Cell Chemical Biology, Volume *23*

Supplemental Information

Global Cysteine-Reactivity Profiling during Impaired Insulin/IGF-1 Signaling in *C. elegans* Identifies Uncharacterized Mediators of Longevity

Julianne Martell, Yonghak Seo, Daniel W. Bak, Samuel F. Kingsley, Heidi A. Tissenbaum, and Eranthie Weerapana

Supplemental Information

Detailed Experimental Procedures:

C. elegans **culture for MS and RNAi experiments**

Strain Maintenance

Worm strains were grown at 15 °C on OP50 *E. coli*-seeded nematode growth medium (NGM) stock plates using standard *C. elegans* techniques[1]. The following strains were used:

daf16;daf-2**:** DR1309 *daf-16(m26) I; daf-2(e1370) III*
daf-2**:** CB1370 *daf-2(e1370) III*
daf-16**:** GR1307 *daf-16(mgDf50) I*
Wild-type: N2

Strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

Preparation of 4 day old *daf-2* and *daf-16;daf-2* worms for MS analysis

Age-synchronization of worms was done by shaking ~3000 gravid adult worms in a solution of sterile water (5.0 mL), KOH (1.0 mL, 5 M), and bleach (4.0 mL) for 5 minutes until only the eggs remained. The eggs were centrifuged for 30 seconds (4 °C, 2500 rpm), the supernatant was removed and the eggs were washed with S Medium (5 x 10 mL). The eggs were resuspended in S Medium (8 mL) and allowed to hatch overnight in a 15 °C incubator. The next day, the hatched L1 worms (~100,000) were aliquoted onto 10 NGM plates and synchronized growth began with the addition of OP50 *E. coli* (100 µL, 100 mg/mL). The worms were grown at 15 °C until the L4 larval stage where they were transferred to 25 floxuridine-containing plates (FUDR, 0.05 mg/mL) to prevent reproduction. The worms were fed OP50 (150 µL), and moved to a 25 °C incubator. The following day was counted as day 1 of adulthood and the worms were grown until they were 4 days old. Additional OP50 was added daily as needed to prevent starvation. After 4 days, the worms were washed off the plates with PBS and any remaining bacteria, eggs, larva, deceased worms, or debris was removed via sucrose gradient separation: worms were washed with 3 x 5 mL cold 0.1 M NaCl and then suspended in 2.5 mL cold 0.1 M NaCl and 2.5 mL cold 60% sucrose in water. This was spun in a 4 °C centrifuge at 3500 rpm for 5 minutes, allowing the age-synchronized worms to float on the sucrose and pelleting the unwanted debris. The worms were carefully removed, washed with 5 x 5 mL PBS, and stored at -80 °C until lysis. The worms were resuspended in 4 mL PBS, sonicated to lyse, and spun at 5000 rpm for 10 minutes to isolate the protein extracts.

Quantitative mass spectrometry analysis using isotopic azobenzene tags: reactive cysteines in *daf-2*

Click chemistry and streptavidin enrichment of probe-labeled proteins

For each MS sample, *daf-16;daf-2* worm lysates (4 x 500 µL, 2 mg/mL) in PBS were aliquoted into 1.5 mL eppendorf tubes. Two tubes were treated with the high concentration of IA-alkyne (100 µM from 100x stock) and the other two tubes treated with the low concentration of IA-alkyne (10 µM from 100x stock) for 1 hour at room temperature. The heavy azobenzene tag (Azo-H; 100 µM) and light azobenzene tag (Azo-L; 100 µM) were added to the samples treated with 100 µM IA-alkyne and 10 µM IA-alkyne, respectively, and conjugated through click chemistry by the addition TCEP (1.0 mM from fresh 50X stock in water), ligand (100 µM from 17X stock in DMSO:t-Butanol 1:4) and $CuSO_4$ (1.0 mM from 50X stock in water). Samples were allowed to react at room temperature for 1 hour. The tubes

were combined pairwise, centrifuged for 10 minutes (5,900 g at 4 °C) to pellet the precipitated proteins, and resuspended in cold MeOH (500 µL) by sonication. The tubes were again combined pairwise, centrifuged, and washed in MeOH, after which the pellet was solubilized in PBS containing 1.2% SDS via sonication and heating (5 min, 80°C). The SDS-solubilized, probe-labeled proteome samples were diluted with PBS (5 mL) for a final SDS concentration of 0.2%. The solutions were incubated with 100 µL streptavidin-agarose beads (Thermo Scientific) at 4 °C for 16 hrs. The solutions were then incubated at room temperature for 3 hrs. The beads were washed with 0.2% SDS/PBS (5 mL) for 10 mins, PBS (3 x 5 mL), and water (3 x 5 mL). The beads were pelleted by centrifugation (1400 g, 3 mins) between washes.

On-bead trypsin digestion and azobenzene cleavage

The washed beads were suspended in 6 M urea/PBS (500 µL) and 10 mM dithiothreitol (DTT) (from 20X stock in water) and placed in a 65 °C heat block for 15 mins. Iodoacetamide (20 mM, from 50X stock in water) was then added and the samples were placed in a 37 °C incubator and agitated for 30 mins. Following reduction and alkylation, the beads were pelleted by centrifugation (1400 g, 3 min) and resuspended in 200 µL of 2 M urea/PBS, 1 mM CaCl2 (from 100X stock in water), and trypsin (2 µg). The digestion was allowed to proceed overnight at 37 °C. The digested peptides were separated from the beads using a Micro Bio-Spin column (BioRad). The beads were washed with PBS (3 x 500 µL) and water (3 x 500 µL) and subsequently transferred to screw-cap eppendorf tubes. The azobenzene cleavage was carried out by incubating the beads with 50 µL of fresh sodium dithionite in PBS (25 mM) for 1 hour at room temperature on a rotator. After centrifugation, the supernatant was transferred to a new eppendorf tube. The cleavage process was repeated twice more with 75 µL of 25 mM dithionite solution and 75 µL of 50 mM dithionite solution to ensure completion, each time combining the supernatants in the eppendorf. The beads were additionally washed twice with water (75 µL). Formic acid (17.5 µL) was added to the samples and stored at -20 °C until mass spectrometry analysis.

Liquid chromatography-mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed on an LTQ-Orbitrap Discovery mass spectrometer (ThermoFisher) coupled to an Agilent 1200 series HPLC. Peptide digests were pressure loaded onto a 250 µm fused silica desalting column packed with 4 cm of Aqua C18 reverse phase resin (Phenomenex). The peptides were then eluted onto a biphasic column (100 µm fused silica with a 5 µm tip, packed with 10 cm C18 and 3 cm Partisphere strong cation exchange resin (SCX, Whatman)) using a gradient 5-100% Buffer B in Buffer A (Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The peptides were then eluted from the SCX onto the C18 resin and into the mass spectrometer using four salt steps as previously described[2]. The flow rate through the column was set to ~0.25 µL/min and the spray voltage was set to 2.75 kV. One full MS scan (FTMS) (400-1800 MW) was followed by 18 data dependent scans (ITMS) of the n^{th} most intense ions with dynamic exclusion disabled.

MS data analysis - peptide identification

The generated tandem MS data were searched using the SEQUEST algorithm[3] against the Uniprot *C. elegans* database. A static modification of +57.02146 on cysteine was specified to account for alkylation by iodoacetamide and differential modifications of +443.2897 (Azo-L tag) and +449.3035 (Azo-H tag) were specified on cysteine to account for probe modifications. SEQUEST output files were filtered using DTASelect2.0.5 and quantification of light:heavy ratios was performed using the CIMAGE quantification package as previously described[4].

Quantitative mass spectrometry analysis using isotopic azobenzene tags: *daf-2* vs. *daf-16;daf-2*

For each MS sample, *daf-2* and *daf-16;daf-2* worms lysates (2 x 500 µL, 2 mg/mL each) were aliquoted into 1.5 mL eppendorf tubes. The tubes were treated with IA-alkyne (100 µM from 100x stock) for 1

hour at room temperature. The Azo-H (100 μM) was added to the *daf-16;daf-2* lysates, and the Azo-L (100 μM) was added to the *daf-2* lysates and conjugated through click chemistry for 1 hour at room temperature. The rest of the procedure is the same as described above.

RNAi-mediated knockdown experiments

RNAi bacterial culture and RNAi feeding plate preparation

RNAi bacterial clones came from the Ahringer Lab RNAi feeding library (provided by the Tissenbaum Lab), which uses the L4440 vector containing T7 promoters and the TetR gene transformed into HT115 (DE3), an RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity and ampicillin resistance[5]. Frozen stocks from the library were streaked on LB agar plates containing ampicillin (100 μg/mL) and tetracycline (12.5 μg/mL) and grown overnight at 37 °C. Single colonies were inoculated and used to make frozen glycerol stocks from which all RNAi plates were made. Frozen stocks were grown overnight at 37 °C in LB media (3.0 mL) with ampicillin and tetracycline. The overnight cultures (200 μL) were added to LB media (20 mL) with ampicillin only and grown for 6 hours at 37 °C. RNAi plates containing ampicillin and IPTG (1.0 mM) were seeded with this RNAi bacterial culture (~800 μL) and allowed to dry overnight in the dark. RNAi plates used for lifespan assays also contained floxuridine (FUDR, 0.1 mg/mL).

Lifespan assays

Worms were cultured for two generations on RNAi plates at 15 °C. For each lifespan assay, 30 second generation L4 worms were transferred to a new RNAi plates (with FUDR) and moved to 20 °C. After 7 days, the worms were censored for “sick” phenotypes (e.g. vulva bursting) and then scored by gently tapping with a platinum wire every 2-3 days. Worms that crawled off the plate or into mold that was excised out of the agar were censored from the analysis. Four replicates of each plate were performed.

Dauer formation assays

daf-2 worms were cultured for two generations on RNAi plates at 15 °C. For each dauer formation assay, 9 second generation L4 worms from each RNAi plate were transferred to 3 new RNAi plates and allowed to lay eggs overnight. The next day, adult worms were removed from the plates and the remaining eggs were moved to a 22.5 °C incubator. After 4 days, the worms were scored for dauer larva and the percentage of dauer larva was compared to L4440 vector-treated *daf-2* control worms. Several temperatures between 20-25 °C were tested to determine which was most appropriate to cause 10-20% dauer arrest in the control worms (not shown).

Evaluation of mRNA levels in L4440 vector- and RNAi-treated *C. elegans*

RNA Extraction

Worms grown on RNAi plates were removed via washing with DEPC-water into an RNAse-free 1.5 mL eppendorf tube. After the worms were allowed to settle, the supernatant was removed and the worms were washed with 1 mL of DEPC-water and rotated at room temperature for 20 minutes to remove excess RNAi bacteria. This washing step was repeated 4 more times. Excess DEPC-water was removed and TRIzol reagent (1.0 mL) was added, briefly agitated, and allowed to incubate at room temperature for 5 minutes. Chloroform (200 μL) was added to the tube, inverted to mix, let sit at room temperature for 3 minutes, and centrifuged for 15 minutes at 4 °C. The top layer was carefully transferred to another eppendorf tube and isopropanol (400 μL) added, vortexed well, and allowed to sit at room temperature for 10 minutes. The tube was centrifuged for 10 minutes at 4 °C and the supernatant was removed, leaving a white pellet which was then washed with a 75% EtOH in DEPC-water solution (200 μL). The tube was centrifuged for 5 minutes at 4 °C, the supernatant was removed, and the RNA pellet was allowed to air dry for ~10 minutes. The pellet was resuspended in DEPC-water (20-30 μL) and RNA concentrations were determined using the Nanodrop.

cDNA formation

RNA stocks were diluted to 500 ng/µL. DEPC-water (9.5 µL), RNA (1.0 µL, 500 ng/µL = 500 ng), and Oligo-dT's (2.0 µL, 100 µM) were combined in an RNAse-free PCR tube. The tube was incubated at 65 °C for 2 minutes and then chilled on ice for 1 minute. M MuLV Reverse Transcriptase 10x Reaction Buffer (2.0 µL), dNTP mix (2.0 µL, 10 mM), DTT (2.0 µL, 100 mM), RNAse Inhibitor (0.5 µL), and M MuLV Reverse Transcriptase (1.0 µL) were added to the sample giving a total volume of 20.0 µL. The tubes were briefly mixed and then incubated at 37 °C for 60 minutes, then 85 °C for 5 minutes to terminate the reaction. The tubes were allowed to sit on ice for 1 minute, and stored at -80 °C.

RT-PCR

The following primers were designed to evaluate LBP-3, K02D7.1, and PMP-3 mRNA levels. PMP-3 was used a control because of its unusually stable expression levels, with little variation between adults, dauer, and L3 larvae, or between wild-type and *daf-2* or *daf-16* mutants. For each gene, the primers were prepared as a mixture of both the forward and reverse primer (10uM each) in DEPC-water.

lbp-3 forward**:** 5'**-**GCTGCTAAAGGAGTGAGCT-3'
lbp-3 reverse**:** 5'**-**CCATTGTTGACCATTTTCATGAC-3'

pmp-3 forward**:** 5'**-**GGCTAACTTATGAAAGTTCCG-3'
pmp-3 reverse**:** 5'**-**GATGAGTGACTCCAGCAAGT-3'

K02D7.1 forward**:** 5'**-**CAATTCACCAACCAACGCTG-3'
K02D7.1 reverse**:** 5'**-**TGAACCGATACAAATCGGGC-3'

For each sample, DEPC-water (16.8 µL), 5x HF Buffer (5.0 µL), primer mix (1.50 µL), cDNA (1.0 µL), dNTPs (0.5 µL, 10 mM), and Phusion polymerase (0.25 µL). The following PCR conditions were used:

	30 cycles				
Initial	Denature	Anneal	Elongation	Final	
95 °C	95 °C	55 °C	68 °C	72 °C	4 °C
2 mins	15 sec	30 sec	30 sec	10 mins	End

Xylene cyanol (5.0 µL) was added to the samples and each sample (5.0 µL) and the Tri-Dye 100 bp DNA ladder were loaded onto a 2% agarose gel. The gel was run at 155 volts for 15 mins and then visualized under UV light.

References

1. Stiernagle, T., Maintenance of C. elegans. *WormBook : the online review of C. elegans biology* **2006**, 1-11.

2. Weerapana, E.; Speers, A. E.; Cravatt, B. F., Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)--a general method for mapping sites of probe modification in proteomes. *Nature protocols* **2007,** *2* (6), 1414-25.

3. Eng, J. K.; McCormack, A. L.; Yates, J. R., An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *Journal of the American Society for Mass Spectrometry* **1994,** *5* (11), 976-89.

4. Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F., Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **2010,** *468* (7325), 790-5.

5. (a) Timmons, L.; Court, D. L.; Fire, A., Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene* **2001,** *263* (1-2), 103-12; (b) Timmons, L.; Fire, A., Specific interference by ingested dsRNA. *Nature* **1998,** *395* (6705), 854.

Supplemental Figures

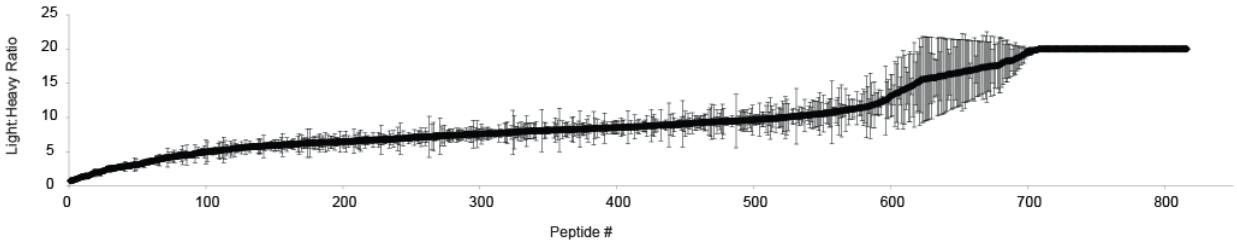

Figure S1. Related to Figure 1 and Table S1. Cysteine reactivity profiling in *C. elegans*. Average heavy:light ratios (R) are shown for 816 cysteine residues identified in four replicates of *daf-16;daf-2* lysates treated with either 10 μM (heavy) or 100 μM (light) IA (Table S1). Error bars represent standard deviation from the mean across the four replicate analyses.

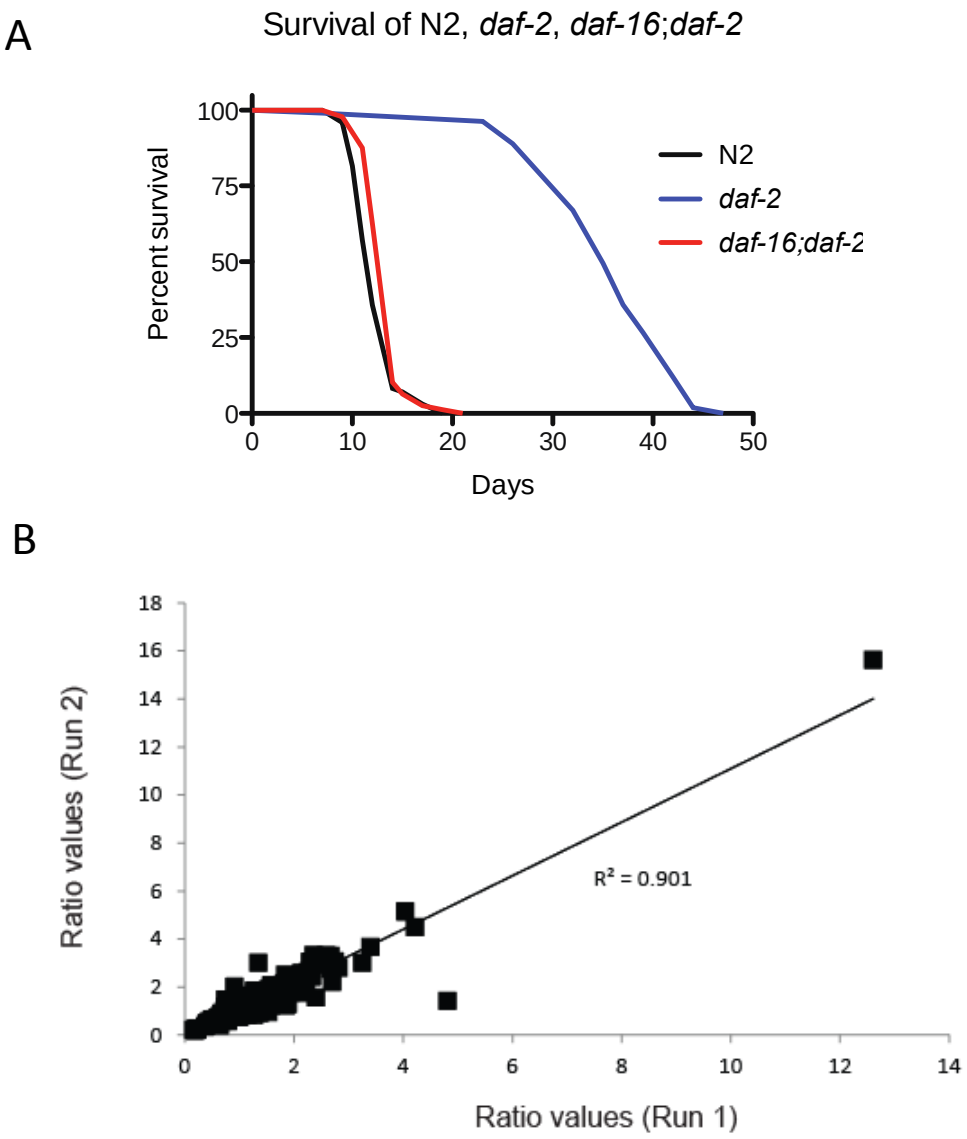

Figure S2. Related to Figure 2 and Table S2. Comparing *daf-2* and *daf-16;daf-2* mutants. (A) Lifespan assays of wild type (N2), *daf-2* and *daf-16;daf-2* mutants. Data obtained from lifespan assays to confirm that *daf-2* mutants show a ~2-fold extension in lifespan relative to *daf-16;daf-2* and N2. (B) Comparing cysteine reactivity in *daf-2* and *daf-16;daf-2* mutants. Correlation of light:heavy ratio (R) values obtained for 338 peptides in two replicates.

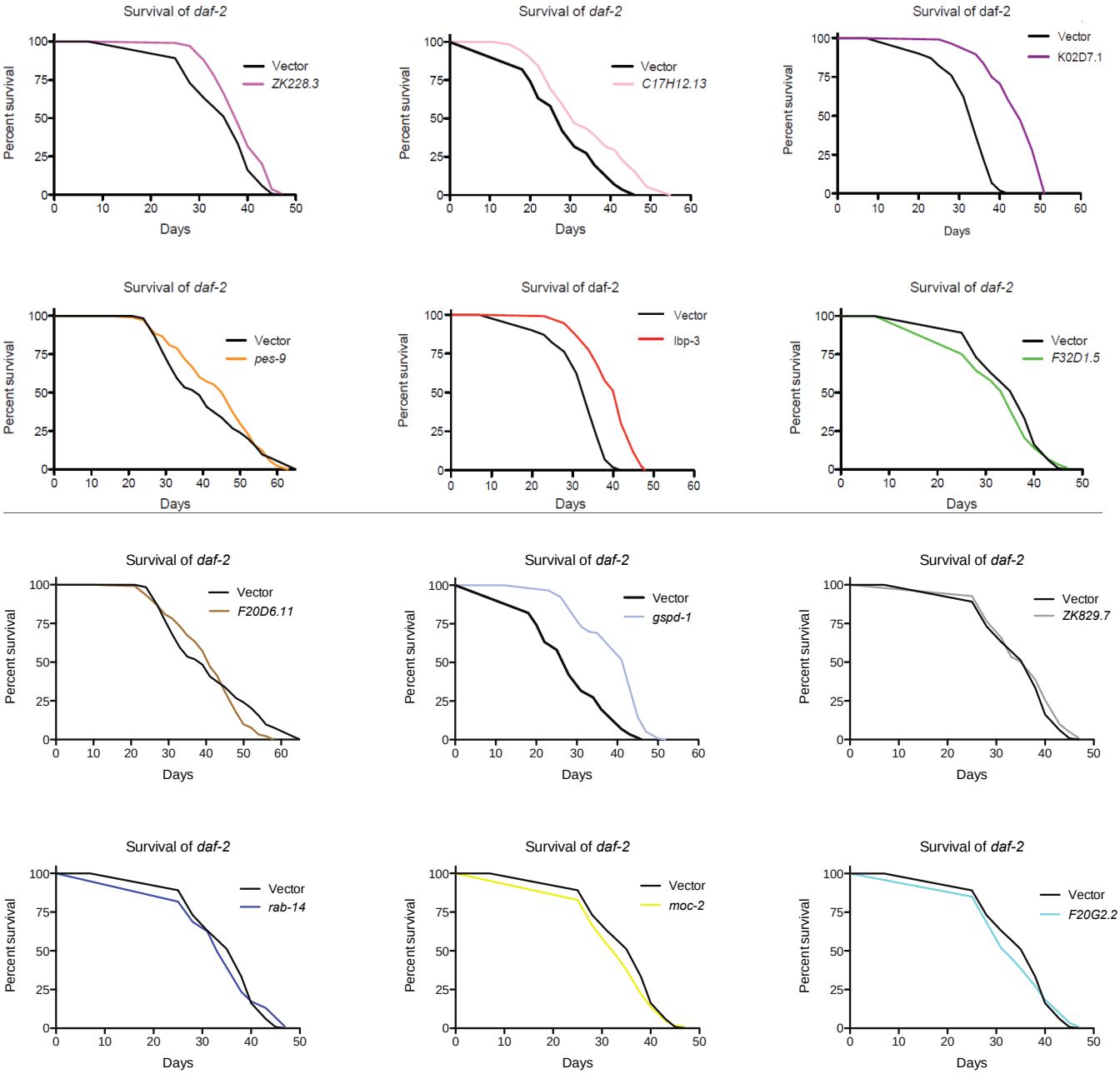

Figure S3. Related to Figure 3. RNAi and lifespan assays. Lifespan assays on RNAi-mediated knockdown of cysteine-containing proteins with the greatest reactivity change between *daf-2* and *daf-16;daf-2* worms. Out of the top 20 proteins with the greatest changes (10 increase, 10 decrease) the Ahringer RNAi library had bacterial strains available to target 17 of them. The 12 proteins with no previous information on the effects their RNAi-mediated knockdown has on longevity were chosen for our lifespan assays.

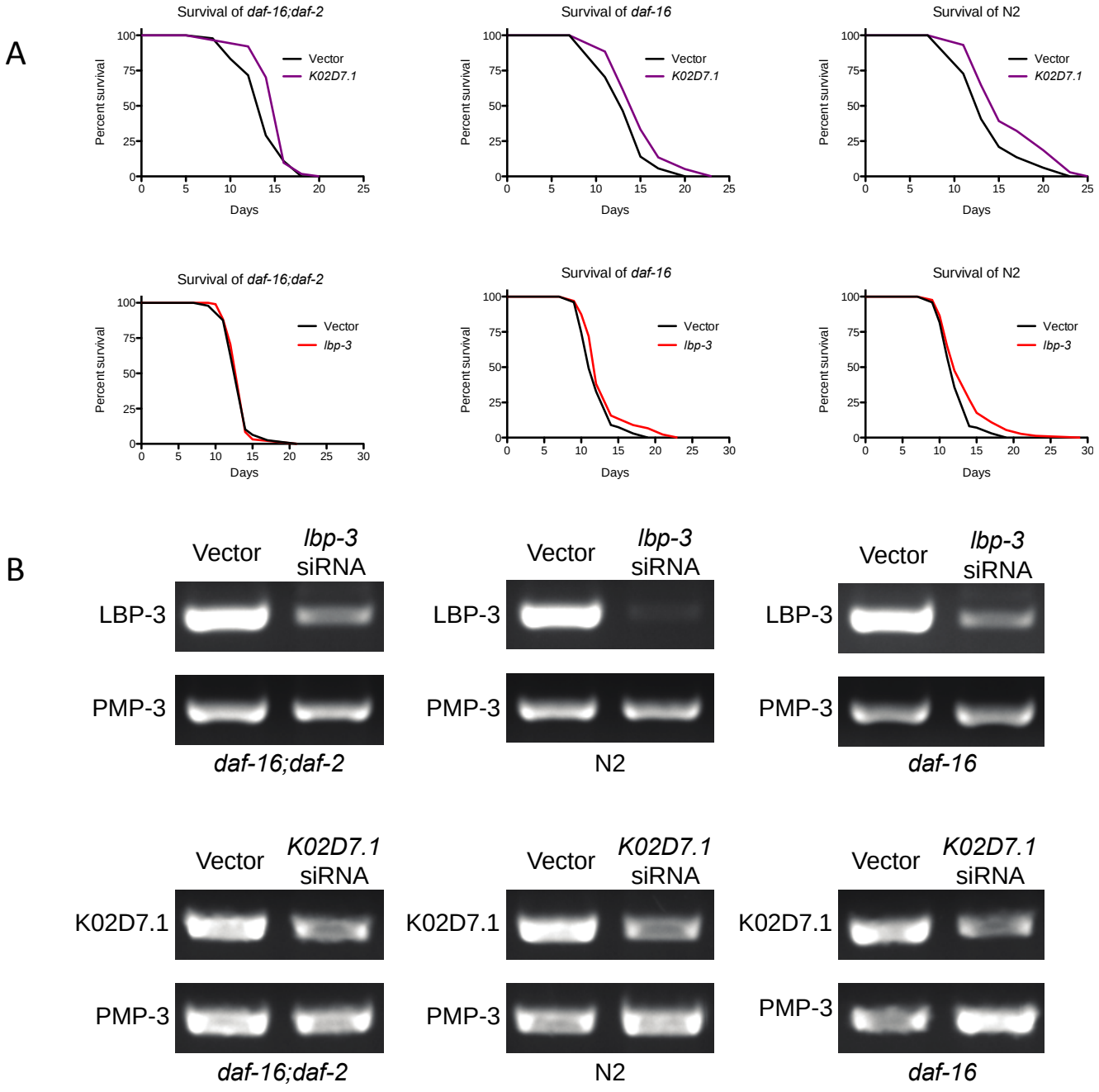

Figure S4. Related to Figure 3. RNAi studies on *lbp-3* and *K02D7.1.* (A) Lifespan assays of RNAi-mediated knockdown of *lbp-3* and *K02D7.1* in the background of *daf-16* mutants, *daf-16;daf-2* double mutants, and wild type (N2) worms. (B) RT-PCR of *daf-16* mutants, *daf-16;daf-2* double mutants, and wild type (N2) worms treated with *lbp-3* or *K02D7.1* siRNA using primers for *lbp-3*, *K02D7.1*, or *pmp-3* as a control.

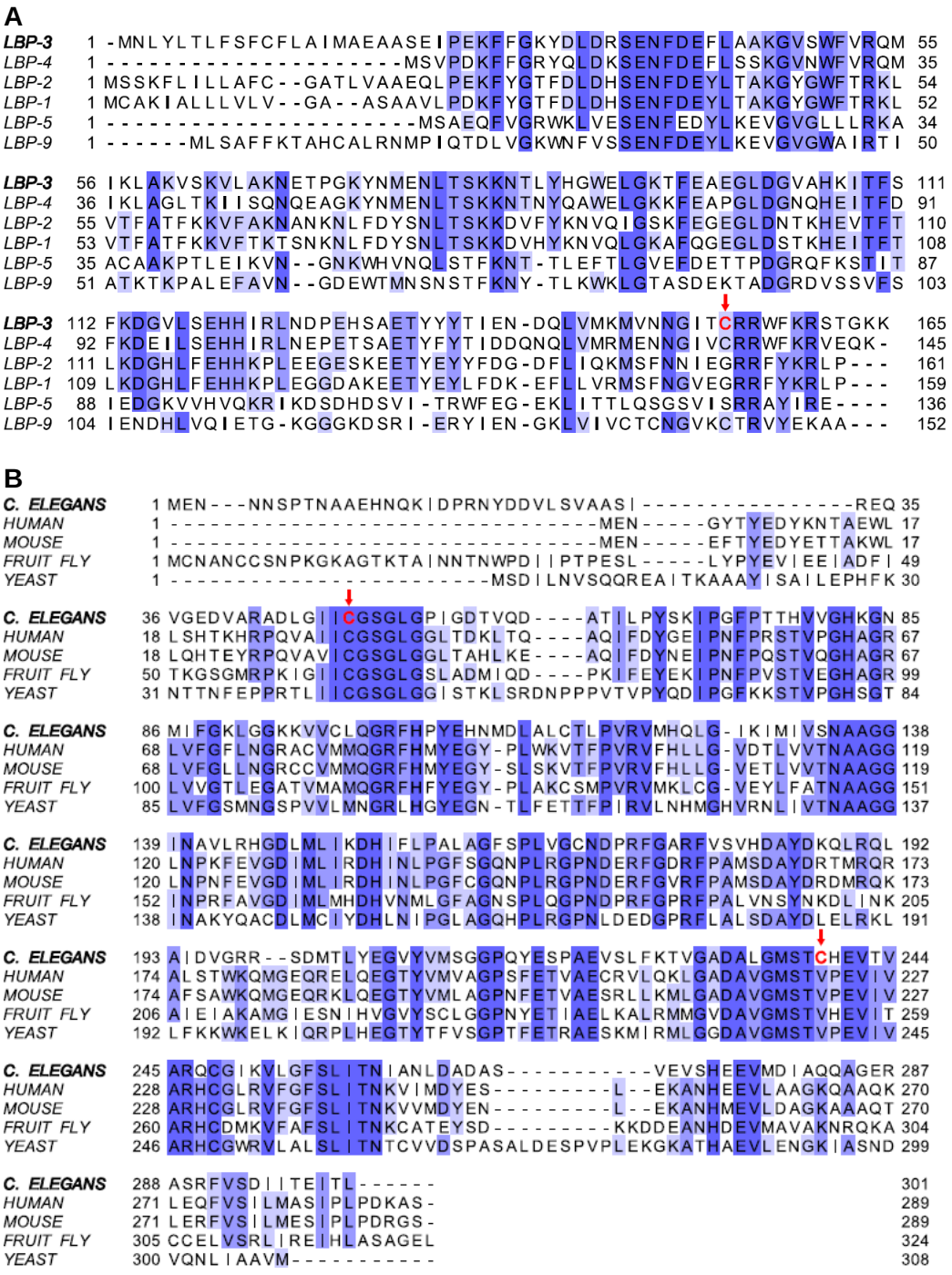

Figure S5. Related to Figure 3. LBP-3 and K02D7.1 (A) Alignment of *C. elegans* LBP-3 with the human FABP family. The reactive cysteine identified in proteomic studies is highlighted in red. (B) Alignment of *C. elegans* K02D7.1 with purine nucleoside phosphorylases from other species. The reactive cysteines identified in proteomic studies are highlighted in red.

Supplemental Tables

Table S1. Related to Figure 1. (attached as an excel file) Cysteine reactivity data. MS analysis showing the 816 cysteine-containing peptides identified in the *daf-16;daf-2* lysates treated with either 10 or 100 μM IA. The cysteines are ranked by reactivity based on their average light:heavy ratio; lower ratios indicate higher reactivity. Identified cysteines with an annotated biological function in *C. elegans* are shown. The closest human homologue for each cysteine-containing protein was determined by performing a BLAST search against the human UniProt database and functional cysteines that are conserved between *C. elegans* and humans are shown. Conservation of the cysteine residue across five species (human, mouse, fly, yeast, mustard) are shown.

Table S2. Related to Figure 2. (attached as an excel file) Comparing *daf-2* and *daf-16;daf-2* lysates. MS analysis showing the 338 cysteine-containing peptides identified in *daf-2* and *daf-16;daf-2* lysates, in order from average light:heavy ratios of < 1 (decreased cysteine reactivity in *daf-2*) to > 1 (increased cysteine reactivity in *daf-2*). All cysteines shown on this table were identified in both biological replicates. Any cysteine with an annotated biological function in *C. elegans* is shown. The closest human homologue for each cysteine-containing protein was determined by performing a BLAST search against the human UniProt database and functional cysteines that are conserved between *C. elegans* and humans are shown. Conservation of the cysteine residue across five species (human, mouse, fly, yeast, mustard) are shown.

Table S3. Related to Figure 2. (attached as an excel file) Comparison to previous redox-proteomics study. Comparison of proteins and cysteines identified in this study to a redox-proteomics study (Kumsta, C., Thamsen, M., and Jakob, U. (2011). Effects of oxidative stress on behavior, physiology, and the redox thiol proteome of Caenorhabditis elegans. Antioxid Redox Signal *14*, 1023-1037). “x” indicates that the cysteine was identified to be oxidized in the Kumsta et al. redox proteomics study.

Table S4. Related to Figure 3. Lifespan and dauer assay data. RNAi was performed on the 12 genes listed and lifespan assays were conducted on all 12 RNAi worms. Median lifespan and % change relative to the corresponding vector control were calculated for each RNAi worm. Please note that these lifespan assays were run in batches, and the vector control showed different median lifespans between batches due to slight variations in growth temperature. For each RNAi worm population the % change in lifespan was calculated based on a vector control cultured within that same batch. All lifespan data was performed as four biological replicates (see detailed data for *lbp-3* and *K02D7.1* in Table S5). RNAi knockdown worms that showed a >15% change in lifespan (*K02D7.1*, *pes-9*, *lbp-3* and *gspd-1*) were subjected to dauer assays and the % change in dauer formation was compared to the corresponding vector control. As before, dauer assays were performed in individual batches resulting in differences between the vector controls. Each RNAi population was compared to the vector control within that batch for determining the % change. Dauer assays were run as three biological replicates.

	Gene	**Median Lifespan**			**Dauer Formation**		
		RNAi	***Vector***	***Change***	***RNAi***	***Vector***	***Change***
Decrease in daf-2	*ZK228.3*	37.4	35.2	6.34%	-	-	-
	C17H12.13	30.3	26.5	14.2%	-	-	-
	K02D7.1	44.4	32.5	36.6%	48.0%	16.4%	31.6%
	pes-9	45.0	38.1	18.3%	24.8%	31.6%	-6.80%
	lbp-3	40.1	32.5	23.4%	29.7%	16.4%	13.3%
	F32D1.5	33.2	35.2	-5.67%	-	-	-
Increase in daf-2	*F20D6.11*	40.4	38.1	6.2%	-	-	-
	gspd-1	41.2	26.5	55.4%	46.3	83.6%	-37.3%
	ZK829.7	35.1	35.2	-0.40%	-	-	-
	Rab-14	32.9	35.2	-6.43%	-	-	-
	Moc-2	32.1	35.2	-8.77%	-	-	-
	F20G2.2	31.6	35.2	-10.3%	-	-	-

Table S5. Related to Figure 3. (attached as an excel file) Lifespan data for *lbp-3* and *K02D7.1* RNAi worms. Data for the number of worms alive on *lbp-3* and *K02D7.1* RNAi plates in each of four replicates for the lifespan assays. Also shown are values for average lifespan of RNAi and vector controls and calculated p-values showing significance.