Reducing translation through eIF4G/IFG-1 improves survival under ER stress that depends on heat shock factor HSF-1 in Caenorhabditis elegans

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Summary
Although certain methods of lowering and/or altering mRNA translation are associated with increased lifespan, the mechanisms underlying this effect remain largely unknown. We previously showed that the increased lifespan conferred by reducing expression of eukaryotic translation initiation factor 4G (eIF4G/IFG-1) enhances survival under starvation conditions while shifting protein expression toward factors involved with maintaining ER-dependent protein and lipid balance. In this study, we investigated changes in ER homeostasis and found that lowering eIF4G/IFG-1 increased survival under conditions of ER stress. Enhanced survival required the ER stress sensor gene ire-1 and the ER calcium ATPase gene sca-1 and corresponded with increased translation of chaperones that mediate the ER unfolded protein response (UPRER). Surprisingly, the heat-shock transcription factor gene hsf-1 was also required for enhanced survival, despite having little or no influence on the ability of wild-type animals to survive ER stress. The requirement for hsf-1 led us to re-evaluate the role of eIF4G/IFG-1 on thermotolerance. Results show that lowering expression of this translation factor enhanced thermotolerance, but only after prolonged attenuation, the timing of which corresponded to increased transcription of heat-shock factor transcriptional targets. Results indicate that restricting overall translation through eIF4G/IFG-1 enhances ER and cytoplasmic proteostasis through a mechanism that relies heavily on hsf-1.

Key words: Caenorhabditis elegans; eIF4G; healthspan; ifg-1; lifespan; proteostasis.

Introduction
Regulation of protein synthesis is essential during development and in response to environmental inputs. Proper regulation insures that cells have the resources required for maintaining cell structure and function.

Excess synthesis or an inability to turn down production during times of stress may cause proteotoxicity due to the inability of the cell to properly fold, refold, and degrade what is manufactured (Sherman & Qian, 2013). Thus, numerous controls are in place at different stages of protein synthesis to insure a proper balance.

In the last decade, a number of studies determined that genetic suppression of translation results in increased lifespan in model organisms including yeast, worms, flies, and mammals (Kaeberlein & Kennedy, 2011; Kyriakakis et al., 2015). Translation is a cellular process with inputs from major longevity-regulating pathways including the target of rapamycin (TOR) and insulin/IGF-1 pathways. TOR integrates intracellular energy and nutrient signals to coordinate multiple cellular processes, including translation (Kapahi et al., 2010). The insulin/IGF-1 pathway coordinates development as well as challenges to homeostasis through intercellular hormone signaling and the FOXO transcription factor DAF-16 (abnormal dauer formation; Kenyon, 2010). Inhibition of the insulin/IGF-1 pathway in Caenorhabditis elegans decreases ribosomal subunits resulting in reduced protein synthesis (Depuydt et al., 2013). In addition to genetic models of lifespan extension, dietary restriction also induces translation inhibition. In fact, reducing translation by limiting dietary intake of the essential amino acid methionine, but not total calories, extends lifespan (Miller et al., 2005). While translation inhibition may not be essential for every genetic or environmental model of lifespan extension, the frequency with which it is involved in longevity models and the rigor of the response across species tested suggests has led to the proposal that translation plays a universal role in the aging process (Tavernarakis, 2008).

A possible explanation for why attenuating translation increases lifespan is enhanced proteostasis due to improved translation fidelity and/or turnover. Recent support for part of this idea was demonstrated by a study which showed that slowing down translation was sufficient to increase fidelity of protein folding, even among mutant proteins with a propensity for misfolding (Merin et al., 2012). On the other hand, other studies have shown that a shift in mRNA translation preference (i.e., differential translation) accompanying an overall reduction of protein synthesis is a key component to increased lifespan in yeast (Steffen et al., 2008, C. elegans (Rogers et al., 2011), and Drosophila (Zid et al., 2009). Differential mRNA translation has long been known to be important in response to cellular stressors (Holcik & Sonenberg, 2005). Additional clues to the mechanisms underlying longevity effects are likely to come from a better understanding of associated cellular changes, which may be dependent on the manner in which translation is modulated and which require an understanding of its major regulators.

Although controlled at multiple levels, translation begins with, and is most frequently rate-limited by, the initiation stage (Hershey et al., 2012). A major determinant of regulation at this stage is the cap-binding complex, which is made up of the eukaryotic translation initiation factor eIF4G, the eIF4A RNA helicase, and the 5’ mRNA cap-binding protein eIF4E. eIF4G acts as a nexus for translation by bringing in other translation factors and helping recruit the small (40S) ribosomal subunit. Although eIF4E or eIF4G can become limiting for cap-mediated translation, only eIF4G is also involved in cap-independent translation...
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Reducing eIF4G in mammalian cells is sufficient to induce transformation (Fukuchi-Shimogori et al., 1997), while in humans, overexpression of eIF4G is associated with breast, pharyngeal, and lung cancer (Bauer et al., 2002; Cromer et al., 2003; Braunstein et al., 2007). Opposing effects of eIF4G inhibition and overexpression on longevity and disease, along with its impact on the translome, led us to investigate the intracellular processes downstream of translational remodeling that are important for maintaining organismal homeostasis.

Taking genes found to be differentially translated when eIF4G/IFG-1 is reduced, we performed an ontological analysis of cellular components and found that protein synthesis of factors that are part of vesicle trafficking and the endomembrane system are promoted. This system involves factors that are manufactured by, and participate in endoplasmic reticulum (ER)-associated processes. The ER is a site of protein synthesis, which can become perturbed in response to misfolded proteins and/or ER calcium imbalance. Stress in this organelle induces the unfolded protein response (UPRER), which was previously shown to regulate longevity in a cell-nonautonomous manner in C. elegans (Taylor & Dillin, 2013). The UPRER is guided by ER stress sensors IRE-1 (inositol-requiring enzyme 1), PERK/PEK-1 (protein kinase RNA-like ER kinase 1) and ATF-6 (activation transcription factor 6). Thus, we tested the impact of reducing ifg-1 under ER stress conditions initiated by tunicamycin, which blocks glycosylation and activates the UPRER. Results showed improved survival in a manner dependent on the gene encoding an ER calcium ATPase, sca-1. This gene is essential for ER homeostasis in C. elegans (Yan et al., 2006) and was previously shown to be required for extended lifespan when ifg-1 is reduced (Rogers et al., 2011).

Additionally, we tested the known sensors of ER stress to determine their requirement for enhanced survival when ifg-1 is attenuated. We found that increased protection under ER stress conditions was dependent on the IRE-1 branch of the UPRER. Neither ATF-6 nor the translation inhibiting kinase PEK-1 was required for increased ER stress tolerance when ifg-1 was reduced. Surprisingly, we also found a requirement for the heat-shock factor HSF-1 in enhanced ER stress tolerance, a transcription factor that regulates the cytoplasmic heat-shock response (HSR). Tests for resistance to thermal stress showed that RNAi targeting ifg-1 required several days of suppression, despite strongly reduced IFG-1 protein levels after only 2 days. Interestingly, the delayed protective response was accompanied by increased constitutive expression of HSF-1 targets. Enhanced constitutive expression of heat-shock factor gene expression, including the temporal delay, was only observed for certain translational interventions tested. From a focused prospective, results indicate that changes in the cytoplasmic HSR are critical to enhanced ER function when translation is modulated by reducing ifg-1. From a broader perspective, findings from tests using multiple methods of translation attenuation suggest a new basis for discerning translation-mediated pathways of stress resistance.

Results

Restricting translation by lowering ifg-1 improved survival under ER stress

Previous analysis showed that increased lifespan resulting from reduced ifg-1 expression in C. elegans was at least partially dependent on several translationally upregulated genes (Rogers et al., 2011). Those most important for increased lifespan are also known to be important for maintaining ER homeostasis and include the ER calcium regulator gene sca-1 and a gene encoding the transcriptional coactivator mediator complex subunit mdt-15. Attenuated expression of either is able to induce the UPRER (Yan et al., 2006; Hou et al., 2014). A novel cellular component-based gene ontological (GO) analysis of genes that were differentially translated when ifg-1 is reduced via RNAi (Rogers et al., 2011) revealed that cellular processes involving vesicle transport and the endomembrane system were significantly overrepresented (Fig. 1A; Table S1, Supporting information). Furthermore, 75% of the differentially translated genes in these GO categories had increased translation according to changes in the association of mRNA with ribosomes. Given the role of the ER in protein synthesis and maintenance of proteostasis, we investigated the effect of reduced ifg-1 expression under conditions that induce the UPRER.

Animals were fed double-stranded (ds)RNA corresponding to ifg-1 or a control vector starting at adulthood followed by exposure to the glycosylation-inhibiting drug tunicamycin. Survival was significantly improved in animals with ifg-1 RNAi, with protective effects also observed in mutants bearing the ifg-1 loss-of-function allele cx729279 (Fig. 1B; Table S2, Supporting information). Baseline expressions of ER stress sensor genes ire-1, atf-6, and pek-1 were unchanged in ifg-1 RNAI-treated animals (Fig. 1C) and ifg-1(cx729279) mutants in the absence of stress (Fig. S1A, Supporting information). However, in animals subjected to ifg-1 RNAI, a small but significant increase in ire-1 transcript levels was observed in response to 24 h of exposure to tunicamycin (Fig. 1C), whereas the ifg-1 mutant showed only a small increase in pek-1 and decrease in atf-6 under the same conditions (Fig. S1A, Supporting information). Thus, lowering ifg-1 increased survival in response to ER stress without major changes in ER stress sensor transcript levels compared with RNAi control animals in a 24-h window after treatment with tunicamycin.

To see whether knockdown of ifg-1 changed expression of ER stress sensors with time, measurements were made at day 10 of adulthood, which is closer to the time that a difference in survival was observed between test and control animals. Interestingly, a small but significant increase was observed for each sensor in ifg-1 RNAI-treated animals regardless of whether they were exposed to tunicamycin, with a greater than twofold change in pek-1 by this time (Fig. 1D). These changes were consistent whether a 2-day (Fig. S1B, Supporting information) or 4-day (Fig. 1D) RNAI induction phase was used prior to tunicamycin exposure. These results indicated that lowering translation through ifg-1 resulted in long-term enhanced expression of ER stress sensors even in the absence of external stress.

As ER stress sensor activity can induce both transcriptional and translational effects, we used polysome profiling to assess changes in global and differential translation. Upon reaching adulthood, animals were fed dsRNA for 4 days prior to profiling. ifg-1 RNAI dramatically decreased total translation and increased free ribosomal subunits

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Fig. 1 Reduced translation through ifg-1 promoted survival under ER stress. (A) Cellular component GO analysis of differentially translated genes associated with suppressing ifg-1 (q = false discovery rate). Additional information is available in the Experimental procedures section. (B) Survival of N2 wild-type animals fed bacteria expressing control (L4440) or ifg-1 dsRNA starting on the first day of adulthood, followed by exposure to 25 μg mL⁻¹ tunicamycin 2 days later. Ifg-1(cx79279) mutants exposed to tunicamycin were included in the survival assay for comparison. Experiments were performed four or more times with similar results (see Table S2, Supporting information). (C) N2 wild-type animals were fed control or ifg-1 dsRNA for 4 days to allow for full effect of RNAi prior to exposure to 25 μg mL⁻¹ tunicamycin. Relative mRNA levels of atf-6, pek-1, and ire-1 are shown after six or 24 h of treatment (6 h Tun and 24 h Tun, respectively). Samples were normalized to similarly prepared animals exposed to DMSO for 6 h (No Tun). Results are compared with controls (Fig. 2A, left panel). An increase in free ribosomal subunits was observed in both control and ifg-1 RNAi animals 6 h after treatment with tunicamycin that continued to increase by 24 h (Fig. 2A, middle and right panels). This indicated that global translation was reduced throughout this period in response to tunicamycin for animals under control or ifg-1 RNAi. Indeed, attenuation of translation is a conserved response to induction of the UPRER (Ron & Walter, 2007).

To see whether translation suppression in response to tunicamycin was related to the PEK-1/PERK arm of the UPRER, we examined the inhibitory phosphorylation status of its protein target, eIF2α, which is part of the mRNA translation ternary complex and represents a separate (ifg-1-independent) rate-limiting regulator of translation initiation (Holcik & Sonenberg, 2005). eIF2α phosphorylation was lower in animals treated with ifg-1 RNAi than control RNAi prior to tunicamycin exposure (Fig. S2A, Supporting information). Subsequently, both control and ifg-1 RNAI-treated animals showed an upward tendency of eIF2α phosphorylation after 6 h of exposure to tunicamycin that disappeared by 24 h (Fig. S2A, Supporting information). No significant differences in phosphorylation status between ifg-1 and control RNAI animals were observed at six and 24 h. Although a tendency for increased eIF2α phosphorylation after 6 h of tunicamycin treatment was in line with the modest translational reduction in ifg-1 and control RNAI-treated animals observed in polysome profiles, the continued abatement of translation after 24 h in the absence of increased eIF2α phosphorylation suggested that translation may be attenuated by additional factors.

To determine the response of the IRE-1 branch of the UPRER, we measured transcription and translation of its target, xbp-1 mRNA. In its active state, IRE-1 splices immature xbp-1u (unspliced) transcripts, which allows translation of the mature xbp-1s (spliced) transcript and synthesis of the transcription factor that it encodes (Ron & Walter, 2007). Prior to tunicamycin treatment, total transcript expression of xbp-1u was similar prior to stress. Upon exposure to tunicamycin, there was a downward trend in total and translated transcripts of xbp-1u and corresponding increase in xbp-1s for all conditions (Fig. 2B), indicating activation of IRE-1. The bias in expression of xbp-1s abated somewhat by 24 h of tunicamycin treatment, but both total and translated forms of xbp-1s increased in ifg-1 RNAI-treated animals by this time. Despite starting at a higher constitutive (no tunicamycin) level, polysome-associated xbp-1s in ifg-1 RNAI animals increased relative to starting levels by 24 h of tunicamycin exposure (P = 0.0092).
Gene expression of several chaperones and other factors are dependent on the IRE-1/XBP-1 arm of the UPRER. Expression of hsp-4, which is orthologous to the mammalian binding immunoglobulin protein (BiP), is known to be highly dependent on activity of XBP-1. We measured hsp-4 in ifg-1 RNAi-treated animals and observed that its transcripts were more highly associated with polysomes after 24 h of tunicamycin treatment than in control RNAi animals (Fig. 2C, right panel). We also tested the DnaJ domain 7 gene, dnj-7, which encodes the C. elegans ortholog of p58IPK, a peripheral ER protein that functions in ER associated degradation (ERAD) of misfolded proteins (Oyadomari et al., 2006). Analysis showed that tunicamycin induced dnj-7 transcription after 24 h of tunicamycin exposure under both conditions. However, animals with reduced ifg-1 showed a higher increase in polysome-associated dnj-7 (Fig. 2D), suggesting that ERAD may be induced to a greater extent by the 24-h time point. Recently, the protein disulfide isomerase family A member 6 (PDI-6) was shown to control a negative feedback to the ER stress response by binding and inactivating IRE-1 in C. elegans (Eletto et al., 2014). Expression analysis here indicated that, although total transcript levels of pdi-6 rose equally in response to control and ifg-1 RNAi after 24 h of tunicamycin exposure, reducing ifg-1 significantly increased pdi-6 polysome association (Fig. 2B, Supporting information). Such differences in UPRER activation could change the kinetics of the ER stress response and lead to faster resolution and rebalancing of organellar proteostasis. Resolution of the response would be indicated by a return of IRE-1 activity to basal levels, and a tendency for this was observed between the six- and 24-h time points in Fig. 2B by partial reversal of spliced vs. unspliced xbp-1 for animals under ifg-1 RNAi (especially among polysomes; Fig. 2B, right panel).

Fig. 2 Reducing expression of ifg-1 promoted transcriptional and translational upregulation of UPRER-responsive genes under ER stress. (A) Polysome profiles for wild-type N2 adults fed bacteria expressing L4440 control (black dashes) or ifg-1 dsRNA (red dashes) for 4 days prior to exposure to 25 μg ml⁻¹ tunicamycin for 6 and 24 h (6 h Tun and 24 h Tun, respectively). Nematode lysates were separated over a sucrose gradient and absorbance through the gradient was measured at 254 nm (UV254). Portions of the profile corresponding to polysomes are highlighted in blue and were collected from each sample for analysis of translated mRNA. Profiles are representative of four experiments performed. (B) Total and translated (polysome-associated) mRNA levels of xbp-1s and xbp-1u were measured under conditions in (A) (*P < 0.05, **P < 0.001; two-tailed t-test; error bars indicate SEM). (C) Total and translated mRNA levels for hsp-4 were measured under conditions in (A) (**P < 0.0001; two-tailed t-test; error bars indicate SEM). (D) Similar to (C), but for dnj-7 (*P < 0.05, **P < 0.001; two-tailed t-test; error bars indicate SEM). Results in A–D are from the same four biological replicates and were considered significant for P < 0.05.
Differences in response of ER homeostasis regulators upon exposure to tunicamycin when ifg-1 was suppressed suggested that one or more may be required for improved survival. We tested the requirement for expression of sca-1 and mdt-15 for improved survival under ER stress induced by tunicamycin in the ifg-1(cx79279) mutant. sca-1 RNAi abrogated enhanced survival in the ifg-1 mutant compared to sca-1 RNAi-treated N2 wild-type animals (Fig. 3A). Although this result indicated a requirement for sca-1, it is interesting to note that sca-1 RNAi in the wild-type background actually had a small but significant protective effect (Fig. 3A; Table S3, Supporting information). On the other hand, RNAi knockdown of mdt-15 did not abrogate improved survival in the ifg-1 mutant (Fig. 3B; Table S3, Supporting information). This result is consistent with recent evidence indicating that, while mdt-15 is essential for ER homeostasis through changes in the lipid synthesis profile of the ER, it does not alter proteostasis (Hou et al., 2014b). The fact that enhanced longevity through reduced ifg-1 expression is dependent on both sca-1 and mdt-15, but here only required sca-1 for increased survival under ER stress, demonstrated that the dependence for lifespan and stress resistance phenotypes can be uncoupled.

Next, we tested the importance of the three ER stress sensor genes atr-6, pck-1, and ire-1 for enhanced survival upon exposure to tunicamycin in the ifg-1 mutant. Lowering atr-6 or pck-1 expression did not mitigate enhanced survival in the ifg-1 mutant (Fig. 3C; Table S3, Supporting information). Efficacy of RNAi for both genes was confirmed by testing for developmental effects in larval animals (not shown). In two of the three experiments performed, reducing ire-1 collapsed survival of the ifg-1 mutant to that of wild-type animals on ifg-1 RNAi (Fig. 3E; Table S3, Supporting information). Further testing showed that attenuation of the IRE-1 substrate xbp-1 abolished enhanced ER stress resistance of the ifg-1 mutant in all tests (Fig. 3F; Table S3, Supporting information). Together, these data support an essential role for the IRE-1 arm of the UPR in mediating the protective effects exhibited in the ifg-1 mutant.

While unfolded proteins in the ER are known to activate the UPR, unfolded proteins in the cytoplasm trigger the HSR. To determine whether enhanced resistance to ER stress in ifg-1 animals was dependent on changes in the HSR governing cytoplasmic proteostasis, we tested the importance of the hsf-1 gene. Despite little or no dependence on survival under ER stress in wild-type animals, results showed that RNAi targeting hsf-1 abolished the increased survival under ER stress in the ifg-1 mutant (Fig. 3G; Table S3, Supporting information). Analysis of total and translated transcript levels revealed that ifg-1 RNAi-treated animals did not have significantly different expression of hsf-1 than control animals in the absence of stress (Fig. S3, Supporting information). However, ifg-1 RNAi led to preferential translation of hsf-1 subsequent to treatment with tunicamycin without increasing its transcription (Fig. S3, Supporting information). These results show that ifg-1 animals required hsf-1 for improved survival and had increased translation of hsf-1 under ER stress.

HSF-1 and the FOXO transcription factor DAF-16 are key regulators of proteostasis that are required for mediating longevity and stress resistance via the ILS pathway, as previously reviewed (Hartl et al., 2011). Based on results here that identified a requirement for hsf-1 for enhanced survival under ER stress when translation is attenuated, we analyzed the importance of daf-16 under the same conditions. Testing showed improved survival under tunicamycin stress in ifg-1 mutants compared to wild-type animals when daf-16 was targeted via RNAi; although the protective effects were dampened (Fig. 3H; Table S3, Supporting information). This incomplete dependence on daf-16 was similar to its effect on lifespan in this background (Rogers et al., 2011).

We also tested the effects of genetically inhibiting translation at other points in initiation known to increase lifespan. RNAi targeting ife-2 (cap-binding subunit of elf4E), rps-15 (small ribosomal subunit S15), or iftb-1 (beta subunit of elf2, a component of the ternary complex representing another regulatory control point for translation initiation) increased survival in the presence of tunicamycin (Fig. S4; Table S4, Supporting information). These results suggested that enhanced survival under ER stress might be a general response of translation attenuation. We then treated animals with the chemical translation elongation inhibitor, cycloheximide, prior to exposure to tunicamycin, using a concentration of cycloheximide we found slowed growth in developing larvae and suppressed global translation in polysome profiling, phenotypes we observed with RNAi of the translation factor genes above (data not shown). Cycloheximide did not significantly impact median survival under ER stress conditions in either wild-type or the ifg-1 mutant strain (Fig. 3I; Table S3, Supporting information), which suggested that inhibiting translation, per se, is not sufficient to increase survival under ER stress. Thus, targeting translation initiation through conserved regulatory nodes (i.e. the cap-dependent/independent complex and ternary complex) might have promoted survival under ER stress in ways that extend beyond global reduction of translation.

Reducing ifg-1 enhanced thermostolerance and resulted in higher constitutive and induced expression of genes encoding heat-shock factors

Given the dependence on hsf-1 for the protective role of reducing ifg-1 under conditions that elicit an ER stress response, we analyzed differences in survival under heat stress that activates the cytoplasmic HSR. In two previous studies that tested thermostolerance in response to ifg-1 RNAi in adult C. elegans, one study found no protection from thermal stress after 2 days on ifg-1 RNAi (Pan et al., 2007), while the other showed significant protection 4 days after exposure to RNAi (Hansen et al., 2007). We re-examined survival under continuous thermal stress (35 °C) after 2, 5, or 7 days of ifg-1 RNAi. Day one adults on ifg-1 RNAi for 2 days showed no enhanced protection from thermal stress (Fig. 4A; Table S5, Supporting information). However, longer exposure to ifg-1 RNAi resulted in increased thermostolerance (Fig. 4A,D; Table S5, Supporting information). Results here support both studies based on differences in the duration of dsRNA feeding prior to heat stress. To see whether the delay in enhanced thermostolerance was
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Fig. 3 Enhanced survival in the ifg-1 mutant required sca-1, the ire-1 branch of the UPR\(^{16}\), and the cytoplasmic UPR gene hsf-1. Day 1 adults were subjected to RNAi as indicated for 2 days prior to exposure to 25 \(\mu\)g mL\(^{-1}\) tunicamycin and survival was tracked daily. Kaplan–Meier survival curves were compared using Mantel–Cox log-rank test, and average median lifespan for replicates was calculated using two-tailed t-test with Welch’s correction. (A) The survival curve of ifg-1(cxTi9279) fell below that of N2 when each were subjected to sca-1 RNAi (\(P < 0.0001\)). In (B–D), survival was enhanced in ifg-1(cxTi9279) compared to N2 at \(P < 0.0001\) for RNAi test conditions shown. In (E–G), average median survival was not enhanced in ifg-1(cxTi9279) compared to N2 when each were subjected to ire-1 RNAi (\(P = 0.37\)), xbp-1 RNAi (\(P > 0.99\)), and hsf-1 RNAi (\(P = 0.18\)). (H) Survival was enhanced in ifg-1(cxTi9279) compared to N2 when both were subjected to daf-16 RNA (\(P < 0.0001\)). (I) Survival was enhanced in ifg-1(cxTi9279) compared to N2 when day 1 adults were pretreated with cycloheximide for 2 days prior to exposure to 25 \(\mu\)g mL\(^{-1}\) tunicamycin (\(P < 0.001\)). All experiments were performed three times and were considered significant for \(P < 0.05\). See Table S3 (Supporting information) for additional data.

Western blot analysis was performed after 2 days on RNAi and showed that IFG-1 was reduced by 80% (\(P < 0.05\), Wilcoxon test; Fig. 4B). This suggested that slow turnover of IFG-1 did not account for lack of a protective effect observed by that time. We also tested stress tolerance in the ifg-1(cxTi9279) mutant, in which longevity and developmental phenotypes are less severe than ifg-1 RNAI-treated animals. Results indicated markedly enhanced thermotolerance compared to wild-type at both 2 and 7 days of adulthood (Fig. 4C,D; Table S5, Supporting information). Thus, although we cannot completely rule out that the delay may be due to a requirement for even lower levels of IFG-1, the evidence collected does not support this interpretation.

To understand more about how lower IFG-1 led to enhanced thermotolerance and the role of HSF-1, we looked at induction of transcript levels for HSR genes after inhibiting expression of ifg-1 via RNAi for 2 or 7 days. We tested expression of hsp-16.1/hsp-16.11, hsp-16.2, hsp-16.4, and F44E5.4/F44E5.5 and found that after 2 days of ifg-1 RNAi, the induction of HSR genes under heat stress (35 °C) was comparable to control RNAi (Fig. 4E). However, after 7 days, animals on ifg-1 RNAi showed higher constitutive HSR gene expression compared with animals under control RNAi both before and after heating (Fig. 4E). We used the same time points to test expression of F44E5.4/F44E5.5 in animals treated with cycloheximide or RNAi for ife-2, rps-15, or iftb-1. Both rps-15 and iftb-1 resulted in upregulation of this chaperone by day 7, similar to ifg-1 RNAi (Fig. 5S, Supporting information). Interestingly, rps-15 and iftb-1 were also similar to ifg-1 in that they each are essential for translation and RNAi for these genes resulted in larval arrest in developing nematodes (data not shown). In contrast, ife-2, which is required for cap-dependent translation only, did not result in upregulation of heat-shock gene expression (Fig. S5, Supporting information). It is also noteworthy that, as only one of five eIF4E orthologs in C. elegans, ife-2 suppression did not result in any developmental effects, even after multiple generations on RNAi (data not shown) and despite its protective effect in ER stress assays (Fig. S4A, Supporting information). To see whether the ability to inhibit development and reduce translation is required for the effect on heat-shock gene expression, we tested the response to cycloheximide and found that this treatment, similar to ife-2, failed to increase heat-shock gene expression (Fig. S5, Supporting information). Thus, the kinetics of heat-shock factor gene expression, while it potentially helped explain the delayed effect in enhancing thermotolerance under ifg-1 RNAi, also suggested potential differences in response to cap-mediated vs. non-cap-mediated translational targeting.
Genes essential for increased lifespan when \textit{ifg-1} is reduced were not required for enhanced thermotolerance

As demonstrated above, \textit{hsf-1} was required for enhanced survival in the \textit{ifg-1} mutant exposed to tunicamycin (Fig. 3G). To test whether \textit{ifg-1} required \textit{hsf-1} for enhanced thermotolerance, we treated wild-type and \textit{ifg-1} mutant animals with \textit{hsf-1} RNAi for 7 days prior to heat stress. Although reducing \textit{hsf-1} severely decreased heat tolerance, the \textit{ifg-1} mutant demonstrated enhanced survival compared to N2 wild-type animals (Fig. 5A; Table S6, Supporting information), indicating that there is a HSF-1-independent protective effect in the \textit{ifg-1} translation mutant. RNAi of neither \textit{sca-1}, \textit{mdt-15}, nor \textit{ire-1} had an effect on the ability of
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The ifg-1 mutant to enhance thermotolerance (Fig. 5B–D and Table S6, Supporting information). This further uncoupled survival phenotypes, demonstrating that enhanced thermotolerance does not require the same genes that are required for enhanced resistance to ER stress or lifespan. Once again, we tested the effects of cycloheximide as a chemical inhibitor of global translation. For this form of acute stress, thermotolerance was enhanced for wild-type animals treated with cycloheximide for 7 days, unlike its lack of protective effect under tunicamycin stress. Interestingly, cycloheximide did not further enhance survival under thermal stress in the ifg-1 mutant (Fig. 5E; Table S6, Supporting information). This indicated that lowering translation, itself, had a protective effect to protein unfolding stress from heat.

Enhanced HSF-1 activity indicated in Fig. 4E corresponded with enhanced survival when the UPR was induced with tunicamycin. Although null mutants are not viable for ifg-1 or hsf-1 in C. elegans, we assessed the effect of ifg-1 RNAi on lifespan in a hsf-1(sy411) reduced-function background. Results indicate that ifg-1 RNAi was able to extend lifespan in this background (Fig. 5F and Table S7, Supporting information), in contrast to a different method of translation inhibition in another study that found RNAi knockdown of genes encoding ribosomal subunits, including rps-15, failed to extend lifespan in the hsf-1(sy411) background (Seo et al., 2013). Thus, although results in the current study indicated that hsf-1 was required for enhanced proteostasis elicited through attenuation of ifg-1 under ER stress, it was not required for increased lifespan. Conversely, regulators of ER homeostasis were not required for the enhanced cytoplasmic protective effects associated with lowering translation through ifg-1 prior to heat stress.

**Discussion**

Reducing translation increases lifespan in model organisms and dysregulation is associated with age-related diseases in humans, yet we do not fully understand how intracellular processes are altered to mediate these effects. By acting as a rate-limiting modulator of protein synthesis, the cap-binding complex subunit eIF4G is a nexus for translation-mediated gene expression regulation. Previous studies suggest that eIF4G/IFG-1 expression is regulated as an adaptive response to changing environmental conditions. In yeast, treatment with rapamycin or nutrient deprivation rapidly degrades eIF4G in a TOR-dependent manner, but not the other cap-binding complex subunits eIF4E and eIF4A (Berset et al., 1998). In C. elegans, where only one gene encoding eIF4G (ifg-1) exists, adult animals fasted for 2 days show sharply diminished IFG-1 protein expression (Rogers et al., 2011). In mammals, where there are three known forms of eIF4G, depletion of the eIF4GI form phenocopies nutrient deprivation and mTOR inhibition (Ramirez-Valle et al., 2008). Furthermore, mRNAs important for growth and proliferation were preferentially excluded from polysomes (Ramirez-Valle et al., 2008). Thus, lowering eIF4G may act as a ‘switch’ to lower and redirect energy...
expenditure when conditions are not conducive to growth and reproduction.

Here, we investigated how attenuation of this factor remodels intracellular processes and responses to perturbed protein homeostasis. We focused our investigation in the ER based on the observation that lowering ifg-1 shifts translation in favor of genes that encode components of the endomembrane system. We found that attenuating ifg-1 promoted survival under conditions that induce stress in the ER, the organelle responsible for maintaining the endomembrane system. A previous study showed that the preference for translation shifts from the cytosol to the ER when cap-binding complex components (like eIF4G) become limiting (Lerner, 2006). This could explain why components of the endomembrane system are translationally preferred. The UPRER is induced in response to misfolded proteins and/or ER calcium imbalance.

One of the key players in the enhanced ability to withstand ER stress and increase lifespan when ifg-1 is reduced is the ER calcium homeostasis regulator SCA-1. This factor is highly expressed in contractile tissue (Zwaal et al., 2001) where it maintains a high level of calcium in the ER and helps ensure that calcium signaling in the cytoplasm is a transient event. We previously showed that translation and protein of SCA-1 increases in response to lowering ifg-1 in C. elegans (Rogers et al., 2011). Its importance for enhanced stress tolerance and longevity suggest that calcium balance plays a major role in translation-driven changes in proteostasis mediated by IFG-1.

Another ER-specific factor, IRE-1 controls a major arm of the UPRER. ER stress derepresses activation of this factor by freeing it of BIP (encoded in C. elegans by hsp-3 and hsp-4). This, in turn, enables splicing of the retained intron in xbp-1 by IRE-1 and production of the transcription factor XBP-1. Although we did not observe increased activity of IRE-1 when ifg-1 is attenuated according to the splicing status of xbp-1 under nonstressed conditions, we did observe a constitutive increase in overall abundance of xbp-1 in polysomes. Furthermore, we found that enhanced resistance to ER stress requires ire-1 and xbp-1, suggesting that whatever role hsf-1 plays still requires canonical UPRER activation. Interestingly, a study investigating the effects of translation attenuation noted enhanced resistance to tunicamycin stress in yeast strains with ribosomal subunit deletions that did not require Hac1, the yeast ortholog of XBP-1 (Steffen et al., 2012). However, the mechanism(s) underlying enhanced resistance to stress in those yeast mutants may be very different, as we do not know how or whether the stoichiometry of ribosomal subunits changes when ifg-1 is attenuated. For example, differential expression resulting from direct modulation of ribosomal constituents in yeast may be different from that elicited by reducing ifg-1 in C. elegans. In contrast to the protective effects of attenuating translation on ER stress outcomes in our model via ifg-1 in C. elegans and in the Steffen et al. model via ribosomal subunits in yeast, the translation inhibitor cycloheximide was not found to be protective under this condition, despite having a protective effect under thermal stress. Although this might suggest that global translation inhibition is not a major protective factor under the ER stress-inducing conditions used here, it may also be due to a lack of ability to differentially regulate translation, offsetting normally protective effects of overall reduced translation. Additional studies are required to determine similarities and differences in altered translation among different models and methods.

Although finding that hsf-1 gene expression was required for enhanced survival under ER stress when ifg-1 is reduced was surprising, wild-type animals showed no such requirement, suggesting that HSF-1 transcriptional activity must be induced for the effect. In other systems, it was shown that ER stress responses neither automatically activate the HSR (mammalian tissue culture; Putics et al., 2008) nor require HSF-1 for normal survival under ER stress-inducing conditions (yeast; Hou et al., 2014a). However, constitutive activation of the HSR in yeast was able to partially compensate for loss of ire-1, allowing cells to grow in the presence of ER stress (Liu & Chang, 2008). Some evidence for possible cross talk between hsf-1 and the ER is shown in the transcriptional induction of sca-1 to heat shock when ifg-1 is inhibited in the current study. Activating heat-shock transcription factor in response to heat in other systems involves release from cytosolic HSP90, which keeps it sequestered in the cytoplasm prior to stress. Interestingly, we previously observed that ifg-1 RNAi diminished the translation and protein level of DAF-21 (Rogers et al., 2011), the C. elegans ortholog of cytoplasmic HSP90. Although further investigation is required to determine exactly how boosting HSF-1 enhances resistance to ER stress, results of the current study indicate a direct link between enhanced HSR function and amelioration of proteotoxicity in the ER.

In C. elegans, the ability to maintain proteostasis declines rapidly during the first day of adulthood (Ben-Zvi et al., 2009; Labbadia & Morimoto, 2015). Results in the current study indicate that suppressing translation through ifg-1 in adult nematodes dramatically slows the decline in tolerance to heat normally observed in wild-type animals. Another study showed that suppressing translation this way also enhances proteostasis in a polyglutamine model of protein folding disorder in nematodes (Kirstein-Miles et al., 2013). In fact, numerous age-related diseases are considered protein folding disorders resulting from a failure to maintain proteostasis, including Alzheimer’s, Parkinson’s, and Huntington’s. In some instances, chronic activation of the UPRER is also associated with these disorders, as is dysregulation of calcium homeostasis (Thibault et al., 2007). Future studies are required to determine whether suppressing translation through eIF4G can potentially alleviate proteotoxicity in failing cellular systems to recover balance and health in affected tissues.

### Experimental procedures

#### Nematode culture and strains

*Caenorhabditis elegans* strains were cultured at 20 °C and maintained on normal growth medium plates seeded with OP50 unless otherwise noted. Strains used included wild-type N2, SJ4005 zc454 [Phsp-4::GFP, lin-15(765)] V, hsf-1(n441), and ifg-1(cx9279). All strains were permitted to grow at least three generations under normal, unstressed conditions prior to use in experiments.

#### RNAi experiments

RNAi bacteria strains were cultured and utilized as previously described (Kamath et al., 2001). Induction was performed using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in all cases. NGM agar plates prepared this way are referred to as ‘RNAi plates’. Bacteria expressing dsRNA included empty vector L4440 (Addgene, Cambridge, MA, U.S.A.), ifg-1 (M110.4), sca-1 (K11D9.2), mdr-15 (R12B2.5), ire-1(c41C4.4), att-6 (F456E.2), peek-1 (F46C3.1), xbp-1 (R74.3), hsf-1 (YS5310A.12), daf-16 (R13H8.1), ife-2 (R04A9.4), rps-15 (F36A2.6), and ift-1 (K04G2.1) from the Arrhinger library (Source BioScience, Nottingham, U.K.).

#### Lifespan

Synchronized populations of nematodes were obtained from gravid adults treated with bleach to remove eggs. Eggs were permitted to hatch overnight in 10 mL of S Basal (0.1 mM NaCl, 5.74 mM K2HPO4...
Survival under tunicamycin stress was performed similar to a method previously described (Taylor & Dillin, 2013). Strains were synchronized by bleeding and eggs were allowed to grow on OP50. On the first day of adulthood worms were transferred to RNAi plates spotted with HT115 bacteria containing dsRNA as indicated and treated with FUDr as described above. After 2 days, worms were transferred to new RNAi plates containing a final concentration of 25 μM 1 tunicamycin dissolved in DMSO. These plates were spotted with bacteria 1 day prior to addition of tunicamycin, which was added 1 day prior to transferring worms. Control plates lacking tunicamycin contained an equivalent amount of DMSO. Survival was scored beginning at the time of tunicamycin exposure.

Chemical inhibition of translation
Cycloheximide (0.5 mM; Sigma, St. Louis, MO, U.S.A.) was added to RNAi plates containing bacteria bearing L4440 control vector or as otherwise indicated. Animals were exposed to cycloheximide 2 days prior to 25 μM 1 tunicamycin exposure or 7 days prior to shifting to 35 °C.

Western blotting
Frozen worm pellets were ground in 8 M Urea, 2% SDS, 50 mM DTT, 50 mM Tris pH 7.4, with 1× Halt Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA, U.S.A.), allowed to solubilize on ice for 10 min, and debris was pelleted by centrifugation at 12 000 g for 3 min. Protein concentrations were determined using Qubit 2.0 protein assay (Life Sciences, Thermo Scientific, Waltham, MA, U.S.A.). Proteins were separated using 4–12% mini Protean TGX gels (Bio-Rad) and transferred to PVDF membrane. Detection of IFG-1 or phosphorylated eIF2α was performed using anti-IFG-1 polyclonal antibody (a kind gift from Brett Keiper; Contreras et al., 2008) or anti-phospho-eIF2α (Ser51; #9721; Cell Signaling Technology) along with goat anti-rabbit IgG HRP-conjugated secondary antibody (Thermo Scientific, Waltham, MA, U.S.A.). For IFG-1 quantification, antibody against beta-tubulin (Developmental Studies Hybridoma Bank) was used as a loading control with HRP-conjugated goat anti-mouse IgG (Thermo Scientific) used as a secondary antibody. Chemiluminescence was detected using the G: Box imager (Syngene, Cambridge, U.K.). For quantifying phosphorylated eIF2α, data were normalized using total protein quantified by loading into TGX stain-free gels exposed to UV light for five min in a stratallinker prior to blotting. After transfer, the bands were visualized on a UV transilluminator with a fixed exposure time of 1 s. While all bands per lane were used for estimation of total protein loaded, a single representative band is shown in Fig. S1C (Supporting information). Background corrected band intensity was quantified using ImageJ.

RNA isolation, cDNA synthesis, and qRT–PCR
RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol for chloroform extraction. Samples were additionally processed with SurePrep RNA Cleanup and Concentration kit (Fisher BioReagents, Fair Lawn, NJ, USA). The polysome profile RNA isolation included 10 μg glycogen in each sample. A total of 200 ng RNA was reverse-transcribed using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). qRT–PCR was performed in technical duplicate using KAPA SYBR FAST qPCR Master Mx on a LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA). Target gene mRNA was normalized to the housekeeping gene cdc-42, except for instances of polysome analysis, in which compared samples were normalized to the housekeeping gene act-1, which we have found does not change among total and translated pools between N2 and ifg-1, nor before or after exposure to tunicamycin. Changes in gene expression levels were analyzed by using the 2−ΔΔCT method. Primer sequences are provided in Table S8 (Supporting information).
waused for background correction. Broad GO functions were filtered out using the GO FAT option.

Statistical analysis

All statistics were performed using GraphPad Prism 6 software (La Jolla, CA, U.S.A.). Two-tailed t-tests were performed on qRT–PCR samples to assess significance. Significance in instances of multiple comparisons was verified via ANOVA followed by post hoc Tukey test. Kaplan–Meier survival curves were plotted and compared using the Mantel–Cox log-rank test. Western blots were analyzed using a Wilcoxon test.

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Author contributions

AH, JR, SS, SC, and ANR performed experiments. ANR and AH designed experiments and wrote the manuscript.

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Conflict of interest

The authors have no conflict of interests to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Reducing *ifg-1* altered genes important for regulating normal ER function and stress responses.

Fig. S2 Reducing *ifg-1* altered *hsf-1* translation under ER stress conditions.

Fig. S3 Reducing *ifg-1* altered *hsf-1* translation under ER stress conditions.

Fig. S4 Reducing translation longevity regulators *ife-2*, *iftb-1* or *rps-15* promoted survival under ER stress.

Fig. S5 Reduced *iftb-1* or *rps-15*, but not *ife-2* or cycloheximide, constitutively activated expression of the HSR chaperone F44E5.4/F44E5.5 by day 7 of adulthood.

Table S1 David GO term enrichment analysis of differentially translated genes showed five cellular component categories were altered after *ifg-1* RNAi feeding.

Table S2 Survival under 25 mg L$^{-1}$ tunicamycin-induced UPR ER stress was extended by attenuating *ifg-1* expression.

Table S3 Enhanced survival provided by reduced *ifg-1* function under chronic UPR ER stress (25 mg L$^{-1}$ tunicamycin) required certain proteostasis regulators.

Table S4 Reduced expression of *ife-2*, *rps-15*, or *iftb-1* promoted survival under ER stress (25 mg L$^{-1}$ tunicamycin).

Table S5 Thermotolerance was enhanced with an extended period of translation attenuation through *ifg-1* RNAi.

Table S6 Thermotolerance in *ifg-1*(cxTi9279) animals was only partially dependent on *hsf-1*.

Table S7 Lifespan was increased in *hsf-1*(sy441) animals on *ifg-1* RNAi.

Table S8 Sequences of quantitative RT–PCR primers used in this study.