eLife

Reviewed Preprint
v2 • April 8, 2025
Revised by authors

Reviewed Preprint
v1 • October 15, 2024

Cell Biology

Reprograming gene expression in "hibernating" *C. elegans* involves the IRE-1/XBP-1 pathway

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eLife Assessment

This **useful** study reveals that as C. elegans, a poikilothermic ("cold-blooded") animal, adapt to cold (4ºC), they display a drastic reduction in translation (assessed by polysome profiling and SUNSET). The remaining translation (by ribo-seq) correlates with mRNA levels (by RNA-seq), and the changes in gene expression at least partially require IRE-1, an established endoplasmic reticulum stress sensor. The reviewers consider the data assessing global translation and RNA expression upon cold exposure and the data demonstrating the requirement of ire-1 to be **solid**, but the conclusion that "transcription" is the major regulatory step and "lipid changes" can be a signal for IRE-1 activation in cold adapted worms needs substantially more evidence. Overall, this study demonstrated a good correlation between translation and RNA levels and yielded an inventory of gene changes as C. elegans adapt to cold, and will be of general interest to researchers interested in stress response and cold adaptation.

https://doi.org/10.7554/eLife.101186.2.sa3

Abstract

In nature, many animals respond to cold by entering hibernation, while in clinical settings, controlled cooling is used in transplantation and emergency medicine. However, the molecular mechanisms that enable cells to survive severe cold are still not fully understood. One key aspect of cold adaptation is the global downregulation of protein synthesis. Studying it in the nematode *Caenorhabditis elegans*, we find that the translation of most mRNAs continues in the cold, albeit at a slower rate, and propose that cold-specific gene expression is regulated primarily at the transcription level. Supporting this idea, we found that the transcription of certain cold-induced genes is linked to the activation of unfolded protein response (UPR) through the conserved IRE-1/XBP-1 signaling pathway. Our findings suggest that this pathway is triggered by cold-induced perturbations in proteins and lipids within the endoplasmic reticulum, and that its activation is beneficial for cold survival.

Introduction

One constant feature of hibernation is a global reduction of protein synthesis. This is evident from the loss of polyribosomes (also known as polysomes, which are linked to active translation) and the inhibition of key translation factors, such as eIF2α and eEF2, which are required for the initiation and elongation phases of translation, respectively (Chen et al., 2001; Frerichs et al., 1998; Knight et al., 2000; Van Breukelen & Martin, 2001). Likewise, cells from non-hibernating species, including humans, also reduce translation in response to cold (Hofmann et al., 2012). Despite this, many studies infer cold-related gene functions based solely on changes in transcript levels. However, given the general suppression of protein synthesis, the relative contributions of transcriptional versus translational regulation to cold-specific gene expression remain unclear.

Hibernation has been traditionally studied in non-standard animal models like squirrels, bats, or bears. Nonetheless, simpler genetic models are often advantageous in dissecting complex biological phenomena. Thus, to better understand gene expression underlying cold adaptation, we employ the nematode *C. elegans*: a rapid, morphologically simple, and genetically tractable animal model. *C. elegans* thrive in temperate climates, indicating that, in the wild, they can tolerate cold spells (Frezal & Felix, 2015). In the laboratory, deep cooling of *C. elegans* either leads to death when the cooling is rapid (Habacher et al., 2016; Ohta et al., 2014; Robinson & Powell, 2016) or to a dormant state when the cooling is more gradual (Habacher et al., 2016; Ohta et al., 2014). Studying the latter response, which in our laboratory involves shifting *C. elegans* for 2 hours to 10°C and then to 4°C for days, we observed that cold dormancy suppresses aging (Habacher et al., 2016). Incubating nematodes at 4°C also elicits a diapause-like arrest (Horikawa et al., 2024). Moreover, some mechanisms that facilitate *C. elegans* cold survival similarly benefit cold-treated mammalian cells (Pekec et al., 2022). Thus, while *C. elegans* cold dormancy and mammalian hibernation and are not identical, we take the liberty of also referring to the former as *C. elegans* “hibernation”.

Here, we demonstrate that *C. elegans*, like bona fide hibernators, respond to cold by globally reducing mRNA translation. However, the residual translation of individual transcripts generally correlates with their abundance. Since transcription is typically the key determinant of steady-state mRNA levels (Tippmann et al., 2012), our findings suggest that cold-specific gene expression is primarily regulated at the transcriptional level. To validate this, we focused on *lips-11*, a cold-induced gene encoding a putative lipase implicated in unfolded protein response (UPR) (Shen et al., 2005). The UPR consists of three stress-sensing and transducing branches: IRE1, PEK1, and ATF6, all conserved in *C. elegans* (Hetz et al., 2020). Once activated, the UPR restores homeostasis by various means, including through remodeling transcription and translation. We find that, in hibernating *C. elegans*, cold activates the IRE-1 branch of the UPR. This activation happens in response to protein and lipid bilayer stress in the endoplasmic reticulum (ER) and results in the expression of some cold-induced genes, which appears beneficial for hibernating nematodes.

Results

Protein synthesis is globally reduced in hibernating *C. elegans*

In various species and cultured cells, cooling leads to a global reduction of translation (Chen et al., 2001; Frerichs et al., 1998; Hofmann et al., 2012; Knight et al., 2000; Van Breukelen & Martin, 2001). To test if it also applies to *C. elegans*, we used a previously described cooling

paradigm and sampling across different temperatures and time points (**Figure 1A**) (Habacher et al., 2016; Pekec et al., 2022). We combined it with either polysome profiling or SUrface SEnsing of Translation (SUnSET). The former method separates translated mRNAs according to the number of bound ribosomes. The latter involves the incorporation of puromycin into newly synthesized peptides and subsequent detection of the incorporated puromycin by western blotting (Arnold et al., 2014; Schmidt et al., 2009). By polysome profiling, we observed in the cold a massive loss of polyribosomes (multiple ribosomes associated with mRNAs), with a concomitant increase in monosomes (**Figure 1B**). By SUnSET, we observed a stark drop in puromycin incorporation (**Figure 1C** and **Figure 1 – figure supplement 1**; note that puromycin is still incorporated at 4°C, albeit much reduced compared with 20°C). Thus, consistent with observations in other species, severe cooling is accompanied in *C. elegans* by a global reduction of protein synthesis.

Transcription determines cold-specific gene expression

Although global translation decreases in the cold, the translation of specific transcripts still could be regulated, i.e., activated or repressed. To examine this possibility, we combined ribosomal profiling with total RNA sequencing (**Supplementary files 1 and 2**). The biggest changes in the ribosomal occupancy of mRNAs occurred when the animals were shifted from 10°C to 4°C (**Figure 2A – figure supplement 1A**). Thus, most translational remodeling appears to happen during the first day of incubation at 4°C. Focusing on this transition (from 10°C to 4°C on day 1), we observed a strong correlation between changes in mRNA levels and ribosomal occupancy (**Figure 2A**). This observation suggests that, overall, mRNA abundance is the main determinant of cold-specific translation (for gene categories changing the most between 10°C and 4°C day 1, see **Figure 2 – figure supplement 2**). Nonetheless, we observed some exceptions, such as a group of transcripts whose translation appeared reduced in the cold with no concomitant drop in mRNA levels (red dots in **Figure 2A**), suggesting that they may be subjected to cold-specific translation repression. Intriguingly, this group includes mRNAs encoding three fatty acid desaturases (FAT-2, -3, and -4; **Supplementary file 3** and **Figure 2 – figure supplement 1B**). This is surprising, considering that desaturation of membrane lipids has been thought to play an important role in cold adaptation (Hayward et al., 2007).

However, the levels of most mRNAs correlate with the ribosomal occupancy. If the latter accurately describes translation status, the more ribosomes associate with a particular mRNA, the more protein it will yield in the cold. To strengthen this argument, we searched a public *C. elegans* depository (CGC) and identified three strains expressing GFP-fused proteins, whose ribosomal footprints either increase (*cebp-1* and *numr-1*) or not (*hsf-1*) in the cold. Monitoring their expression, we observed the expected rise in the levels of CEBP-1::EGFP and NUMR-1::EGFP, but not HSF-1::EGFP, in the cold (**Figure 2 – figure supplement 3**).

Transcription is typically the key determinant of mRNA levels (Tippmann et al., 2012). Thus, our results suggest that cold-specific gene expression may stem from transcriptional regulation. To test this, we selected one gene, *lips-11*, whose expression increased the most during the shift from 10°C to 4°C (green dot in **Figure 2A**). To test if cold upregulates *lips-11* transcription, we generated a strain expressing a GFP reporter, whose expression depends on the endogenous *lips-11* promoter (P*lips-11*) and the *unc-54* 3'UTR (permitting unregulated expression of the attached open reading frame). We observed that the *lips-11* promoter was sufficient to upregulate GFP fluorescence (**Figure 2B**), suggesting that the expression of at least some genes in the cold is regulated at the transcriptional level.

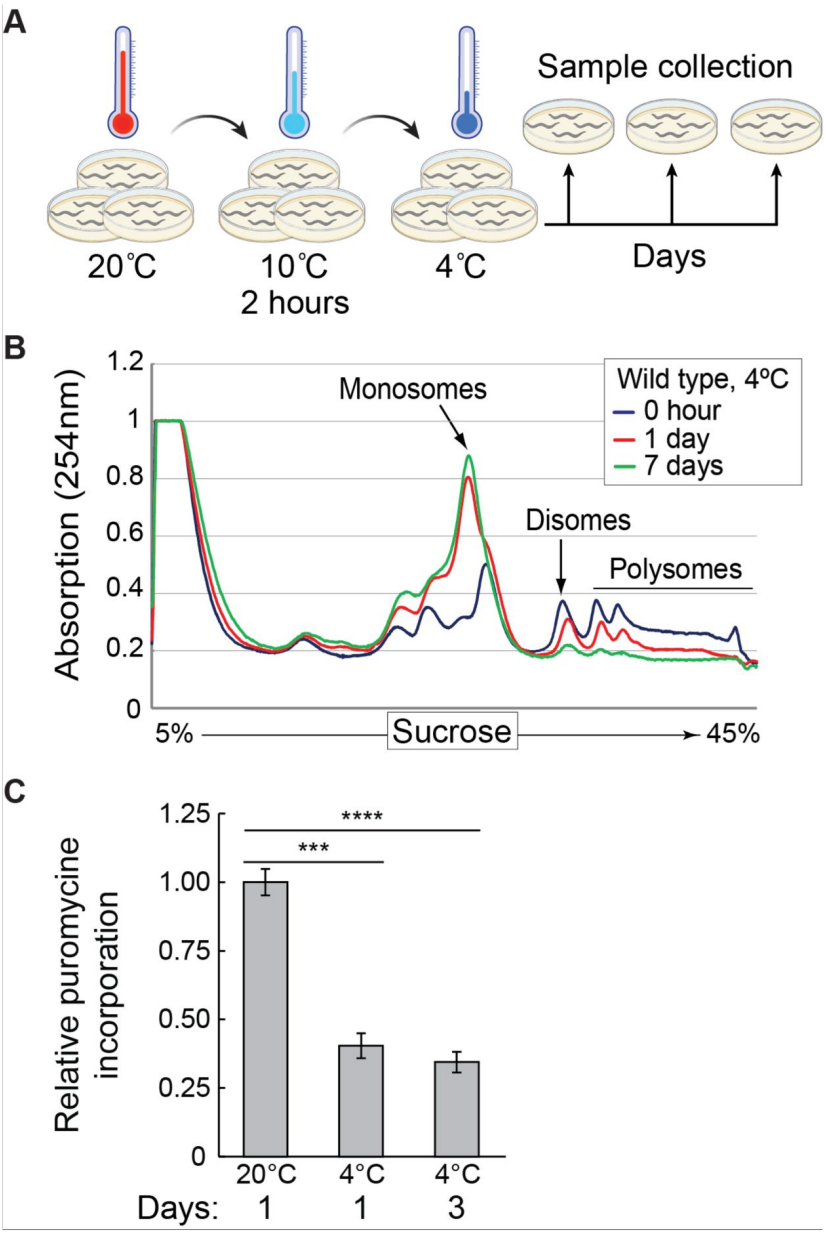


Figure 1.

Global protein synthesis is suppressed in hibernating *C. elegans*.

(**A**) Schematic of the cooling paradigm used in this study. Young adult nematodes, grown at 20°C on multiple plates, are first adapted to the cold at 10°C for 2 hours, and then shifted to 4°C. At indicated time points, the animals are collected and treated in an experiment-specific manner. Created with *BioRender.com*. (**B**) Polysome profiles from wild-type animals treated as shown in (A) and collected from 4°C at the indicated times. Marked are the positions of mono-, di-, and polysomes. Note a strong decrease of large polysomes with a concomitant increase of monosomes in cold, which become more pronounced with a longer cold exposure. (**C**) Protein synthesis evaluated with the SUnSET assay in animals incubated as indicated. The quantification reflects changes in puromycin incorporation (relative to day 1 at 20°C), normalized to actin as loading control, and detected by western blotting. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. ***: $p < 0.001$, ****: $p < 0.0001$.

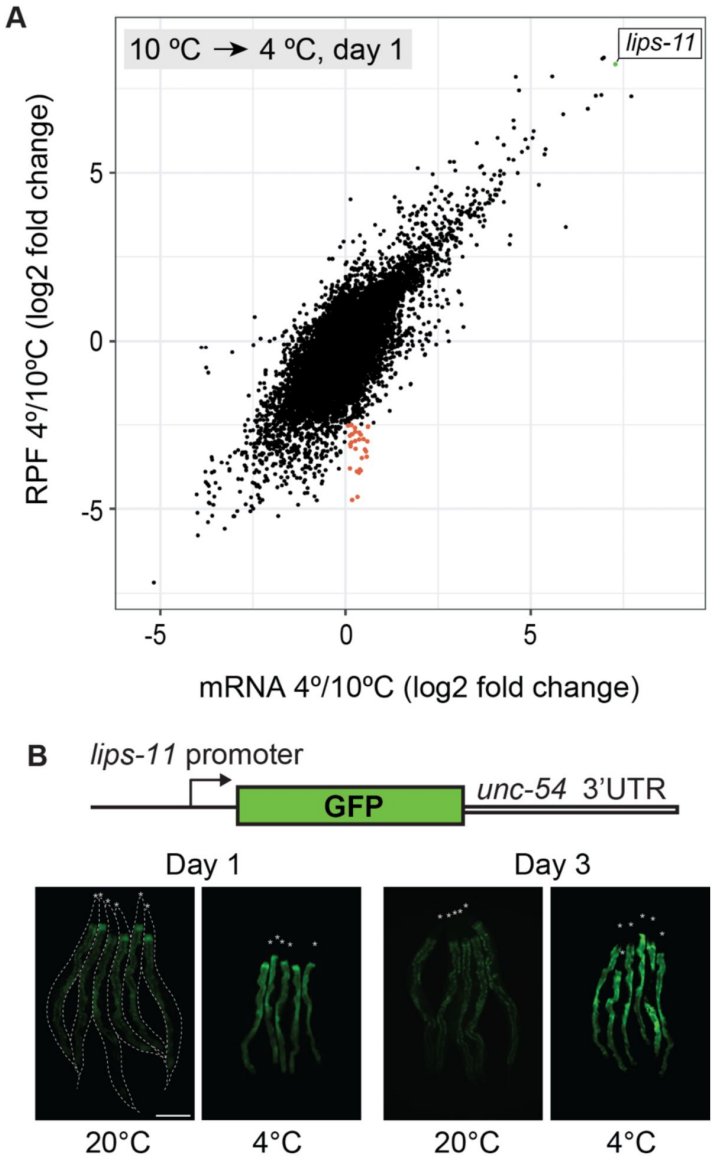


Figure 2.

Transcription may be the main determinant of gene expression in the cold.

(**A**) Changes in mRNA abundance ("mRNA" on the x-axis) and translation (ribosome protected fragment, "RPF" on the y-axis) for all transcripts upon shifting wild-type animals from 10°C to 4°C. Each dot represents the log (base 2) fold change of a single transcript. The most upregulated transcript, *lips-11*, is indicated in green. Overall, note a strong correlation between mRNA levels and translation (Pearson correlation coefficient = 0.7244968). A small subpopulation of transcripts (red) displayed little or no change in mRNA levels but reduced association with ribosomes, suggesting specific translation repression. (**B**) Top: Diagram representing a reporter construct, wherein GFP is expressed under the control of the *lips-11* promoter and *unc-54* 3' UTR. Below are representative fluorescent micrographs, taken at the indicated conditions, of several bundled animals carrying the GFP reporter. The animals are outlined in the control panel and the heads are indicated by asterisks. The scale bar = 200 µm.

Cold-induced transcription of *lips-11* depends on the IRE-1/XBP-1 branch of the UPR

Intriguingly, *lips-11* belongs to genes activated by the UPR during ER stress (UPR^{ER}) (Shen et al., 2005). Indeed, we observed that the P*lips-11* GFP reporter's levels increased upon treating animals with the protein glycosylation inhibitor tunicamycin, a UPR^{ER} inducer (**Figure 3 – figure supplement 1**). This observation prompted us to examine if the induction of *lips-11* in the cold depends on a particular branch of the UPR^{ER}; IRE-1, ATF-6, or PEK-1. Indeed, we observed reduced levels of the *lips-11* reporter upon RNAi-mediated depletion of *ire-1*, but not *atf-6* or *pek-1* (**Figure 3A**). Thus, *lips-11* expression in the cold depends on the UPR^{ER} signal transducer IRE-1.

In response to ER stress, the endoribonuclease domain of IRE-1 is activated and promotes the "splicing" of *xbp-1* mRNA, which gives rise to the functional form of the XBP-1 transcription factor (Calfon et al., 2002). As XBP-1-independent functions of IRE-1 have been also reported, we first examined if *xbp-1* mRNA is processed in the cold. To do this, we used an *xbp-1* splicing reporter strain, wherein the *xbp-1* promoter drives the expression of a genomic *xbp-1* fragment fused to GFP, which is expressed in frame upon the processing by IRE-1 (Ozbey et al., 2020). Importantly, we observed a significant upregulation of XBP-1::GFP in the cold (**Figure 3B and Figure 3 – figure supplement 2A**). Additionally, we used our ribosome profiling data to assess the translation of spliced *xbp-1* mRNA. The observed pattern of ribosomal occupancy was consistent with an increased splicing of *xbp-1* mRNA during cooling (**Figure 3 – figure supplement 2B**). Finally, the cold-induced expression of the *lips-11* reporter depended on XBP-1 (**Figure 3C**). Together, these observations suggest that the upregulation of *lips-11* during cold dormancy depends on the activation of the IRE-1/XBP-1 branch of the UPR^{ER}.

Hibernation specifically activates the IRE-1 branch of the UPR^{ER}

The activation of IRE-1 in the cold suggests that hibernating animals experience ER stress. To confirm this, we utilized a commonly used UPR^{ER} reporter strain, wherein GFP expression is driven by the promoter of *hsp-4*, the *C. elegans* homolog of the ER chaperone BiP (Calfon et al., 2002). Indeed, the expression of this reporter increased in the cold (**Figure 4A**). By following the expression of mitochondrial chaperones *hsp-6* (homolog of HSP70) and *hsp-60* (homolog of HSP60), we additionally examined if cold elicits UPR in the mitochondria (UPR^{mito}) (Yoneda et al., 2004). However, by contrast to *hsp-4*, neither *hsp-6* nor *hsp-60* were induced in the cold (**Figure 4 – figure supplement 1**). Thus, hibernating animals appear to experience stress in the ER but not mitochondria.

While we showed that *lips-11* upregulation is mediated by the IRE-1/XBP-1 pathway, other branches of the UPR^{ER} could be activated in the cold. Testing this possibility, we examined the expression of select UPR^{ER} target genes regulated through the IRE-1 (*dnj-27*, *srp-7*, *C36B7.6*), the ATF-6 (*cht-1*, *ZC168.2*), or the PEK-1 (*cbp-3*, *R02D3.8*) pathway (Shen et al., 2005). Among these transcripts, four displayed increased abundance on day 3 in the cold, relative to the corresponding day 3 animals at 20°C (**Figure 4B**). Notably, 3/4 of them are known targets of the IRE-1 pathway. Further analysis confirmed that the endogenous transcript levels of these IRE-1 responsive genes (including *lips-11*) increased in abundance as early as 1 day in the cold and continued to accumulate after 3 days, relative to reference animals collected at 20°C immediately before cooling (day 0) (**Figure 4 – figure supplement 2**). Importantly, the upregulation of these transcripts in the cold declined in the loss-of-function *ire-1(ok799)* mutants, indicating that their upregulation in the cold depends on IRE-1 (**Figure 4 – figure supplement 2**). Together, these findings suggest that cooling triggers the UPR^{ER}, resulting in IRE-1-dependent gene expression.

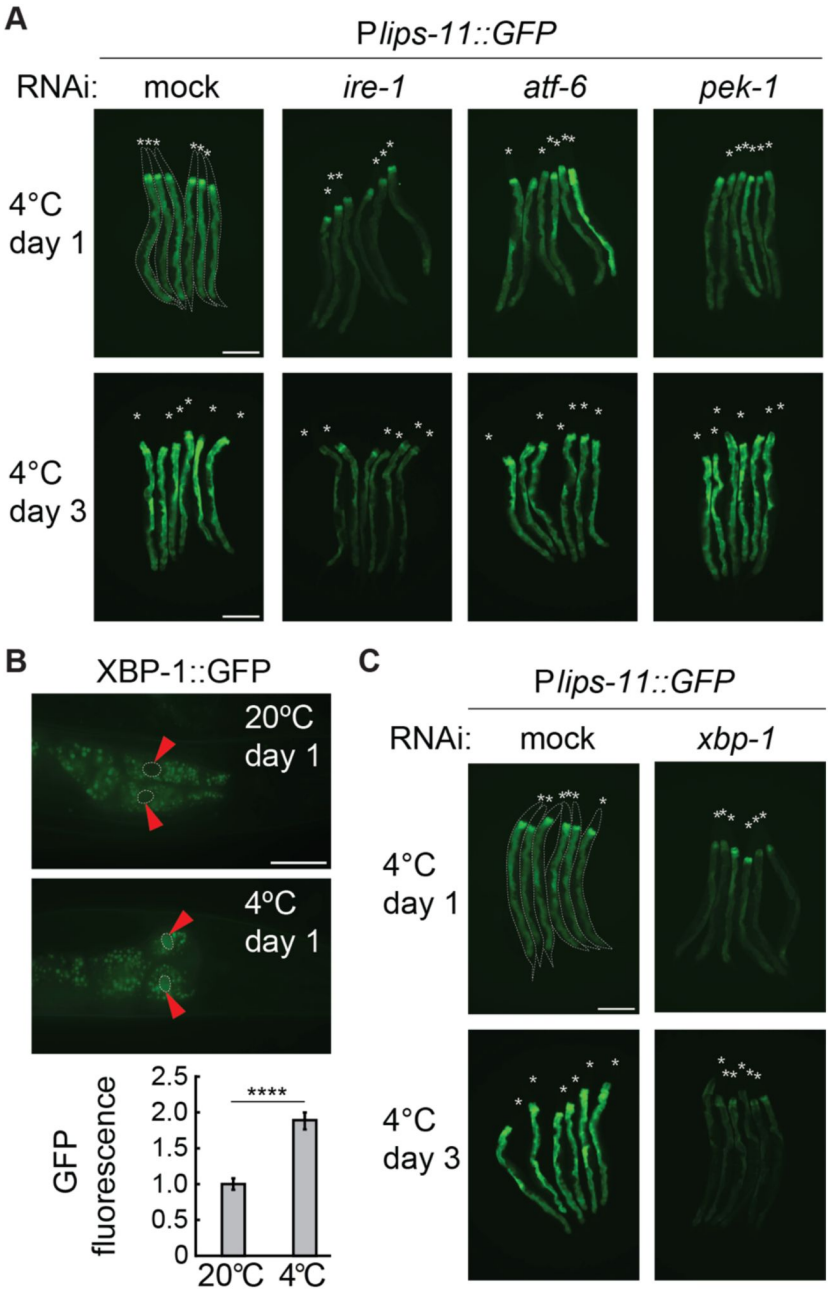


Figure 3.

Cold-induced transcription of *lips-11* depends on the IRE-1/XBP-1 pathway.

(**A**) Micrographs of several bundled animals expressing the P*lips-11::GFP* reporter, taken at the indicated conditions. Note a strong decrease in GFP expression after 1 and 3 days at 4°C upon RNAi-mediated knockdown of *ire-1*, but not of *atf-6* or *pek-1*. The animals are outlined in the top-left panel and the heads are indicated by asterisks (A and C). The scale bar = 200 µm. (**B**) Top: Representative micrographs of adult animals expressing the P*xbp-1::xbp-1::GFP* splicing reporter at the indicated temperatures and time points. GFP is expressed in frame only upon removal of the IRE-1-regulated intron in *xbp-1* mRNA. Arrowheads indicate the outlined nuclei of the most anterior pair of intestinal cells. The scale bar = 40 µm. Below: the corresponding quantification of the nuclear GFP relative to 20°C. Between two and five nuclei were analyzed per animal, in at least fifteen animals per condition. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. ****: $p < 0.0001$. (**C**) Micrographs of mock or *xbp-1* RNAi-treated P*lips-11::GFP* reporter animals, kept for 1 or 3 days at 4°C. The scale bar = 200 µm.

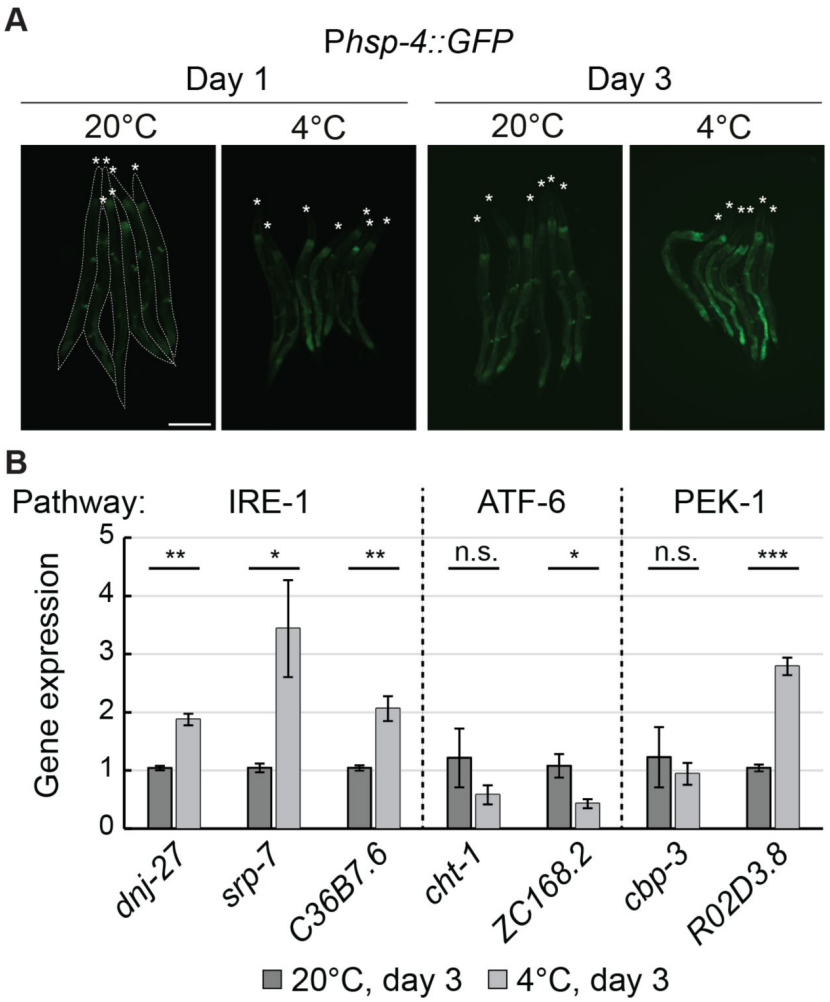


Figure 4.

Cold specifically activates UPR^{ER} through IRE-1.

(**A**) Micrographs of several bundled animals carrying the P*hsp-4::GFP* reporter, taken at the indicated temperature and time. The animals are outlined in the left and the heads are indicated by asterisks. The scale bar = 200 µm. (**B**) RT-qPCR analysis of changes in mRNA levels for known UPR^{ER} target genes after 3 days at 4°C, relative to 3 days at 20°C. Dashed lines separate genes that are regulated by different pathways (IRE-1, ATF-6, and PEK-1). Note that the mRNA levels of all IRE-1 responsive genes are significantly upregulated at 4°C. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

The cold-induced UPR^{ER} stems from both protein misfolding and lipid disequilibrium

ER stress and subsequent activation of the UPR^{ER} are typically associated with misfolded proteins accumulating in the ER lumen. To test if cold aggravates protein misfolding, we employed another reporter strain, wherein YFP is fused to a mutated form of the *C. elegans* cathepsin L-like protease (CPL-1$^{W32A, Y35A}$), which does not fold properly and accumulates during ER stress (Efstathiou et al., 2022). We found that the levels of misfolded CPL-1 modestly increased after one day in the cold but then returned to basal levels after a longer cold exposure (**Figure 5**). Thus, disturbed ER proteostasis may, at least transiently, trigger the activation of UPR^{ER} in hibernating animals.

Apart from protein misfolding, lipid disequilibrium is also known to trigger UPR^{ER} (Halbleib et al., 2017; Tam et al., 2018; Volmer et al., 2013). The IRE-1 pathway is specifically activated when the ratio between phosphatidylcholine (PC) and phosphatidylethanolamine (PE) decreases, or when the levels of unsaturated fatty acids become insufficient (Ariyama et al., 2010; Hou et al., 2014; Thibault et al., 2012). In the latter case, reduced content of unsaturated fatty acids (FAs) results in a decreased ER membrane fluidity, which is thought to increase the oligomerization and thus the activation of IRE-1 (Halbleib et al., 2017). Thus, we asked if dietary supplementation of unsaturated FAs or choline (crucial for PC synthesis) affects the IRE-1 activity in the cold. We first validated the effectiveness of FA supplementation by confirming a previous report, that adding unsaturated FAs prevents the *hsp-4* induction in *fat-6* RNAi-depleted animals (Hou et al., 2014) (**Figure 6 – figure supplement 1**). Following a similar FA supplementation procedure, we observed no reduction in the expression of the *lips-11* reporter in the cold (**Figure 6 – figure supplement 2**). Thus, insufficient lipid desaturation does not seem to be the main trigger of UPR^{ER} in the cold. By contrast, supplementing the diet with choline reduced the expression of both *lips-11* and *hsp-4* reporters in hibernating animals (**Figure 6A-B**). Moreover, we found that choline supplementation was beneficial for cold survival (**Figure 6C**). Together, these experiments suggest that cold-induced activation of UPR^{ER} could be triggered by sensing disruptions in both protein and lipid homeostasis, with the latter related to insufficient levels of PC rather than unsaturated FAs.

IRE-1 is important for a robust cold survival

If activating IRE-1 signaling is important during cooling, then inhibiting this pathway may be expected to impair cold survival. At standard temperature, the loss-of-function *ire-1(ok799)* mutants appear superficially wild-type. In the cold, however, these mutants displayed a modest but significant impairment of cold survival (**Figure 7A**). Thus, the IRE-1 signaling improves cold survival, presumably by activating its downstream targets. One obvious candidate is LIPS-11, but we found that its RNAi-mediated depletion had no obvious impact on cold survival, suggesting that LIPS-11 either plays no essential role in cold survival or functions redundantly with other IRE-1/XBP-1 targets.

Several previous studies reported IRE-1-dependent genes activated in response to protein misfolding (UPR^{PT}; (Shen et al., 2005)), lipid bilayer stress (UPR^{LBS}; (Koh et al., 2018)), or cold when using a different cooling paradigm (Dudkevich et al., 2022). To examine if these genes were also activated by cold treatment in our study, we extracted them from the publications (**Supplementary file 4**) and examined their overlap with the cold-induced transcripts from **Figure 2A**. Curiously, we observed little overlap between the different gene sets (**Figure 7 – figure supplement 1** and **Supplementary file 5**). The UPR^{PT} and the UPR^{LBS} are already known to regulate largely distinct targets (Koh et al., 2018). Also, the limited overlap between those genes and IRE-1-dependent genes from Dudkevich et al. suggests that IRE-1-mediated gene expression is largely context-dependent, which could also apply to IRE-1-dependent gene expression reported here.

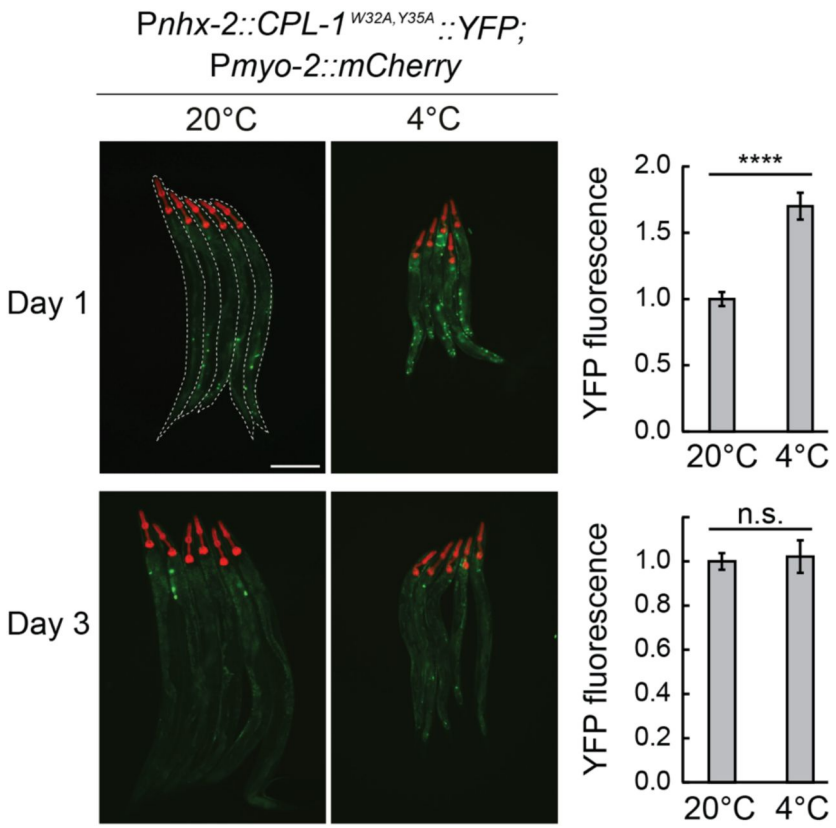


Figure 5.

Misfolded protein levels increase transiently during cold exposure.

Left: representative micrographs, taken at the indicated conditions, of bundled animals carrying the CPL-1^{W32A,Y35A}::YFP misfolding reporter. The head of each animal is highlighted by the pharyngeal expression of mCherry, driven by the *myo-2* promoter. Animals are outlined in the top-left panel. The scale bar = 200 µm. Right: the corresponding quantification of YFP fluorescence at 4°C after 1 or 3 days, relative to 20°C. The YFP fluorescence was analyzed from whole animals, with a minimum of 38 animals per condition. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. ns: $p > 0.05$, ****: $p < 0.0001$.

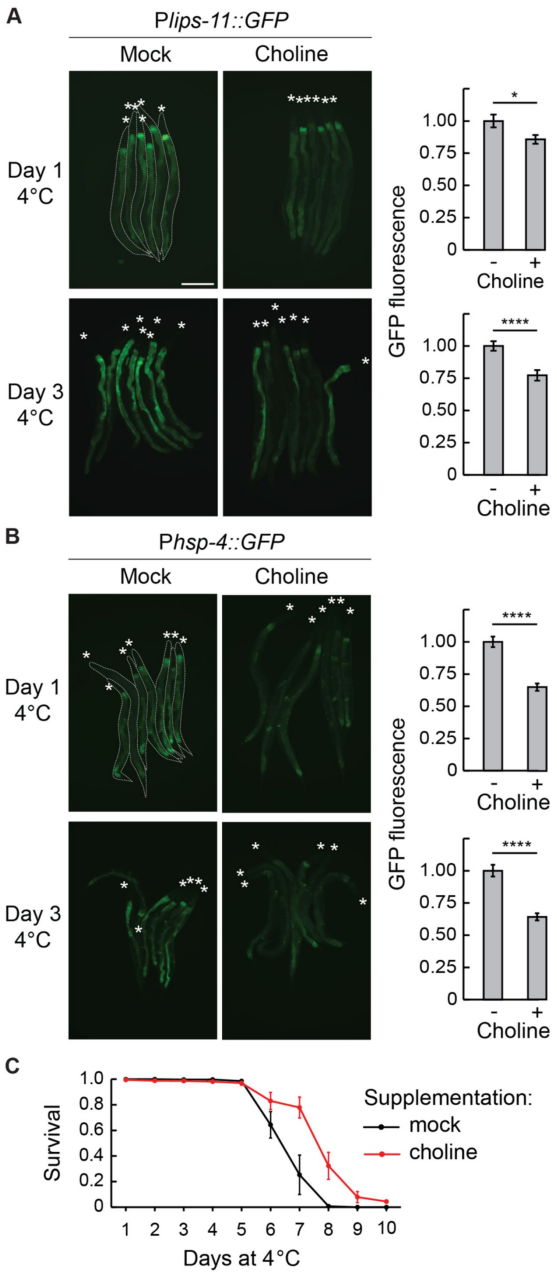


Figure 6.

Choline supplementation suppresses UPR^{ER} and improves cold survival.

(**A**) Left: micrographs taken after 1 or 3 days at 4°C of bundled P*lips-11::GFP* reporter animals on a mock or 50 mM choline supplemented diet. Animals are outlined in the top-left panel and the heads are indicated with asterisks (A and B). The scale bar = 200 µm. Right: the corresponding quantification of intestinal GFP fluorescence after 1 or 3 days at 4°C on a choline supplemented diet, relative to a mock diet. GFP fluorescence was analyzed from a minimum of 38 animals per condition. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. *: $p < 0.05$, ****: $p < 0.0001$. (**B**) Left: Micrographs taken after 1 or 3 days at 4°C of bundled P*hsp-4::GFP* reporter animals on a mock or 50 mM choline supplemented diet. The scale bar = 200 µm. Right: the corresponding quantification of intestinal GFP fluorescence after 1 or 3 days at 4°C on a choline supplemented diet, relative to a mock diet. GFP fluorescence was analyzed from a minimum of 28 animals per condition. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. ****: $p < 0.0001$. (**C**) Survival of wild-type animals on a mock or choline-supplemented diet. Error bars indicate the SEM of three biological replicates. A minimum of 200 animals were scored per time point. Wilcoxon signed-rank test was used for statistical analysis; $p = 0.02$.

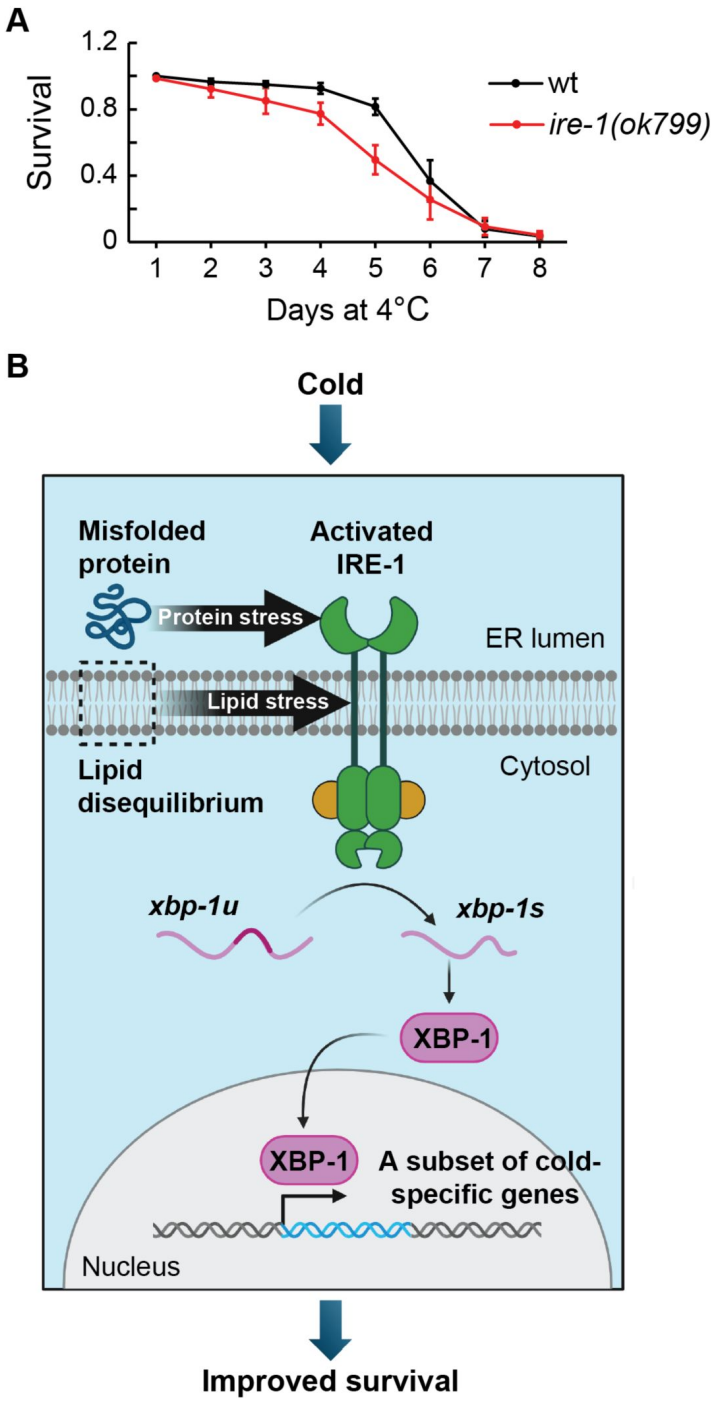


Figure 7.

The IRE-1 pathway facilitates *C. elegans* survival during cold dormancy.

(**A**) Survival of wild-type and *ire-1(ok799)* animals at 4°C. Error bars indicate the SEM from 6 biological replicates. A minimum of 800 animals were scored per time point. Wilcoxon signed-rank test was used for statistical analysis; p = 0.03. (**B**) A model for the IRE-1 function in hibernating nematodes. Cold exposure leads to increased protein misfolding and lipid disequilibrium in the ER. These two stressors trigger the activation of IRE-1, which promotes the downstream processing of *xbp-1u* to *xbp-1s* mRNA. The functional XBP-1 transcription factor enters the nucleus to promote transcription of specific genes, including those facilitating cold survival. Created with *BioRender.com*.

Summarizing, our findings suggest that cold-induced protein and lipid stress in the ER specifically induces the IRE-1 branch of the UPR. Following the IRE-1-mediated processing of *xbp-1* mRNA, the XBP-1 transcription factor activates its target genes. These include *lips-11*, but additional targets seem necessary to explain how IRE-1 benefits cold survival (**Figure 7B**). This model does not rule out additional players. For example, hibernating animals are sensitive to reactive oxygen species (ROS) (Pekec et al., 2022), and ROS can activate IRE-1-dependent antioxidant response mediated by the transcription factor SKN-1/Nrf2 (Hourihan et al., 2016). Whether this pathway is activated in the cold remains to be tested. Similarly, additional pathways may work alongside IRE-1 to regulate gene expression in hibernating animals. Intriguingly, we noticed that *zip-10* mRNA, encoding a transcription factor promoting organismal death during cold shock (Jiang et al., 2018), also goes up during cold dormancy. However, since the animals survive unabated, future studies will clarify if it plays a role in hibernating nematodes.

Discussion

Consistent with findings from other models, our results show that also *C. elegans* responds to severe cold by globally decreasing protein synthesis. Since translation is one of the most energy-consuming biological processes, this reduction likely helps preserve cellular energy reserves. Additionally, translational regulation is reversible, which may allow for the rapid restoration of protein synthesis once animals are returned to temperatures conductive to growth and development. In poikilotherms, such as *C. elegans*, whose body temperature fluctuates with the environment, reducing global translation presumably helps the animals survive until temperatures rise again. The same applies to homeotherms capable of temporal heterothermy, meaning animals that adjust their body temperature in a circadian or seasonal circle. In other homeotherms, including humans, a global reduction of protein synthesis may serve a protective role in surviving accidental hypothermia or aiding the repair of peripheral cold injuries affecting extremities and exposed skin.

Despite the general reduction of protein synthesis during cold exposure, our results suggest that most available mRNAs are translated, albeit at a slower rate. This implies that cold-specific gene expression could be regulated through transcription or mRNA stability. In the latter case, the enrichment of specific mRNAs could result either from the degradation of specific transcripts at 20°C (allowing them to accumulate in the cold) or from the selective degradation of some transcripts in the cold, making others relatively more abundant. While we cannot rule out this possibility for certain transcripts, we favor a simpler model in which cold-specific gene is generally regulated at the transcriptional level. Supporting this hypothesis, our findings indicate that the IRE-1/XBP-1 pathway induces transcription of at least some genes in hibernating animals.

Curiously, a different cooling protocol (shifting animals from 15°C to 2°C) also activates IRE-1 signaling, but in this case, it occurs independently of *xbp-1* processing (Dudkevich et al., 2022). In this case, IRE-1 activation is observed in neurons but remodels lipid metabolism in other tissues, likely to balance saturated and unsaturated FAs. Supporting this idea, dietary supplementation with unsaturated FAs bypasses the need for IRE-1 activation. This aligns with previous findings showing that desaturated FAs are crucial for survival at low but physiologically tolerable temperatures like 15°C (Svensk et al., 2013). In contrast, the IRE-1 signaling described in our study involves *xbp-1* processing, and dietary supplementation with unsaturated FAs does not prevent IRE-1 activation. A possible explanation for these differences is that animals were grown at different starting temperatures (15°C versus 20°C) before cooling.

C. elegans exhibits profound physiological differences between these two temperatures. For example, the above-mentioned demand for unsaturated FAs is heightened at 15°C (Svensk et al., 2013), and the cold-sensitive TRPA-1 channel functions at 15° but not at 20°C (Xiao et al., 2013). Additionally, while nematodes arrest development at 4°C, they continue developing–

albeit slowly–at 9°C or higher (Horikawa et al., 2024). These observations suggest that *C. elegans* may activate IRE-1 through distinct mechanisms and with different outcomes depending on their physiological states and different cooling regimes.

What triggers IRE-1 activation in this study? Using the CPL-1$^{W32A, Y35A}$ protein folding sensor, we observed transient protein misfolding on day 1 of cold exposure, but not on day 3. Since protein synthesis is strongly reduced in the cold, the burden of misfolded proteins may similarly decrease over time, suggesting that additional cues drive UPR^{ER} activation at later stages of hibernation. Our finding that supplementing unsaturated FAs did not prevent IRE-1 activation in hibernating nematodes aligns with previous research showing that inhibiting *C. elegans* fatty acid desaturases has little impact on their ability to survive severe cold (Murray et al., 2007). Combined with our observation that some FA desaturases may undergo additional translational repression in the cold, these results suggest that desaturated FAs are not limiting during *C. elegans* cold dormancy.

In contrast, choline supplementation reduced IRE-1-dependent expression during both early and later stages of hibernation, suggesting that changes in one or more choline derivatives contribute to ER stress. Since choline is essential for PC synthesis, a major component of biological membranes (Kent, 1990), the IRE-1 pathway may monitor a PC-sensitive aspect of ER biology. For example, it could detect alterations in transmembrane channels or peripheral membrane-binding proteins, whose activities depend on the physical properties of membrane lipids (Allende et al., 2004; Janmey & Kinnunen, 2006). However, choline also plays other roles, including in neurotransmitter synthesis and methylation metabolism. Thus, we cannot yet rule out the possibility that the protective effects of choline supplementation stem from functions outside PC synthesis.

Regardless of the exact role of IRE-1 signaling in *C. elegans* cooling, there is evidence suggesting that its connection to cold extends beyond nematodes. In cultured human neurons, moderate hypothermia activates the ER stress response and induces all three branches of the UPR, including IRE1 (Rzechorzek et al., 2016). The same study suggests that cooling-induced UPR^{ER} may be neuroprotective. Whether this response also occurs *in vivo* and under deep hypothermia remains to be determined. If so, its manipulation could lead to improved procedures in organ transplantation and emergency medicine, where deep cooling induces a poorly understood state of preservation supporting vital organ functions of trauma patients (Kutcher et al., 2016).

Methods

C. elegans strains and maintenance

Unless stated otherwise, animals were maintained as previously described, grown at 20°C on 2% Nematode Growth Media (NGM) agar plates, seeded with the *E. coli* OP50 bacteria. All strains used in this study are listed in Supplementary file 6. Synchronized animals were obtained by extracting embryos from gravid adults with a bleaching solution (30% (v/v) sodium hypochlorite (5% chlorine) reagent (ThermoFisher Scientific; 419550010), 750 mM KOH). The embryos were left to hatch in the absence of food into arrested L1 larvae by overnight incubation at room temperature in M9 buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 86 mM NaCl, 1 mM $MgSO_4$).

Cooling procedure

The cooling procedure was as previously published (Habacher et al., 2016; Pekec et al., 2022). In short, synchronized L1 larvae were grown at 20°C until becoming young adults. The animals were then adapted to the cold for 2 hours at 10°C, before being transferred to 4°C.

RNA-seq, polysome and ribosome profiling

Animals were pre-grown at 20°C, then moved to 10° for 2 h, and then incubated at 4°C. The reference 20°C animals were collected at the time when others were moved to 10°C (day 0). The RNA-seq, polysome profiling, and ribosome profiling were performed from two biological replicates as previously described (Arnold et al., 2014; Scheckel et al., 2012).

Computational processing of RNA-seq and ribosome profiling

Both RNA-seq and ribosome profiling were processed with the ribo-seq pipeline in ORFik (v1.22.2). The code for this pipeline can be found at *https://www.bioconductor.org/packages/release/bioc/vignettes/ORFik/inst/doc/Ribo-seq_pipeline.html*. This code with minor alterations (species, names of files, samples, footprint lengths, etc.) adapted to our use case and libraries are available upon request. The pipeline was run with default parameters indicated in the code above. It wraps several steps: stripping adapters using fastp (v0.23.4) with adapter sequence "TGGAATTCTCGGGTGCCAAGG", filtering contaminants (e.g. rRNA) and mapping reads with STAR (v2.7.11b) to the WBcel235 assembly and estimating expression (RPKM) using DESeq2(v1.42.1). Tracks used to display coverage for **Figure 3 – figure supplement 2B** were created from the mapped BAM files using igvtools (v2.14.0) and the coverage was normalized to library size.

Gene set enrichment analysis

Gene set enrichment was performed on the RNA-seq expression of all genes using the R Bioconductor package clusterProfiler (v4.10.1) and org.Ce.eg.db (v3.18.0). The top 10 categories from both the enriched and suppressed sets were visualized using enrichPlot (v1.22.0).

Western blot analysis

Animals were harvested from plates, washed thrice in M9 buffer and pelleted before being snap-frozen in liquid nitrogen. Protein extracts were prepared by grinding the pellet with a mortar and pestle in the presence of liquid nitrogen and dissolving in Lysis Buffer (50 mM HEPES (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 5% glycerol (w/vol), 1 mM PMSF, 7 mg/ml cOmplete Proteinase Inhibitor Tablets (Roche, 11697498001)). Debris were removed by centrifugation at 16,100 x g for 20 minutes at 4°C. Protein concentrations were measured by Bradford Assay (Bio-Rad). The required amount of 4x NuPAGE™ LDS Sample Buffer (Invitrogen, NP0007) and 10x NuPAGE Sample Reducing Agent (Invitrogen, NP0004) was added to the protein samples, followed by an incubation at 70°C for 10 minutes. Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane by wet transfer. Membranes were washed thrice for 5 minutes with PBS-T, blocked for 1 hour in Intercept (TBS) Blocking Buffer (LI-COR, 927-60001), and incubated overnight at 4°C with primary antibodies diluted in the same blocking buffer. The following primary antibodies were used: 1:10,000 monoclonal mouse anti-puromycin (Merck, MABE343), and 1:5,000 polyclonal rabbit anti-actin (Abcam, ab8227). Detection was carried out with IRDye 680RD-conjugated goat anti-mouse secondary antibody (LI-COR Biosciences, 926-68070) or IRDye 800CW-conjugated goat anti-rabbit secondary antibody (LI-COR Biosciences, 926-32211) and infrared imaging (LI-COR Biosciences, Odyssey CLx).

SUnSET assay

A total of 12,000 synchronized L1 larvae were grown at 20°C and then cooled as described earlier or kept continuously at 20°C. The animals were kept on multiple large 15 cm^2 plates for each sample to obtain sufficient material without crowding. The SUnSET assay was carried out essentially as previously described (Arnold et al., 2014), with modifications to assay protein synthesis at 4°C. In brief, animals were washed twice in S-basal, resuspended in 4 ml S-medium, and transferred to a 50 ml Erlenmeyer. An overnight culture of *E. coli* OP50 was 10x concentrated in S-medium and 750 µl was added to the animals together with 250 µl of 10 mg/ml puromycin

(Millipore Sigma, P8833). The animals were grown for 4 hours at 200 rpm before harvesting. Animals were washed thrice with S-basal, pelleted, and snap frozen in liquid nitrogen. Lysates were prepared as described in the western blot procedures and 40 µg of total protein was loaded per well. The incorporation of puromycin into nascent peptides was measured by normalizing band intensities from anti-puromycin to anti-actin antibodies. Three biological replicates were used for the final quantification.

Construction of the *lips-11::GFP* reporter strain

The P*lips-11::GFP::unc-54* 3'UTR construct was generated via the MultiSite Gateway Technology (Thermo Fisher Scientific, 12537-023). The *lips-11* promoter (1140 bp) and GFP (867 bp) were amplified from *C. elegans* genomic DNA and a plasmid carrying GFP::H2B (pCM1.35) (Merritt et al., 2008), before being inserted into the entry vectors pDONRP4P1R and pDONR221, respectively (oligos are listed in Supplementary file 6). The resulting entry vectors were recombined along with the entry vector pCM5.37, carrying the *unc-54* 3'UTR (699 bp) (Merritt et al., 2008), and the destination vector pCFJ150 (Frokjaer-Jensen et al., 2008), carrying chromosome II integration sites together with the *unc-119* (+) gene, resulting in the expression clone P*lips-11::GFP::unc-54* 3'UTR. Transgenic animals were obtained via single-copy integration into the *ttTi5605* locus on chromosome II by injecting EG4322 (*ttTi5605 II; unc-119(ed3) III*) animals with the expression clone (Frokjaer-Jensen et al., 2008).

Microscopy

Immediately prior to imaging, animals were anesthetized in a drop of 5 mM levamisole in M9 buffer on a 2% (w/v) agarose pad, clustered, covered with a cover slip and immediately imaged with the Zeiss AxioImager Z1 microscope. Micrographs were acquired with an Axiocam MRm REV2 CCD camera using the Zen software (Zeiss) and processed with Image J. The specific area that was analyzed for fluorescent intensities, as well as the number of measurements, is indicated in each figure legend.

Induction of ER stress by tunicamycin treatment

Animals were grown at 20°C on NGM agar plates seeded with *E. coli* OP50. At the 2-day-old adult stage, animals were shifted to plates containing 20 µg/ml tunicamycin (Millipore Sigma, T7765), prepared from a 1 mg/ml stock solution dissolved in DMSO. Animals were then grown for 6 hours at 20°C in the presence of tunicamycin. Control animals were treated similarly on plates containing the same volume of DMSO.

Generation of iOP50 RNAi bacteria

Plasmids targeting *ire-1*, *xbp-1*, *pek-1*, or *atf-6* were extracted from overnight cultures of *E. coli* HT115 bacteria derived from the Ahringer library using the QIAprep Spin Miniprep Kit (QIAGEN, 27104) (Kamath et al., 2003). *E. coli* OP50 bacteria were rendered RNAi competent and chemically competent as previously described (Neve et al., 2020). The resulting iOP50 bacteria were transfected with 1 µl of the purified plasmids derived from the Ahringer library for downstream gene-specific RNAi or with the plasmid vector L4440 for mock RNAi (Fire et al., 1998; Kamath et al., 2003).

RNAi

Gene-specific knockdown was achieved by feeding the animals with bacteria carrying plasmids expressing double-stranded RNA, sourced from either the Vidal or Ahringer libraries (Fraser et al., 2000; Kamath et al., 2003; Rual et al., 2004). Overnight cultures of *E. coli* HT115 (for RNAi at 20°C) or *E. coli* iOP50 bacteria (for RNAi at 4°C) were induced for 1 hour with 1 mM IPTG and seeded on NGM agar plates containing 1 mM IPTG and 50 µg/ml carbenicillin. Plates were additionally supplemented with fatty acids as described below for experiments combining RNAi-

mediated gene knockdown and dietary supplementation. Synchronized L1 larvae were grown on the RNAi-inducing agar plates at 20°C until reaching the 1-day-old adult stage and were either adapted to the cold as described and kept for 1 or 3 days at 4°C or kept at 20°C for the same duration. Bacteria containing the 'empty' L4440 vector were utilized as a mock RNAi control for all experiments.

Dietary supplementation

For choline supplementation assays, a working stock of 200 mg/ml choline (Millipore Sigma, C7527) in water was added to autoclaved NGM media at 55°C to a final concentration of 50 mM, except in the cold survival assay where the final concentration was 25 mM. Fatty acid supplemented plates were prepared as previously described with adaptations (Deline et al., 2013). In brief, working stocks of 100 mM were prepared by dissolving palmitic acid (Millipore Sigma, P9767), oleic acid (Millipore Sigma, O7501), and linoleic acid (Millipore Sigma, L8134) in 50% ethanol. Fatty acid sodium salts were added to autoclaved NGM medium containing 0.1% Tergitol (NP40) to a final concentration of 0.8 mM. Fatty acid supplemented plates were covered with foil to prevent light oxidation. All plates were seeded with an overnight culture of *E. coli* OP50 and dried for 3 days before use.

The assay for *C. elegans* cold survival

Cold survival experiments were performed as previously described (Habacher et al., 2016; Pekec et al., 2022). In brief, a minimum of 150 synchronized L1 larvae were grown and adapted to the cold as previously described for each time point. Animals were sampled at the indicated time points and their survival was scored after 24 h recovery at 20°C; those animals that were unresponsive to touch were considered dead. Cold survival was assessed from a minimum of three independent biological replicates, with a minimum of 200 animals used to assess the viability at each indicated time point.

RT-qPCR

Approximately 6,000 animals were subjected to RNA extraction as previously described (Arnold et al., 2014), animals were either collected immediately before cold adaptation (20°C day 0) or were adapted to the cold and kept for 1 or 3 days at 4°C or kept at 20°C for the same time prior to collection. Subsequently, genomic DNA was removed by DNase treatment and the quality of the RNA was assessed using the NanoDrop Spectrophotometer. Reverse transcription was performed by using the SuperScript^{TM} IV First – Strand Synthesis System with random primers, following the protocol from the suppliers. RT-qPCR was performed with 2.5 µl of 1:5 diluted cDNA, 0.25 µl of 10 µM gene-specific primers (Supplementary file 6) and 2 µl of the HOT FIREPol EvaGreen qPCR Mix (Solis BioDyne, 08-36-00001) in a LightCycler 96 qPCR machine.

Data availability

RNA sequencing information from this research can be found in the GEO repository under the following accession numbers: GSE269587 (RNA-seq) and GSE269589 (Ribo-seq).

Additional information

Funding

The research leading to these results received funding from the Norwegian Financial Mechanism 2014–2021 operated by the Polish National Science Center under the project contract nr UMO-2019/34/H/NZ3/00691. Some of the strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

Author contributions

Melanie L. Engelfriet: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft and revision.

Yanwu Guo: Conceptualization, Formal analysis, Investigation, Methodology. Andreas Arnold: Investigation, Formal analysis, Methodology, Validation.

Eivind Valen: Formal analysis, Visualization.

Rafal Ciosk: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Resources, Writing – original draft and revision.

Supplementary figures

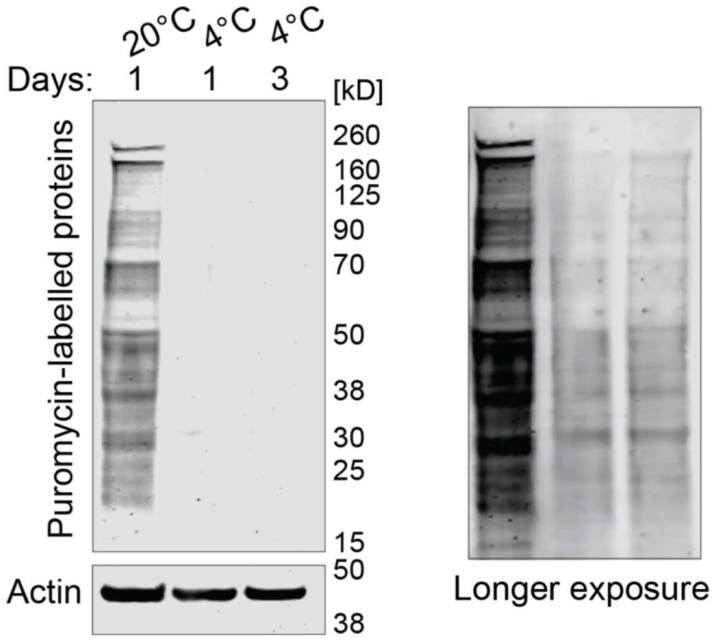


Figure 1 – figure supplement 1.

Protein synthesis evaluated with the SUnSET assay in animals incubated as indicated. Puromycin incorporation was detected by western blot. Actin (ACT-1) was used as a loading control. A longer exposure of the blot on the right shows that puromycin incorporation, although reduced, continues at 4°C.

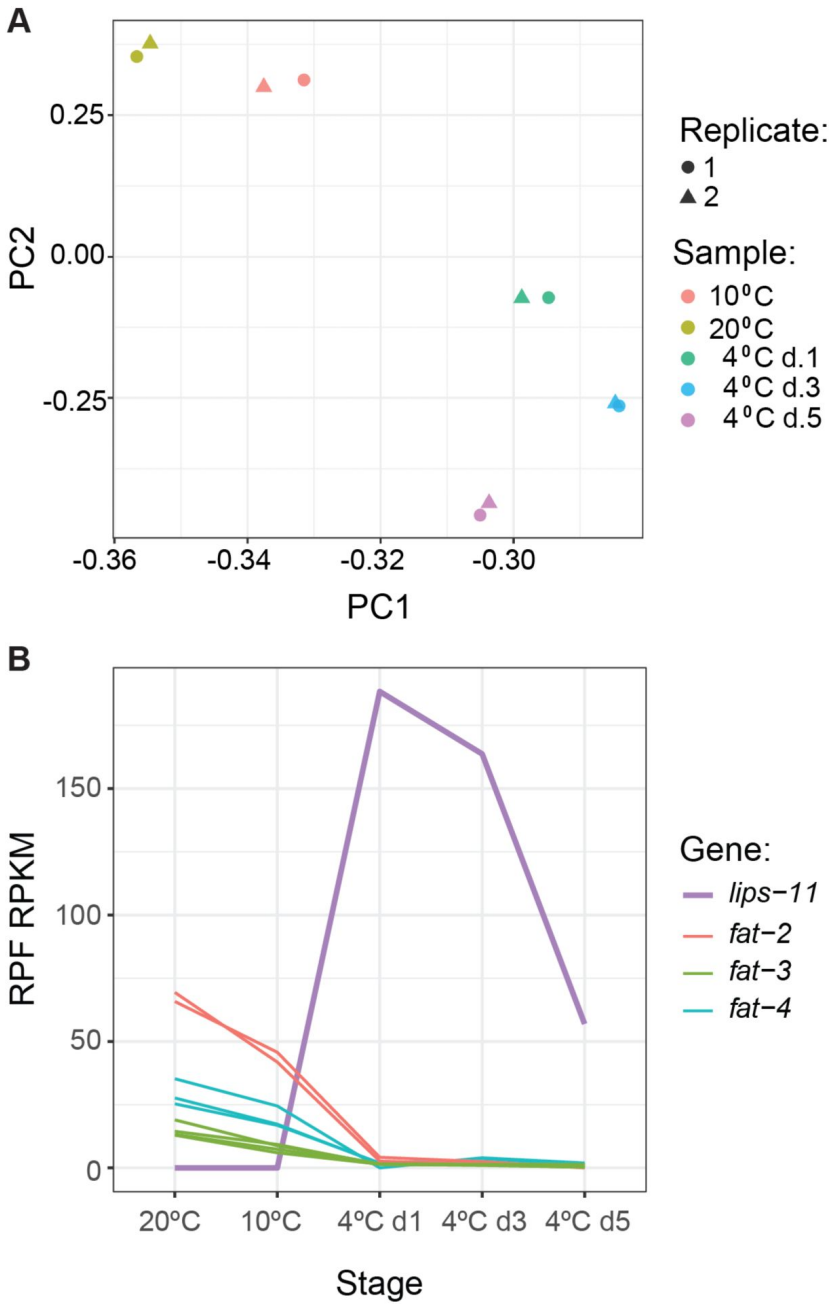


Figure 2 – figure supplement 1.

(**A**) PCA plot of ribosome profiling samples and replicates based on mRNA ribosome protected fragment (RPF) normalized according to reads per kilobase exon per million reads (RPKM). Note co-clustering of replicates and temperatures. (**B**) Changes in translation for selected genes as a function of temperature changes and exposure.

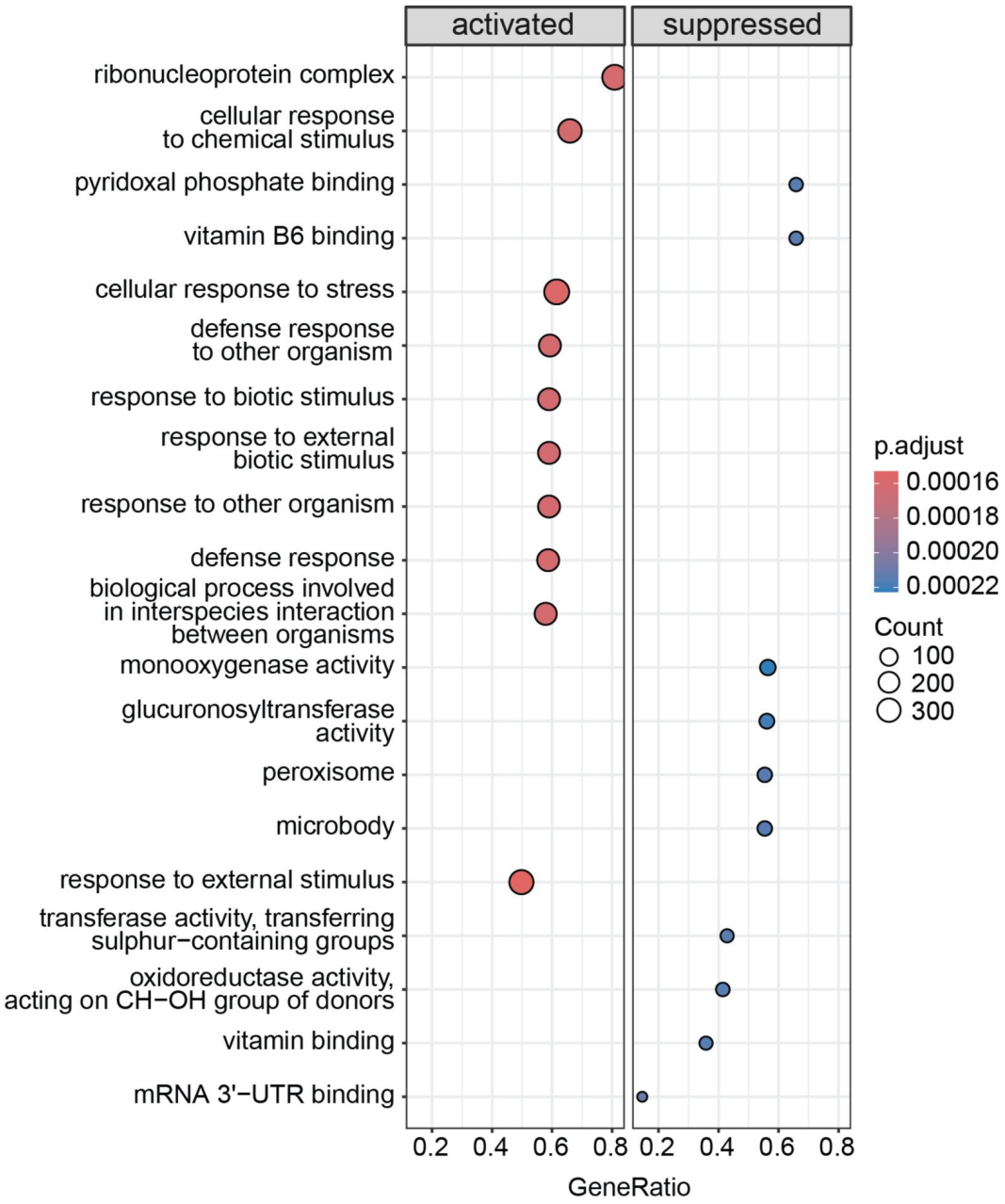


Figure 2 – figure supplement 2.

Gene set enrichment analysis depicting the top 10 categories that are activated or suppressed between 10°C and 4°C on day 1 in wild-type animals.

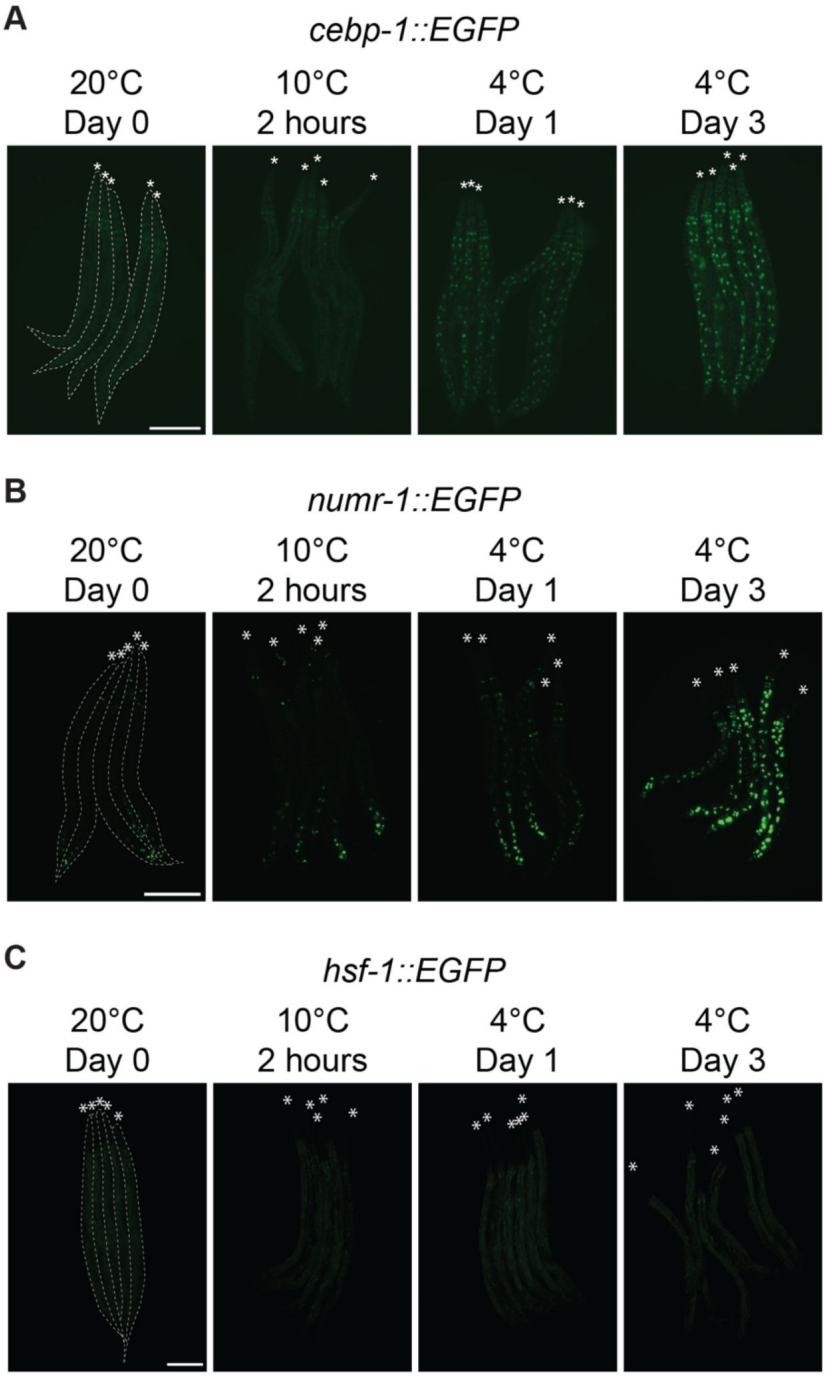


Figure 2 – figure supplement 3.

Micrographs of animals expressing EGFP fused to CEBP-1 (**A**), NUMR-1 (**B**), or HSF-1 (**C**), taken at the indicated time and temperature. Animals are outlined in the left panels and the heads are indicated with asterisks (A-C). The scale bar = 200 μm.

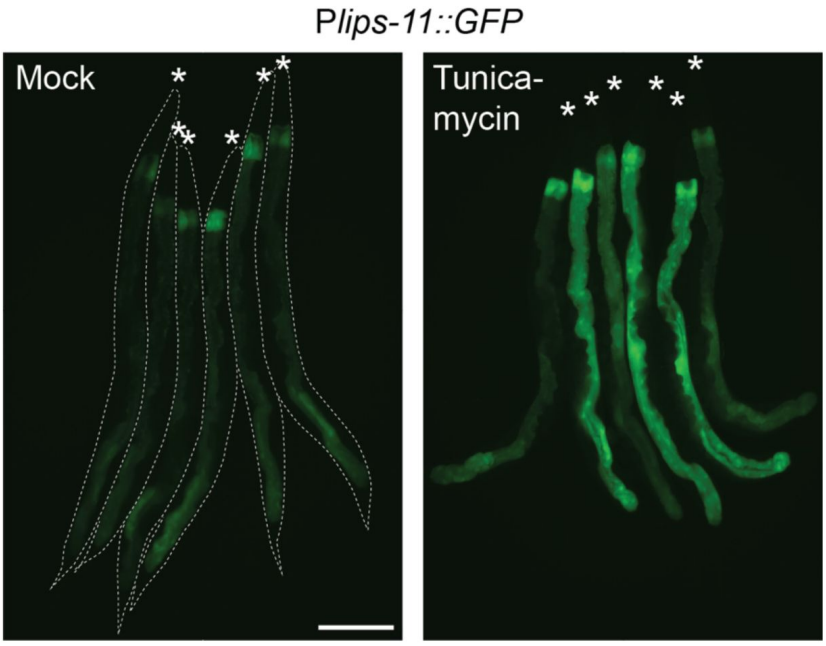


Figure 3 – figure supplement 1.

Micrographs of bundled P*lips-11::GFP* reporter animals after 6 hours of treatment with DMSO (mock) or tunicamycin at 20°C. Animals are outlined in the left panel and the heads are indicated with asterisks. The scale bar = 200 µm.

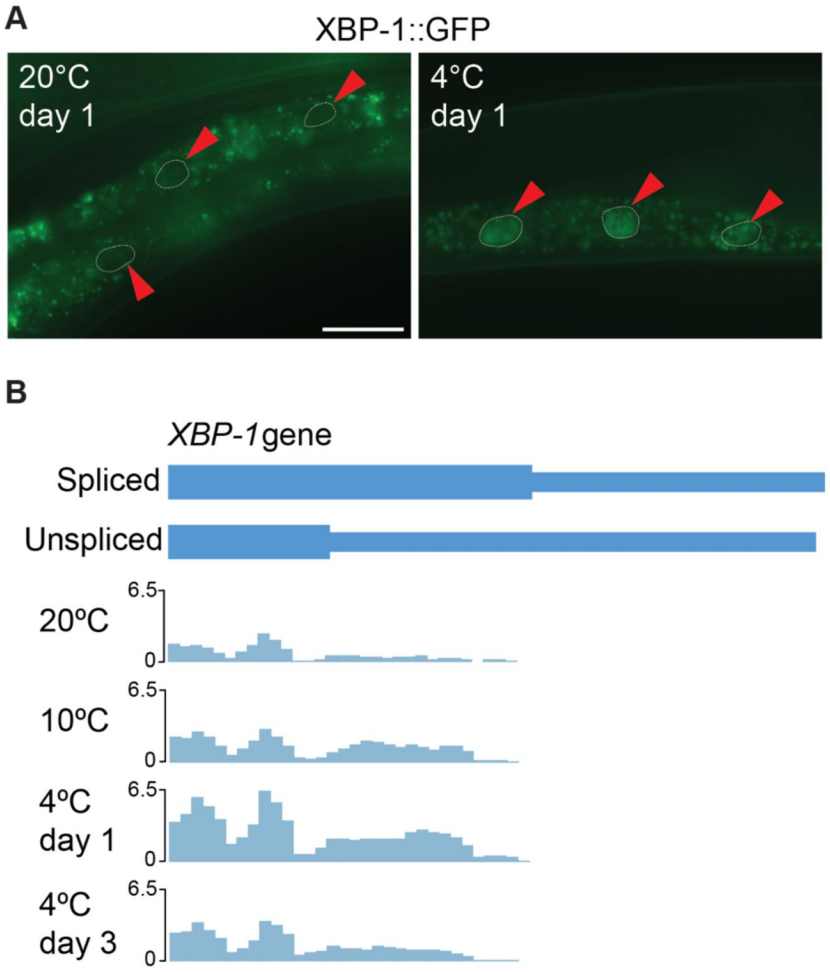


Figure 3 – figure supplement 2.

(**A**) Micrographs depicting the middle part of adult animals expressing the P*xbp-1::xbp-1::GFP* splicing reporter at the indicated temperatures and time points. GFP is expressed in frame only upon removal of the IRE-1-regulated intron in *xbp-1* mRNA. Arrowheads indicate outlined intestinal nuclei. The scale bar = 40 µm. (**B**) Coverage of ribosome protected fragments (RPF) over the two isoforms of *xbp-1* showing increased translation of the extended (Spliced) proteoform after 2 hours at 10°C and subsequent days at 4°C as compared to 20°C day 0. All tracks are on the same scale, normalized to their respective library sizes and multiplied by a scale factor (10^6).

Figure 4 – figure supplement 1.

(**A**) Micrographs of bundled P*hsp-6::GFP* UPR^{mito} reporter animals, taken at the indicated time and temperature. Animals are outlined in the top-left panel and the heads are indicated with asterisks. The scale bar = 200 µm. (A and B). (**B**) Micrographs of bundled P*hsp-60::GFP* UPR^{mito} reporter animals, taken at the indicated time and temperature.

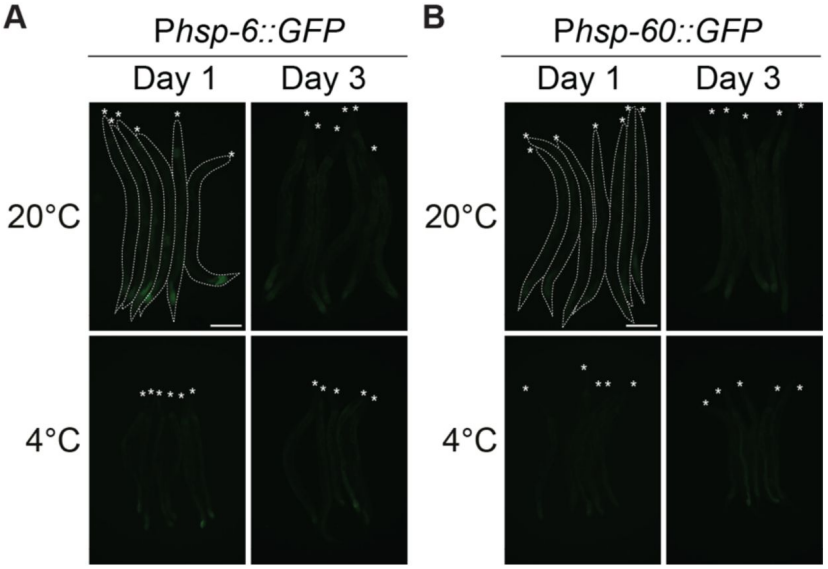
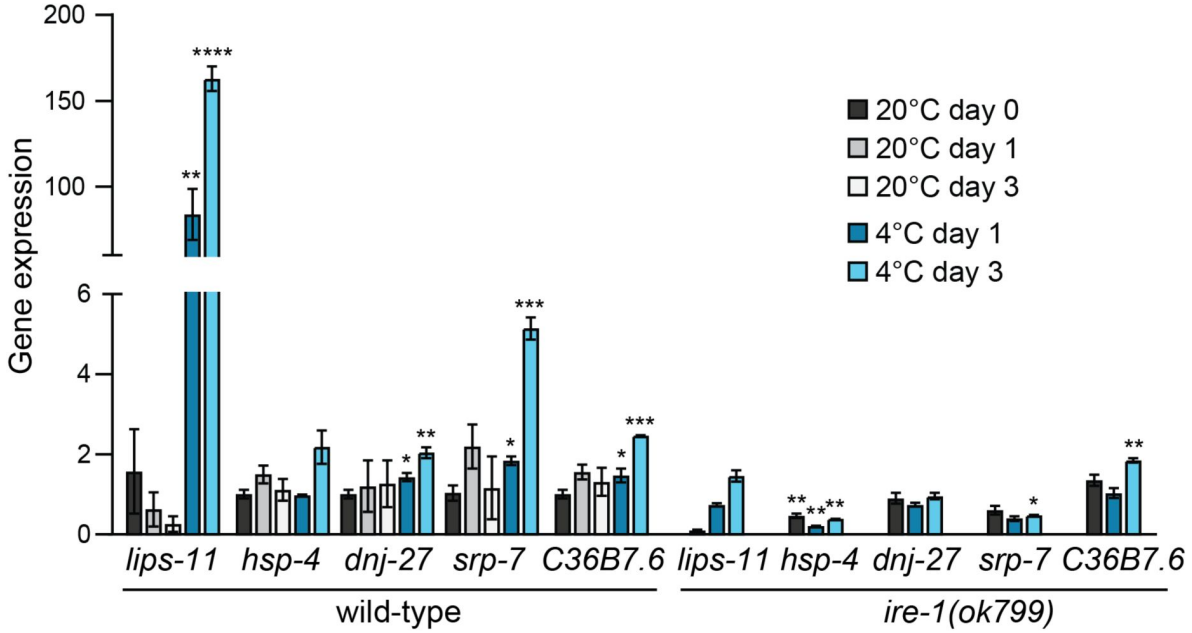


Figure 4 – figure supplement 2.

RT-qPCR analysis of changes in mRNA levels for known IRE-1 target genes in wild-type and *ire-1(ok799)* animals after 1 or 3 days at 20°C or 4°C, relative to wild-type animals at 20°C day 0 (harvested immediately prior to cold adaptation at 10°C). Note that the mRNA levels of most IRE-1 responsive genes are significantly upregulated at 4°C in wild type and remain unchanged or are significantly downregulated in *ire-1(ok799)* mutants. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. Unmarked bars: $p > 0.05$ (not significant), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.



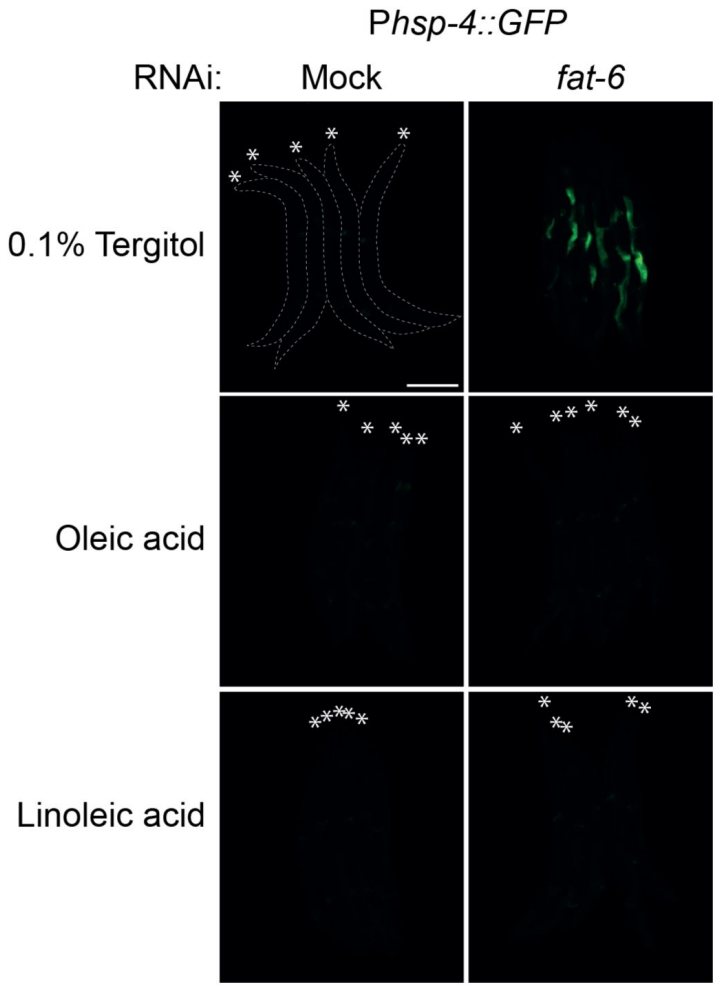


Figure 6 – figure supplement 1.

Micrographs of bundled animals expressing the P*hsp-4::GFP* reporter, subjected to either mock or *fat-6* RNAi and supplemented with either 0.1% tergitol (mock), 0.8 mM oleic acid, or 0.8 mM linoleic acid. Note a strong increase in the GFP reporter upon RNAi-mediated knockdown of *fat-6* in animals on the mock diet, which is suppressed by the supplementation with either oleic or linoleic acid. The animals are outlined in the top-left panel and the heads are indicated by asterisks. The scale bar = 200 μm.

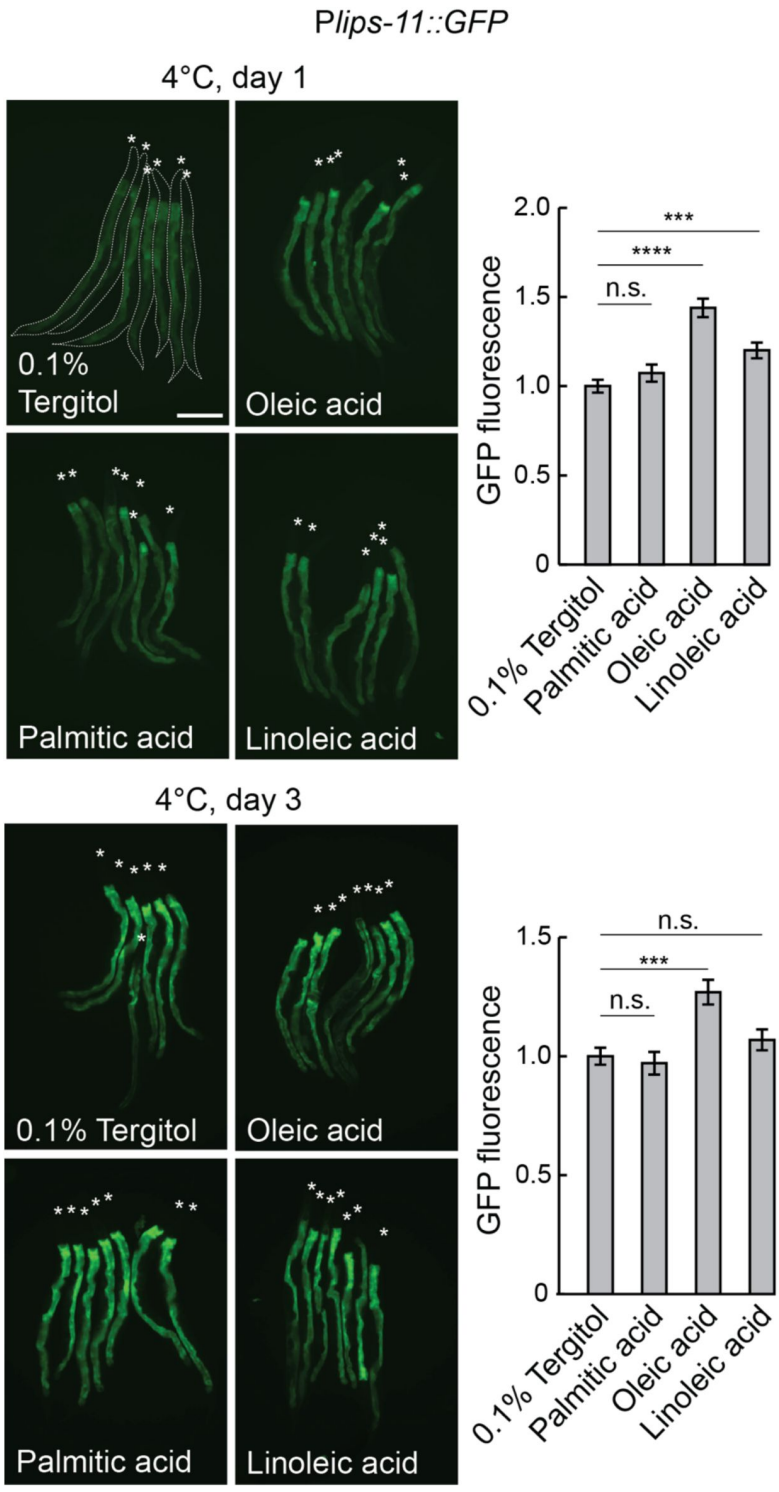


Figure 6 – figure supplement 2.

Left: Micrographs taken after 1 or 3 days at 4°C of bundled P*lips-11::GFP* reporter animals on the diet supplemented with 0.1% tergitol (mock), 0.8 mM oleic acid, 0.8 mM palmitic acid, or 0.8 mM linoleic acid. Animals are outlined in the top-left panel and the heads are indicated with asterisks. The scale bar = 200 µm. Right: the corresponding quantification of intestinal GFP fluorescence after 1 or 3 days at 4°C on different diets, relative to the mock diet. The GFP fluorescence was analyzed from 21 to 31 animals per condition. Error bars indicate the SEM of three biological replicates. Unpaired two-sided t-test was used for statistical analysis. N.s.: $p > 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$.

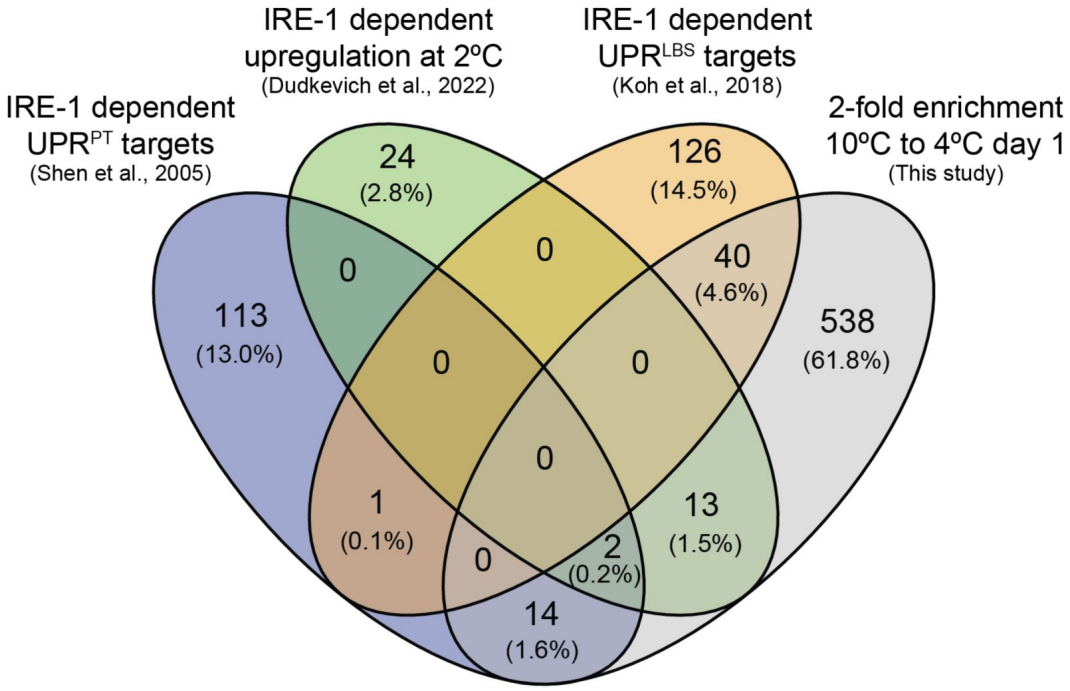


Figure 7 – figure supplement 1.

Four-way Venn diagram depicting the overlap between genes with a minimum 2-fold enrichment (10°C to 4°C day 1) and previously reported IRE-1 responsive genes that are upregulated either during the UPR^{PT} (Shen et al., 2005), upon shifting animals from 15°C to 2°C (Dudkevich et al., 2022), or during the UPR^{LBS} (Koh et al., 2018).

Acknowledgements

We thank Dimos Gaidatzis and the Functional Genomics and Computational Biology facilities at the Friedrich Miescher Institute for Biomedical Research for the initial genomic analysis. We also thank Agnieszka Chabowska-Kita and the Laboratory of Animal Model Organisms (Institute of Bioorganic Chemistry PAS) for constructing the *lips-11* reporter strain, and Solfrid Lindhjem Kvinnesland (Department of Biosciences, UiO) for analyzing cold survival upon dietary choline supplementation.

Additional files

Supplementary file 1. *Total RNA-seq data of wild-type animals kept at the different indicated temperatures.*

Supplementary file 2. *Ribo-seq data of wild-type animals kept at the different indicated temperatures.*

Supplementary file 3. *Genes highlighted in Figure 2A in red.*

Supplementary file 4. *IRE-1 responsive genes from published datasets used to generate the Venn diagram in Figure 7 – figure supplement 1. The IRE-1 dependent cold genes were selected from Table S2 and Table S3 (Dudkevich et al., 2022): the genes were selected if upregulated in wild-type animals but not ire-1(ok799) mutants at 2°C.*

Supplementary file 5. *Shared genes from the Venn diagram in Figure 7 – figure supplement 1.*

Supplementary file 6. *The C. elegans strains (A) and oligos (B) used in this study.*

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Reviewer #2 (Public review):

Summary:

This study investigates cold induced states in C. elegans, using polysome profiling and RNA seq to identify genes that are differentially regulated and concluding that cold-specific gene regulation occurs at the transcriptional level. This study also includes analysis of one gene from the differentially regulated set, lips-11 (a lipase), and finds that it is regulated in response to a specific set of ER stress factors.

Strengths:

(1) Understanding how environmental conditions are linked to stress pathways is generally interesting.
(2) The study used well-established genetic tools to analyze ER stress pathways.

Weaknesses:

(1) The conclusions regarding a general transcriptional response are based on a few genes, with much of the emphasis on lips-11, which does not affect survival in response to cold.

(2) Definitive conclusions regarding transcription vs translational effects would require the use of blockers such as alpha-amanitin or cyclohexamide. Although this may be beyond the scope of the study, it does affect the breadth of the conclusions that can be made.

(3) Conclusions regarding the role of lipids are based on supplementation with oleic acid or choline, yet there is no lipid analysis of the cold animals, or after lips-1 knockdown. Although choline is important for PC production, adding choline in normal PC could have many other metabolic impacts and doesn't necessarily implicate PC without lipidomic or genetic evidence. Although they note the caveats, their evidence falls short of proving a role in PC production.

https://doi.org/10.7554/eLife.101186.2.sa2

Reviewer #3 (Public review):

Summary:

The authors sought to understand the molecular mechanisms that cells use to survive cold temperatures by studying gene expression regulation in response to cold in C. elegans. They determined whether gene expression changes during cold adaptation occur primarily at the transcriptional level and identified specific pathways, such as the unfolded protein response pathway, that are activated to possibly promote survival under cold conditions.

Strengths:

Effective use of bulk RNA sequencing (RNA-seq) to measure transcript abundance and ribosome profiling (ribo-seq) to assess translation rates, providing a comprehensive view of gene expression regulation during cold adaptation. This combined approach allows for correlation between mRNA levels and their translation, thereby offering evidence for the authors' conclusion that transcriptional regulation is the primary mechanism of cold-specific gene expression changes.

Weaknesses:

Many aspects of the weakness have been addressed by the revision. Still, the weak cold sensitivity phenotype observed in ire-1 mutants suggests the ER-UPR pathway's role is likely minor, modulatory or there is an unknown compensatory mechanism responsible for surviving cold.

https://doi.org/10.7554/eLife.101186.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1:***
>
> *(...) some concerns with interpretations and technical issues make several major conclusions in this manuscript less rigorous, as explained in detail in comments below. In particular, the two major concerns I have: 1) the contradiction between the strong reduction of global translation, with puromycin incorporation gel showing no detectable protein synthesis in cold, and an apparently large fraction of transcripts whose abundance and translation in Fig. 2A are both strongly increased. 2) The fact that no transcripts were examined for dependance on IRE-1/XBP1 for their induction by cold, except for one transcriptional reporter, and some weaknesses (see below) in data showing activation of IRE-1/XBP-1 pathway. The conclusion for induction of UPR by cold*

> *via specific activation of IRE-1/XBP-1 pathway, in my opinion, requires additional experiments.*

Relating to the first point, the results of puromycin incorporation and ribosome profiling are not contradictory. The former shows *absolute* changes in translation, i.e. changes in how much protein the cell is producing, while the latter shows relative changes between the produced proteins, i.e. how the cell prioritizes its protein production. An observed up-regulation in ribosome profiling does not necessarily mean (but could) that the corresponding protein goes up in absolute terms (units produced per time). Instead, it implies that out of the population of all translating ribosomes, a larger fraction is translating (prioritizing) this particular mRNA relative to other mRNAs. The second point is addressed later in the response.

> *Major concerns:*
>
> *(1) Fig. 1B shows polysomes still present on day 1 of 4ºC exposure, but the gel in Fig. 1C suggests a complete lack of protein synthesis. Why?*

We realized that the selected gel exposure may give the false impression of a complete lack of puromycin incorporation at 4ºC. To avoid confusion, we now show in Figure 1 – figure supplement 1 the original gel image next to its longer exposure. The quantification of puromycin incorporation remains in Fig. 1C (it is based on 3 biological replicates and only one replicate is shown in the corresponding supplement). We hope it is now clear that there is an ongoing puromycin incorporation/translation at 4ºC, albeit much reduced compared with 20ºC.

> *What is then the evidence that ribosomal footprints used in much of the paper as evidence of ongoing active translation are from actual translating rather than still bound to transcripts but stationary ribosomes, considering that cooling to 4ºC is often used to 'freeze' protein complexes and prevent separation of their subunits? The authors should explain whether ribosome profiling as a measure of active translation has been evaluated specifically at 4ºC, or test this experimentally.*

While the ribosomal profiling alone might not prove ongoing translation, the residual puromycin incorporation does (see the longer gel exposure in Figure 1 – figure supplement 1). To strengthen this argument, we selected two additional genes (*cebp-1* and *numr-1*) whose ribosomal footprints increase in the cold, and whose GFP-fusions were available from the CGC. Monitoring their expression, we observed the expected increase in the cold (see Figure 2 – figure supplement 3 A-B). The ongoing translation in the cold is also in line with our previous study (Peke et al., 2022), where we observed de novo protein synthesis of other proteins under the same cooling conditions as in this study.

> *They should also provide some evidence (like Western blots) of increases in protein levels for at least some of the strongly cold-upregulated transcripts, like lips-11.*

As explained above, we addressed it by additionally examining two strains expressing GFP-fused proteins, whose translation in the cold is predicted to increase according to our ribosomal profiling data. See the new Figure 2 – figure supplement 3 A-B.

> *As puromycin incorporation seems to be the one direct measure of global protein synthesis here, it conflicts with much of the translation data, especially considering that quite a large fraction of transcripts have increased both mRNA levels and ribosome footprints, and thus presumably increased translation at 4ºC, in Fig. 2A.*

We hope the above explanations put this concern to rest.

> *Also, it is not clear how quantitation in Fig. 1C relates to the gel shown, the quantitation seems to indicate about 50-60% reduction of the signal, while the gel shows no discernable signal.*

A above, see a longer western blot exposure in Figure 1 – figure supplement 1 and note that the quantification is based on three biological replicates.

> *(2) It is striking that plips-11::GFP reporter is induced in day 1 of 4ºC exposure, apparently to the extent that is similar to its induction by a large dose of tunicamycin (Fig. 3 supplement),*

We did not intend to compare the extend of induction between cold and tunicamycin treatment. The tunicamycin experiment was meant to confirm that, as suggested by expression data from Shen et al. 2005, *lips-11* is upregulated upon UPR activation.

> *...but the three IRE-1 dependent UPR transcripts from Shen 2005 list were not induced at all on day 1 (Fig. 4 supplement). Moreover, the accumulation of the misfolded CPL-1 reporter, that was interpreted as evidence that misfolding may be triggering UPR at 4ºC, was only observed on day 1, when the induction of the three IRE-1 targets is absent, but not on day 3, when it is stronger. How does this agree with the conclusion of UPR activation by cold via IRE-1/XBP-1 pathway?*

In the originally submitted supplemental figure, we compared mRNA levels between day 1 animals at 20ºC versus 4ºC. However, as argued later by this reviewer, it may be better to use day 0 animals at 20ºC as the reference (since at 20ºC the animals will continue producing embryos). Thus, we repeated the RT-qPCR analysis with additional time points (and genes relevant to other comments). This analysis, now in Figure 4 – figure supplement 2, shows that these mRNAs (*dnj-27*, *srp-7*, and *C36B7.6*) increased already at day 1 in the cold compared with the reference 20ºC animals on day 0, and their levels increased further on day 3.

> *It is true that the authors do note very little overlap between IRE-1/XBP-1-dependent genes induced by different stress conditions, but for most of this paper, they draw parallels between tunicamycin-induced and cold induced IRE-1/XBP-1 activation.*

We carefully re-examined the manuscript to ensure that we do not draw parallels between cold and tunicamycin treatment. The three genes (*dnj-27*, *srp-7*, and *C36B7.6*) were taken from Shen et al. because that study reported *lips-11* as an IRE-1-responsive gene, which we realized thanks to the Wormbase annotation of *lips-11*. Examining the three genes in our expression data, *srp-7* (like *lips-11*) is also upregulated more than 2-fold, while the other two genes go up but less than 2-fold. As mentioned by the reviewer, we note little overlap between the different stress conditions suggesting that the response is context dependent. Additional differences may arise if, as we hypothesize, UPR is activated in the cold in response to both protein and lipid stress. Note that the 2-fold cutoff used in the previous Figure 7 – figure supplement 1 was (erroneously) on the log2 scale, so showed genes upregulated at least 4-fold. We now corrected it to 2-fold. While there are now a few more overlapping genes, the overall conclusion, that there is little overlap between different conditions, did not change. We now list the shared genes in the new Supplementary file 5.

> *The conclusion that "the transcription of some cold-induced genes reflects the activation of unfolded protein response (UPR)..." is based on analysis of only one gene, lips-11. No other genes were examined for IRE-1 dependence of their induction by cold, neither the*

> *other 8 genes that are common between the cold-induced genes here and the ER stress/IRE-1- induced in Shen 2005 (Venn diagram in Figure 7 supplement), nor the hsp-4 reporter. What is the evidence that lips-11 is not the only gene whose induction by cold in this paper's dataset depends on IRE-1? This is a major weakness and needs to be addressed.*
>
> *Furthermore, whether induction by cold of lips-11 itself is due to IRE1 activation was not tested, only a partial decrease of reporter fluorescence by ire-1 RNAi is shown. A quantitative measure of the change of lips-11 transcript in ire-1 and xbp-1 mutants is needed to establish if it depends on IRE-1/XBP-1 pathway.*

We now examined by RT-qPCR if the induction of the three genes from Shen at al. (*dnj-27*, *srp-7*, and *C36B7.6*), as well as *lips-11* and *hsp-4* depends on IRE-1. In the new Figure 4 – figure supplement 2, we show that the upregulation of all these genes is reduced in the cold in the *ire1* mutant (although in the wild type, the increase of *hsp-4* mRNA appeared to be non-significant, despite the observed upregulation of the *hsp-4* GFP reporter).

> *The authors could provide more information and the additional data for the transcripts upregulated by both ER stress and cold, including the endogenous lips-11 and hsp-4 transcripts: their identity, fold induction by both cold and ER stress, how their induction is ranked in the corresponding datasets (all of these are from existing data), and do they depend on IRE-1/XBP-1 for induction by cold?*

As above, the dependence of endogenous *lips-11* and *hsp-4* on IRE-1 is now shown in the new Figure 4 – figure supplement 2, and the shared genes from Figure 7 – figure supplement 1 are listed in the new Supplementary file 5. We did not perform additional analysis comparing various data sets, as we felt that understanding the differences between IRE-1-mediated transcription outputs across different conditions goes well beyond this study.

> *Without these additional data and considering that the authors did not directly measure the splicing of xbp-1 transcript (see comment for Fig. 3 below), the conclusion that cold induces UPR by specific activation of IRE-1/XBP-1 pathway is premature.*

To address the splicing of endogenous *xbp-1*, we examined our ribosome profiling data for the translation of spliced *xbp-1*, and found that the spliced variant is more abundant in the cold. This data is now shown in Figure 3 – figure supplement 2B.

> *There are also technical issues that are making it difficult to interpret some of the results, and missing controls that decrease the rigor of conclusions:*
>
> *(1) For RNAseq and ribosome occupancy, were the 20ºC day 1 adult animals collected at the same time as the other set was moved to 4ºC, or were they additionally grown at 20ºC for the same length of time as the 4ºC incubations, which would make them day 2 adults or older at the time of analysis? This information is only given for SUnSET: "animals were cultivated for 1 or 3 additional days at 4ºC or 20ºC".*

In the RNAseq experiments, the 20ºC animals were collected at the same time as the others were moved to 10ºC (and then 4ºC), so they were *not* additionally grown at 20ºC. We make it now clear in Methods.

> *This could be a major concern in interpreting translation data: First, the inducibility of both UPR and HSR in worms is lost at exactly this transition, from day 1 to day 2 or 3 adults, depending on the reporting lab (for example Taylor and Dillin 2013, Labbadia and Morimoto, 2015, De-Souza et al 2022).*

As explained above, the 20ºC animals were collected at the same time as the others were moved to 4ºC. Then, we reported before that ageing appears to be suppressed in animals incubated at 4ºC (Habacher et al., 2016; Figure S1C). Thus, it terms of their biological age, cold-incubated animals appear to be closer to the 20ºC animals at the time they are moved to the cold (day 0). Thus, the ageing-associated deterioration in UPR inducibility mentioned above presumably does not apply to cold-incubated animals, which is in line with the observed IRE-1-dependent upregulation of several genes in day 3 animals at 4ºC.

> *How do authors account for this? Would results with reporter induction, or induction of IRE-1 target genes in Fig. 4, change if day 1 adults were used for 20ºC?*

Our analysis in Figure 4 – figure supplement 2 now includes 20ºC animals at day 0, 1, and 3.

> *Second, if animals at the time of shift to 4ºC were only beginning their reproduction, they will presumably not develop further during hibernation, while an additional day at 20ºC will bring them to the full reproductive capacity. Did 4ºC and 20ºC animals used for RNAseq and ribosome occupancy have similar numbers of embryos, and were the embryos at similar stages?*

As explained above, the reference animals at 20ºC were young adults containing few embryos. Indeed, at 4ºC the animals do not accumulate embryos. Although we cannot say that for all genes, note that the genes analysed in Figure 4 – figure supplement 2 increase in abundance also when compared with the day 3 animals kept at 20ºC.

> *(2) Second, no population density is given for most of the experiments, despite the known strong effects of crowding (high pheromone) on C. elegans growth. From the only two specifics that are given, it seems that very different population sizes were used: for example, 150 L1s were used in survival assay, while 12,000 L1s in SUnSET. Have the authors compared results they got at high population densities with what would happen when animals are grown in uncrowded plates? At least a baseline comparison in the beginning should have been done.*

None of the experiments involved crowded populations. In the SUnSET experiments, we just used larger and more plates to obtain sufficient material.

> *(3) Fig. 3: it is unclear why the accepted and well characterized quantitative measure of IRE1 activation, the splicing of xbp-1transcript, is not determined directly by RT-PCR. The fluorescent XBP-1spliced reporter, to my knowledge, has not been tested for its quantitative nature and thus its use here is insufficient. Furthermore, the image of this fluorescent reporter in Fig. 3b shows only one anterior-most row of cells of intestine, and quantitation was done with 2 to 5 nuclei per animal, while lips-11 is induced in entire intestine. Was there spliced XBP-1 in the rest of the intestinal nuclei? Could the authors show/quantify the entire animal (20 intestinal cells) rather than one or two rows of cells?*

As explained above, we now included the analysis of *xbp-1* splicing in Figure 3 – figure supplement 2B. As for the fluorescent reporter, it is difficult to measure all gut nuclei since part of the gut is occluded by the gonad. Nonetheless, we do see induction of the reporter in other gut nuclei and show now additional examples from midgut in Figure 3 – figure supplement 2A.

> *(4) The differences in the outcomes from this study and the previous one (Dudkevich 2022) that used 15ºC to 2ºC cooling approach are puzzling, as they would suggest two quite different IRE-1 dependent programs of cold tolerance. It would be good if authors*

> *commented on overlapping/non-overlapping genes, and provided their thoughts on the origin of these differences considering the small difference in temperatures.*

Indeed, there seem to be substantial differences between different temperatures and cooling paradigms. While understanding the *C. elegans* responses to cold is still in its infancy, one possible explanation for the observed differences is that we used different starting growth temperatures. While the initial populations in our study were grown at 20ºC, Dudkevich et al. used 15ºC. Worms display profound physiological differences between these two temperatures. For example, Xiao et al. (2013) showed that the cold-sensitive TRPA-1 channel is important at 15ºC but not 20ºC. Thus, the trajectories along which worms adapt to near freezing temperature may vary depending on their initial physiological state (and perhaps the target temperature, as we used 4ºC and they 2ºC). We now expanded argumentation on this topic in Discussion. I should also say that we planned on testing NLP-3 function in our paradigm, but our request for strains remained unanswered.

> *Second, have the authors performed a control where they reproduced the rescue by FA supplementation of poor survival of ire-1 mutants after the 15ºC to 2ºC shift? Without this or another positive control, and without measuring change in lipid composition in their own experiments, it is unclear whether the different outcomes with respect to FAs are due to a real difference in adaptive programs at these temperatures, or to failure in supplementation?*

While we did not re-examine the findings by Dudkevich et al., we did include now another positive control. As reporter by Hou et al. (2014), supplementing unsaturated FAs rescues the induction of the *hsp-4* reporter in *fat-6* RNAi-ed animals. Although we were able to reproduce that result (Figure 6 – figure supplement 1), the same supplementation procedure did not suppress the *lips11* reporter (Figure 6 – figure supplement 2).

> *(5) Have the authors tested whether and by how much ire-1(ok799) mutation shortens the lifespan at 20ºC? This needs to be done before the defect in survival of ire-1 mutants in Fig. 7a can be interpreted.*

The lifespan at standard cultivation temperature was examined by others (Henis-Korenblit et al., 2010; Hourihan et al., 2016), showing that *ire-1(ok799)* mutants live shorter. However, while some mechanism that prolong lifespan may also improve cold survival, the two phenomena are not identical and whether IRE-1 facilitates longevity and cold survival in the same or different way remains to be seen.

> ***Reviewer #2**:*
>
> *(1) The conclusions regarding a general transcriptional response are based on one gene, lips-11, which does not affect survival in response to cold. We would suggest altering the title, to replace "Reprograming gene expression: with" Regulation of the lipase lips-11".*

We now examined IRE-1 dependent induction of additional genes – see Figure 4 – figure supplement 2. While we do not know what fraction of cold-induced genes depends on IRE-1, we feel that our findings justify the statement that that gene expression in the cold *involves* the IRE1/XBP-1 pathway (title) or that that the transcription of *some/a subset of* cold-induced genes depend on this pathway (in abstract, model, and discussion).

> *(2) There is no gene ontology with the gene expression data.*

We now included the top 10 most enriched and suppressed gene categories between 10ºC and 4ºC (since the biggest change happens between these conditions, as shown in Figure 2 – figure

supplement 1A). This is now included in the Figure 2 – figure supplement 2.

> *(3) Definitive conclusions regarding transcription vs translational effects would require use of blockers such as alpha amanatin or cyclohexamide.*

As explained also for reviewer 1, we confirmed now that at least some genes, whose translation is upregulated based on the ribosome profiling, are indeed upregulated in the cold at the protein level (Figure 2 – figure supplement 3A-B). Thus, the increase in ribosomal occupancy seems to accurately reflect increased translation. Since mRNA levels correlate overall with the ribosomal occupancy, it appears that the mRNA levels are the main determinants of the translation output. Because the *lips-11* promoter is sufficient to upregulate the GFP reporter in the cold, it further suggests that the regulation happens at the transcription level. It is true that at this point we cannot completely rule out the effects of mRNA stability, which we clearly acknowledge in the discussion.

> *(4) Conclusions regarding the role of lipids are based on supplementation with oleic acid or choline, yet there is no lipid analysis of the cold animals, or after lips-1 knockdown.*

We agree that this is an important direction for future studies but feel that lipidomic analysis goes beyond the scope of current work.

> *Although choline is important for PC production, adding choline in normal PC could have many other metabolic impacts and doesn't necessarily implicate PC without lipidomic or genetic evidence.*

We agree and acknowledge it now in Discussion: “However, choline also plays other roles, including in neurotransmitter synthesis and methylation metabolism. Thus, we cannot yet rule out the possibility that the protective effects of choline supplementation stem from functions outside PC synthesis.”

> ***Reviewer #3:***
>
> *The study has several weaknesses: it provides limited novel insights into pathways mediating transcriptional regulation of cold-inducible genes, as IRE-1 and XBP-1are already well-known responders to endoplasmic reticulum stress, including that induced by cold.*

We presume the reviewer refers to the study by Dudkevich et al. (2022). As explained in our manuscript, there are important differences between that study and ours in how the IRE-1 signalling is utilized and to what ends.

Additionally, the weak cold sensitivity phenotype observed in ire-1 mutants casts doubt on the pathway's key role in cold adaptation. The study also overlooks previous research (e.g.PMID: 27540856) that links IRE-1 to SKN-1, another major stress-responsive pathway, potentially missing important interactions and mechanisms involved in cold adaptation.

We state in the manuscript that the IRE-1 pathway plays a modest but significant role in cold adaptation and state in the Fig. 7 model and Discussion that additional pathways work alongside IRE-1 to drive cold-specific gene expression.

> ***Recommendations for the authors:***
>
> ***Reviewer #1:***
>
> *Minor comments:*

> *(1) Fig. 2B - reporter expression seems to be already present in the intestine of 20ºC animals. What is the turnover rate of GFP in the intestine and how is it affected by the temperature shift? If GFP degradation is inhibited, could it explain the increase in signal in 4ºC animals, rather than increased transcription? This seems to be true for the hsp-4 transcriptional reporter, as the GFP fluorescence appears to increase during 4ºC incubation (Fig. 4a), but the hsp-4 message levels are only increased after 1 day but not in later days at 4ºC, based on the RNAseq in provided dataset. How well do changes in lips-11 reporter fluorescence correspond to the changes in the endogenous lips-11 transcript?*

Note that increased GFP fluorescence is accompanied by increased mRNA levels. In addition to the RNAseq data, we now also examined changes of the endogenous *lips-11* transcript by RTqPCR and observed its strong (and IRE-1 dependent) upregulation in the cold– see Figure 4 – figure supplement 2. Moreover, we now included two other examples of GFP-tagged proteins whose fluorescence increases in the cold, concomitant with increased mRNA levels and ribosomal occupancy (Figure 2 – figure supplement 2A-B).

> *(2) Descriptions of methods to measure different aspects of translation are very abbreviated and in some places make it difficult to understand the paper. One example - what is RFP in Fig. 2a?*

We replaced now “RFP” with “RPF” (ribosome protected fragment) and the abbreviation is explained firsts time it is used.

> *(3) How was the effectiveness of RNAi at 4ºC validated?*

As explained in Methods, we subjected animals to RNAi long before they were transferred to 4ºC, so the corresponding protein is depleted prior to cooling.

> *(4) Several of the conclusions on translation and ribosomal occupancy are written in a somewhat confusing way. For example, the authors state that "shift from 10ºC to 4ºC had a strong effect" when describing "impact on translation (ribosomal occupancy)" (page 4), but in the next sentence, they state "a good correlation between mRNA levels and translation (Figure 2A)". Was ribosomal occupancy normalized to the transcript abundance?*

We do not perceive any discrepancy between the two statements. The former refers to the difference between time points, where we observed the largest change in both the transcriptome and ribosomal occupancy from 10ºC to 4ºC (as can be inferred in the PCA plot in Figure 2 - figure supplement 1). The latter refers to the observation that changes in mRNA levels mirrored, in most of cases, similar changes in the ribosomal occupancy.

The ribosomal occupancy was not normalized, as that would essentially normalize the y-axis (ribosomal occupancy) with the x-axis (mRNA), and so express changes in “translational efficiency” as a function of changes in mRNA abundance. While this type of analysis can also reveal interesting biological phenomena, it would explore a different question.

> *(5) "For most transcripts ... increased the abundance of a particular protein appears to correlate depend primarily on the abundance of its mRNA" (page 5). This is an overstatement, the protein levels were not quantified.*

As explained above, we now additionally monitored the expression of two GFP-tagged proteins (CEBP-1 and NUMR-1). Monitoring their expression, we observed the expected increase in GFP fluorescence in the cold (see Figure 2 – figure supplement 3 A-B). While we

did not examine them also by western blot, these observations are in line with our conclusions.

> *(6) The statement "Since transcription is the main determinant of mRNA levels, these results suggest that cold-specific gene expression primarily depends on transcription activation" seems to assume that message degradation doesn't have much of an impact at 4ºC. What is the evidence here? The authors themselves later suggest either transcription or mRNA stability in Discussion.*

While we cannot exclude that mRNA stability of some genes may be affected, this concern is more valid for the messages that go down in the cold. Although we have done it for only selected genes, each time we observed an increase in the mRNA levels, we also observed the corresponding increase in the protein; this study and Pekec et al. (2022). Then, the *lips-11* reporter was designed to monitor the activity of its promoter, which we showed in sufficient to upregulate reporter GFP in the cold. We have now expanded the corresponding paragraph in Discussion, which will hopefully come across as more balanced.

> ***Reviewer #2:***
>
> *(1) Alter title, conclusions to better reflect specific nature of the work.*

We now provided additional data and feel that it justifies our conclusions and title.

> *(2) Use Gene Ontology searches to look at patterns of gene expression in RNA seq data.*

We now show it in Figure 2 – figure supplement 2.

> *(3) Use genetic or lipidomic tools rather than solely adding exogenous lipids.*

We agree that lipidomic analysis is an important direction for future research, but feel that lipidomic analysis and further genetic experiments go beyond the scope of current manuscript.

> ***Reviewer #3:***
>
> *To strengthen the evidence for the role of IRE-1 in cold adaptation, the authors might consider performing additional functional assays, such as testing the effects of IRE-1 and XBP-1 mutations under varying cold conditions and testing the genetic interaction of ire-1 with xbp-1, skn-1, and hsf-1 in cold sensitivities. It is also worth using alternative approaches such as independent alleles of ire-1, knockdowns or tissue-specific knockouts (without potential developmental compensation in global constitutive mutants) to better characterize the contribution of IRE-1 to cold adaptation. Additionally, studies that examine tissue-specific responses to cold exposure could provide important insights, as different tissues may utilize distinct molecular pathways to adapt to cold stress.*

We also tested *ire-1* and *xbp-1* functions by RNAi-mediated depletion. SKN-1 is a good candidate for future studies, but Horikawa at al. (2024) showed that HSF-1 is not required for cold dormancy (at 4ºC); we also show now that HSF-1::GFP does not increase in the cold (Figure 2 – figure supplement 3C).

> *This reviewer also recommends clarifying the novelty of your findings in the context of existing literature, particularly regarding the established roles of IRE-1 and XBP-1 in responding to endoplasmic reticulum stress.*

The entry point of this study was to clarify a long-standing problem in hibernation research, i.e., the apparent discrepancy between a global translation repression and de novo gene expression observed in the cold. By connecting cold-mediated expression of some genes to the IRE-1/XBP1 pathway, we strengthen the argumentation for transcription-mediated gene regulation in hibernating animals. We did go the extra mile to test the possible reason behind the activation of UPR^{ER} in the cold but feel that a deeper analysis deserves a separate study.

> *The term "hibernation" should be avoided or reworded since the study does not provide direct behavioral or physiological evidence for hibernation-like states; instead, the manuscript could refer to "cold-induced responses" or "adaptations to cold temperatures."*

The term “hibernation” was used before even in the context of the *C. elegans* dauer state, which, arguably, is even less appropriate. In addition to a global suppression of translation shown here, we reported before that the same cooling regime suppresses ageing (Habacher et al., 2016; Figure S1C). Incubating at 4ºC also arrests *C. elegans* development (Horikawa et al., 2024). Thus, while the worm and mammalian hibernation are certainly not equivalent – which we clearly spell out – we like to use “hibernation” interchangeably with “cold dormancy” to draw attention to a fascinating aspect of *C. elegans* biology. Still, we use now quotation marks in the title to avoid misunderstanding.

> *The discussion could be strengthened by addressing the relevance of prior studies, such as those linking IRE-1 to SKN-1 (PMID: 27540856), TRPA-1 (PMID: 23415228), ZIP-10 (PMID: 29664006), HSF-1 (PMID: 38987256) in cold adaptation and elaborating on how your findings provide new*

The IRE-1/SKN-1 and ZIP-10 papers are now mentioned when describing the model in Figure 7. The TRP-1 and HSF-1 papers are cited when discussing physiological differences between different cold temperatures. Consistent with our studies, the HSF-1 paper shows that nematodes enter a dormant state at 4ºC (but at 9ºC and higher temperatures continue developing). Importantly, HSF-1 promotes the development at 9ºC but is not important for the arrest at 4ºC. We also shown now in Figure 2 – figure supplement 3C that HSF-1 does not go up at 4ºC.

https://doi.org/10.7554/eLife.101186.2.sa0