It’s All in Your Mind: Determining Germ Cell Fate by Neuronal IRE-1 in *C. elegans*

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Abstract

The *C. elegans* germline is pluripotent and mitotic, similar to self-renewing mammalian tissues. Apoptosis is triggered as part of the normal oogenesis program, and is increased in response to various stresses. Here, we examined the effect of endoplasmic reticulum (ER) stress on apoptosis in the *C. elegans* germline. We demonstrate that pharmacological or genetic induction of ER stress enhances germline apoptosis. This process is mediated by the ER stress response sensor IRE-1, but is independent of its canonical downstream target XBP-1. We further demonstrate that ire-1-dependent apoptosis in the germline requires both CEP-1/p53 and the same canonical apoptotic genes as DNA damage-induced germline apoptosis. Strikingly, we find that activation of ire-1, specifically in the ASI neurons, but not in germ cells, is sufficient to induce apoptosis in the germline. This implies that ER stress related germline apoptosis can be determined at the organism level, and is a result of active ire-1 signaling in neurons. Altogether, our findings uncover ire-1 as a novel cell non-autonomous regulator of germ cell apoptosis, linking ER homeostasis in sensory neurons and germ cell fate.

Introduction

Apoptosis, also known as programmed cell death (PCD), is a highly conserved fundamental cellular process that provides a self-elimination mechanism for the removal of unwanted cells. PCD is critical for organ development, tissue remodeling, cellular homeostasis and elimination of abnormal and damaged cells [1,2]. The apoptotic machinery that actually executes cell death is intrinsic to all cells and can be activated in response to extracellular or intracellular cues. These are thought to be mediated by cell death receptors or by cytotoxic stress respectively [3].

In *C. elegans*, 131 somatic cells invariably undergo apoptosis during hermaphrodite development [4,5]. In contrast, in the adult *C. elegans*, only germ cells undergo apoptotic cell death. These cell deaths can be either physiological or stress-induced [6,7]. So far, stress-induced germ cell apoptosis has been associated with DNA damage, pathogens, oxidative stress, osmotic stress, heat shock and starvation [7–9]. These apoptotic events are restricted to germ cells at the pachytene stage which are located in the loop region of the gonad [6], where oogenesis transition normally occurs [10]. The physiological germ cell apoptosis pathway acts during oogenesis and is thought to act either as a part of a quality control process, preferentially removing unfit germ cells from the gonad, or as a resource re-allocation factor important for maintaining oocyte quality or simply as a gonad homeostatic pathway removing excess germ cells [6,11,12]. Both somatic and germ cell apoptosis rely on the highly conserved core apoptotic machinery comprised of the Caspase-3 homolog *ced-3*, the Apaf-1 homolog *ced-4* and the anti-apoptotic Bcl-2 homolog *ced-9* [6,13–16].

All germ cell apoptosis, physiological and stress-induced, relies on the core apoptotic machinery [7–9]. However, different upstream genes activate the core apoptotic machinery in the germline in response to different stresses. For example, DNA damage-induced germ cell apoptosis involves the proteins EGL-1, CED-13, and the DNA damage response protein p53 homolog CEP-1 [9,17–19]. In contrast, oxidative, osmotic, heat shock and starvation stresses induce germ cell apoptosis through a CEP-1 and EGL-1 independent pathway and rely on the MEK-1 and SEK-1 MAPKs instead [7].

The endoplasmic reticulum (ER) fulfills many essential cellular functions, including a role in the secretory pathway, in lipid metabolism and in calcium sequestration. Accordingly, ER homeostasis is essential for proper cellular function [20]. A specialized, conserved cellular stress response, called the unfolded protein response (UPR), is in charge of detecting ER stress and adjusting the capacity of the ER to restore ER homeostasis. In *C. elegans*, as in humans, three proteins located at the ER membrane sense ER stress and activate the UPR: the ribonuclease inositol-requiring protein-1 (IRE-1), the PERK kinase homolog PEK-1.
and the activating transcription factor-6 (ATF-6) [21]. Of the three, Ire-1 is the major and most highly conserved ER stress sensor. In response to ER stress, Ire-1 activates the ER stress-related transcription factor XBP-1, which induces the transcription of genes that help restore ER homeostasis [22–24]. Accordingly, ire-1 and xbp-1 deficiencies perturb ER homeostasis [25,26].

Although Ire-1 typically protects cells, upon excessive and prolonged ER stress, Ire-1 can also trigger cell death, usually in the form of apoptosis [27,28]. For example, Ire-1 can lead to activation of the cell death machinery via JNK and caspase activation [29,30] or by mediating decay of critical ER-localized mRNAs through the RIDD pathway, tipping the balance in favor of apoptosis [31]. These functions of Ire-1 are independent of XBP-1 [29–33].

Highly proliferating cells with a high protein and lipid biosynthetic load are thought to rely on ER function to a greater extent than other cells. This together with the general sensitivity of the germ line to cellular stresses prompted us to investigate the affects of ER stress on germ cell fate. Strikingly, we discovered that ER stress does not simply kill the germ cells by not meeting their biosynthetic demands. Instead, we found that ER stress initiates a signaling cascade in neurons that regulates germ cell survival non-autonomously. Thus, our findings reveal that germ cell sensitivity to ER stress conditions can be regulated at an organismal level and implicates signaling between tissues.

Results

Disruption of ER homeostasis induces germ cell apoptosis

To investigate whether ER stress induces apoptosis in the C. elegans germline, we first assessed the number of apoptotic corpses in the gonads of animals treated with tunicamycin, a chemical ER stress inducer which blocks N-linked glycosylation. Apoptotic corpses in the gonad were identified by staining with the vital dye SYTO12 and by their discrete cellularization within the germline syncytium. We found that tunicamycin treatment increased the number of apoptotic germ cells present in wild-type gonads by approximately 3 fold compared to control DMSO treatment from day-1 to day-3 of adulthood (Figure 1A–B). Thus, the genetic analysis clearly implicates the apoptotic machinery that mediates DNA damage-induced germ cell apoptosis.

The strong changes in CEP-1 levels observed in tfg-1 RNAi-treated animals suggest that ER stress controls CEP-1 activation within the germ cells. One possible explanation for the involvement of genes implicated in DNA damage-induced germ cell apoptosis is that ER stress indirectly damages DNA, which in turn leads to CEP-1 activation and DNA damage-induced germ cell apoptosis. However, whereas a previous study implicated the intestinal kri-1 gene in non-autonomous regulation of ionizing-radiation induced germ cell apoptosis [37], we found that tfg-1 RNAi treatment efficiently induced germ cell apoptosis in kri-1-deficient animals (Figure 3C). This genetic uncoupling between the requirements for ionizing-radiation induced germ cell apoptosis and ER stress-induced germ cell apoptosis suggest that ER...
stress does not simply induce DNA damage which in turn leads to germ cell apoptosis. To further substantiate this conclusion, we examined directly whether the DNA damage response is activated in the germ cells of ER stressed-animals treated with tfg-1 RNAi. To this end, we followed the nuclear aggregation of HUS-1::GFP, which encodes a DNA damage checkpoint protein that relocates to distinct nuclear foci upon induction of DNA damage [38]. Whereas nuclear HUS-1::GFP aggregates were clearly observed in the germ cells of DNA-damaged rad-51 RNAi treated animals (P < 0.001 compared to control RNAi), HUS-1::GFP aggregates were not detected in tfg-1 RNAi treated animals (P = 0.78 compared to control RNAi, Figure 3D). Thus, ER stress activates CEP-1/p53 to induce germ cell apoptosis without generating DNA damage.

**Conditions that disrupt ER homeostasis induce germ cell apoptosis via the ER stress sensor ire-1**

We next asked whether any of the canonical ER stress sensing genes was implicated in ER stress-induced germ cell apoptosis. To this end, we examined mutants deficient in the ER stress-response sensor genes that comprise the UPR: ire-1, pek-1 or atf-6. This array of mutants was treated with DMSO or with tunicamycin and germ cell apoptosis was scored by SYTO12 labeling. We found that similarly to its effect in wild-type animals, tunicamycin treatment increased the number of germ line corpses in atf-6 and pek-1-deficient animals by approximately 3 fold from day-1 to day-3 of adulthood (P < 0.001, Figure 1A,C). In contrast, ER stress induced by tunicamycin treatment failed to increase germ cell apoptosis in ire-1 mutants (Figure 1A,B). Therefore, the insensitivity of the germline to tunicamycin is unique to ire-1-deficient animals and not seen in animals deficient in other UPR sensors.

ire-1 mutants are abnormal in terms of their gonad anatomy and their reproductive capacity: ire-1 mutants have approximately 2 fold less progeny and 2 fold less mitotic germ cells within their proliferative zones compared to ire-1(+) wild-type animals (P < 0.001, Figures S3A,D). Thus, we wondered whether these abnormalities affected the ability of their germ cells to undergo apoptosis in general or whether they were specifically defective in their ability to undergo apoptosis in response to ER stress.

First we assessed germline apoptosis in ire-1(−) mutants under normal growth conditions, from day-0 (L4) to day-3 of adulthood. At all timepoints, we detected approximately half the amount of germ line corpses in ire-1(−) gonads compared to ire-1(+) wild-type gonads, as assessed by SYTO12 labeling and by the CED-1::GFP engulfment marker (Figure 2A–C). The low levels of germ cell apoptosis persisted in ire-1 mutants treated with vps-18 RNAi (Figure 2D), a treatment that impairs germ cell corpse clearance [39]. However, normalization of the number of apoptotic corpses to the number of mitotic germ cells resulted in comparable levels of germ cell corpses in ire-1 mutants and in non-stressed wild-type animals (P = 0.12, Figure S3C). This indicates that in spite of their reproductive abnormalities, physiological germ cell apoptosis in ire-1(−) and ire-1(+) animals is comparable.

Next, we assessed stress-induced germline apoptosis in ire-1 mutants. We found that although manipulations that disrupt ER homeostasis fail to increase germ cell apoptosis in ire-1 mutants (Figure 1B,1D–E), DNA damage and oxidative stress conditions did increase germ cell apoptosis in ire-1 mutants (P < 0.001 compared to non-stressed ire-1 mutants, Figure 1D–E). This resulted in a similar level of germline apoptosis as in stressed wild-type animals upon normalization of the number of apoptotic corpses to the number of mitotic germ cells (P = 0.44 for DNA damage and P = 0.42 for oxidative stress, Figure 1D–E). Thus, in spite of the reproductive abnormalities of ire-1 mutants, the germ cells of these mutants undergo stress-induced apoptosis similarly to wild-type animals, however not in response to ER stress. The inability of ire-1 mutants to increase germline apoptosis specifically in response to perturbations in ER homeostasis suggests that IRE-1 may be a critical mediator of ER stress-induced germ cell apoptosis.

IRE-1 function in ER stress-induced germ cell apoptosis is independent of its canonical downstream target XBP-1

The most established mode of action of IRE-1 under ER stress conditions is via the activation of the UPR-related transcription factor XBP-1 [22–24]. Therefore, if IRE-1 enabled ER stress-induced germ cell apoptosis via its downstream target xbp-1, then the number of germline corpses detected in xbp-1(−) mutants should remain low under ER stress conditions, similarly to ire-1(−) mutants.

In order to test this, we first examined germ cell apoptosis in xbp-1(tm2457) null mutants. Surprisingly, in contrast to ire-1(−) mutants, we consistently detected increased germ cell apoptosis in xbp-1(−) gonads compared to wild-type gonads under normal growth conditions. A 2.5 fold increase in the number of germ line corpses in xbp-1(−) mutants was detected by SYTO12 labeling of gonads from day-1 to day-3 of adulthood compared to wild-type animals (P < 0.001, Figure 2A–B). A similar observation was apparent by using the CED-1::GFP engulfment marker (Figure 2C). The 2.5 fold increase in the number of germ cell corpses was still apparent in engulfment defective vps-18 RNAi-treated animals (Figure 2D) and upon normalization to the number of mitotic germ cells located in the proliferative zone (Figure S3C). Thus, in contrast to ire-1 mutants and wild-type animals, xbp-1 mutants exhibit a high basal level of germ cell apoptosis.

We hypothesized that the increase in germline apoptosis in xbp-1 mutants may be due to perturbed ER homeostasis in these animals [25,26]. If so, then it should be mediated via ire-1, similarly to other ER stress conditions that induce germ cell apoptosis. Accordingly, we found that in an ire-1(−) background, the xbp-1 mutation did not increase germ cell apoptosis. This observation was consistent along different time points spanning from day-0 (L4) to day-3 of adulthood (Figure 2A–B). This also persisted upon normalization to the number of mitotic germ cells.
Neuronal IRE-1 Determines Germline Apoptosis

A

wild type

tfg-1(RNAi)

xbp-1(-)

ire-1(-)

tfg-1(RNAi); ire-1(-)

ire-1(-); xbp-1(-)

B

No. of germline corpses / gonad arm

L4 Day 1 Day 2 Day 3

wild type
tfg-1 (RNAi)
xbp-1(-)
ire-1(-)
tfg-1(RNAi); ire-1(-)
ire-1(-); xbp-1(-)

C

CED-1::GFP

No. of germline corpses / gonad arm

control RNAi
tfg-1 (RNAi)
xbp-1 (RNAi)
ire-1 (RNAi)

D

vps-18(RNAi)

No. of germline corpses / gonad arm

wild type
xbp-1 (tm2457)
xbp-1 (tm2482)
ire-1 (ok799)
located in the proliferative zone (Figure S3B–C). This indicates that the reduced amount of mitotic cells in the gonad of ire-1 mutants can be uncoupled from the inability of their germ cells to undergo ER stress-associated apoptosis.

The finding that xbp-1 deficiency per se promotes ire-1-dependent ER stress-induced germ cell apoptosis suggests that xbp-1 is dispensable for increasing germ cell apoptosis in response to ER stress. Consistent with this, we found that tunicamycin treatment further increased germ cell apoptosis in xbp-1 mutants \( (P<0.001, \text{Figure 1A–B}) \). Altogether, these results lend further support to the notion that ire-1 is a critical signaling molecule in mediating ER stress-induced germine apoptosis, whereas its’ downstream canonical target xbp-1 is not. Furthermore, since ER function is compromised both in ire-1 and in xbp-1 deficient mutants \([25,26]\), the differential ability to induce germ cell apoptosis in these mutants suggests that germ cell apoptosis may be the result of active Ire-1 signaling, rather than simply a consequence of ER dysfunction.

In mammalian cells, activation of IRE1 can cell-autonomously activate JNK via the adapter protein TRAF. Consequently, IRE1-mediated activation of JNK initiates proapoptotic signaling, independently of XBP1 \([29]\). Thus, we examined whether the C. elegans homologs of TRAF and JNK proteins were required for ire-1/ER stress-induced apoptosis in C. elegans, which is also independent of xbp-1. To this end, tfg-1 mutants or mutants deficient in all three C. elegans JNK homologs were treated with control or tfg-1 RNAi. We found that tfg-1 RNAi increased germ cell apoptosis independently of the tfg-1 and the JNK-like genes (Figure 3E). Thus, since ER stress can effectively induce germ cell apoptosis in the absence of xbp-1, tfg-1 and JNK homologs, the signaling mediated by Ire-1, in this case, must be executed by an alternative xbp-1-independent output of Ire-1.

**ER stress in the ASI sensory neurons regulates germ cell apoptosis cell non-autonomously**

Next, we examined whether ER stress triggers programmed cell death autonomously within the germ cells, or non-autonomously from the soma. To test this, we used tfg-1 RNAi to induce ER stress specifically in the germine or in the soma. To induce ER stress primarily in the germ cells, mutants in the rrf-1 gene, encoding an RNA-directed RNA polymerase (RdRP) homolog required for most somatic RNAi but not for germline RNAi \([40]\), were treated with tfg-1 RNAi. No increase in the amount of germine corpses was observed as a result of tfg-1 RNAi treatment in rrf-1 mutants \( (P=0.19, \text{Figure 4A}) \). To induce ER stress specifically in the soma, mutants in the ppw-1 gene, which is required for efficient RNAi in the germine \([41]\), were treated with tfg-1 RNAi. This resulted in a 4.5 fold increase in the amount of apoptotic corpses in the gonads \( (P<0.001, \text{Figure 4A}) \). Thus, ER stress in the soma, rather than in the germ cells, is sufficient for the induction of germ cell apoptosis.

Does germ cell apoptosis occur upon disruption of ER homeostasis in the entire soma or does it occur in response to ER stress in a particular part of the soma? To answer this, ER stress was induced locally in specific somatic tissues. This was achieved by treating animals expressing functional RNAi machinery only in specific tissues with tfg-1 RNAi and assessing germ cell apoptosis in these animals. We found that tfg-1 RNAi treatment did not increase germ cell apoptosis in animals which respond to RNAi only in the intestine, in the muscle, in the hypodermis, in the uterine or in the distal tip cells \( (P>0.1 \text{ in each one of these strains, Figure 4B}) \). In contrast, tfg-1 RNAi treatment increased germ cell apoptosis by approximately 7 fold in animals which respond to RNAi specifically in the neurons \( (P<0.001, \text{Figure 4A}) \).

Next, we examined whether ER stress-induced germline apoptosis is under pan-neuronal control or under the control of specific neurons. To this end, we introduced ER stress-inducing tfg-1 RNAi into animals expressing functional RNAi machinery specifically in the cholinergic, glutamatergic, GABAergic, dopaminergic or in a subset of sensory neurons. Importantly, we found that tfg-1 RNAi treatment increased germ cell apoptosis only in animals whose sensory neurons responded to RNAi (Figure 4C).

Among the sensory neurons whose exposure to ER stress increased germline apoptosis were the ASI neurons, which have been previously implicated in the regulation of germ cell proliferation and maturation \([42]\). Hence, we examined whether ER stress in the ASI sensory neurons alone is sufficient for the induction of germ cell apoptosis in the gonad. To this end, we first assessed germline apoptosis in daf-28(sa191) mutants, which produce the toxic insulin peptide which induces ER stress specifically in the ASI neurons \([43]\). We found that germ cell apoptosis in the gonads of daf-28(sa191) mutants was increased by approximately 4 fold compared to wild-type animals \( (P<0.001, \text{Figure 4D}) \). Importantly, germ cell apoptosis was not increased in a daf-28(tm2308) null strain, which is deficient in daf-28 and does not produce the toxic insulin peptide which induces ER stress \( (P=0.15, \text{Figure 4D}) \). tfg-1 RNAi treatment of the two daf-28 mutant strains increased the number of germine corpses in daf-28(tm2308) null strain \( (P<0.001) \), but did not further increase germine apoptosis in the daf-28(sa191) strain \( (P=0.09, \text{Figure 4D}) \). tfg-1 RNAi treatment did not alter ASI overall morphology as assessed by the expression pattern of a GFP reporter driven by an ASI-specific promoter (Figure S4A).

Together, these findings suggest that expression of the toxic form of DAF-28 and tfg-1-deficiency increase germ cell apoptosis by similar means; most likely by causing ER stress and activating the UPR in the ASI neurons.
Neuronal IRE-1 Determines Germline Apoptosis

A

No. of germline corpses / gonad arm

wild type  ced-3(-)  ced-4(-)  cep-1(-)  egl-1(-)  ced-13(-)

control RNAi  tgf-1(RNAi)

B

Relative CEP-1::GFP fluorescence

control RNAi  rad-51(RNAi)  tgf-1(RNAi)

C

No. of germline corpses / gonad arm

wild type  kri-1(-)  pmk-1(-)  sek-1(-)

D

% gonads with HUS-1::GFP aggregates

control RNAi  rad-51(RNAi)  tgf-1(RNAi)

E

No. of germline corpses / gonad arm

wild type  kgb-1(-)  kgb-2(-)  jnk-1(-)  trf-1(-)
Activation of IRE-1 in ASI neurons is sufficient to induce germ cell apoptosis.

We have demonstrated that in the absence of the ER stress sensor ire-1, ER stress does not increase germline apoptosis. We further demonstrated that ER stress in the ASI sensory neurons is sufficient to induce germ cell apoptosis. Thus, we next examined whether it is also sufficient to express ire-1 in the soma, and specifically in the ASI neurons, to restore germ cell apoptosis in response to ER stress.

To this end, we restored ire-1 expression in the entire soma, pan-neuronally or specifically in the ASI/ASJ neurons of ire-1(−) mutants. This was achieved using multi-copy ire-1 transgenes under ire-1, rgef-1 and daf-28 promoters respectively. Since the expression of multi-copy transgenes is normally suppressed in germline cells [44], and due to the specificity of their promoters, these transgenes restore ire-1 expression within different parts of the soma but not in the germline. We found that expression of each of these ire-1 transgenes completely restored the increase in germline apoptosis in response to treatment with tfg-1 RNAi (P<0.001), compare white and black bars within each strain in Figure 5A. Similarly, we restored ire-1 expression in muscle cells and in the PVD and OLL neurons using multi-copy ire-1 transgenes under myo-3 and ser-2 promoters respectively. No increase in germline apoptosis in response to tfg-1 RNAi treatment was apparent in these two transgenic lines compared to control RNAi treatment (P>0.1, Figure 5A). The fact that not all ire-1 transgenes induced apoptosis supports the notion that ire-1-induced germline apoptosis is not the result of leaky expression of the transgenes in other tissues. Altogether, this implies that not all tissues and not all neurons are involved in the regulation of this process.

Next, we asked whether increasing IRE-1 levels to a greater extent may be sufficient for inducing germline apoptosis even in the absence of ER stress. To this end, we overexpressed ire-1 transgenes in various tissues or cells of ire-1(+)/wild-type animals. This was achieved by using multi-copy ire-1 transgenes under ire-1, rgef-1, daf-28 and daf-7 promoters. This is consistent with the interpretation that some activation of IRE-1 is achieved merely by its over-expression, as has been previously observed in yeast and in mammalian cells [45,46]. We found that this artificial activation of IRE-1 in the soma, pan-neuronally or specifically in the ASI/ASJ neurons of ire-1(+) animals was sufficient to induce high levels of germ cell apoptosis (P<0.001, compare white bars with transgenic animals to that of wild-type animals in Figure 5B). No increase in germ-cell apoptosis was observed upon overexpression of an ire-1 transgene in muscle cells or in the AY neurons in ire-1(+) animals (P>0.1, Figure 5B). These findings support the claim that the rescuing activity of the ire-1 transgenes stems from their expression in specific neurons.

IRES RNAi treatment of ire-1(+) animals over-expressing the ire-1 transgenes in the soma, in the neurons and specifically in the ASI/ASJ neurons did not further increase germline apoptosis (P>0.5, compare white and black bars within the strains, Figure 5B). This suggests that IRE-1 overexpression and tfg-1-deficiency increase germline apoptosis by similar means, i.e. by activating IRE-1. Taken together, our data demonstrate that activation of ire-1 specifically in the ASI neurons, either by ER stress in the ASI neurons or by IRE-1 overexpression, can non-autonomously regulate germ cell apoptosis. Furthermore, since over-expression of transgenic IRE-1 is sufficient for its artificial activation in a manner that is independent of ER stress, this further suggests that active IRE-1 signaling in the ASI neurons per se, rather than neuronal ER stress or ER dysfunction, is the cause of germ cell apoptosis.

Discussion

Understanding the molecular events that regulate the life-death decision of cells is of fundamental importance in cell biology research, cell development, cancer biology and disease biology [47]. In this study, we gained new and fascinating insights into the complex coupling between ER stress in the nervous system and germ cell apoptosis.

We report for the first time that germ cells undergo apoptosis in response to ER stress. We find that activation of the ER stress response gene ire-1 is required and sufficient to induce germ cell apoptosis in response to several ER stress-inducing conditions. Strikingly, we find that germ cell fate is regulated non-autonomously by ER stress and/or through IRE-1 activation specifically in the ASI neurons. This implies that ER homeostasis and UPR signaling in the germ cells themselves is not a factor in determining their fate, ruling out the possibility that these apoptotic events are part of a quality control process that removes "stress-damaged" germ cells from the gonad [6,11,12]. Furthermore, this assigns a central neuroendocrine role for the ASI neuron pair in coupling between stress sensing and the onset of germ cell apoptosis. This is in addition to other central physiological processes in C. elegans, such as dauer formation [48,49] and longevity [50,51], that are also controlled by the sensory ASI neuron pair. Interestingly, another pair of sensory neurons, the ASJ neurons, has been previously implicated in the protection of germ cells from apoptosis under hypoxic conditions [52]. Thus, depending on the stress condition, different neurons can shift germ cell fate from survival to death or vice versa.

How might IRE-1 activation in the ASI neurons dictate germ cell survival or death? One possibility is that defects associated with ire-1 deficiency and/or ire-1 activation indirectly abrogate the communication between the neurons and the gonad. However, several lines of evidence undermine this hypothesis: (1) We find that ER stress-induced germ cell apoptosis proceeds normally in animals with a severely defective nervous system (Figure 5B,C). This implies that germline apoptosis does not result from a generic neuronal defect. (2) ire-1 deficiency is associated with germline abnormalities which include a significant reduction in the number of mitotic germ cells and in reduced progeny number. However, these gonad-related defects do not confer generic resistance to stress-induced apoptosis as the germ
Neuronal IRE-1 Determines Germline Apoptosis

A

rrf-1(-)  ppw-1(-)  neurons only RNAi

control RNAi

tfg-1 RNAi

No. of germline corpses, gonad arm

control RNAi

tfg-1 RNAi

B

WT  RNAi defective  intestine only RNAi  muscle only RNAi  hypodermis only RNAi  uterine only RNAi  DTC only RNAi

No. of germline corpses, gonad arm

C

No. of germline corpses, gonad arm

cholinergic neurons  glutamatergic neurons  GABAergic neurons  dopaminergic neurons  sensory neurons

D

daf-28(tm2308)  daf-28(sa191)

control RNAi

tfg-1 RNAi

No. of germline corpses, gonad arm

daf-28 (tm2308)  daf-28 (sa191)
cells of ire-1 mutants do undergo apoptosis in response to a variety of stresses. Furthermore, a mutation in xbp-1, which improved the reproductive abnormalities of ire-1 mutants, did not restore responsiveness to ER stress induced germ cell apoptosis in ire-1; xbp-1 double mutants, thus uncoupling the two. (3) Whereas the comparison of germ cell apoptosis in ire-1 and wild-type animals may be confounded by the basal discrepancy of their reproductive systems, this concern does not exist in the analysis of ire-1 overexpressing strains, whose gonad appears to be normal (P = 0.1 for Pire-1::ire-1 and Pdaf-7::ire-1 compared to wild-type animals Figure S3A,D). Similarly, this concern does not exist in the intra-strain comparisons of germ cell apoptosis within the ire-1(−) strain under control and stress conditions.

If ire-1 misregulation in the ASI neurons does not indirectly abrogate the communication between the neurons and the gonad, how might it dictate germ cell survival or death? IRE-1 is a dual-activity enzyme, bearing both kinase and endoribonuclease activities and a propensity to self-aggregate at the ER membrane in response to ER stress. The most characterized mode of action of IRE-1 is the activation of its downstream transcription factor XBP-1 [53]. Significantly less characterized are XBP-1 independent targets of IRE-1, that include activation of the cell death

**Figure 4. ER stress specifically in sensory neurons is sufficient to induce germline apoptosis.** (A) Bar graph and representative fluorescence micrographs (400-fold magnification) of SYTO12-stained germ cell corpses in day-2 adults. Arrows point at SYTO12 stained germ cell corpses. Bar graph presents average number +/- SEM of apoptotic corpses per gonad arm in the indicated genotypes (n = 60 per genotype). Asterisks mark Student’s t-test values of P < 0.001 of tfg-1 RNAi treated animals compared to the corresponding control RNAi treated animals. Note that in animals in which RNAi functions only in the neurons, tfg-1 RNAi increased the amount of germ cell corpses to a greater extent than in pww-1 mutants or wild-type animals. This may be due to more efficient tfg-1 RNAi uptake in the neurons of these animals, whose neurons over-express SID-1 [76] (B,C) Bar graph presents average number +/- SEM of SYTO12-labeled germ cell corpses of day-2 adults of the indicated genotypes (n = 60 gonads per genotype). Asterisks mark Student’s t-test values of P < 0.001 of tfg-1 RNAi treated animals (black bars) compared to the corresponding control RNAi treated animals (white bars). See Materials and methods for strains details. (D) Representative fluorescence micrographs (400-fold magnification) and bar graph of SYTO12-stained germ cell corpses in day-2 daf-28 mutant strains treated with control RNAi (white bars) or tfg-1 RNAi (black bars). Bar graph presents average number +/- SEM of apoptotic corpses per gonad arm in the indicated genotypes (n = 70 per genotype). tm2308 is a deletion mutation in the daf-28 gene. sa191 is a point mutation that interferes with the posttranslational processing of DAF-28. Asterisks mark Student’s t-test values of P < 0.001 of tfg-1 RNAi treated animals compared control RNAi treated animals.

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**Figure 5. Over-expression of ire-1 in the ASI pair of amphid neurons is sufficient to induce germline apoptosis independently of ER stress.** (A–B) Bar graph presents average number of apoptotic corpses per gonad arm in day-2 ire-1(−) or ire-1(+) animals expressing an ire-1 transgene driven by the indicated promoters (n = 100 gonads per genotype). Animals were treated with control RNAi (white bars) or with tfg-1 RNAi (black bars). Asterisks mark Student’s t-test values of P < 0.001 of each strain treated with tfg-1 RNAi compared to its’ control RNAi treatment. Error bars indicate SEM. (A) Germ cell apoptosis in ire-1(−) mutants expressing an ire-1 rescuing transgene in different tissues is presented. The ire-1 rescuing transgene is expressed in somatic cells (Pire-1::ire-1), in neurons (Prgef-1::ire-1, Pdaf-28::ire-1 and Pser-2::ire-1, which drive expression in all neurons, in the ASI/ASJ neurons or in the PVD and OLL neurons respectively) and in muscle cells (Pmyo-3::ire-1). (B) Germ cell apoptosis in ire-1(+) animals overexpressing an ire-1 transgene in different tissues is presented. The ire-1 transgene was expressed in somatic cells (Pire-1::ire-1), in neurons driven by the promoters of rgef-1 (pan-neuronal expression), daf-28 (expressed in the ASI/ASJ neurons), daf-7 (expressed only in the ASI neurons) and ttx-3 (expressed in the AIY interneurons) and in muscle (Pmyo-3::ire-1).

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machinery via JNK/TRAF signaling and degradation of ER-localized mRNAs that encode secreted and membrane proteins in a process called RIDD [29,30,32,54–58]. Since we find that ER stress can effectively induce germ cell apoptosis in the absence of xbp-1, trf-1 and JNK homologs, the signaling mediated by IRE-1 in this case may be executed by the RIDD pathway or via a novel, yet undescribed, xbp-1-independent output of IRE-1.

We propose that activation of IRE-1 in the neurons (either as a result of ER stress or merely by its over-expression) actively regulates the production of a germ cell regulatory signal. In principle this may be a germ cell proapoptotic signal produced by the neurons upon IRE-1 activation. Alternatively, this may be a germ cell anti-apoptotic signal that is down-regulated by IRE-1 upon its activation. This ASI-regulated signal, whose identity and nature remain to be elucidated, propagates in the animal and affects the gonad where it acts upstream to the p53 homolog cep-1, activating the same apoptotic machinery in the germ cells as the DNA damage response, without inducing DNA damage in the germ cells (Figure 6). This indicates the existence of a new pathway that can activate CEP-1 independently of DNA damage upon activation of neuronal IRE-1.

Interestingly, in adult animals, exposure to ER stress or activation of IRE-1 in the soma induce apoptosis only in germ cells, as we did not detect any apoptotic corpses outside of the gonad of these animals. This is in contrast to the developing embryo, where exposure to ER stress can induce apoptosis in the soma [35,59]. We propose that as the organism completes development, its ability to respond or execute programmed cell death upon exposure to ER stress is maintained in mitotic germ cells while being selectively abrogated in the post-mitotic soma, as has been demonstrated for their ability to execute apoptosis in response to DNA-damage [60]. This resistance of the soma is important in terms of survival of the animal as it prevents cell death of somatic tissues that lack stem cell pools and regenerative capacity, while allowing cell death of immortal germline cells at times of stress.

What could be the advantage in diluting the germ cell pool when neurons “feel” ER-stressed (i.e. when IRE-1 is activated naturally by ER stress or artificially by overexpression)? Recent studies demonstrate a tight inverse correlation between germ-cell proliferation and the maintenance of somatic proteostasis and longevity [61–63]. This inverse correlation is thought to be due to a limitation of resources shared by the germline and the soma and due to altered metabolic and cellular repair mechanisms in the soma that are enabled upon germ cell loss. Previous studies implicated the nervous system in systemic and hierarchical control of cellular stress responses elsewhere in the soma to maintain organismal homeostasis [66–72]. Our data further imply that neurons also have the ability to communicate with the germ cells to promote their death in response to stress in the ER. This, in turn, may orchestrate a proapoptosis switch in the soma at the expense of a replenishable germ cell pool in times of stress. This adds a new layer of complexity to our understanding of how protein homeostasis is regulated and coordinated across tissues in multicellular organisms.

Materials and Methods

Cell corpse assay

For single time-point experiments, the number of apoptotic germ cells was scored in day-2 animals stained with SYTO12 (Molecular Probes) as previously described [6]. For time course experiments, the number of SYTO12-labeled apoptotic corpses per gonad arm was scored in animals from day-0 (L4) to day-3 of adulthood. Where indicated, the average number of apoptotic corpses was normalized to the number of mitotic germ cells within the proliferative zone of the gonads, determined by section analysis of DAPI-stained gonads.
Oxidative stress treatment

Day-1 adult animals were placed in 200 μl of M9 (control) or 10 mM paraquat (oxidative stress) for 1.5 h at 20°C. After the incubation period, 1 ml of M9 was added to dilute the paraquat. Animals were then transferred to eppendorfs with SYTO12 staining for 4.5 hrs. Animals were allowed to recover on plates for 40 min. Finally, the animals were mounted and observed under the microscope to determine cell corpse numbers.

Gonad dissection and DAPI staining

Gonads of day-1 adults were dissected, fixed, and stained with DAPI as previously described [10].

RNA interference

Bacteria expressing dsRNA were cultured overnight in LB containing tetracycline and ampicillin. Bacteria were seeded on NGM plates containing IPTG and carbenicillin. RNAi clone identity was verified by sequencing. Eggs were placed on plates and synchronized from day-0 (L4). The efficacy of the unpaired Student's t test.

Gonad dissection and DAPI staining

Animals were anaesthetized on 2% agarose pads containing 2 mM levamisol.

Fluorescence microscopy and quantification

Images were taken with a CCD digital camera using a Nikon 90i fluorescence microscope. For each trial, exposure time was calibrated to minimize the number of saturated pixels and was kept constant through the experiment. The NIS element software was used to quantify mean fluorescence intensity as measured by intensity of each pixel in the selected area.

Statistical analysis

Error bars represent the standard error of the mean (SEM) of at least 3 independent experiments. P values were calculated using the unpaired Student’s t test.

Strains and transgenic lines

The following lines were used in this study: N2, CF2012: phek-1(ck275) X, CF2988: afd-6(ok351) X, CF2473: ire-1(ok799) II, CF3208: xbp-1(tm2457) III, SHK62: ire-1(ok799) II; xbp-1(tm2457) III, MD701: Ptim-7;ced-1::gfp V, xbp-1(tm2482) III, CF2185: ced-3(n1289) IV, MT547: ced-4(n1162) III, TJ1: cep-1(gk138) I, MT3753: egl-1(n1084n3082) V, FX536: ced-13(n356) X, WS1433: hua-1(op241) I; unc-119(ed3) III, oph-34, CF2052: kri-1(ok1251) I, KU25: pmk-1(nk5) IV, AU1: sek-1(ag1) X, CF3030: kgb-1(um3) kgb-2(ck361) pmk-1(gk7) IV, NS2937: trf-1(nr2014) III, CF2260: zcs1/Pphsp-4::gfp V, CL2166: Pgst-4::gfp[dvxs19] V, CF1553: mls154 [pAD76] Pod-3::gfp[pod-6] S, SJ4100: Pphsp-6::gfp(zcIs13) V, TIG125: Phsp-16.2::gfp [dat-1p::rde-1::SL2::sid-1 C]; eri-1(mg366) IV; rde-1(ne219) V; lin-15B(n744) X - Cholinergic neuron-specific RNAi strain. XE1375: wpSl12 II [unc-17p::rde-1::SL2::ubc-1+; unc-119++; rde-1(ok799)] II; rde-1(ok799) II; lin-15B(n744) X - Cholinergic neuron-specific RNAi strain. XE1581: wpSl10 II [func-7p::rde-1::SL2::ubc-1+; unc-119++; rde-1(ok799)] II; rde-1(ok799) II; lin-15B(n744) X - Cholinergic neuron-specific RNAi strain.

Plasmid construction

Prgef-1::ire-1 - ire-1 CDNA was cloned under the 3.5 kb rgef-1 (F25B3.3) promoter and injected at 5 ng/μl with Pmyo-3::mCherry at 50 ng/μl.

Pire-1::ire-1 - ire-1 CDNA was cloned under the 4.5 kb ire-1 (C41C4.4) promoter in the L3691 vector and injected at 25 ng/μl with rol-6 at 100 ng/μl. Pttx-3::ire-1 - was created by cloning the ire-1 CDNA into a Pttx-3 vector [74] using KpnI/SphI.

Pdaf-7::gfp and Pdaf-7::ire-1 -daf-7 promoter fragment [49] was cloned into pPD95.75 (gift from A. Fire, Carnegie Institute) using SphI/XbaI to createdaf-7p::gfp transcriptional fusion. The gfp fragment was then replaced by ire-1 cDNA using Xmal/AfII to make dna-1p::rde-1::SL2::sid-1 (C41C4.4) promoter and injected at 5 ng/μl with dna-1p::gfp and PM9 (rol-6) at 20 ng/μl each.

ser-2prom-3::ire-1 - was created by cloning ire-1 cDNA under ser-2prom-3 fragment [75] using Xmal/AfII, ser-2prom-3::ire-1 was injected at 10 ng/μl with xt3::mCherry at 40 ng/μl.

Supporting Information

Figure S1 ttg-1 deficiency specifically activates the ER stress response. Representative fluorescence micrographs (400-fold magnification) of adult transgenic animals expressing a GFP reporter fused to make dna-1p::rde-1::SL2::sid-1 (C41C4.4) promoter and injected at 5 ng/μl with dna-1p::gfp and PM9 (rol-6) at 20 ng/μl each.

Figure S2 ER stress does not increase germ cell apoptosis in ced-3 deficient animals. Representative fluorescence micrographs (400-fold magnification) of SYTO12-stained germ cell corpses in...
day-2 adults. Arrows point at SYTO12-labeled germ cell corpses. ER stress was induced by inactivation of the UPR gene xbp-1 or by blocking protein export from the ER by inactivation of ire-1 (UPR is constitutively activated in ire-1-deficient animals, see Figure S1). No germ cell corpses were detected in either ced-3(n3286), xbp-1(ok1257); ced-3(n3286) or ire-1(n783); ced-3(n3286) backgrounds. Bar graph shows average number +/- SEM of apoptotic corpses per gonad arm (n = 40 per genotype). Asterisk marks Student’s t-test value of P < 0.001 compared to ced-3-deficient animals. (TIF)

Figure S3 Effects of different ire-1 expression levels on the reproductive system. (A) Bar graph presents amount of mitotic germ cells per gonad arm scored in DAPI-stained dissected gonads from day-1 adults of the indicated genotypes (n = 50 gonads per genotype). Note that the xbp-1 mutation did not significantly increase the levels of apoptotic corpses in the gonads of ire-1(ok799) mutants (P = 0.29). Note that xbp-1 mutants and xbp-1; ire-1 double mutants have similar amounts of mitotic germ cells (P = 0.072, see panel A). (B) Bar graph presents the fold change in the normalized amount of apoptotic corpses per gonad arm compared to wild-type animals. The amount of apoptotic corpses (presented in B) was normalized to the average number of mitotic germ cells in each of the indicated genotypes (presented in A). Asterisk marks Student’s t-test values of P < 0.001. (D) Bar graph presents average progeny number scored in 15 animals per genotype. Asterisk marks Student’s t-test value of P < 0.001 compared to wild-type animals. Error bars represent SEM. All animals in panel D contained a daf-28:gfp transgene in their background. (TIF)

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Figure S4 Uncoupling general neuronal dysfunction and the responsiveness to ER stress. (A) Representative fluorescence micrographs (400-fold magnification) of GFP-expressing ASI neurons driven by the daf-7 promoter. The overall pattern of the ASI neurons was similar in control RNAi and the fie-1 RNAi treated animals. (B–C) Bar graph and representative fluorescence micrographs (400-fold magnification) of germ line corpses in unc-31(e2311), unc-64(e246) unc-31(e2928) and eat-4 (e53) day-2 mutants are presented. The average number of apoptotic corpses per gonad arm was scored by SYTO12 staining (n = 40 animals per genotype). Note that although these strains have a severely defective nervous system, they display normal basal levels of germline apoptosis, which increase in response to ER stress. These results uncouple general neuronal dysfunction and the responsiveness to ER stress-induced germline apoptosis. (TIF)

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Author Contributions
Conceived and designed the experiments: MLF SHK. Performed the experiments: MLF AHC. Analyzed the data: MLF SHK. Contributed reagents/materials/analysis tools: MS YS HEB. Wrote the paper: MLF SHK.
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