Stress increases in exopher-mediated neuronal extrusion require lipid biosynthesis, FGF, and EGF RAS/MAPK signaling

Jason F. Cooper, Ryan J. Guasp, Meghan Lee Arnold, Barth D. Grant, and Monica Driscoll

Published September 2, 2021.

In human neurodegenerative diseases, neurons can transfer toxic protein aggregates to surrounding cells, promoting pathology via poorly understood mechanisms. In Caenorhabditis elegans, proteostressed neurons can expel neurotoxic proteins in large, membrane-bound vesicles called exophers. We investigated how specific stresses impact neuronal trash expulsion to show that neuronal exopher production can be markedly elevated by oxidative and osmotic stress. Unexpectedly, we also found that fasting dramatically increases exophergenesis. Mechanistic dissection focused on identifying nonautonomous factors that sense and activate the fasting-induced exopher response revealed that DAF16/FOXO-dependent and -independent processes are engaged. Fasting-induced exopher elevation requires the intestinal peptide transporter PEPT-1, lipid synthesis transcription factors Mediator complex MDT-15 and SREBP-1/SREPB1, and fatty acid synthase FASN-1, implicating remotely initiated lipid signaling in neuronal trash elimination. A conserved lipid synthesis enzymatic pathway that acts downstream of, or in parallel to, lipid signaling also promotes fasting-induced neuronal exopher elevation. A germline-based model of stress proteostasis reveals that fasting-induced exopher production (4). Animals expressing toxic HTTQ128-CFP in touch neurons that extruded aggregates in exophers retained better touch sensitivity than transgenic HTTQ128-CFP animals in which the neurons did not produce exophers (4), suggesting that exopher production that helps clear the neuron of toxic aggregates is neuroprotective, at least in the short term.

A key mystery in the biology of exophers is the precise nature of the cellular conditions and stresses that induce or elevate exopheresis. To address this question, we systematically tested external, physiological stresses for the capacity to influence the expulsion of cellular trash. Our data 1) show a clear link between specific, environmental stresses and neuronal exopher production (namely food withdrawal, osmotic stress, and oxidative stress);

Significance

In neurodegenerative disease, protein aggregates spread to neighboring cells to promote pathology. The in vivo regulation of toxic material transfer remains poorly understood, although mechanistic understanding should reveal previously unrecognized therapeutic targets. Proteostressed Caenorhabditis elegans neurons can concentrate protein aggregates and extrude them in membrane-encased vesicles called exophers. We identify specific systemic stress conditions that enhance exopher production, revealing stress-type, stress-level, and temporal constraints on the process. We identify three pathways that promote fasting-induced exopheresis: lipid synthesis, FGF/RAS/MAPK, and EGF/RAS/MAPK. In establishing an initial molecular model for transistissue requirements for fasting-induced exopher elevation in neurons, we report molecular insights into the regulation of aggregate transfer biology, relevant to the fundamental mysteries of neurodegenerative disease.

Author contributions: J.F.C., R.J.G., M.L.A., B.D.G., and M.D. designed research; J.F.C., R.J.G., and M.L.A. performed research; J.F.C., R.J.G., and M.L.A. contributed new agents/analytic tools; J.F.C., R.J.G., M.L.A., B.D.G., and M.D. analyzed data; and J.F.C., R.J.G., B.D.G., and M.D. wrote the paper. The authors declare no competing interest.

This article is a PNAS Direct Submission. This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence may be addressed. Email: driscoll@biology.rutgers.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2101410118/-/DCSupplemental.

PNAS 2021 Vol. 118 No. 36 e2101410118

https://doi.org/10.1073/pnas.2101410118
20) emphasize that there exists a “permissive” window for exopher production in early adult life during which external stresses can elevate exopher levels; and 3) identify a previously unrecognized level of stress that can induce exophers, such that, beyond this upper stress limit, exopher genesis is not observed. We quantitated exopher production when mCherryAg2 animals were fed four different E. coli strains: E. coli OP50, which is the standard C. elegans food source (12); HT115, a strain that lacks RNAaseIII and is used in RNAi studies (13); HB101, a food source that promotes larger body size and faster development (14); and NA22, a strain that fosters enhanced growth in liquid culture (15). We noted no major differences among the different strains in common laboratory use, all of which support the transcription factors MDT-15 and SBP-1/SREBF2, and their target FASN-1/fatty acid synthase, as well as to epidermal growth factor (EGF) and fibroblast growth factor (FGF)/RAS/MAPK signaling pathways. Our data reveal how stress conditions might promote the spread of aggregates from neurons to their neighbors and suggest pathways that might be targeted to regulate analogous processes, with implications for addressing human neurodegenerative disease.

Results

Food Quality and Food Availability Dramatically Influence Exopher Production. A common stress that C. elegans encounter in their natural environment is variable food type and/or food abundance, which can have a significant impact on C. elegans gene expression, development, metabolism, and longevity (7–9). In the laboratory, C. elegans eat a diet of Escherichia coli spread on agar plates, and food sources can be easily manipulated. We first asked whether neuronal exopherogenesis levels are sensitive to food source, quantitating ALMR exophers produced by neurons expressing an mCherry protein (strain ZB4065 bzIs166[P_meca-mCherry1]), which is avidly expelled as exopher cargo (10) (see Fig. 1 for an example). For clarity’s sake we refer to assay strain ZB4065 as mCherryAg2 in the text hereafter. Exophers are typically produced by young adult animals, peaking at days 2 to 3 of adulthood and returning close to baseline by adult day 4 (Ad4) (4, 11). We therefore measured exophers produced each day, Ad1 to Ad4, to compare both daily relative levels and temporal profiles.

We quantitated exopher production when mCherryAg2 animals were fed four different E. coli strains: E. coli OP50, which is the standard C. elegans food source (12); HT115, a strain that lacks RNAaseIII and is used in RNAi studies (13); HB101, a food source that promotes larger body size and faster development (14); and NA22, a strain that fosters enhanced growth in liquid culture (15). We noted no major differences among the different E. coli strains in common laboratory use, all of which support the basic pattern of peak exopher production around Ad2 that returns to the baseline around Ad4 (SI Appendix, Fig. S1A).

Complete food withdrawal markedly induces exophers. We reared mCherryAg2 animals at 20 °C and at Ad1 (onset of egg laying) moved animals to unseeded plates at 20 °C for either 3 or
6 h, counting ALMR exophers shortly thereafter. Continuously fed control mCherryAg2 animals generate exophers at levels that range from 5 to 20% of animals, and in this study, baseline was close to 5% on Ad1. Subsequent to a 3-h fast, we found exophers in more than 20% ALMRs; a 6-h fast increased average exopher numbers ~5- to 10-fold to nearly 50% of ALMRs examined (Fig. 1B). Fasting also causes an increase in multiple-exopher events, in which more than one exopher is generated (SI Appendix, Fig. S1B). We conclude that food withdrawal can have a rapid and dramatic impact on the extrusion of exophers in animals expressing a noxious mCherry reporter.

**Fasting Modulates Exophogenesis in Early Adult Life.** At 20 °C, in the presence of food, the young adult wave of exopher production falls narrowly within the first 4 d of adulthood (SI Appendix, Fig. S1A). We were therefore curious as to whether the fasting-induced elevation of exophers was restricted to the Ad1 to Ad4 timeframe or, alternatively, whether food withdrawal experienced at any time point could be effective for inducing exopher production. We subjected animals from a single, synchronized population to 6-h fasting regimens as L4 larvae as well as on Ad1 to Ad10; we were curious as to how exopher production might influence the timing or levels of adult exopher production. We reared synchronized L4 stage into three parallel cultures that were thereafter subjected to a controlled, hypoxic environment. We subjected Ad1 to Ad5 mCherryAg2 animals to 0.1% oxygen for 6 h and monitored ALMR exophers after the removal of animals from the chamber.

We raised animals under standard growth conditions until the L4 stage and shifted cultures to paraquat plates, measuring 250 mM concentrations of sucrose, glucose, sorbitol, and NaCl. At Ad1, we introduced mCherryAg2 animals to these osmotic stresses for 6 h and scored ALMR exophers thereafter (Fig. 2A). We found that a 6-h exposure to osmotic stress increased exopher production ~4x above baseline. Since each solute-induced stress resulted in a similar elevation of exophers, the exopher response is likely grounded in osmotic stress itself, rather than the chemical nature of the specific osmolytes.

We also quantitated exopher levels under conditions of long-term, hyperosmotic stress during adult life (Fig. 2B). We raised animals under standard growth conditions until the L4 stage and then moved animals to 250 mM sucrose, glucose, sorbitol, or NaCl plates for adult life. We first measured exopher levels 24 h after initiating osmotic stress and tested the same population on Ad1 to Ad4 thereafter. For all solutes tested, osmotic stress resulted in an ~5x increase in exophers over the baseline at peak and shifted the measured peak of exopher onset forward 1 d. Notably, exopher levels returned to baseline at Ad4, even in the presence of osmotic stressors. We conclude that both transient and extended exposure to hyperosmotic stress can elevate exopher production substantially in early adult life and infer that hyperosmotic conditions rapidly generate a trigger that elevates exophers.

**Osmotic Stress and Oxidative Stress Can Induce Exopher Production.** Osmotic stress introduces proteostasis challenges (20), and thus, we were curious as to how exoper production might respond to osmotic stress conditions. We first made agar plates designed to introduce osmotic stresses based on standard C. elegans conditions, testing 250 mM concentrations of sucrose, NaCl, and glucose. We subjected Ad1 to Ad5 mCherryAg2 animals to these osmotic stresses for 6 h and scored ALMR exophers thereafter (Fig. 2A). We found that a 6-h exposure to osmotic stress increased exopher production ~4x above baseline. Since each solute-induced stress resulted in a similar elevation of exophers, the exopher response is likely grounded in osmotic stress itself, rather than the chemical nature of the specific osmolytes.

We also quantitated exopher levels under conditions of long-term, hyperosmotic stress during adult life (Fig. 2B). We raised animals under standard growth conditions until the L4 stage and then moved animals to 250 mM sucrose, glucose, sorbitol, or NaCl plates for adult life. We first measured exopher levels 24 h after initiating osmotic stress and tested the same population on Ad1 to Ad4 thereafter. For all solutes tested, osmotic stress resulted in an ~5x increase in exophers over the baseline at peak and shifted the measured peak of exopher onset forward 1 d. Notably, exopher levels returned to baseline at Ad4, even in the presence of osmotic stressors. We conclude that both transient and extended exposure to hyperosmotic stress can elevate exopher production substantially in early adult life and infer that hyperosmotic conditions rapidly generate a trigger that elevates exophers.
at Ad7. Thus, paraquat-mediated oxidative stress can extend the time period of permissive exophergenesis by 2 d, raising the possibility that paraquat might induce signals that normally promote exopher production. Overall, data from three mitochondrial ROS-generating compounds indicate that chemically-induced oxidative stress can increase exopher formation.

Excessive Stress Decreases Exopher Production. While initially testing the ability of stressors to modulate exopher production, we often utilized a dose–response approach. Our studies revealed a striking commonality regardless of stressor type: excessive stress suppresses exopherogenesis. For example, 6-h exposure of 240 μM juglone (Fig. 2D), 25 μM rotenone (Fig. 2E), or 25 mM paraquat (Fig. 2F) reduces exopher levels, even though lower levels of these ROS stressors enhance exopher production. The same pattern emerges under osmotic stress conditions; 6-h exposure to 500 mM concentrations of sucrose, glucose, sorbitol, and NaCl suppresses exopher levels, compared to 250 mM concentrations of each of these solutes (Fig. 2C). Although at Ad1 ALMR exophers modestly increase with 6-h exposures to increasing temperatures up to 30 °C, 6 h at 37 °C causes a collapse in exophergenesis (SI Appendix, Fig. S2F).

We generated additional evidence in support of the idea that excessive stress can inhibit exopher formation by exposing Ad1 mCherryAg2 animals to combined two-stress conditions that, by themselves, individually enhance exopher production. For example, whereas a 6-h fast elevates exophers (Figs. 1–F and 2G), cointroducing osmotic stress with fasting (which also normally elevates exophers; Fig. 2 A and B) suppresses exopher levels (Fig. 2G). We also found a combined inhibitory effect for fasting + oxidative stress (Fig. 2H).

Furthermore, temperature (SI Appendix, Fig. S2G) and anoxia (Fig. 2I and SI Appendix, Fig. S2H), stresses that did not affect exophergenesis on their own (SI Appendix, Fig. S2 A, B, and D), could suppress the effects of fasting on exopher production. Data suggest that under extreme stress neurons either cannot meet molecular requirements for exopher production or might enact mechanisms that actively suppress exophergenesis (see Discussion).

In summary, food withdrawal, osmotic stress, and oxidative stress can enhance exopher production, although above specific
The Robust Increase in Exophers in Response to Food Withdrawal Occurs by DAF-16/Foxo-Dependent and DAF-16/Foxo-Independent Mechanisms. With a goal of defining molecular mechanisms by which stresses elevate neuronal exopher production, we sought to define genetic requirements for the fasting-dependent induction response. We elected to focus on the fasting response because of the robust and highly reproducible level of induction associated with 6 h food withdrawal (Fig. 1 B and C), combining genetic mutant and RNAi strategies. To probe the mechanism by which fasting elevates neuronal exopher production, we first tested mutants for well-characterized, stress-activated transcription factors: heat shock factor 1 hsf-1/HSF1, required for transcription of heat shock responder genes and proteostasis (22); hypoxia inducible factor hif-1/HIF1, required for hypoxia stress responses (23); hli-30/TFEB, required for starvation resistance and lysosomal integration with metabolism (24); skn-1/NRF2, which promotes response to oxidative stress and xenobiotic challenge (25, 26); and daf-16/FOXO, which is activated by low-nutrient pathway signaling and functions in a range of stress-protective responses, including proteostasis (27).

We constructed mCherryAg2 strains with viable mutant alleles of each transcription factor and subjected mutants to 6 h food deprivation on Adl, measuring ALMR exophers therefrom (Fig. 3A). We observed the significant elevation of exophergenesis in hsf-1, hsf-1, hli-30, and skn-1 backgrounds in response to food withdrawal, indicating that these transcription factors are not critical for the induction of exophers in response to fasting. In contrast, the daf-16 null mutant exhibited a partial defect in the food withdrawal response, with exopher levels clearly increasing over baseline in the absence of food (P < 0.001) but never reaching the levels observed in wild-type (WT) animals (P < 0.001) (Fig. 3A). The partial effect in the daf-16 null mutant background is consistent with a model in which a daf-16-dependent process mediates one component of the fasting response but that a daf-16-independent process works in parallel to elevate exophers when food is withdrawn.

We also tested whether autophagy, a pathway activated by food limitation, might be critical for the fasting-induced increase in exophers. We used RNAi approaches to knockdown autophagy genes lgg-1, aeg-7, and bcc-1 in mCherryAg2 animals and scored for exophers on Adl (SI Appendix, Fig. S3A). Since all three disruptions in the autophagy pathway failed to suppress fasting-induced exopher elevation, we infer that engagement of autophagy functions is not required for fasting-induced exopher increase. Pharmacological inhibition of autophagy with 1 mM spautin or of the proteosome with 10 mM MG132, combining genetic mutant and RNAi strategies. To probe the mechanism by which fasting elevates neuronal exopher production, we first tested mutants for well-characterized, stress-activated transcription factors: heat shock factor 1 hsf-1/HSF1, required for transcription of heat shock responder genes and proteostasis (22); hypoxia inducible factor hif-1/HIF1, required for hypoxia stress responses (23); hli-30/TFEB, required for starvation resistance and lysosomal integration with metabolism (24); skn-1/NRF2, which promotes response to oxidative stress and xenobiotic challenge (25, 26); and daf-16/FOXO, which is activated by low-nutrient pathway signaling and functions in a range of stress-protective responses, including proteostasis (27).

We constructed mCherryAg2 strains with viable mutant alleles of each transcription factor and subjected mutants to 6 h food deprivation on Adl, measuring ALMR exophers therefrom (Fig. 3A). We observed the significant elevation of exophergenesis in hsf-1, hsf-1, hli-30, and skn-1 backgrounds in response to food withdrawal, indicating that these transcription factors are not critical for the induction of exophers in response to fasting. In contrast, the daf-16 null mutant exhibited a partial defect in the food withdrawal response, with exopher levels clearly increasing over baseline in the absence of food (P < 0.001) but never reaching the levels observed in wild-type (WT) animals (P < 0.001) (Fig. 3A). The partial effect in the daf-16 null mutant background is consistent with a model in which a daf-16-dependent process mediates one component of the fasting response but that a daf-16-independent process works in parallel to elevate exophers when food is withdrawn.

We also tested whether autophagy, a pathway activated by food limitation, might be critical for the fasting-induced increase in exophers. We used RNAi approaches to knockdown autophagy genes lgg-1, aeg-7, and bcc-1 in mCherryAg2 animals and scored for exophers on Adl (SI Appendix, Fig. S3A). Since all three disruptions in the autophagy pathway failed to suppress fasting-induced exopher elevation, we infer that engagement of autophagy functions is not required for fasting-induced exopher increase. Pharmacological inhibition of autophagy with 1 mM spautin or of the proteosome with 10 mM MG132 (SI Appendix, Fig. S3B) did not block the fasting-induced elevation of exophers, in further support that autophagy and proteosome contributions are not critical for fasting-induced exopher increase.

PEPT-1 Intestinal Di-/Tripeptide Transporter, Mediator Complex MDT-15 and Binding Partner SBP-1, and Lipid Synthesis Gene FASN-1 are Required for Neuronal Exopher Elevation by Fasting. To better characterize the pathways involved in fasting-induced exopher production, we compiled a list of known genes implicated in starvation and feeding in the C. elegans literature and screened for fasting-induced exopher elevation when the candidate genes were knocked down using RNAi (28) (see SI Appendix, Table S1 for a list of candidates tested). Note that the strain we targeted with feeding RNAi, mCherryAg2, should permit efficient RNAi knockdown in all tissues except neurons, because neurons do not express a double-stranded RNAi transporter, sid-1, required for the efficacious knockdown in feeding RNAi (29). Utilizing this experimental design, we expected to identify genes operative in the nonautonomous initial events in the sensation and signaling of fasting stress to the touch neurons, rather than genes involved in neuron-intrinsic exophergenesis.

We tested positive clones from the first round of the RNAi screen in tripllicate to identify intestinal peptide transporter pept-1, lipid synthesis-implied Mediator complex factor mdt-15, MDT-15 binding partner sbp-1/SREBF2, and fatty acid synthase fasn-1, as required for robust exopher elevation in response to fasting (Fig. 3B).

pept-1 encodes a conserved intestinal di-/trimino acid transporter implicated in C. elegans nutrient sensing (30). Loss of the pept-1 function increases the intestinal absorption of free fatty acids from ingested bacteria, such that short- and medium-chain fatty acids are highly increased in the mutant, and de novo synthesis of long-chain and polyunsaturated fatty acids is greatly decreased (31). Our data suggest that sudden withdrawal of food causes a metabolic reconfiguration that signals for enhanced exopher production and that the sensing of food limitation requires PEPT-1 transporter activity.

MDT-15 has been shown to promote health and longevity by orchestrating many of the metabolic changes that occur in response to short-term fasting (32). MDT-15 encodes a subunit of the transcriptional coregulator Mediator complex that is required to express fatty acid metabolism genes and fasting-induced transcripts (32–35), heavy metal and xenobiotic detoxification genes (32, 36), and oxidative stress genes (37). SBP-1, the homolog of the mammalian sterol regulatory element-binding protein (SREBF2) transcription activator that regulates fatty acid homeostasis, is a known partner of MDT-15, and together, MDT-15 and SBP-1 promote the expression of lipid synthesis genes (32). FASN-1 encodes the sole C. elegans fatty acid synthase, and its expression is regulated by MDT-15/SBP-1 (38, 39). Together, MDT-15, SBP-1, and FASN-1 may act to promote the synthesis of a lipid-based factor that signals for, or is otherwise required for, neuronal exopher production under fasting stress.

Expression of lipid synthesis genes in multiple tissues may contribute to fasting-induced exopher elevation. To address where the lipid synthesis gene group required for fasting-induced exopher elevation acts, we took a tissue-specific RNAi knockdown approach. We worked with strains that expressed mCherryAg2 in touch neurons but were defective in either the double-stranded RNA (dsRNA) transporter sid-1 (neurons, muscle, pharynx, intestines, and hypodermis) or RISC complex factor rde-1 (germline and vulva), and we reintroduced sid-1 or rde-1 using tissue-specific promoters to drive expression and restore RNAi knockdown capability only in the rescued tissue. The promoters we used to restore expression (and therefore RNAi targeting) in specific tissues were the following: pan-neuronal npef-1, muscle myo-3, pharynx myo-2, intestine vha-6, hypodermis hyp7 semo-1, vulva lin-31, and germline sun-1. This test set of seven tissue-specific RNAi lines enabled us to target most cells of the animal. We fasted animals to ask whether RNAi disruption in individual tissues is sufficient to disrupt the exopher induction response, which would indicate that expression in that targeted tissue is necessary in the response. We found that although whole body knockdown of pept-1, mdt-15, sbp-1, and fasn-1 could disrupt the fasting-induced exopher elevation (Fig. 3B), no tissue-specific knockdown was effective in blocking this response (SI Appendix, Fig. S4A). Data are consistent with a model in which multiple tissues can contribute the required lipid biosynthesis, although we cannot rule out that RNAi targeting was ineffective or missed necessary cells.

An FGF-Activated RAS/ERK Pathway Acts in the Fasting-Induced Transsolute Induction of Neuronal Exophers. Food limitation stresses engage multiple signaling pathways that can activate animal defenses, including RAS/MAPK pathways that transduce developmental...
Lipid synthesis and FGF-activated RAS/ERK signaling are required for the induction of neuronal exophers in response to fasting. For all panels, bars are SEM, ***P < 0.001, **P < 0.01, *P < 0.05, and CMH statistics. Feeding RNAi was initiated at the L4 larval stage and continued until Ad2. (A) The daf-16 null mutant has diminished exopher production in response to fasting. We tested mutants defective in major stress-responsive transcription factors (daf-16/FOXO, has-1/HSF-1, hif-1/HIF1, hhl-30/TFE3 and skn-1/NRF2) in the mCherryA2 background, Ad2: exopher counts 6x trials, 50 animals per trial, and CMH difference between WT control and daf-16 deletion mutant ***P < 0.001. (B) Lipid synthesis is implicated in the fasting-induced boost in exophogenesis. RNAi knockdown of pept-1, mdt-15, sbp-1, and fasn-1 beginning at L4 in the mCherryAg2 strain, 6 h fast followed by exopher assay on Ad2, control is empty vector RNAi. We compared WT fed versus fasted P < 0.001 CMH, the others are not significant: 3x trials and 50 animals per trial. (C) Schematic of C. elegans MAPK signaling pathways targeted in genetic tests for fasting-induced exopher elevation: p38 MAPK signaling (purple), JUN/FOS MAP kinase signaling (orange), EGF-mediated MAPK signaling (green), and FGF-mediated MAPK signaling (blue). Black boxes highlight a common function in both EGF and FGF MAPK signaling. (D) RNAi knockdown of most components of the p38 and JUN/FOS signaling cascades does not impair fasting-induced exopher increases. The strain was mCherryAg2; RNAi was initiated at the L4 stage; and 6 h fast was followed by exopher counts at Ad2: 3x trials and 50 animals per trial. mek-1(RNAi) stood out as exceptional in failing to induce exophers. (E) Loss-of-function mutants for JUN/FOS signaling do not suppress exopher production. kgb-1; mCherryAg2 and jun-1; mCherryAg2 mutants were fasted for 6 h at Ad2 before exopher counts: 5x trials and 50 animals per trial. (F) An egl-17/FGF–mediated MAPK signaling cascade is necessary for a fasting-induced increase in exopher production. RNAi for the indicated genes was initiated at the L4 stage on strain mCherryAg2, and at Ad2, animals were fasted 6 h and then scored for exophers. RNAi knockdown of let-23, ksr-2, and FGF ligand let-756 did not disrupt the fasting exopher response 3x trials and 50 animals per trial.
and stress responses to activate specific transcription programs (40, 41). We tested members of three canonical, well-characterized *C. elegans* MAPK signaling pathways—the PMK-1/p38 pathway that functions in some innate immunity and oxidative stress responses; the JNK pathway, which, among other activities, functions in intermittent fasting programs (42); and the RAS/ERK pathway, which, among other things, affects vulval precursor fate and vulval development response to starvation stress (43) (pathways summarized in Fig. 3C).

The core MAP kinases in the p38 pathway are *nsy-1*/MAPKKK and *pmk-1*/MAPK (40). RNAi directed against *nsy-1* and *pmk-1* genes was ineffective in blocking the fasting induction of neuronal exophers (Fig. 3D), and thus, we do not find evidence supporting the engagement of the *pmk-1*/p38 stress pathway in fasting-induced exopher elevation.

In *C. elegans*, an adult intermittent fasting protocol of 2 d without food followed by 2 d of food extends lifespan via an MLK-1, MEK-1, and KGB-1 JNK pathway that converges on AP-1 (JUN-1, FOS-1)—mediated transcription (42). We find that *kgb-1* (RNAi), *kgb-1*, and *jun-1* genetic mutations do not block the fasting-induced elevation of exopher production (Fig. 3D and E). This result, coupled with published data examining a transcriptional time course following food withdrawal that revealed a distinct transcription pattern for 3 to 6 h after fasting, as compared to the more chronic starvation of 9 h and longer (44), suggest a prolonged response to short-term food withdrawal that we characterize here does not operate via the characterized, intermittent fasting pathway. Still, the positive *mek-1*/MAPKK and partial *mlk-1*/MAPK outcome (*mlk-1*/RNAi) fasted versus WT fasted, not significant; *mlk-1* (RNAi) fasted versus *mlk-1* (RNAi) fed, *P* < 0.01 (Fig. 3D), suggest possible pathway involvement or cross-talk involving these kinases. As evidence for more definitive engagement of the RAS/ERK pathway was evident in our studies (Fig. 3F), we focused on examining MAP kinases in more detail.

RAS/ERK signaling in *C. elegans* (reviewed in refs. 40 and 41; see Fig. 3C) can involve the EGF or FGF activation of receptor tyrosine kinases that interact with adaptor proteins such as SEM-5/GRB2 or SOC-1/GAB1, which recruit guanine nucleotide exchange factor SOS-1 to activate small GTPase LET-60/RAS. LET-60 interacts with GRB2 or SOC-1/GAB1, which recruit guanine nucleotide exchange factors that interact with adaptor proteins such as SEM-5/see Fig. 3.

The core MAP kinases in the p38 pathway are *nsy-1*/MAPKKK and *pmk-1*/MAPK (40). RNAi directed against *nsy-1* and *pmk-1* genes was ineffective in blocking the fasting induction of neuronal exophers (Fig. 3D), and thus, we do not find evidence supporting the engagement of the *pmk-1*/p38 stress pathway in fasting-induced exopher elevation.

In *C. elegans*, an adult intermittent fasting protocol of 2 d without food followed by 2 d of food extends lifespan via an MLK-1, MEK-1, and KGB-1 JNK pathway that converges on AP-1 (JUN-1, FOS-1)—mediated transcription (42). We find that *kgb-1* (RNAi), *kgb-1*, and *jun-1* genetic mutations do not block the fasting-induced elevation of exopher production (Fig. 3D and E). This result, coupled with published data examining a transcriptional time course following food withdrawal that revealed a distinct transcription pattern for 3 to 6 h after fasting, as compared to the more chronic starvation of 9 h and longer (44), suggest a prolonged response to short-term food withdrawal that we characterize here does not operate via the characterized, intermittent fasting pathway. Still, the positive *mek-1*/MAPKK and partial *mlk-1*/MAPK outcome (*mlk-1*/RNAi) fasted versus WT fasted, not significant; *mlk-1* (RNAi) fasted versus *mlk-1* (RNAi) fed, *P* < 0.01 (Fig. 3D), suggest possible pathway involvement or cross-talk involving these kinases. As evidence for more definitive engagement of the RAS/ERK pathway was evident in our studies (Fig. 3F), we focused on examining MAP kinases in more detail.

RAS/ERK signaling in *C. elegans* (reviewed in refs. 40 and 41; see Fig. 3C) can involve the EGF or FGF activation of receptor tyrosine kinases that interact with adaptor proteins such as SEM-5/ GRB2 or SOC-1/GAB1, which recruit guanine nucleotide exchange factor SOS-1 to activate small GTPase LET-60/RAS. LET-60 activates ERK, which enter the nucleus to phosphorylate transcription factors that execute phosphorylation and activates MPK-1/ERK. MPK-1 can then phosphorylate and activate MPK-1/ERK. MPK-1 can then phosphorylate and activate MPK-1/ERK. MPK-1 can then phosphorylate and activate MPK-1/ERK. MPK-1 can then

Lipid Biosynthesis Acts Upstream of RAS/ERK Activation to Stimulate Exopher Induction. *

let-60(n1046gf)* is a well-characterized, gain-of-function RAS allele that constitutively activates MAPK signaling (45). Our RNAi data, identifying the FGF and EGF/RAS/MAPK pathways as required for fasting-induced exopher induction, predict that the *let-60(gf)* allele should induce higher exopher levels, even in the absence of starvation. To test this model, we constructed a *let-60(n1046gf)*;mCherryAg2 strain and measured exopher levels at Ad2. We find that exophers are indeed elevated in the *let-60(n1046gf)* background in the absence of fasting, confirming that RAS activation enhances exopher production (Fig. 4B). *let-60(n1046gf)* has been used extensively in epistasis pathway ordering (45), and thus we pursued an epistasis approach to clarify the relationship between the lipid synthesis gene group and the FGF/RAS/MAPK pathways (Fig. 4C). We reasoned that if the genes involved in lipid synthesis act downstream or in parallel to the RAS/MAPK pathway that elevates exophers, RNAi knockdown of the lipid synthesis genes in the *let-60(gf)* background would have no effect on exopher elevation. Our data thus suggest that *let-60* and *iso-1* are required in both the hypodermis and in the germline for fasting-induced exopher induction.

**Lipid Biosynthesis Acts Upstream of RAS/ERK Activation to Stimulate Exopher Induction.** *let-60(n1046gf)* is a well-characterized, gain-of-function RAS allele that constitutively activates MAPK signaling (45). Our RNAi data, identifying the FGF and EGF/RAS/MAPK pathways as required for fasting-induced exopher induction, predict that the *let-60(gf)* allele should induce higher exopher levels, even in the absence of starvation. To test this model, we constructed a *let-60(n1046gf)*;mCherryAg2 strain and measured exopher levels at Ad2. We find that exophers are indeed elevated in the *let-60(n1046gf)* background in the absence of fasting, confirming that RAS activation enhances exopher production (Fig. 4B). *let-60(n1046gf)* has been used extensively in epistasis pathway ordering (45), and thus we pursued an epistasis approach to clarify the relationship between the lipid synthesis gene group and the FGF/RAS/MAPK pathways (Fig. 4C). We reasoned that if the genes involved in lipid synthesis act downstream or in parallel to the RAS/MAPK pathway that elevates exophers, RNAi knockdown of the lipid synthesis genes in the *let-60(gf)* background would have no effect on exopher elevation. Our data thus suggest that *let-60* and *iso-1* are required in both the hypodermis and in the germline for fasting-induced exopher induction.
should suppress constitutive exopher production. Alternatively, if the lipid synthesis branch acts upstream of LET-60/RAS activation, disruption of the upstream genes should not change the exopher elevation associated with let-60(gf) (Fig. 4C). We therefore repeated RNAi knockdowns of genes in the lipid synthesis and FGF/RAS/MAPK pathways in the let-60(gf) mCherryAg2 background, quantifying the impact on exopher formation. As expected for known downstream kinases in the RAS pathway, the RNAi disruption of mek-2/MAPKK and mkk-1/MAPKK suppressed the let-60(gf) phenotype (Fig. 4D). In contrast, genes encoding egl-17/FGF, egl-15/FGF, and soc-1 that act upstream of let-60/RAS did not suppress the let-60(gf)/RAS phenotype, consistent with the known action of these genes upstream of RAS in the signaling pathway.

Importantly, RNAi directed against pept-1, and the lipid biosynthesis genes mdt-15, sbp-1, and fasn-1 did not suppress elevated exopher levels in the let-60(gf) background (Fig. 4D). We conclude that the lipid biosynthesis branch is likely to act upstream of the FGF/RAS/MAPK to promote exopher elevation.

Lipid and FGF signaling may act upstream of EGF signaling. We also used an epistasis strategy to begin to address how lipid biosynthesis and FGF pathways might relate to the EGF pathway in fasting-induced exopher elevation. We performed the RNAi knockdown of required lipid synthesis genes and of upstream FGF pathway-specific genes egl-17/FGF, egl-15/FGF, and soc-1 on the neuronal let-23(gf) strain in which exopher levels are elevated, reasoning that if a critical lipid or FGF pathway is normally activated downstream of neuronal EGRF activation, perturbation of lipid or FGF pathway-specific genes would block the let-23(gf) exopher elevation under well-fed conditions. Our RNAi-dependent disruptions of either pathway, however, did not suppress neuronal let-23(gf) high-exopher levels (Fig. 4E), suggesting that the essential lipid and FGF signaling required for fasting-induced exopher induction normally occurs upstream of neuronal EGF signaling. Although these studies constitute only the first rudimentary tests required to establish pathway details (caveats discussed in more detail in SI Appendix, Fig. S4), data suggest a basic framework for mechanistic evaluation (Fig. 4F).

Overall, we identify three pathways that are required for fasting-induced exopher elevation in stressed touch neuron: lipid synthesis, an FGF/MAPK pathway, and an EGF/MAPK pathway that can act in neurons. Our data suggest a model in which different stressors that influence neuronal exopher production act downstream of neuronal contents upon the introduction of fasting stress. Upon food withdrawal, di-tripeptide transporter PEPT-1 plays a role in nutrient sensing and a lipid-based stress signal (the generation of which depends on MDT-15/SBP-1 transcriptional activity and fatty acid synthase FASN-1) is produced. The lipid-dependent process could act directly to nonautonomously influence exopher production in the touch neurons or could trigger/activate the required FGF/RAS/MAPK and/or EGF MAPK signaling and the likely consequent downstream transcription. Downstream, or in parallel, RAS/MAPK activity in hypodermis and germline contributes to the stress-sensing tissue network, establishing that nonautonomous signaling directs exopher production in the touch neurons. FGF signaling pathway genes are known to be highly activated downstream of neuronal EGFR activation, perturbation of general, rather than fasting-specific, mechanisms are engaged.

Although many details of this complex lipid-EGF-EGF signaling network remain to be further elucidated, our data provide documentation that aging and proteostasis-relevant stresses engage multiple pathways that can act over multiple tissues to influence a dramatic expansion of neuronal contents. Conserved signaling molecules can modulate a process of fundamental interest in neuronal proteostasis, relevant to the understanding of neuronal degeneration.

Discussion

Maintaining neuronal proteostasis is a critical goal for healthy brain aging and a fundamental challenge for diseased neurons in a range of neurodegenerative diseases (47). A recently identified facet of Alzheimer’s disease, Parkinson’s disease, and other neurodegenerative diseases is the transfer of aggregates to neighboring cells, which can seed aggregate spread and promote pathology (48, 49). In vivo dissection of the aggregation of protein aggregate spread is challenging to investigate in mammalian brain, but it is clear that the understanding of mechanisms that regulate autonomous and nonautonomous aggregate expansion in relation to other neuroprotective strategies is of considerable importance in addressing potential treatment. C. elegans touch neuron exopher production, which increases with high proteostress (4), models several aspects of aggregate/organellar transfer biology. We find that the production of neuronal exophers can be dramatically responsive to specific stress conditions, being enhanced by food withdrawal, oxidative stress, and osmotic stress but influenced relatively little by temperature or hypoxia. We also demonstrate the temporal restriction of stress-induced exopher production to the first 3 d of adult life, and we document a “stress ceiling” phenomenon, in which the highest levels of individual stress, or a combination of two distinct noninhibitory stresses, suspend exopher production. Finally, we show that fasting-induced exopherosis is dependent on nonautonomous lipid biosynthesis, FGF-activated RAS/MAPK, and EGF-activated RAS/MAPK signaling pathways. Although details remain to be filled in regarding the complex interactions of the signaling steps, a major point is that environmental and genetic factors can be manipulated nonautonomously to regulate the expulsion of offensive aggregates from neurons. Given the importance of aggregate management in aging and neurodegenerative disease and the poorly understood biology of in vivo aggregate transfer, exopher-related mechanisms may suggest new strategies toward the manipulation of the analogous process in higher organisms.

Fasting, Oxidative Stress, and Hypersomotic Stress Can Markedly Elevate Neuronal Exopher Production. Acute food withdrawal, oxidative stress, and hypersomotic stress elevate exopher production, but temperature elevation and hypoxia/anoxia are relatively ineffective at provoking similar responses. Starvation (8, 50), oxidative stress (51), and osmotic stress (52) perturb proteostasis and share ROS elevation (53, 54). The future dissection of the intersection of the multiple physiologic conditions common to these three stresses should provide insight into molecular mechanisms that promote exopherosis. Likewise, physiological differences in stress responses to temperature and hypoxia, which are not potent inducers of exophers, may help distinguish particular conditions that are specifically correlated with exopher induction.

Elevated Stress Levels, Which May Be Attained from Combined Exopher-Inducing Stimuli, Can Inhibit Exopher Production. Exopher production, comprising the release of a large, membrane-surrounded vesicle filled with cellular contents, has the appearance of an energetically costly incident that involves the dynamic loss of organelles and aggregates. Our working model posits that exopherosis is invoked when the levels of damaged organelles and proteins surpass the neuronal capacity for internal degradation. Consistent with this idea, increasing oxidative challenge and increasing hypersomotic exposure both increase exopherosis. Interestingly, conditions of extreme osmotic and oxidative stress markedly suppress the formation of exophers. Moreover, combining two stresses, either of which is sufficient to promote exopher production when introduced alone, can result in exopher suppression. This combinatorial effect can also occur with stress stimuli that themselves do not significantly induce exophers, such as anoxia and modestly elevated temperature. Together, these observations reveal a molecular summing of stress signals that appear to flip the “off” switch for exopherosis. The suppression of exopher production under conditions of extreme stress may be...
caused by energy exhaustion, a molecular repression mechanism, or grievous loss of homeostasis, leading to physiological dysregulation.

**Temporal Restrictions on Exopher Production Are Not Readily Changed by Exogenous Stresses.** Exopher production follows a distinctive and reproducible temporal profile in early adult life (4, 11). In the Ag2mCherry strain, exophers are not produced in larval development but begin to be detected after animals reach reproductive maturity, typically peaking in numbers around Ad2 and returning to low-baseline detection by Ad4. Data included here underscore that the temporal pattern is generally maintained, despite the continued or introduced presence of stresses. In other words,
Presses definitively elevate exopher production, but for the most part, these presses do not extend the period of exopher production later into adult life. Our findings thus define a limited temporal window in which exopher production can be modulated by stresses and suggest the existence of physiological states permissive (or restrictive) for exopher production. A link to reproduction likely defines this permissive period. For some stimuli (parquat, rote- none, osmotic stress, and a shift to 25°C), we do report a capacity to move the peak day of exopher production ahead to Ad1 or at least to markedly enhance Ad1 levels above nonstressed controls. We infer that these stimuli reach the molecular threshold for exopher triggering faster than other conditions.

Both daf-16–Dependent and daf-16–Independent Mechanisms Mediate Fasting-Induced Exopher Elevation. The fasting-induced elevation of exopher levels does not require stress transcription factors HSF-1, HIF-1, HLH-30/TFE3, or SKN-1/NRF2 but does depend in part on DAF-16/FOXO, a conserved stress-responsive transcription factor that drives the expression of food-sensitive, oxidative stress resistance and proteostasis genes (27, 44, 55) and is known to exert autonomous and nonautonomous impacts on stress resistance and longevity (56). Our data implicate a FOXO family member in regu- lation of neuronal aggregate expulsion. Our data do not rule out whether HSF-1, HIF-1, HLH-30/TFE3, or SKN-1/NRF2 might function redundantly in the exopher response to fasting.

How DAF-16 interacts with fat biosynthesis pathways and RAS/ERK signaling in exopher induction remains to be clarified. DAF-16 can control the expression of mdt-15 and has been previously implicated in transtissue benefits by interactions with MDT-15 (57). DAF-16 also intersects with FGF, ERK, and lipid biosynthesis pathways and vice versa (for example, refs. 58–61). The future definition of how DAF-16 integrates with these signaling pathways and the identification of the transcription factor that mediates the DAF-16–independent component of the fasting-induced exopher response will add molecular understanding to what appears to be a complex regulatory network.

Involvement of Lipids in Fasting-Induced Neuronal Exopher Stimulation. Mediator complex subunit MDT-15 is a transcriptional coregulator involved in lipid metabolism (34), response to fasting (32, 62), and oxidative, stress-induced expression of detoxification genes associ- ated with the exposure to reagents like parquat (63). The require- ment for mdt-15 in fasting-induced, neuronal exopherogenesis adds a new facet to the MDT-15 integration of multiple, tran- scriptional regulatory pathways (32), expanding the known roles of MDT-15/SBP-1 to include the activation of extrusion of remote neuronal aggregates in exophers. SBP-1/SREBF2 acts with MDT-15 to promote the expression of lipid metabolism genes (33, 34), and fatty acid synthase fast-1 can be regulated by these (38, 39, 62, 64, 65). The requirement for multiple genes involved in lipid synthesis in fasting-induced exopher increase suggests that a lipid-based signal may be issued to ultimately direct or modulate neuronal trash expulsion. An equally plausible model is that lipid-dependent machinery is required for upstream signaling that promotes exopherogenesis.

FGF/RAS/MAPK Signaling Mediates the Exopher Induction Pathway. Our findings identify FGF ligand egl-17 (but not FGF ligand let-756), FGF receptor egl-15, FGF pathway specific soc-1, and pathway components let-60, mek-2, ksr-1, and mpk-1 as required for the fasting induction of exophers. These data reveal a specific FGF/RAS/ERK signaling pathway that enhances neuronal exo- pher production when food is withdrawn. Since the candidate RNAi screen for the factors required for fasting-induced exopher elevation was conducted in a strain background that is not readily permissive for neuronal RNAi effects and since RNAi knockdown of pathway components (specifically in neurons) does not block fasting-induced exopher increase, FGF/ERK signaling likely takes place outside of the touch receptor neurons to exert a regulatory role on neuronal exopher production. An interesting potential site of FGF action is the hypodermis, which is necessary for some MEK-2/MAPK-1 MAPK signaling. Such signaling is permissive for fasting-induced exopher pro- duction and the hypodermis is a known site of expression of FGF/R pathway components (SI Appendix, Fig. S4B). Detailed, cell-specific expression studies will be required to test this model.

The conserved FGF pathway executes numerous roles in mammalian development and homeostasis (66). Interestingly, mammalian FGF21 acts as a global starvation signal that, among other things, impacts lipid metabolism. Although most FGF21 studies feature extended starvation and mouse–human differ- ences have been noted (67), FGF21 is one of the most up- regulated rat liver genes under the conditions of an 8-h fast (68). FGF21 can cross the blood–brain barrier to change hypo- thalamic neuron gene expression (69). Overall, the implication of FGF/ERK pathways in the response of neurons to food limita- tion across diverse metazoans suggests a mechanism that may be ancient and raises the possibility that the FGF branch of these pathways might activate extracellular trash expulsion mecha- nisms within mammalian neurons. If so, FGF signaling might be considered as a target for the therapeutic elimination of stored neuronal aggregates.

EGF RAS/MAPK signaling is also engaged in fasting-induced exopher elevation. The knockdown of the sole C. elegans EGF ligand lin-3 in whole animal, or only in germline, impaired fasting-induced exopher elevation.

Although RNAi knockdown of the EGFR let-23 and EGF pathway-specific ksr-2 in whole animal or only neurons was not effective, expressing activated, gain-of-function EGFR receptor allele let-23(sa62) in neurons resulted in elevated exopher levels in the absence of fasting. Epistasis studies suggest that EGFR activation in neurons could occur as a downstream target of lipid biosynthesis and FGF signaling. Although definitive establishment of fasting-induced EGFR activation in neurons remains (experimental caveats in interpretation of data are discussed in detail in SI Appendix, Fig. S4C), data are consistent with a role for EGF signaling originating in the germline as an inducer of EGFR-activated responses in neurons that promote exopher production. Future studies will need to confirm neuron requirements and to ad- dress whether EGFR directly activates exopherogenesis in touch neu- rons or engages additional neurons as intermediary signaling centers. It is important that the germline serves as an EGF source needed for fasting-induced exopher elevation. Indeed, in studies of exopher production under standard growth conditions, we have defined a role for germline in the production of young adult exophers. A key point here is that food-sensing, nonautonomous growth factor signaling across generations can influence seemingly extreme neuronal proteostasis activity.

EGF- and FGF-dependent processes cooperate in develop- ment. For example, EGF signaling activates FGF production and a downstream FGF pathway required in vulval epithelial fate specification (70). Stimulation conditions can influence the signal- ing level for the EGF/RAS/ERK pathway that specifies C. elegans vulval cell fates—either starvation or pept-1(RNAi)—can enhance RAS/MAPK signaling during vulval fate specification (43). Our documentation of FGF signaling in food limitation responses that elevate neuronal proteostasis outcomes identifies a second C. elegans EGF- and FGF-regulated signaling pathway that responds to food limitation. Why food limitation might in- duce neuronal trash elimination is unclear. One possibility is that exopherogenesis [which we track in single neurons in our study but is likely to also occur in other neurons and cells (41)] might serve as a mechanism to discard superfluous, neuronal proteins and organelles for degradative recycling in neighboring cells as re- sources become limited.
Implications of Conserved Exopher-Like Biology for Aging and Neurodegeneration. Our study defines the basic framework by which metabolic stresses engage a distributed network that influences a significant neuronal expulsion phenomenon. Evidence is accumulating that exopher-like extrusion capabilities are not limited to stressed C. elegans neurons [mammalian examples in refs. 71 and 72]. For example, a recent comprehensive study of mitochondrial expulsion by muscle cardiomyocytes revealed numerous analogies between C. elegans exophers and mouse mitochondrial expulsion models (72). Although elaborating details of molecular homologies remain for the future, that related biology is likely to be conserved holds significant implications of interest with regard to mammalian aging and neurodegeneration. 1) It is interesting that stresses, particularly associated with aging (i.e., oxidative stress) or proteostasis impairment (i.e., osmotic stress), are especially potent in inducing C. elegans exopher elevation; the disruption of exopher-related biology may contribute generally to the decline/dysfunction in aging neurons across phyla. 2) Likewise, the direct demonstration that stress extremes, or the summation of distinct, nonconsequential stresses, can effectively shut down the exopher response suggests a type of potential excessive stress impairment relevant to pathological mechanisms. 3) Our data establish that exopher production can be responsive to specific, conserved biochemical signaling, such that chemical strategies for inducing, or limiting, the expulsion of neurotoxic material by exploiting exopher-related mechanisms in mammals might be considered targets for therapeutic manipulation.

Materials and Methods

Method details are included with SI Appendix. Protocols for distinguishing and scoring touch neuron exophers are outlined in detail in ref. 10. RNA libraries and protocols described in detail in refs. 28, 29, 73, and 74.

Strains. The strains used in this study were the following:

**ZB4065 bz5166 [mec-4;CherryAg2]** II; also referred to as mCherryAg2

**ZB5160 bz5166 [mec-4;CherryAg2]** II; hs-1(54y441) I

**ZB5161 bz5166 [mec-4;CherryAg2]** II; kgb-1(un33) IV

**ZB5162 bz5166 [mec-4;CherryAg2]** II; skn-1(zg15) IV

**ZB5163 bz5166 [mec-4;CherryAg2]** II; jun-1(kc551) I

**ZB5164 bz5166 [mec-4;CherryAg2]** II; hif-1(a44) V

**ZB5165 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I

**ZB5166 [mec-4;CherryAg2]** II; daf-16(mu86) I; rgef-1(f542) V; let-23(sa62) V

**ZB5117 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; sid-1(qt9) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

**ZB5298 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

**ZB5300 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

**ZB5301 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

**ZB5303 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

**ZB5304 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

**ZB5305 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

**ZB5306 bz5166 [mec-4;CherryAg2]** II; let-60(n1046) I; hif-1(a44) V; let-23(sa62) V; daf-16(mu86) I; let-56(n1046) I; mCherry[ promoter-less control] V

1. J. Labbadia, R. I. Morimoto, The biology of proteostasis in aging and disease. Ann. Rev. Biochem. 84, 435–464 (2015).

2. A. Kurtishi, B. Rosen, K. S. Patil, G. W. Alves, S. G. Møller, Cellular proteostasis in C. elegans. Annu. Rev. Cell Dev. Biol. 34, 345–568 (2018).

3. I. Melentijevic et al., C. elegans neurons jettison protein aggregates and mitochondria under neurotoxic stress. Nature 542, 367–371 (2017).

4. G. van Niel, G. D’Angelo, G. Raposo, Shedding light on the cell biology of extracellular vesicles. Nat. Rev. Mol. Cell Biol. 19, 213–228 (2018).

5. M. Chaffe et al., The neural circuit for touch sensitivity in Caenorhabditis elegans. J. Neurosci. 5, 956–964 (1985).

6. S.-J. Lee, C. Kenyon, Regulation of the longevity response to temperature by thermosensory neurons in Caenorhabditis elegans. Curr. Biol. 19, 715–722 (2009). Correction in: Curr. Biol. 19, 798 (2009).

7. M. Santra, A. K. Dill, A. M. R. de Graaf, Proteostasis collapse is a driver of cell aging and death. Proc. Natl. Acad. Sci. U.S.A. 116, 22173–22178 (2019).

8. E. M. Fawcett, J. M. Hoy, J. K. Johnson, D. L. Miller, Hypoxia disrupts proteostasis in Caenorhabditis elegans aging. Cell 149, 92–101 (2015).

9. D. J. Dues et al., Aging causes decreased resistance to multiple stresses and a failure to activate specific stress response pathways. Aging (Albany NY) 8, 777–795 (2016).

10. E. C. Lee et al., Abnormal osmotic avoidance behavior in C. elegans is associated with increased hypertonic stress resistance and improved proteostasis. PLoS One 11, e0154156 (2016).

11. I. Korovila et al., Proteostasis, oxidative stress and aging. Redox Biol. 13, 550–567 (2017).

12. A.-L. Hu, T. C. Murphy, C. Kenyon, Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science 300, 1142–1145 (2003).

13. H. Jiang, R. Guo, J. A. Powell-Coffman, The Caenorhabditis elegans hif-1 gene encodes a bHLHPAS protein that is required for adaptation to hypoxia. Proc. Natl. Acad. Sci. U.S.A. 98, 7916–7921 (2001).

14. E. J. O’Rourke, G. Rukun, Mxl-3 and HLF-3 transcriptionally link lipopolysaccharide and autophagy to nutrient availability. Nat. Cell Biol. 15, 668–676 (2013).

15. J. A. An, T. K. Blackwell, SKN-1 links oxidative stress response. Genes Dev. 17, 1882–1893 (2003).

16. A. J. Przybysz, K. F. Choe, L. J. Roberts, K. Strange, Increased age reduces DAF-16 and SKN-1 signaling and the hormetic response of Caenorhabditis elegans to xenobiotic juglone. Mech. Ageing Dev. 130, 357–369 (2009).

17. H. A. Tissenbaum, DAF-16: FOXO in the context of C. elegans. Curr. Top. Dev. Biol. 127, 1–21 (2018).

18. R. S. Kamath, J. Ahiringer, Genome-wide RNAi screening in Caenorhabditis elegans. Methods 30, 313–321 (2003).

19. A. Calixto, D. Chelur, I. Topalidou, X. Chen, M. Chaffe, Enhanced neuronal RNAi in C. elegans using SID-1. Nat. Methods 7, 554–559 (2010).

20. B. Meissner, M. Boll, H. Daniel, R. Baumeister, Deletion of the intestinal peptide transporter affects insulin and TOR signaling in Caenorhabditis elegans. J. Biol. Chem. 279, 36739–36745 (2004).

Cooper et al. Stress increases in exopher-mediated neuronal extrusion require lipid biosynthesis, FGF, and EGF RAS/MPK signaling

PNAS | 11 of 12
https://doi.org/10.1073/pnas.2101401118
31. B. Spanier et al., How the intestinal peptide transporter PEPT-1 contributes to an obesity phenotype in Caenorhabditis elegans. PLoS One 4, e4279 (2009).

32. S. Taubert, M. Hansen, M. R. Van Gilst, S. B. Cooper, K. R. Yamamoto, The Mediator subunit MDT-15 confers metabolic adaptation to ingested material. PLoS Genet. 4, e1000221 (2008).

33. F. Yang et al., An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. Nature 422, 700–704 (2006).

34. S. Taubert, M. R. Van Gilst, M. Hansen, K. R. Yamamoto, A Mediator subunit, MDT-15, integrates regulation of fatty acid metabolism by NHR-49-dependent and -independent pathways in C. elegans. Genes Dev. 20, 1137–1149 (2006).

35. D. Lee et al., MDT-15/MDT15 permits longevity at low temperature via enhancing lipidostasis and proteostasis. PLoS Biol. 17, e3000415 (2019).

36. N. Shomer et al., Mediator subunit MDT-15 and nuclear receptor HIZR-1/HNF4 cooperate to regulate toxic metal stress responses in Caenorhabditis elegans. PLoS Genet. 15, e1008558 (2019).

37. G. Y. S. Goh et al., The conserved Mediator subunit MDT-15 is required for oxidative stress responses in Caenorhabditis elegans. Aging Cell 13, 70–79 (2014).

38. F. Meja-Martinez et al., The NOL-3/SREBP-1 axis is responsible for glucose-dependent fat accumulation in C. elegans. Genes (Basel) 8, 307 (2017).

39. D. Lee et al., SREBP and MDT-15 protect C. elegans from fatty-acid-induced accelerated aging by preventing accumulation of saturated fat. Genes Dev. 29, 2490–2503 (2015).

40. M. V. Sundaram, RTK/Ras/ERK signaling. Wormbook, 1–19 (2006).

41. M. V. Sundaram, Canonical RTK-Ras-ERK signaling and related alternative pathways. Wormbook, 1–38 (2013).

42. S. Honjo, T. Yamamoto, M. Uno, E. Nishida, Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C. elegans. Nature 457, 726–730 (2009).

43. S. Grimbert, A. M. Vargas Velazquez, C. Braendle, Physiological starvation promotes Caenorhabditis elegans vulval induction. G3 (Bethesda) 8, 3069–3081 (2018).

44. M. Uno et al., A fasting-responsive signaling pathway that extends life span in C. elegans. Cell Rep. 3, 79–91 (2013).

45. G. J. Beitel, S. G. Clark, H. R. Horvitz, Caenorhabditis elegans ras gene let-60 acts as a switch in the pathway of vulval induction. Nature 348, 503–509 (1990).

46. J. Serizay et al., Distinctive regulatory architectures of germline-active and somatic genes in C. elegans. Genome Res. 30, 1752–1765 (2020).

47. C. L. Klaips, G. G. Jayaraj, F. U. Harl, Pathways of cellular aging and disease. J. Cell Biol. 217, 51–63 (2018).

48. S. Nath et al., Spreading of neurodegenerative pathology via neuron-to-neuron transmission of α-amyloid. J. Neurosci. 32, 8676–8777 (2012).

49. C. I. Nussbaum-Kramer, K.-W. Park, L. Li, R. Melki, R. I. Morimoto, Spreading of a prion domain from cell-to-cell by vesicular transport in Caenorhabditis elegans. PLoS Genet. 9, e1003351 (2013).

50. M. Larance et al., Global proteomics analysis of the response to starvation in C. elegans. Mol. Cell. Proteomics 14, 1598–2001 (2015).

51. L. Tomanek, Proteomic responses to environmentally induced oxidative stress. J. Exp. Biol. 218, 1867–1879 (2015).

52. K. Burkevitz, K. Choe, K. Strange, Hypertonic stress induces rapid and widespread protein damage in C. elegans. Am. J. Physiol. Cell Physiol. 301, C566–C576 (2011).

53. J. Tao, Q.-Y. Wu, Y.-C. Ma, Y.-L. Chen, C.-G. Zou, Antioxidant response is a protective mechanism against nutrient deprivation in C. elegans. Sci. Rep. 7, 43547 (2017).

54. G. Miller, N. Suzuki, S. Ciftci-Yilmaz, R. Mittler, Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 33, 453–467 (2010).

55. C. T. Murphy, P. J. Hu, Insulin/insulin-like growth factor signaling in C. elegans. acts-upbook. 1–42 (2015).

56. N. Shemes, L. Meshnik, N. Shipigl, A. Ben-Zvi, Dietary-induced signals that activate the gonadal longevity pathway during development regulate a proteostasis switch in Caenorhabditis elegans adulthood. Front. Mol. Neurosci. 10, 254 (2017).

57. P. Zhang, M. Judy, S.-J. Lee, C. Kenyon, Direct and indirect gene regulation by a life-extending FOXO protein in C. elegans: Roles for GATA factors and lipid gene regulators. Cell Metab. 17, 85–100 (2013).

58. M.-T. Château, C. Araiz, S. Descamps, S. Galas, Klotho interferes with a novel FGF-signalling pathway and insulin/FGF-like signalling to improve longevity and stress resistance in Caenorhabditis elegans. Aging (Albany NY) 2, 567–581 (2010).

59. T. Okuyama et al., The ERK-MAPK pathway regulates longevity through SKN-1 and insulin-like signaling in Caenorhabditis elegans. J. Biol. Chem. 285, 30274–30281 (2010).

60. F. R. G. Amrit et al., DAF-16 and TCER-1 facilitate adaptation to germline loss by restoring lipid homeostasis and repressing reproductive physiology in C. elegans. PLoS Genet. 12, e1005788 (2016).

61. N. O. Burton et al., Insulin-like signalling to the maternal germline controls progeny response to osmotic stress. Nat. Cell Biol. 19, 252–257 (2017).

62. G. Y. S. Goh et al., NHR-49/HNF4 integrates regulation of fatty acid metabolism with a protective transcriptional response to oxidative stress and fasting. Aging Cell 17, e12743 (2018).

63. Q. Hu, D. R. D’Amora, L. T. MacNeil, A. J. M. Walhout, T. J. Kubieski, The Caenorhabditis elegans oxidative stress response requires the NHR-49 transcription factor. G3 (Bethesda) 8, 3857–3863 (2018).

64. K.-Z. Lee, M. Kriaeva, M. Han, N. Pujol, J. J. Ewbank, The fatty acid synthase fasn-1 upstream of WNK and Ste20/GCK-VI kinases to modulate antimicrobial peptide expression in C. elegans epidermis. Virulence 1, 113–122 (2010).

65. T. Nomura, M. Horikawa, S. Shimamura, T. Hashimoto, K. Sakamoto, Fat accumulation in Caenorhabditis elegans is mediated by SREBP homolog SBE-P-1. Genes Nutr. 5, 17–27 (2010).

66. F. M. Fisher, E. Maratos-Flier, Understanding the physiology of FGF21. Annu. Rev. Physiol. 78, 223–241 (2016).

67. H. Staijer, M. Keuper, L. Berti, M. Hrabe de Angelis, H.-U. Häring, Fibroblast growth factor 21-metabolic role in mice and men. Endocr. Rev. 38, 468–488 (2017).

68. Y. Ozaki et al., Rapid increase in fibroblast growth factor 21 in protein malnutrition and its impact on growth and lipid metabolism. Br. J. Nutr. 114, 1410–1418 (2015).

69. H. Huschou, W. Pan, A. J. Kastin, The fasting polypeptide FGF21 can enter brain from blood. Peptides 28, 2382–2386 (2007).

70. M. Cui, M. Han, Cis regulatory expression requirements for vulval cell-specific expression of the Caenorhabditis elegans fibroblast growth factor gene egl-17. Dev. Biol. 257, 104–116 (2003).

71. C.-H. O. Davis et al., Transcellular degradation of axonal mitochondria. Proc. Natl. Acad. Sci. U.S.A. 111, 9633–9638 (2014).

72. J. A. Nicolas-Avila et al., A network of macrophages supports mitochondrial homeostasis in the heart. Cell 183, 94–109.e23 (2020).

73. J.-F. Rual et al., Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. Genome Res. 14, 2162–2168 (2004).

74. R. S. Kamath et al., Systematic functional analysis of the Caenorhabditis elegans phenome using RNAi. Nature 421, 231–237 (2003).

75. M. Turek et al., Muscle-derived exophers promote reproductive fitness. EMBO Rep. 22, e52071 (2021).