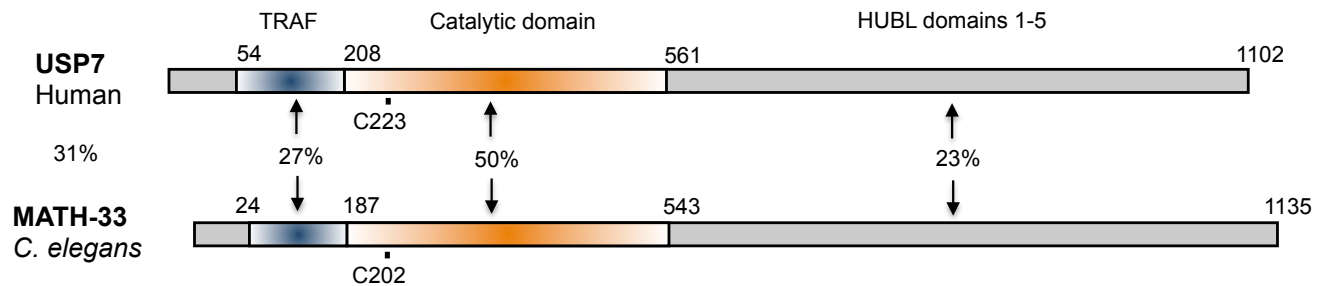**Figure S6, Related to Figure 6; Amino acid sequence alignment of human USP7 with *C. elegans* MATH-33.** The isoform 1 of human USP7 (UniProt Q93009-1) was aligned with *C. elegans* MATH-33 (UniProt Q7JKC3). Percent sequence identity of proteins and individual domains are presented. TRAF, substrate-binding domain. C223, C202, active site cysteine residues. HUBL, USP7/HAUSP ubiquitin-like domain.

Supplementary Table S1. Related to Figure 4 and Figure 5

Strain	Mean Lifespan ± SEM (Days)	p Value	Total Animals Died/Total*
experiment 1			
daf-2(e1370)	54.0 ± 0.8		94/100
daf-16(mu86); daf-2(e1370)	14.3 ± 0.4	<0.001[a]	86/105
daf-2(e1370); math-33(tm3561)	14.2 ± 0.4	<0.001,[a] 0.8889[b]	82/110
daf-2(e1370); rle-1(cxTi510); math-33(tm3561)	23.6 ± 0.9	<0.001,[a] <0.001[c]	74/96
daf-2(e1370); rle-1(cxTi510)	41.4 ± 1.4	<0.001,[a] <0.001[d]	61/69
experiment 2			
daf-2(e1370)	43.5 ± 1.3		95/100
daf-16(mu86); daf-2(e1370)	13.8 ± 0.3	<0.001[a]	97/100
daf-2(e1370); math-33(tm3561)	13.5 ± 0.4	<0.001,[a] 0.9414[b]	95/100
daf-2(e1370); rle-1(cxTi510); math-33(tm3561)	19.2 ± 0.8	<0.001,[a] <0.001[c]	97/100
daf-2(e1370); rle-1(cxTi510)	32.7 ± 1.9	<0.001,[a] <0.001[d]	80/90
daf-16(mu86)	12.0 ± 0.3		93/99
math-33(tm3561)	11.1 ± 0.3	0.1213[e]	90/100

*The total number of observations equals the number of animals that died plus the number censored. Animals that crawled of the plate, exploded, or bagged were censored at the time of the event. The log-rank (Mantel-Cox) test was used for statistical analysis.

[a] Compared to *daf-2(e1370)* animals

[b] Compared to *daf-2(e1370); daf-16(mu86)* animals

[c] Compared to *daf-2(e1370); math-33(tm3561)* animals

[d] Compared to *daf-2(e1370); math-33(tm3561); rle-1(cxTi510)* animals

[e] Compared to *daf-16(mu86)* animals

Supplementary Table S2. Related to Figure 4 and Figure S4

Strain	Treatment	Mean Lifespan ± SEM (Days)	Percent change[a]	p Value[d]	Total Animals Died/Total[b]
experiment 1: *math-33* knock down from hatching[c]					
daf-2(e1370)	vector	41.6 ± 1.3	---		95/101
	math-33 dsRNA	30.9 ± 0.7	-25.7	<0.001	97/100
N2	vector	19.2 ± 0.3	---		94/97
	math-33 dsRNA	17.1 ± 0.3	-10.9	<0.001	99/101
experiment 2: *math-33* knock down from hatching[c]					
daf-2(e1370)	vector	63.6 ± 1.2	---		88/108
	math-33 dsRNA	42.3 ± 0.6	-33.5	<0.001	86/120
N2	vector	19.6 ± 0.5	---		87/120
	math-33 dsRNA	16.2 ± 0.4	-17.3	<0.001	96/120
daf-16(mu86)	vector	14.9 ± 0.4	---		78/120
	math-33 dsRNA	14.9 ± 0.3	0.0	0.2681	99/120

[a] Percent change in lifespans of experimental animals versus control animals examined at the same time.

[b] The total number of observations equals the number of animals that died plus the number censored. Animals that crawled of the plate, exploded, or bagged were censored at the time of the event.

[c] Animals were grown at 15ºC during larval stages and shifted to 20ºC at day 1 of adulthood to prevent dauer formation.

[d] p-values were calculated for control and experimental animals examined at the same time. Control animals were grown on HT115 bacteria harboring the RNAi plasmid vector. The log-rank (Mantel-Cox) test was used for statistical analysis.

Supplementary Table S3. Related to Figure 5 and Table 1

rel-1 inactivation is able to partially rescue dauer formation of *daf-2(e1370); math- 33(tm3561)* mutant animals

Genotype	% L2 arrest	% predauer (L2D arrest)	% dauers	% L3 - L4	% adults	No. animals scored (trials)
N2; vector	0.4	0.0	0.0	0.8	98.8	237 (1)
math-33(tm3561); vector	3.5	0.0	0.0	2.5	94.0	202 (1)
rle-1(cxTi510); vector	0.0	0.0	0.0	9.8	90.2	143 (1)
daf-2(e1370) vector	0.0	3.3	96.7	0.0	0.0	276 (2)
daf-16(mu86); daf-2(e1370); vector	0.0	0.0	0.0	11.2	88.8	179 (2)
daf-2(e1370); math-33(tm3561); vector	49.6	22.0	0.4	6.0	22.0	232 (2)
daf-2(e1370); math-33(tm3561); daf-16 dsRNAi	52.7	15.9	0.0	13.1	18.3	464 (2)
daf-2(e1370); rle-1(cxTi510); math-33(tm3561); vector	3.0	96.2	0.0	0.8	0.0	132 (2)
daf-2(e1370); rle-1(cxTi510); math-33(tm3561); daf-16 dsRNAi	8.4	42.1	0.5	10.5	38.5	429 (2)

Dauer/larval arrest phenotype was scored at 24.5°C (see Methods). Animals that resembled a pre-dauer L2 stage (L2D) appear darker due to accumulation of intestinal fat granules.

We observed that a high percentage of *daf-2(e1370); math-33(tm3561)* animals arrested at the L2 larval stage or as pre-dauers. Further reduction of *daf-16* activity in *daf-2(e1370); math-33(tm3561)* animals using RNAi did not prevent the formation of L2 and pre-dauer arrested stages indicating that these larval arrests are likely not caused by residual DAF-16 activity.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

***C. elegans* strains and generation of transgenic lines.** All strains were maintained at 15ºC using standard *C. elegans* methods (Brenner, 1974) and fed on *Escherichia coli* OP50. The strains used were wild-type N2 Bristol, KK1056 *math-33(tm3561)*, CF1553 *muIs84[pAD76(sod-3::GFP)]*, AGD915 *daf-16(mu86); daf-2(e1370); rle-1(cxTi510); muIs84[pAD76(sod-3::GFP)]*, KB-6 *rle-1(cxTi510)*, CF1085 *daf-16(mu86); daf-2(e1370)*, CF1041 *daf-2(e1370)*, CF1588 *daf-16(mu86); daf-2(e1370); muIs84[pAD76(sod-3::GFP)]*, AGD521 *daf-16(mu86); daf-2(e1370); muIs109[GFP::daf-16a cDNA + Podr-1::RFP]*, CF1038 *daf-16(mu86)*, AGD915 *daf-2(e1370); rle-1(cxTi510); math-33(tm3561); muIs84[pAD76(sod-3::GFP)]*, AGD843 *daf-16(mu86); daf-2(e1370); muIs84[pAD76(sod-3::GFP)]; uthIs262[Pdaf-16::tetra::daf-16a; pRF4(rol-6)]*, AGD1095 *daf-2(e1370); rle-1(cxTi510); muIs84[pAD76(sod-3::GFP)]*, AGD1093 *math-33(tm3561); muIs84[pAD76(sod-3::GFP)]*, AGC576 *daf-2(1370); math-33(tm3561)*, AGD1160 *daf-2(e1370); math-33(tm3561); muIs84[pAD76(sod-3::GFP)]*; AGD1002 *daf-16(mu86); daf-2(e1370); math-33(tm35610); muIs109[GFP::daf-16a cDNA; Podr-1::RFP]*, CF1580 *daf-2(e1370); muIs84[pAC76(sod-3::GFP)]*, TWH3 *daf-2(e1370); rle-1(cxTi510)*, TWH4 *daf-2(e1370); rle-1(cxTi510); math-33(tm3561)* AGD1030 *daf-2(e1370); uthEx674[Pmath-33::tomato]*.

Extrachromosomal array carrying transgenic strains were generated using standard microinjection methods (Mello et al., 1991). For generation of *daf-16(mu86); daf-2(e1370); muIs84[pAD76(sod-3::GFP)]* animals overexpressing Tetra-tagged *daf-16a*, pNL209_P*daf-16::*Tetra-*daf16a* plasmid DNA was injected with the pRF4(*rol-6*) construct. For generation of *daf-2(e1370); uthEx674*[P*math-33::*tdTomato] nematodes*,* p260_P*math-33::*tdTomato plasmid DNA was injected. Individual transgenic F$_1$ progeny were selected on the basis of

the roller phenotype or tdTomato expression. Transgenic F$_2$ animals were isolated to establish independent lines. Integrated Tetra-tagged *daf-16* expressing transgenic lines were generated using gamma irradiation. Nematodes carrying an integrated transgene were out-crossed six times.

Generation of plasmids. To construct a plasmid expressing N-terminal tagged Tetra-*daf-16a* driven by the endogenous *daf-16* promoter, the Tetra-tag was amplified from pCaSpeR-hs-act-tetra (Yang et al., 2006) by PCR and cloned into pNL209 (Libina et al., 2003). The promoter region of *math-33* containing a 1.7 kbp fragment upstream of the *math-33* coding region was amplified from a fosmid vector (WRM0612cE12) by PCR and subcloned with tdTomato into the pPD95.77 vector (Fire Lab kit) to construct a P*math-33*::tdTomato transcriptional reporter. To generate N-terminal tagged 3xFLAG-*math-33, math-33* was subcloned from pIC26-*math-33* (McCloskey and Kemphues, 2012) into the pKC expression vector (Heimbucher et al., 2007) containing a 3xFLAG tag upstream of the polylinker region. A *math-33(C202S)* point mutation was created by a PCR-based mutagenesis approach. 3xFLAG-*math-33* and 3xFLAG-*math-33(C202S)* were further cloned into pAcG2T (BD Biosciences). The constructs for bacterial expression of the His-tagged catalytic core of Usp2 (Usp2-cc) and His-tagged *daf-16* were previously described (Renatus et al., 2006; Tao et al., 2013). *daf-16a* from pNL209 (Libina et al., 2003) was subcloned into the pHA-MEX expression vector (Dualsystems Biotech). pCMV-FLAG-*rle-1* and pCMV-FLAG-*rle-1 C34S* were described previously (Li et al., 2007). The ubiquitin construct used, which expresses eight tandem copies of His-tagged ubiquitin under the control of the CMV promoter, has been previously described (Treier et al., 1994).

RNAi treatment. RNAi-treated strains were fed *E. coli* (HT115) containing an empty vector or *E. coli* expressing double-stranded RNA (dsRNA) against the gene of interest. Unless indicated, worms were fed bacteria expressing dsRNA from hatching (L1). The *math-33* RNAi clone was from Julie Ahringer's RNAi library (Kamath et al., 2003). The RNAi clone against *daf-16* was described previously (Dillin et al., 2002).

Statistical analysis. JMP8 software was used for statistical analysis of lifespan studies. Using the log-rank (Mantel-Cox method) p-values were calculated for individual lifespan experiments, each consisting of control and experimental animals examined at the same time. Prism 6 software was used for Two Way ANOVA and unpaired two-tailed Student's t-test to analyze quantitative RT–PCR, immunoblot and immunostaining quantification results. The mean normalized levels and standard deviation (STDEV) of at least three biological repeats were presented.

Preparation of *C. elegans* lysates for Tandem Affinity Purification and immunoblotting. Semi-synchronized nematodes populations (AGD843, CF1588) were generated using L1 larva of freshly starved cultures. L1 larva were separated from gravid hermaphrodites and larger larval stages by three rounds of sedimentation, resulting is in a nematode pool highly enriched for L1 larva. 500K - 1.000K L1 larva were grown on 150 mm high growth NGM plates at 15ºC, then shifted to 20ºC at the late L4/ day 1 of adulthood stage for 2 to 6 hr and harvested. Nematodes were homogenized in a mortar with liquid nitrogen and resuspended in ice-cold lysis buffer [50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton-X-100, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF supplemented with protease inhibitor tablets (Sigma) and phosphatase inhibitors (Calbiochem)]. The homogenate was centrifuged at 16.000 g. The

supernatant was mixed with glycerol (10% v/v), flash frozen in liquid nitrogen and stored at -80ºC. FLAG and His based Tandem Affinity Purification was performed as described previously (Yang et al., 2006).

For immunoblotting 3,000 semi-synchronized worms for each strain were lysed by boiling in SDS loading buffer. 30 μg of cleared lysates were resolved by SDS-PAGE, immunoblotted and analyzed with antibodies against DAF-16 (Oh et al., 2006), MATH-33 (McCloskey and Kemphues, 2012), GFP (Roche) and alpha-tubulin (Sigma). Immunoblots were quantified using the Odyssey system (LI-COR Biosciences). Signal intensities were normalized to the *a*-tubulin signal as the reference and displayed relative to a control.

Mass spectrometry and MudPIT proteomic analysis. Precipitated proteins were redissolved in 8M urea - 100 mM Tris(hydroxyethylamine), pH 8.4 and subjected to MudPIT proteomic analysis as previously described (Washburn et al., 2001) with the following modifications. 4-step MudPIT was performed where each step corresponds to 0, 10, 40, and 100% buffer C. An LTQ XL mass spectrometer was used as previously described (Fonslow et al., 2011). Protein identification was done with Integrated Proteomics Pipeline (IP2, http://www.integratedproteomics.com/). Tandem mass spectra were extracted to MS2 files from raw files using Raw Extract 1.9.9 (McDonald et al., 2004) and were searched using ProLuCID (Xu et al., 2006) against a Wormbase *C. elegans* protein database (version 197, downloaded 11/26/2008) with reversed sequences appended. The search space included all fully- and half-tryptic peptide candidates. Carbamidomethylation (+57.02146) of cysteine was considered as a static modification. Peptides candidates were filtered to achieve 1% FDR at the protein level, considering only proteins with 3 peptides using DTASelect (Cociorva et al., 2007; Tabb et al., 2002).

RNA isolation and quantitative RT–PCR. Total RNA was isolated from semi-synchronized populations of approximately 3,000 worms. Worms were grown on 15ºC and shifted to 25ºC at the L4 stage for 24 hr. RNA isolation and quantitative RT–PCR was performed as described previously (Vilchez et al., 2012). SybrGreen real-time qPCR experiments were performed with a 1:20 dilution of cDNA using an ABI Prism 79000HT (Applied Biosystems) following the manufacturer's instructions.

Data were analyzed with the Standard Curve method using the geometric mean of *cdc-42, pmp-3* and *Y45F10D.4* as endogenous control (Hoogewijs et al., 2008). Primers for the quantification of *sod-3, mtl-1, hsp12.6* and *daf-16* isoforms have been described previously (Kwon et al., 2010; Li et al., 2008; Panowski et al., 2007).

Reporter assays. *Psod-3::GFP* reporter: For analyzing the effects of reduced IIS animals were shifted to 25ºC at the L4 stage for 24 hr unless stated otherwise. For oxidative stress experiments L1 larval stages were exposed to 0.3 mM paraquat for 48 hr. *Phsp4::GFP* reporter: L3 larval stages were exposed to 25 ng/ml tunicamycin for 4 hr and analyzed 24 hr following treatment. *Phsp16.2::GFP* reporter: Animals were shifted to 33.5ºC at the L4 larval stage for 15 minutes and analyzed 20 hr following heat stress. *Phsp6::GFP* reporter: L3 larval stages were exposed to 0.5 mM paraquat for 24 hr. For testing *math-33* knockdown effects on reporter activity, animals were treated with vector only or *math-33* dsRNA for at least two generations.

RNA sequencing. Worms were grown as semi-synchronized populations at 15ºC and shifted to 25ºC at the L3/L4 stage for 22 hr or kept at 15ºC. Total RNA was isolated from

approximately 3,000 worms using an RNeasy Kit (Qiagen) with in-column DNase digestion. The RNA quality was verified to have an RNA integrity number (RIN) greater than 8.0 through using a Bioanalyzer (Agilent). Stranded mRNA-seq (mRNA sequencing) libraries were prepared from poly(A) RNA after oligo(dT) selection. Sequence reads were generated on an Illumina HiSeq 2500 system in High Output Mode for 100 PE and mapped to an annotated *C. elegans* genome (WS220/UCSC ce10) by using STAR 2.4.0j aligner (Dobin et al., 2013), after removing the low quality reads and the 3'-adaptor sequences. The gene expression levels, represented by fragments per kilo base per million reads (FPKM), were calculated by using HOMER v4.7 (*homer.salk.edu/*). The statistical significance of gene regulation was performed in edgeR, and the genes with FDR<0.05 were considered statistical significant. The heatmaps of FC (fold changes) were illustrated in MeV (Multi Experiment Viewer). A FPKM cut-off of 0.1 was applied to all the samples, and an additional FPKM of 1 was applied to samples originating from *daf-2(ts1370)* animals for visualization purposes.

Immunoprecipitation assays. Transfected HEK293-T cell pellets were lysed in RIPA buffer supplied with a protease inhibitor cocktail (Roche). 1 mg of cell lysates were immunoprecipitated with 1.5 μg of anti-DAF16 (cN-20) antibody (Santa Cruz) overnight at 4°C. Dynabeads Protein G (Life Biotechnology) were added and beads were washed five times with the following buffer: 10 mM Tris pH 7.8, 150 mM NaCl and 0.1% NP40. Bound proteins were eluted by boiling with SDS loading buffer and subjected to immunoblot analysis with anti-FLAG (Sigma Aldrich) or anti-DAF16 antibody (Oh et al., 2006). To perform immunoprecipitation assays in *C. elegans,* animals expressing DAF-16::GFP were grown on 15ºC and shifted to 25°C for 24 hr. Animals were harvested, washed in M9, homogenized in a mortar with liquid nitrogen and resuspended in ice-cold lysis buffer [50

mM HEPES (pH 7.8), 300 mM NaCl, 0.1 mM EDTA, 0.5 mM EGTA, 2 mM MgCl$_2$, 10% glycerol, Complete (Roche), Phosphatase Inhibitors (Roche), 2 mM PMSF, 1 mM β-mercaptoethanol, 20 mM NEM, 50 μM MG132]. Extract was cleared at 20.000 g, salt concentration was diluted to 200 mM NaCl and DAF-16::GFP was immunoprecipitated using a GFP-Trap resin (ChromoTek). The resign was washed 6 times with 50 mM HEPES (pH 7.8), 150 mM NaCl, 0.1 mM EDTA, 0.5 mM EGTA, 2 mM MgCl$_2$, 10% glycerol, 2 mM PMSF, protease inhibitors (aprotinin, leupeptin, pepstatin), 0.5 mM β-mercaptoethanol, 20 mM NEM. Samples were eluted by boiling with SDS loading buffer and subjected to immunoblot analysis with anti-MATH-33 (McCloskey and Kemphues, 2012) or anti-DAF16 antibody (Oh et al., 2006).

Protein expression and purification from insect cells and bacteria. Recombinant baculoviruses expressing GST-3xFLAG-MATH-33 and GST-3xFLAG-MATH-33(C202S) were generated from plasmids described above using the ProGreen expression system (AB vector) and grown according to supplier's guidelines. Full-length GST-3xFLAG-MATH-33 and GST-3xFLAG-MATH-33(C202S) were expressed in Hi5 cells grown in suspension in a medium containing 60% insect-XPRESS (Lonza) and 40% Sf-900 (Life Technologies) supplemented with 10% FBS. Cells were lysed in 50 mM HEPES, pH 7.6, 400 mM NaCl, 1% NP40, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF supplemented with protease inhibitor tablets (Sigma) as described previously (Canning et al., 2004). Proteins were purified using anti-FLAG M2 affinity gel (Sigma) and 50 mM HEPES, pH 7.6, 300 mM NaCl, 5% glycerol, 0.5 mM β-mercaptoethanol, 1 mM PMSF supplemented with protease inhibitor tablets (Sigma) was used for washes. Elution was performed with 100 μg/ml 3xFLAG peptides (Sigma). Recombinant MATH-33 was further purified by size-

exclusion chromatography using a Superdex 200 column (GE Healthcare). The His-tagged catalytic core of USP2 (USP2-cc) was isolated from bacteria and subjected to size-exclusion chromatography.

Ubiquitin-AMC and di-ubiquitin based assays. Deubiquitylating enzyme activity was determined using Ub-AMC (Boston Biochem) as described previously (Dang et al., 1998). 10 nM recombinant MATH-33 and MATH-33(C202S) purified from Hi5 insect cells were assayed with 500 nM Ub-AMC in 50 mM HEPES, pH 7.6, 150 mM NaCl and 2 mM DTT. Fluorescence was monitored by a TECAN Infinite M1000 PRO microplate reader. Di-Ubiquitin hydrolysis reactions were performed at 37ºC using 5 µM K-48 linked di-ubiquitin (Boston Biochem) and 60 nM MATH-33 or MATH-33(C202S) in 50 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM DTT and 5% glycerol.

Detection of DAF-16 ubiquitylation in *C. elegans*: Animals expressing DAF-16::GFP were grown asynchronously at 15°C, shifted for 24 hr to 25°C, and finally shifted back to 15°C for 8 hr. Animals were harvested, washed in M9, resuspended in 50 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM $MgCl_2$, 300 mM KCl, 1 mM EDTA, 10% glycerol, Complete (Roche), 2 mM PMSF, 20 mM NEM, 100 µM MG132, and lysed by bead-beating. NP-40 was then added to a final of 0.1% (v/v). Extract was cleared at 20.000 g and DAF-16::GFP was immunoprecipitated using GFP-Trap resin (Chromotek). Samples were eluted with hydroxyurea buffer at 65°C (Knop et al., 1999) and analyzed by SDS-PAGE and immunoblotting using anti-ubiquitin antibody (FK2, Millipore). Equal amounts of GFP::DAF-16 were loaded to compare ubiquitylation levels of DAF-16.

GST pull-down experiments. Pull-down assays were performed using the TALON Metal Affinity Resin (Clontech) according to manufacturer's instructions. 800 ng of purified, bacterially derived, His-tagged DAF-16 was incubated with 400 ng GST or GST-MATH-33/C202S fusion proteins purified from insect cells and bound to glutathione beads in binding buffer (150 mM HEPES, pH 7.6, 300 mM NaCl, 0.1% NP40, 0.5 mM EGTA, 10% glycerol, 1 mM DTT, 1 mM PMSF supplemented with protease inhibitor tablets) for 2 hr at 4ºC. Beads were washed 5 times with binding buffer and analyzed by SDS-PAGE.

Immunostaining and microscopy. Nematodes were picked as L4 larvae and grown overnight at 25°C. Nematodes were washed by being picked briefly into a water droplet and then transferred to a polylyisine coated slide and compressed with a coverslip. Enough compression was applied to, in some cases, extrude the gonad but to leave the gut intact (Guo and Kemphues, 1995). An affinity purified anti-MATH-33 guinea pig antibody (McCloskey and Kemphues, 2012) and a goat anti-guinea pig Alexa-Fluor 488 were used for immunostaining of MATH-33. A guinea pig anti-DAF-16 antibody (gift from the Sylvia Lee lab, unpublished) and an anti-guinea pig Alexa Fluor 488 antibody (Invitrogen) were used for staining of DAF-16. A goat anti-GFP (Rockland Immunochemicals) and a donkey anti-goat AF488 antibody were used for observing DAF-16::GFP. The nuclear to cytoplasmic ratio was quantified by comparing the mean pixel intensity of anti-MATH-33 fluorescence in a 0.11 x 0.11 μm region over a gut nucleus to a same-size adjacent region outside the nucleus. Images were obtained on a Zeiss 510 microscope with a 63x oil immersion lens or Leica DM5500B microscope with either a 40x (quantification) or a 60x objective.

Oil Red O staining. Oil Red O staining was conducted as described (Noble et al., 2013). Synchronized animals were shifted to the restrictive temperature (25ºC) after reaching the L2 larval stage to avoid dauer formation. Nematodes at the L3/L4 larval stage were used for Oil Red O staining.

Stress Assays. Oxidative stress survival assays were performed as described (Taylor and Dillin, 2013). Nematodes at day 1 of adulthood were transferred to plates containing 40 mM paraquat. Heat-stress and innate immunity assays were conducted as described (Wolff et al., 2006). Heat stress was performed at 35.5ºC.

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