FSHR-1/GPCR Regulates the Mitochondrial Unfolded Protein Response in *Caenorhabditis elegans*

Sungjin Kim* and Derek Sieburth*,^{1,†}

*Zilkha Neurogenetic Institute, Keck School of Medicine and [†]Department of Physiology and Neuroscience, University of Southern California, Los Angeles, California 90033

ABSTRACT The mitochondrial unfolded protein response (UPR^{mt}) is an evolutionarily conserved adaptive response that functions to maintain mitochondrial homeostasis following mitochondrial damage. In *Caenorhabditis elegans*, the nervous system plays a central role in responding to mitochondrial stress by releasing endocrine signals that act upon distal tissues to activate the UPR^{mt}. The mechanisms by which mitochondrial stress is sensed by neurons and transmitted to distal tissues are not fully understood. Here, we identify a role for the conserved follicle-stimulating hormone G protein-coupled receptor, *FSHR-1*, in promoting UPR^{mt} activation. Genetic deficiency of *fshr-1* severely attenuates UPR^{mt} activation and organism-wide survival in response to mitochondrial stress. *FSHR-1* functions in a common genetic pathway with *SPHK-1*/sphingosine kinase to promote UPR^{mt} activation, and *FSHR-1* regulates the mitochondrial association of *SPHK-1* in the intestine. Through tissue-specific rescue assays, we show that *FSHR-1* functions in neurons to activate the UPR^{mt}, to promote mitochondrial association of *SPHK-1* in the intestine, and to promote organism-wide survival in response to mitochondrial stress. We propose that *FSHR-1* functions cell nonautonomously in neurons to activate UPR^{mt} upstream of *SPHK-1* signaling in the intestine.

KEYWORDS FSHR-1; paraquat; sphingosine kinase; UPR^{mt}

THE mitochondrial unfolded protein response (UPR^{mt}) functions to maintain mitochondrial protein homeostasis in response to mitochondrial dysfunction caused by mitochondrial DNA damage, incorrect mitochondrial protein folding, or impaired oxidative phosphorylation. Failure to appropriately control and maintain protein homeostasis in the mitochondria is associated with the development of numerous diseases, neurodegeneration, and ageing (Durieux *et al.* 2011; Liu *et al.* 2014; Pellegrino *et al.* 2014; Nargund *et al.* 2015; Fiorese *et al.* 2016; Martinez *et al.* 2017). The UPR^{mt} is initiated when mitochondrial proteostasis is disrupted, the detection of which by mitochondrial and cytosolic factors leads to epigenetic modifications and transcriptional responses in the nucleus that restore mitochondrial function. A critical sensor and activator of the UPR^{mt} in *Caenorhabditis elegans* and in mammals is the leucine zipper transcription factor ATSF-1/ATF5 (Fiorese *et al.* 2016). *ATFS-1* is normally

targeted to mitochondria where it is degraded, but upon mitochondrial stress, mitochondrial import is disrupted and *ATFS-1* is targeted instead to the nucleus where it regulates the expression of a cascade of genes including the conserved HSP70-like chaperone, *hsp-6*, which is targeted to the mitochondria to restore protein folding (Nargund *et al.* 2012). The intestinal UPR^{mt} can be activated by mitochondrial stress originating either cell autonomously in the intestine or cell nonautonomously in the nervous system. Mitochondrial stress in neurons activates the UPR^{mt} in the intestine through the release of neuropeptides, serotonin, and/or Wnt ligands (Berendzen *et al.* 2016; Shao *et al.* 2016; Zhang *et al.* 2018).

Genetic screens for additional factors that activate the UPR^{mt} have revealed important roles for mitochondrial ceramide produced by *SPTL-1*/serine palmitoyltransferase and sphingosine-1-phosphate (S1P) produced by *SPHK-1*/sphingosine kinase in the activation of the UPR^{mt} (Liu *et al.* 2014; Kim and Sieburth 2018a). *SPHK-1* recruitment to mitochondria from cytoplasmic pools may serve as an early signal to activate UPR^{mt} (Kim and Sieburth 2018a). *SPHK-1* mitochondrial recruitment is positively regulated by mitochondrial stress originating either from the intestine or from the nervous system. Neuronal mitochondrial stress activates

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¹Corresponding author: Zilkha Neurogenetic Institute, University of Southern California, 1501 San Pablo St., Los Angeles, CA 90033. E-mail: sieburth@usc.edu

intestinal SPHK-1 by a mechanism that involves neuropeptide, but not serotonin, signaling (Kim and Sieburth 2018a). Neuropeptides exert their biological functions primarily through activating G protein-coupled receptors (GPCRs) on target cells to trigger downstream signaling events (Frooninckx *et al.* 2012). However, the specific neuropeptides and the GPCRs functioning in SPHK-1-mediated UPR^{mt} activation have not been identified.

FSHR-1 is a GPCR-containing extracellular Leucine-Rich Repeats (LRRs) protein that is homologous to the follicle-stimulating hormone receptor family (Powell *et al.* 2009). FSHR-1 plays a critical role in activating innate immunity in response to infection by pathogenic bacteria, and functions in the intestine to promote protection against pathogenic infection and to regulate antimicrobial gene expression. (Cho *et al.* 2007; Powell *et al.* 2009; Miller *et al.* 2015). Interestingly, infection by pathogenic bacteria leads to multiple cellular responses in the intestine, including the activation of the UPR^{mt} (Liu *et al.* 2014; Pellegrino *et al.* 2014).

In this study, through the analysis of *fshr-1* null mutants, we show that FSHR-1 positively regulates UPR^{mt} activation and promotes the mitochondrial association of SPHK-1 in the intestine. Through tissue-specific rescue experiments, we find that FSHR-1 functions in the nervous system to exert its function in UPR^{mt} activation. We propose that FSHR-1 is part of a neuroendocrine signaling network that functions to regulate the UPR^{mt} through intertissue signaling.

Materials and Methods

C. elegans strains

Strains used in this study were maintained at 22° following standard methods. Young adult hermaphrodites derived from the wild-type reference strain N2 Bristol were used for all experiments. The following mutant strains were used. SJ4100: *zcls13*[*Phsp-6::GFP*], OJ4113: *vjls138*[*Pges-1::sphk-1::gfp*], OJ4143: *vjls148*[*Pges-1::tomm-20::mCherry*], ST66: *ncls17*[*Phsp-16.2::gfp*], OJ2329: *vjls208*[*Pgst-4::gfp*], *uthls375*[*unc-119p::cco-1 HP + rol-6(su1006)*](Zhang *et al.* 2018), OJ997: *sphk-1(ok1097)*, GR2250: *mgl573*[*cyp-14A4p::gfp*], and KP3397: *fshr-1(ok778)*. The *sphk-1(ok1097)* and *fshr-1(ok778)* strains were outcrossed at least six times with wild-type animals prior to analysis.

Molecular biology

fshr-1 complementary cDNA (cDNA) was cloned from *C. elegans* wild-type cDNA and then inserted into the pPD49.26 expression vector using standard molecular biology techniques. The following plasmids were generated: *pSK55*[*Pges-1::fshr-1*], *pSK56*[*Prab-3::fshr-1*], and *pSK98*[*Prgef-1::fshr-1*]. Sequence files of plasmids are available upon request.

Transgenic lines

Transgenic strains were generated by injecting expression constructs (10–25 ng/μl) and the co-injection marker

pJQ70 [*Pofm-1::mCherry*, 40 ng/μl], *KP#708*[*Pttx-3::rfp*, 40 ng/μl], or *KP#1106* [*Pmyo-2::gfp*, 10 ng/μl] into N2 worms or the indicated mutants using standard techniques (Mello *et al.* 1991). At least three lines for each transgene were tested and a representative transgene was used for further experiments. The following transgenic lines were made: *vjEx1448*[*Prab-3::fshr-1*], *vjEx1449*[*Pges-1::fshr-1*], *vjEx1000*[*Pges-1::gfp*], and *vjEx1831*[*Prgef-1::fshr-1*].

Microscopy and analysis

Fluorescence microscopy experiments were performed following previous methods (Kim and Sieburth 2018a). Briefly, L4-stage or young adult worms were immobilized by using 2,3-butanedione monoxime (BDM, 30 mg/ml; Sigma [Sigma Chemical], St. Louis, MO) in M9 buffer then mounted on 2% agarose pads for imaging. To quantify the fluorescence intensity of *Phsp-6::GFP* or *Pgst-4::GFP*, Z stacks of the intestine posterior to the vulva were selected as a representative region because of low basal expression in the absence of stress. Images were captured with the Nikon (Garden City, NY) eclipse 90i microscope equipped with a Nikon PlanApo 40×, or 60× or 100× objective (NA = 1.4), and a PhotometricsCoolSnap ES2 or a Hamamatsu Orca Flash LT + CMOS camera. Metamorph 7.0 software (Universal Imaging/Molecular Devices) was used to capture serial image stacks, and the maximum intensity was measured (Kim and Sieburth 2018b). Intensity quantification analysis was performed on the same day to equalize the absolute fluorescence levels between samples within the same experimental set. For Figure 1A, each sample cluster of worms was captured using a 4× objective lens for the representative images as shown in Figure 1A, then captured by a 40× objective lens for fluorescence intensity quantification.

RNA interference

A feeding RNA interference (RNAi) knockdown assay was performed following established protocols (Kamath and Ahringer 2003). Briefly, gravid adult animals were placed on RNAi plates seeded with HT115(DE3) bacteria transformed with L4440 vector containing a genomic fragment of the gene to be knocked down (or empty L4440 vector as a control) to collect eggs, then removed after 4 hr to obtain an age-matched synchronized worm population. Young adult animals were used for subsequent assays.

Stress induction assays

For drug-induced stress, transgenic L4 animals were transferred to fresh NGM plates seeded with HB101 bacteria, then 80 μl of stock solutions dissolved in M9 buffer of paraquat were added to plates for a final concentration of 0.4 mM paraquat. After 24 hr, adults were selected for fluorescence microscopy analysis. For imaging of animals that had been subjected to RNAi-induced knockdown, L4 animals grown on RNAi plates were transferred to new RNAi plates to obtain synchronized animals and imaged 24 hr later. For *Pgst-4::GFP* imaging, young adult animals were incubated with 5 mM

Figure 1 FSHR-1 promotes UPR^{mt} activation. (A) Representative low-magnification images of several wt adults or *fshr-1* mutants expressing the *zcls13* [*Phsp-6::GFP*] transgene (GFP driven by the *hsp-6* promoter), following treatment with M9 (control) or PQ for 24 hr. (B and C) Left: representative high-magnification images of intestines posterior to the vulva in young adult wt animals or *fshr-1* mutants expressing *Phsp-6::GFP* following treatment with M9 (control) or PQ (B), or DMSO (control) or antimycin (C) for 24 hr. Right: average GFP fluorescence intensity is quantified. (D) Left: representative images of *Phsp-6::GFP* fluorescence in intestines of wt or *fshr-1* mutants following e.v. control or *cco-1/cox-5B* RNAi treatment by feeding. Right: average GFP fluorescence intensity is quantified. (E and F) Left: representative intestine images of wt or *fshr-1* mutants expressing the *zcls9* [*Phsp-60::GFP*] transgene following M9 and e.v. (G) (control), or PQ or *spg-7/AFG3L2* RNAi treatment. Right: average GFP fluorescence intensity is quantified. (G) Left: representative images of wt or *fshr-1* mutants expressing the *mgl573* [*Pcp-14A4::gfp*] transgene in the absence or presence of PQ. Right: average GFP fluorescence intensity is quantified. (H) Left: representative images of posterior intestines from animals expressing the antioxidant reporter transgene [*Pgst-4::GFP*] in wt or *fshr-1* mutants in the absence or presence of As. Right: average GFP fluorescence intensity is quantified. (I) Left: representative images of posterior intestines from animals expressing heat shock transgene *ncls17* [*Phsp-16.2::GFP*] in wt or *fshr-1* mutants following 22° (control) or 37° exposure for 20 min. Right: average GFP fluorescence intensity is quantified. Bar represents 10 μ m. Error bars indicate $\pm$ SEM. One-way ANOVA with Bonferroni post-test * P < 0.1; ** P < 0.01; *** P < 0.001. As, arsenite; e.v., empty vector; ns, not statistically significant; PQ, paraquat; RNAi, RNA interference; UPR^{mt}, mitochondrial unfolded protein response; wt, wild-type.

arsenite or 50 mM paraquat in liquid solution (in M9 buffer) for 1 hr, then drug was washed out and worms were transferred onto new NGM plates for 4 hr before imaging. M9 buffer was used as a control for paraquat and arsenite treatment.

For *Phsp-16.2::GFP* imaging, young adult animals were incubated at 22° or 37° for 20 min, and then allowed to recover at 22° for 24 hr prior to imaging. For paraquat survival assays, young adult animals were placed onto NGM plates

containing 10 mM paraquat for 17 hr. After 17 hr, the percentage of surviving animals was counted every 3 hr over the course of 15 hr. Survival assays were done in experimental duplicate for each biological duplicate.

Statistical analysis

We utilized one-way ANOVA with a Bonferroni post-test to evaluate the statistical significance for multiple sample comparisons including Figure 3B (26 hr), while Student's *t*-tests were used for two-sample comparison using Graphpad Prism 8. *P*-values < 0.01 or 0.001 are indicated with asterisks (***P* < 0.01 and ****P* < 0.001, respectively). Error bars in the figures indicate the SE of the mean ($\pm$ SEM). At least 15 animals per sample were analyzed for fluorescence quantification and data points, and averages are presented as shaped box plots in the figures.

Data availability

Strains and plasmids used in this study are available upon request. The authors confirm that all data necessary for confirming the conclusions of the findings are present within the article and figures.

Results

FSHR-1/GPCR signaling regulates the UPR^{mt}

Prior studies found that *fshr-1* mutants have normal life spans (Powell *et al.* 2009), but exhibit enhanced sensitivity to lethality caused by the mitochondrial toxin and UPR^{mt} activator paraquat (Miller *et al.* 2015). Because impairing UPR^{mt} activation causes sensitivity to paraquat-induced lethality (Nargund *et al.* 2012; Gatsi *et al.* 2014; Liu *et al.* 2014; Kim and Sieburth 2018a), we speculated that FSHR-1 may positively regulate the UPR^{mt}. To monitor UPR^{mt} activation, we quantified the intestinal fluorescence of the UPR^{mt} transcriptional reporter *zcls13*, in which GFP is expressed under control of the *hsp-6* promoter (*Phsp-6::GFP*). Paraquat is an oxidant that interferes with electron transport at the inner mitochondrial membrane, and has been used widely to acutely activate the UPR^{mt} in both *C. elegans* and in mammalian cells (Nargund *et al.* 2012; Runkel *et al.* 2013; Fiorese *et al.* 2016; Kim and Sieburth 2018a). Wild-type animals treated with paraquat for 24 hr exhibited a roughly 10-fold increase in *Phsp-6::GFP* expression in the intestine compared to nontreated controls (Figure 1, A and B). In contrast, *fshr-1* mutants showed a slight increase in baseline *Phsp-6::GFP* expression, and either no significant change or a small increase in *Phsp-6::GFP* expression following paraquat treatment (Figure 1, A and B). Antimycin is a mitochondrial electron transport chain (ETC) stressor, and antimycin treatment for 24 hr robustly induces the UPR^{mt} (Liu *et al.* 2014). *fshr-1* mutations attenuated the antimycin-induced increase in *Phsp-6::GFP* expression (Figure 1C). *cco-1* (aka *cox-5B*) encodes the cytochrome *c* oxidase subunit in complex IV, which is the terminal electron acceptor of the ETC, and

RNAi-mediated *cco-1* knockdown is a potent activator of the UPR^{mt} (Nargund *et al.* 2012; Berendzen *et al.* 2016; Merkwirth *et al.* 2016; Tian *et al.* 2016; Kim and Sieburth 2018a). *cco-1* RNAi by feeding, which induces mitochondrial stress in the intestine (Zhang *et al.* 2018), significantly increased *Phsp-6::GFP* expression in wild-type animals but failed to increase *Phsp-6::GFP* expression in *fshr-1* mutants (Figure 1D). *fshr-1* mutations also blocked or attenuated the stress-induced increase in expression of two additional UPR^{mt} reporters: *Phsp-60::GFP* and *Pcyp-14A4::GFP* [Figure 1, E–G, Nargund *et al.* (2012), and Mao *et al.* (2019)].

fshr-1 mutants were not defective in the induction of the antioxidant reporter *Pgst-4::GFP* in response to treatment with the mitochondrial reactive oxygen species (ROS) generator arsenite [Figure 1H; 1.6-fold increase in wild-type and 1.9-fold increase in *fshr-1* mutants by arsenite (Inoue *et al.* 2005; Choe *et al.* 2009; Ruiz-Ramos *et al.* 2009; Prakash *et al.* 2015; Wu *et al.* 2016)]. In addition, *fshr-1* mutants exhibited normal induction of the heat-shock reporter *Phsp-16.2::GFP* in response to heat shock at 37° for 20 min (Figure 1I). Together, these results suggest that FSHR-1 plays a specific role in activating the UPR^{mt} in response to mitochondrial stress.

FSHR-1/GPCR functions cell nonautonomously in neurons to regulate UPR^{mt}

FSHR-1 is expressed primarily in the intestine and in a subset of neurons (Sieburth *et al.* 2005; Cho *et al.* 2007; Cao *et al.* 2017). To determine in which tissue FSHR-1 functions to regulate the UPR^{mt}, we examined *Phsp-6::GFP* induction in transgenic *fshr-1* mutants expressing *fshr-1* cDNA in either the nervous system (using the *rab-3* or the *rgef-1* promoter) or the intestine (using the *ges-1* promoter). Pan-neuronal *fshr-1* either partially (*rab-3* promoter) or fully (*rgef-1* promoter) rescued the paraquat-induced *hsp-6* expression defects of *fshr-1* mutants (Figure 2, A and B). In contrast, intestinal *fshr-1* cDNA expression failed to rescue the paraquat-induced *hsp-6* expression defects of *fshr-1* mutants (Figure 2A). RNAi-mediated knockdown of *fshr-1* by bacterial feeding, which efficiently knocks down genes in the intestine but not in the nervous system (Kamath *et al.* 2001; Timmons *et al.* 2001; Asikainen *et al.* 2005), did not attenuate paraquat-induced *hsp-6* expression, confirming that *fshr-1* does not function in the intestine to regulate the UPR^{mt} (Figure 2D). Taken together, these results suggest that FSHR-1 primarily functions cell nonautonomously in the nervous system to positively regulate UPR^{mt} activation.

The intestinal UPR^{mt} can be activated by cell-autonomous stress originating in the intestine or by cell-nonautonomous stress originating in the nervous system (Nargund *et al.* 2012; Berendzen *et al.* 2016; Merkwirth *et al.* 2016; Zhang *et al.* 2018). *fshr-1* is required for proper UPR^{mt} activation elicited by intestinal stress (Figure 1, D and F). To determine whether *fshr-1* promotes UPR^{mt} activation elicited by neuronal stress, we examined *Phsp-6::GFP* expression in animals in which *cco-1* was selectively silenced in the nervous system. Neuronal *cco-1* knockdown

Figure 2 FSHR-1 functions in the nervous system to regulate the UPR^{mt}. (A and B) Left: representative images of posterior intestines of young adult animals or *fshr-1* mutants expressing the *Phsp-6::GFP* transgene following treatment with M9 (control) or PQ for 24 hr. “*fshr-1* neuronal rescue” denotes *fshr-1* mutants expressing *fshr-1* cDNA under the neuron-specific promoter *rab-3* (A) or *rgef-1* (B). “*fshr-1* intestine rescue” denotes *fshr-1* mutants expressing *fshr-1* cDNA under the intestine-specific promoter *ges-1*. Right: average GFP fluorescence intensity is quantified. (C) Left: representative images of intestines of wt or *fshr-1* mutants expressing *Phsp-6::GFP* following e.v. control or *atfs-1* RNAi treatment by feeding. Right: average GFP fluorescence intensity is quantified. (D) Left: representative images of intestines of wt worms expressing *Phsp-6::GFP* following e.v. control or *fshr-1* RNAi treatment by feeding in the absence or presence of PQ. Right: average GFP fluorescence intensity is quantified. (E) Left: representative images of *Phsp-6::GFP* fluorescence in intestines of wt or *fshr-1* mutants in the absence or presence of transgene *uth1s375[unc-119p::cco-1 HP + rol-6(su1006)]*. Right: average GFP fluorescence intensity is quantified. (F) Left: representative images of intestinal cells of wt or *fshr-1* mutants expressing the mitochondrial marker TOMM-20::mCherry under the intestinal-specific promoter *ges-1*. Right: average mCherry fluorescence intensities are quantified. (G) Left: representative images of animals expressing soluble GFP in the intestine (*Pges-1::GFP*) in wt or *fshr-1* mutants. Right: average GFP fluorescence intensities are quantified. Bar in the panel represents 10 μ m. Error bars indicate $\pm$ SEM. One-way ANOVA with Bonferroni post-test *** $P < 0.001$; # $P < 0.001$ compared to *fshr-1*; *Prab-3::fshr-1*; @ $P > 0.05$ compared to wt; \$ $P > 0.05$ compared to wt; and & $P > 0.05$ compared to wt + PQ. cDNA, complementary DNA; e.v., empty vector; ns, not statistically significant; PQ, paraquat; RNAi, RNA interference; UPR^{mt}, mitochondrial unfolded protein response; wt, wild-type.

by hairpin-mediated RNAi is a potent activator of the intestinal UPR^{mt} (Zhang *et al.* 2018). Neuronal *cco-1* knockdown elicited similar intestinal *Phsp-6::GFP* expression in *fshr-1* mutants as in wild-type controls (Figure 2E). The fold induction was slightly reduced in *fshr-1* mutants

[4.3-fold for *fshr-1*(+) vs. 2.2 fold for *fshr-1*(-)] due to increased baseline *hsp-6* expression in *fshr-1* mutants (Figure 2E). Thus, *fshr-1* appears to be largely dispensable for UPR^{mt} activation when stress originates from the nervous system.

Neuronal *FSHR-1* regulates baseline *hsp-6* and *hsp-60* expression

Under nonstressed conditions, *fshr-1* mutants exhibited small but significant increases in *Phsp-6::GFP* expression in the intestine compared to wild-type controls (Figure 1, A–D), and larger increases in *Phsp-60::GFP* expression (Figure 1, E and F) that could reach levels similar to those seen under stressed conditions (Figure 1E). The increase in *Phsp-6::GFP* expression was blocked by RNAi-mediated knockdown of *atfs-1* (Figure 2C) and was restored to wild-type levels by pan-neuronal, but not by intestinal, *fshr-1* cDNA expression (Figure 2, A and B), suggesting that the UPR^{mt} is activated even in the absence of stress in *fshr-1* mutants. Together, these results indicate that neuronal *FSHR-1* functions cell nonautonomously to keep UPR^{mt} activity low in the absence of stress.

Mitochondria marked by the mitochondrial localization signal of TOMM-20 fused to mCherry (*TOMM-20::mCherry*) are distributed in a highly reticulated pattern within the cytoplasm of intestinal cells [expressed under the *ges-1* promoter; Figure 2E, Palikaras *et al.* (2015), and Kim and Sieburth (2018a)]. *fshr-1* mutants exhibited a normal overall pattern of *TOMM-20::mCherry* fluorescence in intestinal cells (Figure 2E), but the average fluorescence intensity of *TOMM-20::mCherry* associated with mitochondria was significantly increased by about threefold in *fshr-1* mutants compared to wild-type controls (Figure 2F). In control experiments, we found that *fshr-1* mutants exhibited no change in the fluorescence intensity of soluble GFP expressed under the *ges-1* promoter (Figure 2G), suggesting that transgene expression was not altered in *fshr-1* mutants. The increase in *TOMM-20* fluorescence intensity in *fshr-1* mutants could reflect either an increase in mitochondrial mass, a decrease in *TOMM-20* turnover, or an increase in *TOMM-20* targeting to the outer mitochondrial membrane. Alterations in mitochondrial mass, morphology, or mitochondrial protein import have been linked to UPR^{mt} activation (Ungvari *et al.* 2011; Houtkooper *et al.* 2013; Lerner *et al.* 2013; Mouchiroud *et al.* 2013; Mao *et al.* 2019).

FSHR-1 and *SPHK-1* function in a common pathway to activate the UPR^{mt}

SPHK-1 functions in the intestine to activate the UPR^{mt} in response to a variety of mitochondrial stressors, including paraquat (Kim and Sieburth 2018a). To test whether *FSHR-1* functions in a common pathway with *SPHK-1* to activate the UPR^{mt}, we examined genetic interactions between *sphk-1* and *fshr-1* mutants. *sphk-1* mutants exhibited a significant reduction in paraquat-induced expression of *Phsp-6::GFP* (89% reduction), which is similar to that exhibited by *fshr-1* mutants [92% reduction; Figure 3A and Kim and Sieburth (2018a)]. Double mutants lacking both *sphk-1* and *fshr-1* exhibited increased baseline expression of *hsp-6*, which was similar to that of *fshr-1* mutants, and had defects in paraquat-induced *hsp-6* induction that were no more severe than those seen in single mutants (93% reduction, Figure 3A).

sphk-1 mutants exhibit reduced survival rates when exposed to toxic levels of paraquat (Kim and Sieburth 2018a), which are similar to those of *fshr-1* mutants (Figure 3B). The survival rates of double mutants lacking both *sphk-1* and *fshr-1* were no more severe than those of either single mutant (Figure 3B). Expression of *sphk-1* cDNA in the intestine fully rescued the increased paraquat-induced lethality of *sphk-1* mutants (Kim and Sieburth 2018a). Expression of *fshr-1* cDNA in the nervous system fully rescued the increased paraquat-induced lethality of *fshr-1* mutants, whereas expression of *fshr-1* cDNA in the intestine partially restored paraquat sensitivity to *fshr-1* mutants [Figure 3B and Miller *et al.* (2015)]. Taken together, *FSHR-1* and *SPHK-1* may function in a common pathway to activate the UPR^{mt}, and to promote organism-wide protection from mitochondrial stress-induced lethality. Moreover, *FSHR-1* functions in the nervous system to activate the UPR^{mt} and promote survival, whereas *SPHK-1* functions exclusively in the intestine.

FSHR-1/GPCR regulates mitochondrial association of *SPHK-1* in the intestine

SPHK-1 localizes to intestinal mitochondria and is recruited to mitochondria from cytosolic pools by mitochondrial stress (Kim and Sieburth 2018a). *SPHK-1::GFP* fusion proteins, which are fully functional in rescuing the UPR^{mt} defects of *sphk-1* mutants, colocalize with *TOMM-20::mCherry* (Figure 4A). Mitochondrial stress induces a significant increase in mitochondrial *SPHK-1::GFP* fluorescence, as well as an increase in the number of intestinal cells in which *SPHK-1::GFP* adopts a mitochondrial distribution pattern [referred to as mitochondrial occupancy; Figure 4B and Kim and Sieburth (2018a)]. To determine whether *FSHR-1* regulates *SPHK-1* mitochondrial association, we examined the mitochondrial abundance of *SPHK-1::GFP* fusion proteins before and after paraquat exposure. *fshr-1* mutants exhibited two defects in *SPHK-1::GFP* mitochondrial association. First, *fshr-1* mutants exhibited a nearly twofold reduction in *SPHK-1::GFP* mitochondrial association compared to wild-type controls in the absence of paraquat (Figure 4B). Second, the paraquat-induced increase of mitochondrial *SPHK-1::GFP* association was completely abolished in *fshr-1* mutants (Figure 4B). Thus, *FSHR-1* positively regulates *SPHK-1* association with mitochondria under nonstressed conditions and *FSHR-1* is required for stress-induced *SPHK-1* mitochondrial targeting.

Neuronal *fshr-1* cell-nonautonomously regulates mitochondrial association of *SPHK-1* in the intestine

To determine in which tissue *FSHR-1* functions to regulate the mitochondrial association of *SPHK-1*, we quantified *SPHK-1::GFP* mitochondrial fluorescence in *fshr-1* mutants expressing *fshr-1* cDNA in either the nervous system or in the intestine. We found that pan-neuronal expression of *fshr-1* cDNA in *fshr-1* mutants restored both the mitochondrial occupancy and fluorescence intensity of *SPHK-1::GFP* to wild-type levels in the absence of stress. Similarly, neuronal *fshr-1*

Figure 3 *FSHR-1* functions in a common pathway with *SPHK-1* to activate the *UPR^{mt}*. (A) Left: representative images of *Phsp-6::GFP* expression in the intestines of wild-type, *sphk-1* mutants, *fshr-1* mutants, and *sphk-1;fshr-1* double mutants in the absence or the presence of PQ for 24 hr. Right: average GFP fluorescence intensity is quantified. (B) Survival rate curves of the indicated strains on plates containing 10 mM PQ. Bar represents 10 μ m. One-way ANOVA

with Bonferroni post-test: @ $P < 0.001$ compared to *sphk-1*; & $P < 0.001$ compared to *sphk-1*; # $P < 0.001$ compared to *fshr-1*; \$ $P < 0.001$ compared to *fshr-1*; and ^ $P < 0.001$ compared to *sphk-1;fshr-1*; ns: $P > 0.05$ compared to *fshr-1;FSHR-1* neuron rescue; * $P < 0.05$ compared to wt; and *** $P < 0.001$ compared to wt. ns, not statistically significant; PQ, paraquat; *UPR^{mt}*, mitochondrial unfolded protein response; wt, wild-type.

cDNA also restored normal paraquat-induced *SPHK-1::GFP* mitochondrial recruitment to *fshr-1* mutants. In contrast, intestinal *fshr-1* cDNA expression failed to rescue the *SPHK-1::GFP* fluorescence defects of *fshr-1* mutants (Figure 4B). These results suggest that *FSHR-1* functions exclusively in the nervous system to positively regulate baseline *SPHK-1* abundance in the intestine in the absence of stress, and to promote paraquat-induced *SPHK-1* mitochondrial association.

Discussion

The nervous system coordinates various stress responses by releasing diffusible factors that act upon distal tissues to activate cellular defense programs (Nussbaum-Krammer and Morimoto 2014; Berendzen *et al.* 2016; Shao *et al.* 2016). Here, we show that the conserved GPCR *FSHR-1* is part of an integrated organism-wide response to mitochondrial stress that functions to activate the *UPR^{mt}* in the intestine. *fshr-1* promotes *UPR^{mt}* activation in response to either acute exposure to mitochondrial toxins or to chronic mitochondrial dysfunction, and functions in a common pathway with *sphk-1* to promote survival in response to toxic mitochondrial stress and *UPR^{mt}* activation. *fshr-1* positively regulates the mitochondrial association of *SPHK-1* in the intestine in the absence of stress, as well as stress-induced *SPHK-1* mitochondrial recruitment. *fshr-1* functions in the nervous system, but not in the intestine, to promote *UPR^{mt}* activation and stress-induced *SPHK-1* mitochondrial recruitment. Finally, neuronal *fshr-1* regulates intestinal mitochondrial homeostasis in the absence of stress by a mechanism that may involve regulating mitochondrial mass and/or mitochondrial protein abundance. We propose a model whereby neuronal *fshr-1* signaling keeps *UPR^{mt}* activity low in the absence of stress and positively regulates the *UPR^{mt}* in response to intestinal stress. The underlying mechanism by which *fshr-1* regulates the *UPR^{mt}* may be by establishing or maintaining proper mitochondrial homeostasis in the intestine.

fshr-1 functions cell nonautonomously in the nervous system to activate the *UPR^{mt}*; however, our genetic evidence suggests that it is not likely to function in one of the

previously identified “mitokine” pathways that signal neuronal stress to the intestine. Disruption of these pathways (e.g., *egl-20*/Wnt or *flp-2*/neuropeptide) blocks the ability of stress originating in the nervous system to promote intestinal *UPR^{mt}* activation (Shao *et al.* 2016; Kim and Ewbank 2018; Zhang *et al.* 2018). We found that *fshr-1* is required for *UPR^{mt}* activation in response to intestinal stress but not neuronal stress. Thus, *fshr-1* is not likely to be a component of a neuronal mitokine pathway but instead may promote *UPR^{mt}* activation by a mechanism that does not involve the direct response to neuronal stress.

Previous studies have established an important role for *FSHR-1* in activating the innate immune response following infection with pathogenic bacteria. *FSHR-1* promotes both survival following infection and the behavioral avoidance response to pathogenic bacteria, and *FSHR-1* activates a number of antimicrobial and antioxidant genes in response to pathogens (Powell *et al.* 2009; Miller *et al.* 2015). *FSHR-1* is proposed to not participate in the detection of the pathogenic bacteria themselves, but instead to participate in the detection of cellular damage (e.g., ROS) resulting from bacterial infection (Miller *et al.* 2015). Pathogenic bacterial infection is also a potent activator of the *UPR^{mt}*, and *UPR^{mt}* activation by pathogens promotes survival by inducing the expression of innate immune genes (Pellegrino *et al.* 2014). We speculate that *fshr-1* may activate the innate immune response by contributing to activation of the *UPR^{mt}* in response to a broad array of insults (e.g., pathogenic infection, ROS, and mitochondrial dysfunction). Interestingly, we found that intestinal *fshr-1* does not regulate the *UPR^{mt}*, yet intestinal *fshr-1* expression protects *fshr-1* mutants from paraquat toxicity, suggesting that activation of the *UPR^{mt}* may not be necessary to elicit resistance to paraquat. Paraquat is an ROS generator that also activates the intestinal antioxidant response, and *fshr-1* promotes the expression of antioxidant genes (Powell *et al.* 2009; Miller *et al.* 2015). Thus, the paraquat sensitivity of *fshr-1* mutants may arise from defects in the antioxidant response in the intestine.

FSHR-1 also regulates intestinal mitochondrial homeostasis in the absence of stress. Whether *FSHR-1* does so by

Figure 4 FSHR-1 regulates mitochondrial association of SPHK-1. (A) High-magnification images of SPHK-1::GFP (left), TOMM-20::mCherry (middle), and merge (right) in wt animals coexpressing *Pges-1::sphk-1::gfp* and *Pges-1::tomm-20::mCherry* transgenes. (B) Left: representative images of the indicated mutants expressing intestinal SPHK-1::GFP in the absence or presence of PQ for 24 hr. *fshr-1* neuron and intestine rescue denote transgenic *fshr-1* mutants expressing *fshr-1* cDNA under the pan-neuronal promoter *rab-3* or the intestinal promoter *ges-1*, respectively. Middle: average fluorescence intensities in the indicated strains expressing SPHK-1::GFP are quantified. Right: mitochondrial occupancy of indicated transgenes is quantified in

the absence or presence of PQ. H, M, or L indicates the percentage of animals showing mitochondrially localized SPHK-1::GFP in more than two-thirds of the intestine (H), one-half of the intestine (M), or only in a few intestinal cells (L). Bar represents 2 μ m. One-way ANOVA with Bonferroni post-test $**P < 0.01$; $***P < 0.001$; $@P > 0.05$ compared to wt; $#P > 0.05$ compared to *fshr-1* mutants; $$P > 0.05$ compared to wt + PQ; and $^P > 0.05$ compared to *fshr-1* + PQ. cDNA, complementary DNA; ns, not statistically significant; PQ, paraquat; wt, wild-type.

regulating mitochondrial mass or mitochondrial protein import, or both, is not clear based on our quantitative imaging of mitochondrial markers. Interestingly, FSHR signaling negatively regulates mitochondrial biogenesis in mammals (Liu *et al.* 2017). Enhanced mitochondrial biogenesis is correlated with UPR^{mt} activation. Inducers of mitochondrial biogenesis, such as nicotinamide riboside and poly(ADP-ribose) polymerase inhibitors (PARPi), disrupt mito–nuclear protein homeostasis resulting in UPR^{mt} activation via the sirtuin 1 (SIRT1) pathway in mammals and *C. elegans*. (Mouchiroud *et al.* 2013). In addition, rapamycin and resveratrol, which increase mitochondrial content, also induce UPR^{mt} activation in *C. elegans* (Ungvari *et al.* 2011; Houtkooper *et al.* 2013; Lerner *et al.* 2013). In worms, mutants with defects in the import of mitochondrial proteins into the matrix exhibit increased *hsp-60* expression that is dependent upon *atfs-1* (Nargund *et al.* 2012). We speculate that the defects in paraquat-induced UPR^{mt} activation in *fshr-1* mutants may be due to an underlying defect in the mitochondrial association of SPHK-1 under normal conditions.

Since FSHR-1 functions to protect animals from diverse stressors, the ligand for FSHR-1 is likely to originate from the host rather than from an exogenous source (Powell *et al.* 2009). FSHR-1 shares homology with three human GPCRs—FSHR, TSHR, and LHCGR—by virtue of nine LRRs found in its extracellular domain (Dolan *et al.* 2007). These GPCRs are activated by the heterodimeric glycopeptide hormones FSH α / β , TSH α / β , and LH α / β , respectively. In humans, FSH induces the generation of S1P by stimulating SphK1 activity for granulosa cell proliferation (Hernández-Coronado *et al.* 2016), suggesting potential functional conservation of ligand-activated FSHR-1 signaling. An additional mammalian glycopeptide hormone family

member, thyrostimulin, is a heterodimer composed of the GPB5 α -subunit and the GPA2 β -subunit, which share homology with worm T23B12.8 and FLR-2, respectively (Rocco and Paluzzi 2016). Interestingly, genetic analysis implicates *flr-2* in the neuronal control of intestinal functions (Oishi *et al.* 2009), but whether *flr-2* regulates the UPR^{mt} or innate immunity has not been investigated. Identifying the ligand(s) for FSHR-1 will help to clarify the cell-nonautonomous mechanisms by which FSHR-1 regulates the UPR^{mt} pathway.

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