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

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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 SBP-1/SREPB1, and fatty acid synthase FASN-1, implicating remotely initiated lipid signaling in neuronal trash elimination. A conserved fibroblast growth factor (FGF)/RAS/MAPK signaling pathway that acts downstream of, or in parallel to, lipid signaling also promotes fasting-induced neuronal exopher elevation. A germline-based epidermal growth factor (EGF) signal that acts through neurons is also required for exopher production. Our data define a nonautonomous network that links food availability changes to remote, and extreme, neuronal homeostasis responses relevant to aggregate transfer biology.

exopher | fasting | MAP kinase | stress | neurology

Proteostasis is critical for the function, maintenance, and long-term survival of all cells (1). Successful maintenance of the proteome entails efficacious balancing of protein synthesis and degradation, with coordinated actions of regulated gene expression and translation, the chaperone-folding network, the ubiquitin proteasome system, and autophagy all serving critical functions in overall protein quality control. Neurons are particularly susceptible to proteostasis disruption, and aggregates are a striking and common feature of neuropathology in most human neurodegenerative diseases (2). Recent studies have shown that in diseases characterized by distinctive aggregates, including Alzheimer's disease, Parkinson's disease, and Huntington's disease, aggregates can spread from neurons to neighboring cells, inducing deleterious consequences (3). The mechanisms operative for aggregate spread are unclear and remain a matter of considerable research attention.

We discovered that adult *Caenorhabditis elegans* neurons can select and then extrude aggregated proteins, such as expanded polyglutamine HTTQ128-CFP (related to neurotoxic Huntington's disease protein) and an aggregation-prone, high-expression mCherry reporter (4) (see Fig. 1*A*). Aggregates are sent out of the neuron in strikingly large, membrane-surrounded vesicles [4-μm average diameter; for comparison, the neuron soma is ∼6 μm; this vesicle is two orders of magnitude larger than exosomes that form by a distinct mechanism (5)]. We refer to these massive cell extrusions as "exophers" ("exo" = outside and "pher" = carry away). In the case of the *C. elegans* touch receptor neurons that mediate sensitivity to gentle touch stimuli (6), exophers are passed into the surrounding hypodermis, which can partially degrade their contents. Baseline production of exophers under nonstress growth conditions is low, detected in 5 to 20% of ALMR touch neurons, and occurs at lower rates for other touch neurons. However, we found that exacerbated proteostress (for example, expressing the human $A\beta_{1-42}$ fragment implicated in Alzheimer's disease pathology, disrupting specific autophagy genes via RNA interference [RNAi] or introducing the MG132 proteasome inhibitor) can significantly increase 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 exophergenesis. 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 exophergenesis: lipid synthesis, FGF/RAS/MAPK, and EGF/RAS/MAPK. In establishing an initial molecular model for transtissue 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.

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The authors declare no competing interest.

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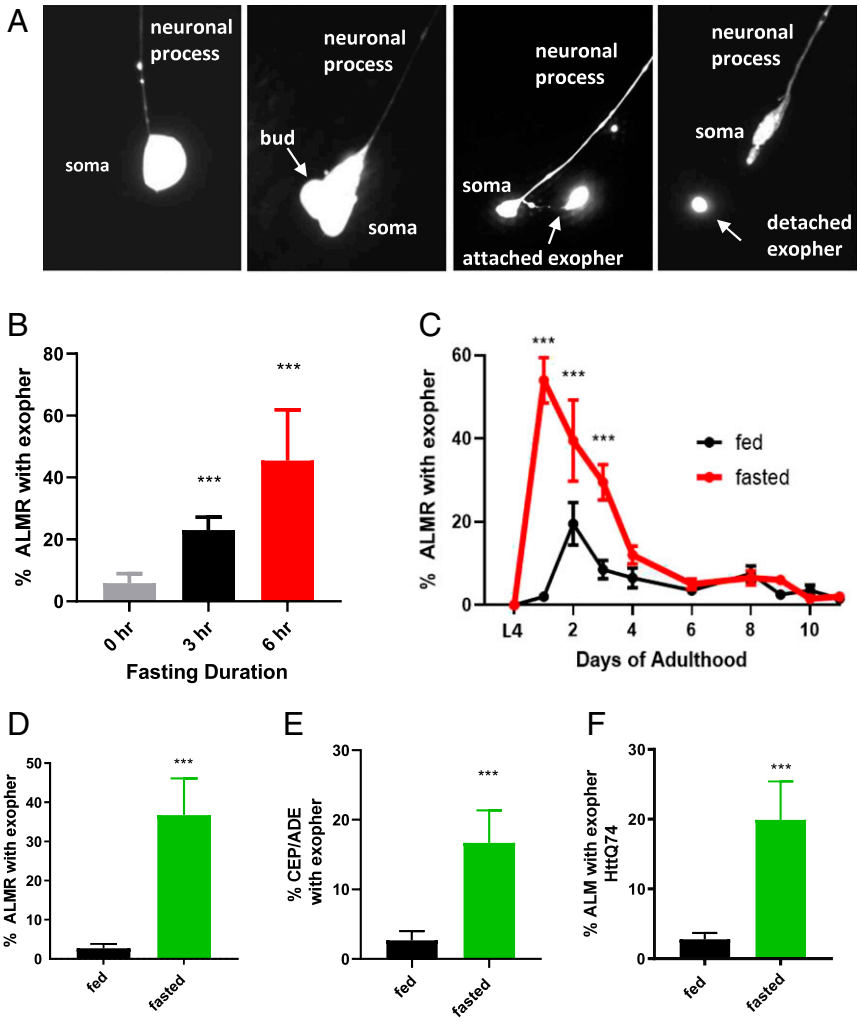

Fig. 1. Transient fasting during early adult life greatly boosts exophergenesis. For all panels, bars are SEM, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, and CMH statistics. (*A*) Neuronal morphologies associated with exopher formation. In vivo image of ALMR neuron exopher formation stages. The strain is ZB4065 *bzIs166*[$P_{mec\text{-}4}$mCherry1], referred to in the text as mCherryAg2. The exopher commonly forms as a nearly soma-sized, budding extrusion, which can initially remain connected to the neuron by a thin, tunneling nanotube-like filament that detaches later. The exopher is then extruded into the hypodermis that surrounds the touch neuron. The extruded exopher can be broken up and taken into the hypodermal lysosomal network (not shown). (*B*) Transient food deprivation can dramatically increase exopher formation. We grew strain mCherryAg2 on *E. coli* OP50, 20 °C, until Ad1 and moved animals to plates that lacked food for the indicated number of hours, followed by immediate scoring for exophers: 50 animals per trial and 4× trials. We noted that fasted animals appear to produce multiple exophers more frequently than fed animals (*SI Appendix*, Fig. S1*B*). (*C*) The impact of fasting on elevated exopher production is limited to the first 3 d of adult life. Animals cultured from a single population were aged to the indicated day of adulthood. We withdrew 50 animals from the OP50 bacteria (red line) for 6 h on the days indicated on the *x*-axis and scored exophers immediately thereafter. mCherryAg2 continuously fed controls are indicated by the black line. Data thus reflect exopher levels resulting from food withdrawal on each of the days indicated, as drawn from a single population: 50 animals per day of sampling and 5× trials. (*D*) Fasting can dramatically increase exopher formation in a GFP model. We grew strain SK4005 zdIs5[$P_{mec\text{-}4}$GFP] on *E. coli* OP50, 20 °C, until Ad1 and moved animals to plates that lacked food for 6 h, followed by immediate scoring for exophers: 50 animals per trial and 3× trials. (*E*) Fasting can dramatically increase exopher formation in dopaminergic neurons. We grew strain BY250 vtIs7[$P_{dat\text{-}1}$GFP] on *E. coli* OP50, 20 °C, until Ad1 and moved animals to plates that lacked food for 6 h, followed by immediate scoring of the CEP and ADE neurons for exophers: 50 animals per trial and 3× trials. (*F*) Fasting can dramatically increase exopher formation in a Huntingtin model. We grew strain ZB5171 *bzSi39*[$P_{mec\text{-}7}$mNG::HttQ74];*bzIs34*[$P_{mec\text{-}7}$mSc::lFD-1] on *E. coli* OP50, 20 °C, until Ad1 and moved animals to plates that lacked food for 6 h, followed by immediate scoring for ALM exophers: 50 animals per trial and 3× trials.

2) 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 limit to the level of stress that can induce exophers, such that, beyond this upper stress limit, exophergenesis is not observed. We linked the fasting-induced increase in exophers (as much as 10-fold elevation in exophers) to activities of the intestinal peptide transporter PEPT-1, 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 exophergenesis levels are sensitive to food source, quantitating ALMR exophers produced by neurons expressing an mCherry protein (strain ZB4065 *bzIs166*[$P_{mec\text{-}4}$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. S1*A*).

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. 1*B*). Fasting also causes an increase in multiple-exopher events, in which more than one exopher is generated (*SI Appendix*, Fig. S1*B*). 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 Exophergenesis 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. S1*A*). 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 measured exopher levels immediately after the 6-h fast on the experimental fasting day (Fig. 1*C*). Our analysis of mCherryAg2 ALMR exophers in this study revealed the following: 1) a 6-h fasting protocol does not induce exopher production during the L4 stage; 2) the 6-h fasting protocol elevates exopher production to peak levels at Ad1 when administered on that day; 3) fasting efficacy is slightly lower when delivered at Ad2 and lower still when delivered at Ad3; and 4) by Ad4, a fasting-induced exopher increase is no longer observed. We conclude that the impact of food withdrawal on enhancing exopher production is limited to a window of time that covers Ad1 to Ad3. The flanking of the permissive phase by exopher-recalcitrant periods suggests the existence of a "licensed" period, in which the expulsion of significant amounts of neuronal material is biologically feasible and during which exophergenesis can be modulated by environmental stress signals. We observed the 6-h fasting–induced elevation of exophers in strains that expressed GFP (Fig. 1*D*) or expanded polyglutamine (Fig. 1*F*) mNG:HttQ74 in touch neurons or expressed GFP in dopaminergic neurons (Fig. 1*E*), indicating that the fasting induction of exophers was not reporter- or cell type–specific.

Temperature and Hypoxia Stresses Are Not Potent Inducers of Exopher Production. Temperature is an environmental factor that dramatically influences the *C. elegans* reproductive life cycle time, lifespan (16), and proteostress (17). We therefore asked whether culture temperature might influence the timing or levels of adult exopher production. We reared animals continuously at 15, 20, and 25 °C from the egg stage and measured exopher levels over the first 4 d of adult life (*SI Appendix*, Fig. S2*A*). Somewhat unexpectedly, exopher levels did not vary substantially with culture temperature. In an alternative experimental design, we reared synchronized populations of animals at 20 °C and then split cultures at the L4 adolescent stage into three parallel cultures that were thereafter maintained at 15, 20, and 25 °C (*SI Appendix*, Fig. S2*B*). We found that, in animals shifted to 25 °C, the peak of exopher production occurred earlier (Ad1) than 20 °C animals, but exopher levels were not significantly elevated, relative to 20 °C. Our data suggest that the experience of a temperature shift, rather than continuous noxious temperature exposure, might exert the strongest impact on levels of exopher production (*SI Appendix*, Fig. S2*C*). That said, the approximate doubling of exopher scores after an L4 shift from 20 to 25 °C is modest in comparison to the 5 to 10× exopher elevation measured for food withdrawal. We conclude that, within the normal confines of *C. elegans* laboratory culture, the temperature at which animals are grown exerts a relatively minor influence on levels of exopher production.

Hypoxia can induce protein aggregation in *C. elegans* models (18), raising the possibility that hypoxia might enhance exopher production. We tested the exposure to 0.1% oxygen using an adjustable hypoxia C-174 chamber (Biospherix) to generate exposures 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 failed to find differences in exophers produced under these hypoxic conditions on any day (*SI Appendix*, Fig. S2*D*). We also tested anoxic conditions induced by replacing oxygen with nitrogen in a sealed anoxia chamber (19), measuring ALMR exophers after 6-h exposure to anoxia (*SI Appendix*, Fig. S2*D*, dark purple line). Exposure to anoxia failed to markedly increase exopher production on any exposure day. While it is possible that longer exposures or different recovery times might alter outcomes, we conclude that hypoxia/anoxia treatments, as delivered, do not elevate exopher levels.

Osmotic Stress and Oxidative Stress Can Induce Exopher Production. Osmotic stress introduces proteostasis challenges (20), and thus, we were curious as to how exopher 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, glucose, sorbitol, and NaCl. At Ad1, we introduced mCherryAg2 animals to these osmotic stresses for 6 h and scored ALMR exophers thereafter (Fig. 2*A*). We found that a 6-h exposure to osmotic stress increased exopher production ~4× 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. 2*B*). 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 ~5× 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.

Oxidative stress is a central player in aging biology and proteostasis (21). We tested whether well-characterized chemical inducers of mitochondrial oxidative stress can influence exopher production in ALMR neurons. We exposed Ad1 mCherryAg2 animals to 6 h of increasing concentrations of juglone (juglone is a plant-derived compound that can induce superoxide production when incorporated into nematode growth medium [NGM]); rotenone, which interacts with mitochondrial electron transport complex I to elevate reactive oxygen species (ROS); and paraquat, which increases mitochondrial superoxide production (Fig. 2 *D*–*F*). For all mitochondrial ROS generators, we observed a dose-dependent increase in exophers at Ad1 (to a point, discussed in *Excessive Stress Decreases Exopher Production*) that ranged from 4 to 6× over untreated controls. We conclude that exposure to mitochondrial ROS induction can enhance exopher production.

We also tested conditions of continuous ROS exposure during adult life. We raised animals under standard growth conditions until the L4 stage and shifted cultures to paraquat plates, measuring ALMR exophers on Ad1 to Ad8 (*SI Appendix*, Fig. S2*E*). We find that continuous paraquat exposure increases exopher levels. Interestingly, exopher levels in the 2-mM paraquat treatment do not begin to fall at Ad3, as is typical for other stresses, but instead remain higher than control levels through Ad6, returning to baseline

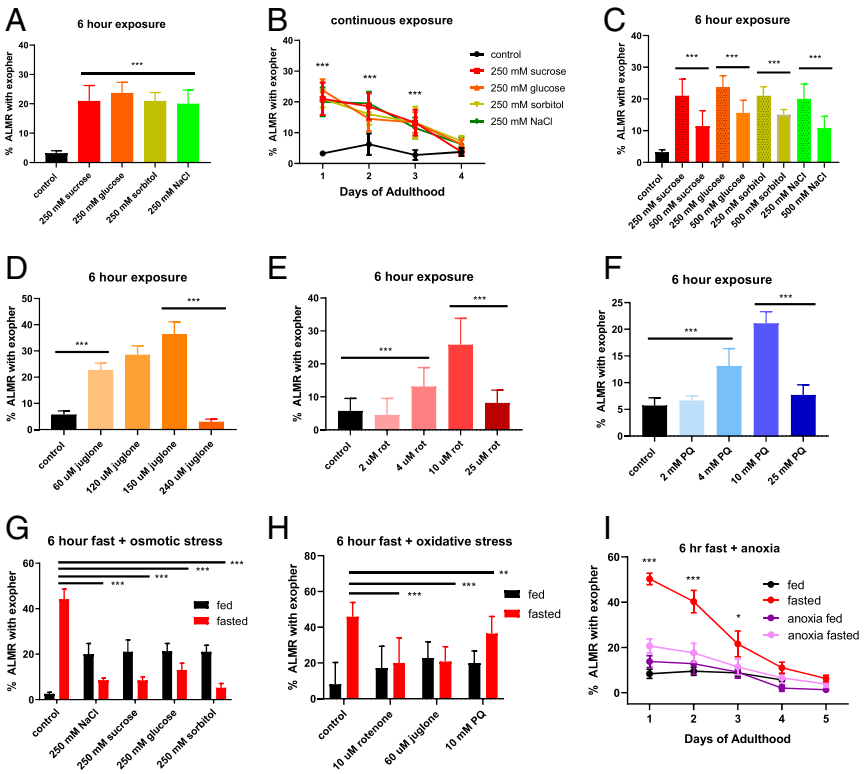

Fig. 2. Osmotic stress and oxidative stress can increase exopher production, but extreme stress levels can decrease exophergenesis. For all panels, bars are SEM, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, and CMH statistics. (*A*) Transient osmotic stress significantly elevates ALMR exophers on Ad1. We exposed mCherryAg2 animals to 250 mM sucrose, glucose, sorbitol, or NaCl on Ad1 and scored for exophers 6 h later: 5× trials and 50 animals per trial. (*B*) Animals chronically exposed to osmotic stress from Ad1 into adulthood produce elevated exophers with early onset peak production. We grew mCherryAg2 animals to the Ad1 stage under standard growth conditions and shifted to 250 mM sucrose, glucose, sorbitol, or NaCl, measuring ALMR exophers in these populations on Ad1 to Ad4: 4× trials and 50 animals per time point. CMH compares osmotic stress media to normal growth media. (*C*) Osmotic stress at 250 mM enhances exopher production more than at 500 mM. We exposed strain mCherryAg2 to 250 and 500 mM sucrose, glucose, sorbitol, and NaCl for 6 h on Ad1 and measured exophers shortly thereafter: 5× trials and 50 animals per trial. (*D–F*) Increasing oxidative stress enhances exopher production to a limit, after which exopher production is suppressed. We grew mCherryAg2 animals to Ad1 at 20 °C, and then, transferred animals to plates housing increasing concentrations of juglone, rotenone (rot), or paraquat (PQ) for 6 h, as indicated, measuring ALMR exophers shortly thereafter (black asterisks compare to control): 7× trials and 50 animals per trial. (*G*) Combined food withdrawal and osmotic stress can suppress exopher induction under conditions that individually induce high-exopher levels. We fasted Ad1 mCherryAg2 animals for 6 h on plates containing the solutes indicated (20 °C) and scored for ALMR exophers shortly thereafter: 3× trials and 50 animals per trial (asterisks compare two indicated conditions at bar ends). (*H*) Combined food withdrawal with oxidative stress can suppress exopher induction under conditions that individually induce high-exopher levels. We fasted Ad1 mCherryAg2 animals for 6 h on juglone, rotenone, and paraquat at the concentrations indicated, scoring for ALMR exophers shortly thereafter: 5× trials and 50 animals per trial (asterisks compare two indicated conditions). (*I*) While anoxia does not increase exophers, combining food withdrawal and anoxic stress can suppress fasting-associated exopher induction. We fasted Ad1 mCherryAg2 animals for 6 h under anoxic conditions, scoring for ALMR exophers shortly thereafter: 4× trials and 50 animals per trial. Note that our anoxia protocol is not effective in limiting fasting-induced exopher elevation (*SI Appendix*, Fig. S2 *D* and *H*).

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 exophergenesis. For example, 6-h exposure of 240 μM juglone (Fig. 2*D*), 25 μM rotenone (Fig. 2*E*), or 25 mM paraquat (Fig. 2*F*) 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. 2*C*). 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. S2*F*).

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 *B–F* and 2*G*), cointroducing osmotic stress with fasting (which also normally also elevates exophers; Fig. 2 *A* and *B*) suppresses exopher levels (Fig. 2*G*). We also found a combined inhibitory effect for fasting + oxidative stress (Fig. 2*H*).

Furthermore, temperature (*SI Appendix*, Fig. S2*G*) and anoxia (Fig. 2*I* and *SI Appendix*, Fig. S2*H*), 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

threshold levels of these stresses (including when two individual, exopher-promoting stresses are combined), exopher production can be suppressed. Thus, in addition to a temporal constraint on when environmental stresses can elevate exopher levels, we demonstrate that there is a limit to the severity of environmental stress capable of inducing the exopher production response.

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 chaperones and proteostasis (22); hypoxia inducible factor *hif-1*/HIF1, required for hypoxia stress responses (23); *hlh-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 insulin 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 Ad1, measuring ALMR exophers thereafter (Fig. 3*A*). We observed the significant elevation of exophergenesis in *hsf-1*, *hif-1*, *hlh-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. 3*A*). 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*, *atg-7*, and *bec-1* in mCherryAg2 animals and scored for exophers on Ad2 (*SI Appendix*, Fig. S3*A*). 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 proteasome with 10 mM MG132 (*SI Appendix*, Fig. S3*B*) did not block the fasting-induced elevation of exophers, in further support that autophagy and proteasome 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 triplicate to identify intestinal peptide transporter *pept-1*, lipid synthesis-implicated 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. 3*B*).

pept-1 encodes a conserved intestinal di-/triamino 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, intestine, 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 *rgef-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. 3*B*), no tissue-specific knockdown was effective in blocking this response (*SI Appendix*, Fig. S4*A*). 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 Transtissue Induction of Neuronal Exophers. Food limitation stresses engage multiple signaling pathways that can activate animal defenses, including RAS/MAPK pathways that transduce developmental

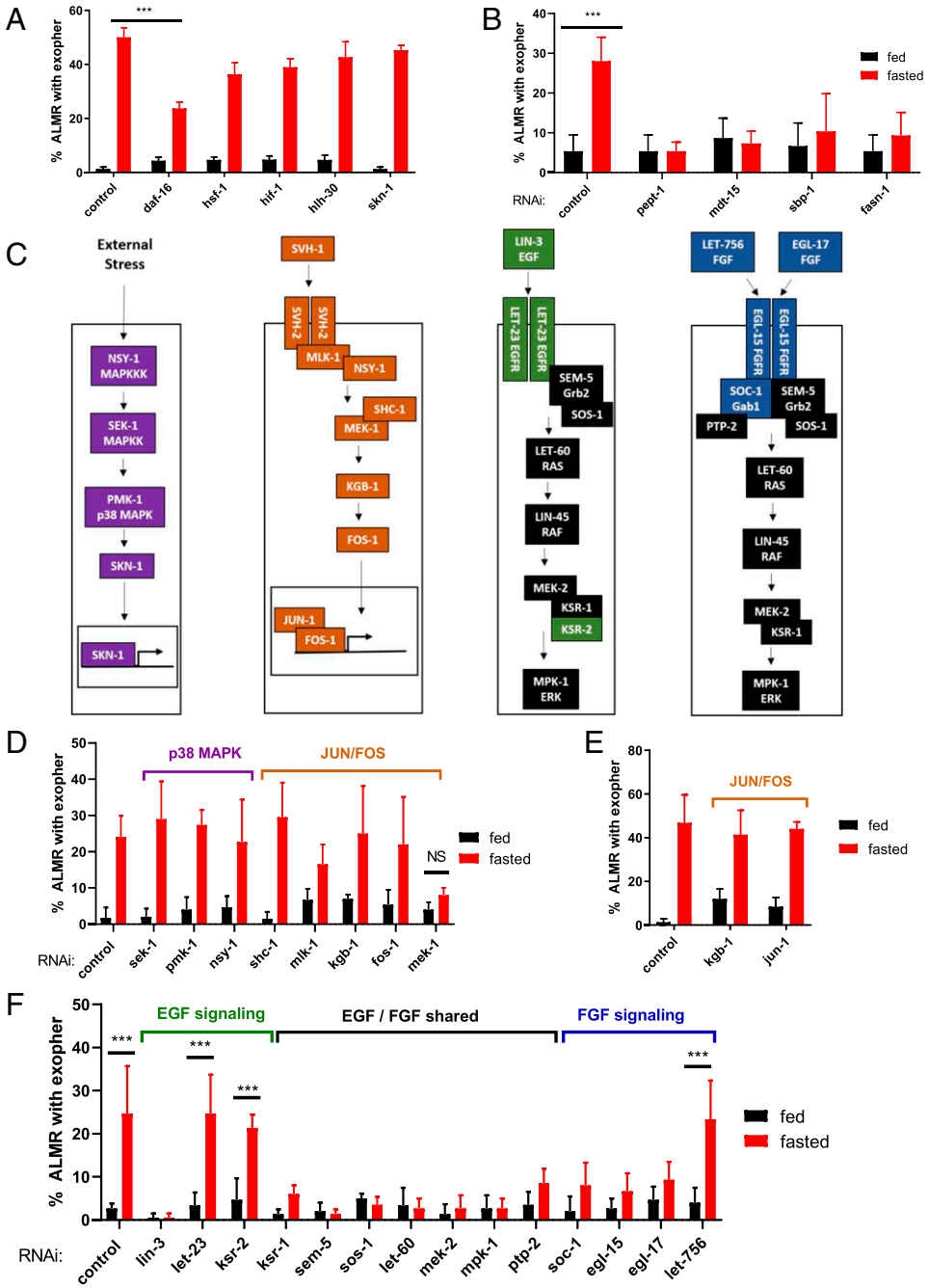

Fig. 3. Lipid synthesis and FGF-activated RAS/ERK signaling are required for the induction of neuronal exophers in response to fasting. For all panels, bars are SEM, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, and CMH statistics. Feeding RNAi was initiated at the L4 larval stage and continued until Ad2. (*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, *hsf-1*/HSF-1, *hif-1*/HIF1, *hlh-30*/TFEB and *skn-1*/NRF2) in the mCherryA2 background, Ad2: exopher counts 6× 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 exophergenesis. 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: 3× 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: 3× 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: 5× 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 3× 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. 3*C*).

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. 3*D*), 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. 3 *D* 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), suggests that the 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. 3*D*), 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. 3*F*), we focused on examining pathway members in more detail.

RAS/ERK signaling in *C. elegans* (reviewed in refs. 40 and 41; see Fig. 3*C*) 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/RAS-GTP activates MAPKKK LIN-45/RAF. Scaffold proteins KSR-1 and/or KSR-2 collect downstream members of the MAPK cascade, so LIN-45/MAPKKK activates MEK-2/MAPKK, which in turn phosphorylates and activates MPK-1/ERK. MPK-1 can then enter the nucleus to phosphorylate transcription factors that execute a transcriptional response. We found RNAi knockdown of multiple members of the conserved core RAS/ERK signaling, namely *let-60*/RAS, *mek-2*/MAPKK, *mpk-1*/ERK, *sem-5*, and *ksr-1*, disrupts the fasting-induced increase of touch neuron exophers (Fig. 3*F*). Given the implication of five core MAPK components in fasting-induced exopher elevation, we conclude that the RAS/ERK pathway plays a critical role in the mechanism by which neurons increase mCherry expulsion (and other cell contents) under food withdrawal stress.

The characterized *C. elegans* EGF and FGF signaling pathways share the aforementioned signaling components in the conserved RAS/ERK pathways but differ in ligands, receptors, KSR-type (EGFR uses both KSR-1 and KSR-2; FGFR exclusively uses KSR-1), and FGF pathway requirement for the adaptor SOC-1/Gab1 (41). We tested MAPK components in an effort to distinguish whether FGF, EGF, or both, contribute to exopher elevation in response to fasting.

We used RNAi approaches to test for a requirement of FGF ligands *egl-17* and *let-756* as well as FGF receptor *egl-15* in fasting-induced exopher elevation. Interventions with ligand *egl-17*/FGF and receptor *egl-15*/FGFR, but not FGF ligand *let-756*, disrupted the capacity to increase exophers in response to 6-h fasting, implicating a specific FGF in exopher regulation biology (Fig. 3*F*). Moreover, we find that FGF pathway-specific *soc-1* (RNAi) diminishes fasting-induced exopher production. Our data, which implicate eight FGF pathway genes (FGF ligand *egl-17*, FGF receptor *egl-15*, FGF pathway-specific *soc-1* and *sem-5*, and core pathway components *let-60*, *mek-2*, *ksr-1*, and *mpk-1*) in the fasting induction of exophers, identify an FGF-activated ERK/MAPK pathway that acts in response to 6-h food withdrawal, as an essential mechanistic step in neuronal exopher increase.

To address the potential source of *egl-17*/FGF and identify the tissue via which FGFR acts to promote fasting-elevated exopher production, we used tissue-specific RNAi approaches to disrupt all identified components in all neurons, muscle, pharynx, intestine, hypodermis, vulva, or germline (*SI Appendix*, Fig. S4*A*).

We were unable to identify single-tissue sources that were required for FGF ligand EGL-17 or FGF receptor EGL-15 activity in fasting-induced exopher elevation, suggesting that either multiple tissues can execute effective FGF signaling, that cells that we were unable to target are responsible, or that unknown technical issues might apply. We did, however, find that knockdown of the most downstream pathway targets, *mek-2* and *mpk-1*, only in the hypodermis or only in the germline (*SI Appendix*, Fig. S4*B*) could disrupt fasting induction. Our data thus suggest that *mek-2* and *mpk-1* are required in both the hypodermis and in the germline for fasting-induced exopher induction.

Germline-Produced EGF is Required for Fasting-Induced Exopher Elevation. Importantly, *mek-2* and *mpk-1* are downstream kinases for both the FGF and the EGF MAPK pathways. Indeed, our RNAi perturbation screens of MAPK pathway members indicated that *lin-3*/EGF also acts in fasting-induced exopher elevation (Fig. 3*F*). Tissue-specific RNAi studies reveal that germline-specific knockdown of *lin-3*/EGF disrupted fasting-induced exopher elevation (while hypodermal knockdown did not) (*SI Appendix*, Fig. S4*C*), implicating the germline production of LIN-3/EGF in a transgenerational influence on neuronal exophergenesis. Knockdown of *mek-2* and *mpk-1* are also needed in the germline for fasting-induced exophergenesis (*SI Appendix*, Fig. S4*B*).

RNAi disruption of EGF receptor *let-23* and EGF pathway-specific *ksr-2* did not eliminate the exopher elevation in response to fasting in our original screen (Fig. 3*F*). This observation raised the possibility that the EGF-responsive pathway might function in neurons (which are generally not as susceptible to RNAi as other tissues). Because *let-23(gf)* alleles caused extensive reproductive system development consequences that complicated the interpretation of exopher production (not shown), we tested the available strains that expressed the EGF pathway-activating EGFR/*let-23(gf)* in specific tissues for elevated exopher levels in the presence of food. We found that the transgenic introduction of EGFR/*let–23(gf)* in neurons (but not muscle or intestine) elevates exophers in the presence of abundant food (Fig. 4*A*), suggesting a neuronal-based EGF pathway in promoting fasting-induced exopher elevation.

Lipid Biosynthesis Acts Upstream of RAS/ERK Activation to Stimulate Exopher Induction. *let-60(n1046)* 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. 4*B*).

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. 4*C*). 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

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. 4*C*). We therefore repeated RNAi knockdowns of genes in the lipid synthesis and FGF/RAS/MAPK pathways in the *let-60(gf)*;mCherryAg2 background, quantitating the impact on exopher formation. As expected for known downstream kinases in the RAS pathway, the RNAi disruption of *mek-2*/MAPKK and *mpk-1*/MAPK suppressed the *let-60(gf)* phenotype (Fig. 4*D*). In contrast, genes encoding *egl-17*/FGF, *egl-15*/FGFR, 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. 4*D*). 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*/FGFR, 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 EGFR 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. 4*E*), 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. 4*F*).

Overall, we identify three pathways that are required for fasting-induced exopher elevation in stressed touch receptor neurons: lipid synthesis, an FGF/MAPK pathway, and an EGF/MAPK pathway that can act in neurons. Our data suggest a model for signaling that influences a dramatic expulsion 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 that nonautonomous signaling directs exopher production in the touch neurons. FGFR signaling pathway genes are known to be highly expressed in the hypodermis (46). Notably, RNAi evidence supports the hypothesis that the same group of genes that mediate fasting-induced exopher increase is required for exopher elevation in response to osmotic stress (*SI Appendix*, Fig. S3*C*), indicating that general, rather than fasting-specific, mechanisms are engaged.

Although many details of this complex lipid-FGF-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 expulsion 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 proteopathies is the transfer of aggregates to neighboring cells, which can seed aggregate spread and promote pathology (48, 49). In vivo dissection of the biology 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 expulsion 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/organelle 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 exophergenesis 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 Hyperosmotic Stress Can Markedly Elevate Neuronal Exopher Production. Acute food withdrawal, oxidative stress, and hyperosmotic 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 genetic and physiological conditions common to these three stresses should provide insight into molecular mechanisms that promote exophergenesis. 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 exophergenesis 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 hyperosmotic exposure both increase exophergenesis. 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 exophergenesis. The suppression of exopher production under conditions of extreme stress may be

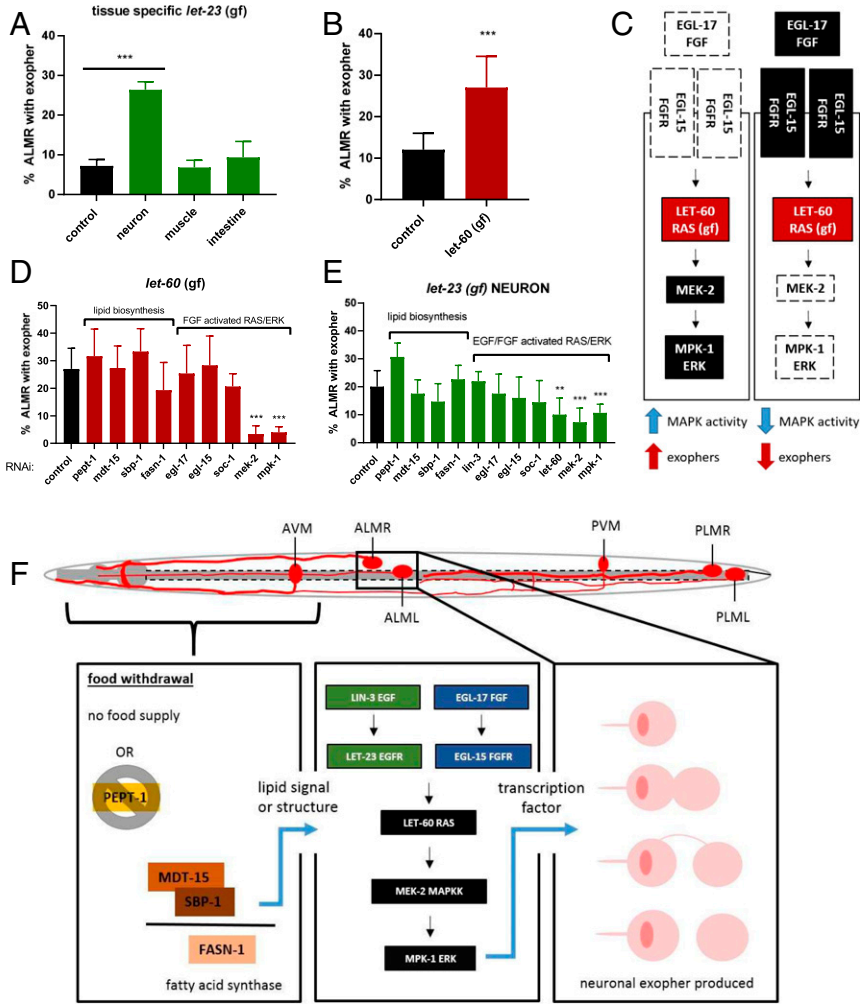

Fig. 4. Molecular pathways that influence fasting-induced exopher elevation. For all panels, bars are SEM, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, and CMH statistics. (*A*) *let-23* (gain of function) increases exopher formation when expressed specifically in neurons. *let23(sa62)* is a gain-of-function allele that activates MAPK signaling. We measured the exopher levels in strains that expressed *let-23(sa62gf)* from neuronal (*unc-119*), intestinal (*vha-6*), or muscle (*myo-3*) promoters in the mCherryAg2 background at Ad2 in the presence of food: 3× replicates and 50 animals per trial. (*B*) *let-60* (gain of function) increases exopher formation under abundant food conditions. *let-60(n1046)* is a gain-of-function RAS allele that activates MAPK signaling. We measured exopher levels in control mCherryAg2 and in *let-60(n1046)*;mCherryAg2 strains at Ad2 in the presence of food: 3× replicates and 50 animals per trial. (*C*) Schematic of the logic of epistasis within the MAPK signaling context. The phenotype of a gain-of-function allele that continuously signals will not be affected by the knockdown of any effector upstream of it within the cascade. On the other hand, any actors downstream of a constitutively active component will affect the phenotype. FGF signaling is used as an example here. (*D*) Lipid synthesis genes act upstream of *let-60(gf)*/RAS to elevate neuronal exopher production. RNAi interventions were initiated at the L4 stage on gain-of-function *let-60*;mCherryAg2 animals. Exophers were scored at Ad2: 3× replicates and 50 animals per trial. Although *mek-2* and *mpk-1*, known to act downstream of *let-60(gf)*, are critical for exopher elevation, the known upstream genes *egl-15*, *egl-17*, and *soc-1* are not, consistent with data that previously ordered the FGF pathway. *pept-1*, *mdt-15*, *sbp-1*, and *fasn-1* do not suppress *let-60(gf)*-induced exophergenesis, indicating that the lipid synthesis genes likely act upstream of *let-60*/RAS. (*E*) Lipid synthesis genes and *lin-3* act upstream of *let-23(gf)* activity when it is expressed in neurons. RNAi knockdown was initiated at the L4 developmental stage on gain-of-function *let-23(sa62);mCherryAg2* animals. Exophers were scored at Ad2: 3× replicates and 50 animals per trial. Cascade components *let-60*, *mek-2*, and *mpk-1* are known to act downstream of *let-23* and effectively abrogate the MAPK signaling leading to exophers, while *lin-3* works upstream of *let-23* and therefore cannot suppress a downstream gain-of-function mutation. RNAi knockdown of *pept-1*, *mdt-15*, *sbp-1*, and *fasn-1* do not suppress neuronal *let-23(gf)*-induced exophergenesis, indicating that the lipid synthesis genes likely act upstream of neuronal *let-23(gf)*. (*F*) Model for signaling that elevates neuronal exopher formation in response to fasting. In the sudden absence of food or when dipeptide transporter PEPT-1 activity is low, a lipid-based signal is produced/released that depends upon MDT-15/SBP-1 transcriptional activity and fatty acid synthase FASN-1. The lipid signal may act on the neurons directly or may contribute to the activation of a required FGF pathway in a relay tissue (unlikely to be neurons) to promote transcription of an essential signal that acts on touch neurons to elevate exopher production. EGF pathway activation in neurons can elevate exopher production; it remains to be determined whether EGF signaling is required in touch neurons per se. In sum, transtissue stress signaling influences remote neuronal trash management through lipid, FGF, and EGF RAS/MAPK signaling. Note that we cannot distinguish between an inducible lipid signal and a static, essential lipid product required for exopher elevation.

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,

stresses definitively elevate exopher production, but for the most part, these stresses 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 (paraquat, rotenone, 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/TFEB, 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 regulation of neuronal aggregate expulsion. Our data do not rule out whether HSF-1, HIF-1, HLH-30/TFEB, 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 biogenesis 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 associated with the exposure to reagents like paraquat (63). The requirement for *mdt-15* in fasting-induced, neuronal exophergenesis adds a new facet to the MDT-15 integration of multiple, transcriptional 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 *fasn-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 exophergenesis.

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 exopher 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 FGFR action is the hypodermis, which is necessary for some MEK-2/MPK-1 MAPK signaling. Such signaling is permissive for fasting-induced exopher production and the hypodermis is a known site of expression of FGFR pathway components (*SI Appendix*, Fig. S4*B*). 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 differences have been noted (67), FGF21 is one of the most upregulated rat liver genes under the conditions of an 8-h fast (68). FGF21 can cross the blood–brain barrier to change hypothalamic neuron gene expression (69). Overall, the implication of FGF/ERK pathways in the response of neurons to food limitation 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 mechanisms 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. S4*C*), 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 address whether EGFR directly activates exophergenesis in touch neurons or engages additional neurons as intermediatory 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 development. For example, EGF signaling activates FGF production and a downstream FGF pathway required in vulval epithelial fate specification (70). Starvation conditions can influence the signaling 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 induce neuronal trash elimination is unclear. One possibility is that exophergenesis [which we track in single neurons in our study but is likely to also occur in other neurons and cells (4)] might serve as a mechanism to discard superfluous, neuronal proteins and organelles for degradative recycling in neighboring cells as resources 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 mouse 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 extreme stress levels, 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 *bzIs166* [P_{mec-4}mCherry1] II, also referred to as mCherryAg2
ZB5160 *bzIs166* [P_{mec-4}mCherry1] II; *hsf-1(sy441)* I
ZB5161 *bzIs166* [P_{mec-4}mCherry1] II; *kgb-1(um3)* IV
ZB5162 *bzIs166* [P_{mec-4}mCherry1] II; *skn-1(zj15)* IV
ZB5163 *bzIs166* [P_{mec-4}mCherry1] II; *jun-1(gk551)* II
ZB5164 *bzIs166* [P_{mec-4}mCherry1] II; *hif-1(ia4)* V
ZB5165 *bzIs166* [P_{mec-4}mCherry1] II; *daf-16(mu86)* I
ZB5166 *bz166* [P_{mec-4}mCherry1] II; *hlh-30(tm1978)* IV
ZB5186 *bzIs166* [P_{mec-4}mCherry1] II; *let-60(n1046)* IV
SK4005 *zdIs5*[P_{mec-4}GFP]
BY250 *vtIs7*[P_{dat-1}GFP]
ZB4599 *let-23(sa62)* II; *bzIs166* [P_{mec-4}mCherry1] II
ZB5184 *bzIs166* [P_{mec-4}mCherry1] II; *sid-1(qt9)* V; *sqIs71* [P_{rgef-1}GFP; P_{rgef-1}*sid-1*;pBS]
ZB5185 *bzIs166* [P_{mec-4}mCherry1] II; *sid-1(qt9)* V; *alxIs6* [P_{vha-6}*sid-1*::sl2::GFP]
ZB5020 *bzIs166* [P_{mec-4}mCherry1] II; *mkcSi13* [P_{sun-1}*rde-1*::*sun-1* 3'UTR + *unc-119*(+)] II; *rde-1(mkc36)* V
ZB5173 *bzIs166* [P_{mec-4}mCherry1] II; *sid-1(qt9)* V; *bzIs153* [P_{myo-2}*sid-1*]
ZB5172 *bzIs166* [P_{mec-4}mCherry1] II; *rde-1(mkc36)* V; *mfIs70*[P_{lin-31}*rde-1*; P_{myo-2}GFP]
ZB5298 *bzIs166* [P_{mec-4}mCherry1] II; *sid-1(qt9)* V; *bzSi33* [P_{myo-2} *sid-1*; P_{myo-2}GFP]
ZB5299 *bzIs166* [P_{mec-4}mCherry1] II; *sid-1(qt9)* V; *bzSi13* [P_{hyp-7}*sid-1*; P_{myo-2}GFP]
ZB5300 *bzIs166* [P_{mec-4}mCherry1] II; *sid-1(qt9)* V; *uthIs237* [P_{myo-3}tomato; P_{myo-3}*sid-1*]
ZB5301 *bzIs166* [P_{mec-4}mCherry1] II; *let-23(sa62)* II; *bzIs155* [P_{myo-2} GFP] (promoter-less control)
ZB5302 *bzIs166* [P_{mec-4}mCherry1] II; *bzIs152* [$P_{unc-119}$*let-23(sa62)*; P_{myo-2}GFP]
ZB5303 *bzIs166* [P_{mec-4}mCherry1] II; *bzIs151* [P_{myo-3}*let-23(sa62)*; P_{myo-2}GFP]
ZB5304 *bzIs166* [P_{mec-4}mCherry1] II; *bzIs153* [P_{vha-6}*let-23(sa62)*; P_{myo-2}GFP]
ZB5171 *bzSi39* [P_{mec-7}HttQ74::mNG]; *bzSi34* [P_{mec-7}mScarlet::IFD-1]

Data Availability. All study data are included in the article and/or *SI Appendix*.

Note Added in Proof. A recent report implicates reproduction in muscle exopher production (75) consistent with a role for germline in nonautonomous regulation of exopher-inducing signals.

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