Insulin/IGF-1 signaling mutants reprogram ER stress response regulators to promote longevity

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When unfolded proteins accumulate in the endoplasmic reticulum (ER), the unfolded protein response is activated. This ER stress response restores ER homeostasis by coordinating processes that decrease translation, degrade misfolded proteins, and increase the levels of ER-resident chaperones. Ribonuclease inositol-requiring protein–1 (IRE-1), an endoribonuclease that mediates unconventional splicing, and its target, the XBP-1 transcription factor, are key mediators of the unfolded protein response. In this study, we show that in *Caenorhabditis elegans* insulin/IGF-1 pathway mutants, IRE-1 and XBP-1 promote lifespan extension and enhance resistance to ER stress. We show that these effects are not achieved simply by increasing the level of spliced *xbp-1* mRNA and expression of XBP-1's normal target genes. Instead, in insulin/IGF-1 pathway mutants, XBP-1 collaborates with DAF-16, a FOXO-transcription factor that is activated in these mutants, to enhance ER stress resistance and to activate new genes that promote longevity.

aging | daf-2 | insulin signaling | unfolded protein response

The endoplasmic reticulum (ER) is the site where newly synthesized secreted and cell-surface proteins fold and mature. ER stress response regulators monitor the flux of proteins through the ER. Increased flux and accumulation of misfolded proteins within the ER creates ER stress, which in turn, triggers signaling pathways collectively termed the unfolded protein response (UPR). The UPR helps restore ER homeostasis by coordinating processes that decrease translation, degrade misfolded proteins, and increase the levels of ER-resident chaperones (1).

In *Caenorhabditis elegans*, three proteins are known to sense ER stress and activate the UPR: the ribonuclease inositol-requiring protein–1 (IRE-1), the PERK kinase homologue PEK-1, and activating transcription factor–6 (ATF-6) (2–5). Of the three, IRE-1 is the most conserved. When activated by ER stress, IRE-1's endonuclease activity is switched on, and it then removes an intron from *xbp-1* (X-box binding protein–1) mRNA through unconventional splicing (2, 4). Spliced *xbp-1* encodes a transcription factor that activates expression of downstream genes, such as genes encoding chaperones and ER-associated degradation proteins (2, 4, 5), that expand the ER's folding capacity and increase degradation of misfolded proteins.

A functional and well balanced ER is critical for maintaining the integrity of the cell and the organism and for protecting the organism from heart disease, neurodegenerative disorders, and diabetes (6–8). Hence, genes that regulate the ER stress response machinery are potential candidate longevity genes. In this study, we have investigated the role of components of the ER stress response in the regulation of aging.

C. elegans mutants carrying reduction-of-function mutations in *daf-2*, which encodes the animal's insulin/IGF-1 receptor, are long-lived. Multiple stress-protective transcription factors, including the FOXO family member DAF-16, are activated in *daf-2* mutants, where they alter the expression of downstream metabolic and stress-protective genes (9–14). The combined activities of these genes produce large changes in lifespan (15, 16). Although many cell-protective genes are induced in *daf-2* mutants, we find that most

known ER stress response genes are not. Nevertheless, in this study, we demonstrate that *ire-1* and *xbp-1* make a large contribution to the long lifespans of *daf-2* receptor mutants and also increase their ER stress resistance. We find that XBP-1 contributes to the long lifespan of *daf-2* mutants, at least in part, by collaborating with DAF-16 to up-regulate the expression of a gene called *dox-1*. In addition, we find that the increased ER stress resistance of *daf-2* mutants, which is dependent on *xbp-1*, also requires DAF-16. Together, our findings suggest that XBP-1 and DAF-16 interact, directly or indirectly, to promote ER stress resistance and to activate new longevity genes in *daf-2* mutants.

Results and Discussion

***ire-1* and *xbp-1* Promote the Longevity of Insulin/IGF-1 Pathway Mutants.** To ask whether ER stress-response proteins contribute to the increased longevity of animals with reduced insulin/IGF-1 signaling, we individually inactivated each of the three ER stress-response genes that comprise the UPR: *ire-1*, *pek-1*, and *atf-6* (2–5). We found that inactivation of *ire-1* by RNAi or by a deletion mutation shortened the lifespan of *daf-2* mutants (Fig. 1A and Fig. S1A), but that inactivation of *atf-6* or *pek-1* did not (Fig. S1 *B* and *C*). In both wild-type and *daf-2*(−) backgrounds, animals subjected to *ire-1* RNAi appeared normal and healthy, whereas *ire-1(ok799)*–null mutants had morphological and behavioral defects. Thus, whereas complete loss of *ire-1* compromised the health of the animal, partial loss of *ire-1*, through RNAi, could shorten lifespan without making the animals appear unhealthy. Together, these findings implicated IRE-1, but not other ER stress–sensing proteins, in the longevity of insulin/IGF-1 pathway mutants.

One could imagine that IRE-1 promotes the longevity of all worms to the same extent that it promotes the longevity of insulin/IGF-1 pathway mutants; however, we found that this was not the case. Indeed, *ire-1* knockdown shortened the lifespan of wild-type animals as well as *eat-2* mutants (which live long as a result of caloric restriction) and *isp-1* mutants (which live long as a result of reduced mitochondrial respiration). However, the extent of lifespan shortening by *ire-1* inactivation was significantly more pronounced for the *daf-2* mutants than for the other strains tested:

**Fig. 1.** *ire-1* and *xbp-1* contribute to the longevity of *daf-2(−)* animals. (*A*) Reduction of *ire-1* activity by the null mutation *ok799* shortened the lifespan of *daf-2(e1370)* mutants more than it shortened the lifespan of wild type. (*B*) The *xbp-1(zc12)* mutation shortened the lifespan of *daf-2(e1370)* mutants more than it shortened wild-type lifespan. (*C*) The *xbp-1(zc12)* mutation did not further shorten the lifespan of *ire-1(−); daf-2(−)* double mutants, suggesting that these genes function in a common pathway. [Compare survival curves of *ire-1(−); daf-2(−)* and *ire-1(−); daf-2(−) xbp-1(zc12)* mutants.] Note that *xbp-1(zc12)* mutation shortened the lifespan of *daf-2(e1370)* mutants, but not as much as did an *ire-1(ok799)* deletion. [Compare survival curves of *ire-1(−); daf-2(−)* and *daf-2(−) xbp-1(zc12)* mutants.] As *xbp-1(zc12)* is likely to eliminate *xbp-1* function, this finding implies that *ire-1* contributes to the longevity of *daf-2* mutants in part through an *xbp-1*–independent pathway. For additional lifespan data, see Table S1. Also, see Fig. S1 for data showing that other ER stress–response genes, namely *abu-11*, *pek-1*, and *atf-6*, are not involved in this pathway.

inactivation of *ire-1* in any of three *daf-2* mutant strains (*e1370*, *e1368*, and *mu150*) shortened lifespan by an average of 40%, whereas inactivation of *ire-1* shortened wild-type lifespan by an average of 22% (Fig. 1*A* and Table S1), and *ire-1* knockdown shortened the extended lifespan caused by the *eat-2* mutation or the *isp-1* mutation by an average of less than 20% (Fig. S1 *E* and *F* and Table S1). We conclude that IRE-1 plays a particularly important role in promoting the longevity of insulin/IGF-1 pathway mutants.

We hypothesized that IRE-1 might contribute to the long lifespan of insulin/IGF-1 signaling mutants by acting through its known target, the transcription factor *xbp-1*, as it does under conditions of ER stress. Consistent with this hypothesis, we found that a putative *xbp-1*–null mutation, which causes truncation of the XBP-1 protein before its DNA binding domain, shortened the lifespan of *daf-2* mutants more than it shortened the lifespan of wild type (Fig. 1*B*) without affecting the apparent health of the animals. Significantly, this *xbp-1* mutation did not further shorten the lifespan of *ire-1; daf-2* double mutants (Fig. 1*C*), suggesting that *xbp-1* did not act independently of *ire-1* to lengthen the lifespan of *daf-2* mutants.

The *ire-1/xbp-1* Pathway Is Set at a Lower Level in *daf-2* Mutants. The simplest scenario to account for the major contribution of the *ire-1/xbp-1* branch of the UPR to the longevity of *daf-2* mutants was that the *ire-1/xbp-1* pathway is activated constitutively in *daf-2* mutants. To test this idea, we examined the level of spliced *xbp-1* mRNA, which correlates with the level of activation of this UPR pathway in wild-type animals. Surprisingly, by using RT-PCR, we found that, in *daf-2(−)* mutants, the level of spliced *xbp-1* mRNA was lower, not higher, than it was in wild-type animals (Fig. 2*A* and Fig. S2). Likewise, the basal expression level of a GFP fusion to the promoter of the XBP-1 target gene *hsp-4*, which encodes an ER chaperone, was lower in *daf-2* mutants than it was in wild type (Fig. 2*B*). Moreover, when exposed to the ER stress–inducing agent tunicamycin (Fig. S3*A*) or to global protein misfolding caused by heat shock (Fig. 2*C*), adult *daf-2* mutants induced less *Phsp-4::gfp*
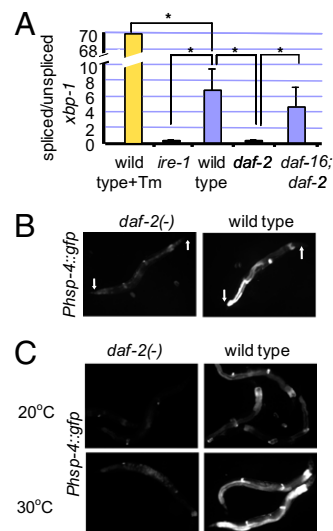

Fig. 2. The *ire-1/xbp-1* pathway is set at a lower level in *daf-2(−)* mutants. (*A*) Bar graph representing the mean ratio of spliced/unspliced *xbp-1* transcripts, multiplied by a factor of 100, in three independent biological experiments. Control lane (yellow bar) presents the *xbp-1* transcripts ratio in wild-type animals treated with tunicamycin. The rest of the bars present steady-state *xbp-1* transcripts ratio in day-1 wild-type, *ire-1*, *daf-2*, and *daf-16; daf-2* animals. These animals have not been treated with tunicamycin (blue bars). Error bars represent SEM. Asterisks mark Student *t* test values of $P < 0.01$. See Fig. S2 for data showing a representative RT-PCR experiment. (*B*) Representative fluorescence micrographs (100-fold magnification) of day-3 adults harboring an integrated *Phsp-4::gfp* transgene in a wild-type or *daf-2 (e1370)* background. *daf-2* mutants expressed lower levels of the *Phsp-4::gfp* transgene than did wild type. wild type, $n = 9$, relative mean fluorescence intensity (m) = 1 ± 0.14; *daf-2(e1370)*, $n = 10$, $m = 0.34 \pm 0.07$, $P = 0.0012$. (*C*) *daf-2(e1370)* mutants expressed less *Phsp-4::gfp* transgene than did wild type even upon heat shock. *Phsp-4::gfp* expression at 20 °C or upon 3 h of heat shock at 30 °C. Arrows point at anterior and posterior ends of the animals.

expression than did wild-type animals. The low level of *Phsp-4::gfp* in *daf-2* mutants could be further reduced upon inactivation of *xbp-1* by RNAi. Thus, the *ire-1/xbp-1* pathway appears to be set at a very low level in *daf-2* mutants, yet is still active enough to influence gene expression.

The *ire-1/xbp-1* Pathway Confers Enhanced ER Stress Resistance to *daf-2* Mutants. Is the lower setting of the *ire-1/xbp-1* pathway in *daf-2* mutants a result of direct inhibition of this pathway, or is it a reflection of a reduced demand on the ER in these mutants? Down-regulation of the *ire-1/xbp-1* pathway (for example, by loss-of-function mutations in these genes) is associated with increased sensitivity to ER stress. If this ER stress response pathway were simply suppressed in *daf-2* mutants, we would expect *daf-2* mutants to be more sensitive to ER stress than are wild-type animals. However, if the lower setting of the *ire-1/xbp-1* pathway were a reflection of a superior ER homeostatic mechanism in *daf-2* animals, we would expect the *daf-2* animals to be more resistant to ER stress. We exposed young animals to toxic concentrations of two ER stress–inducing agents, tunicamycin and DTT, and found that *daf-2* mutants were more resistant to both treatments than were wild-type animals (Fig. 3 and Fig. S3*B*). We interpret this to mean that the lower setting of the *ire-1/xbp-1* pathway in *daf-2* mutants reflects an improved ER homeostasis state compared with wild type. Moreover, this improved state of ER homeostasis is not achieved by up-regulating *xbp-1* mRNA splicing.

Could it be that alternative mechanisms that do not rely on the *ire-1/xbp-1* pathway are responsible for the improved ER homeostasis in *daf-2* mutants? We addressed the possibility that the al-
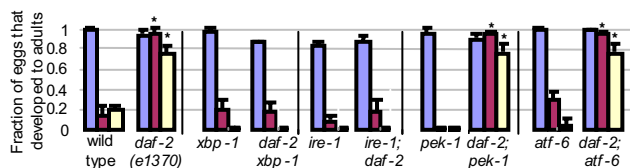
Fig. 3. *daf-2(−)* mutants are resistant to ER stress. Eggs from wild-type or *daf-2 (e1370)* animals containing *xbp-1(zc12)*, *ire-1(ok799)*, *pek-1(ok275)*, or *atf-6 (ok551)* mutations were grown in the presence of 0, 2, or 5 μg/mL tunicamycin (corresponding to the blue, red, and off-white bars in the graph). Percentage of eggs that developed into mature adults was scored. Student *t* test values were calculated between *daf-2(+)* and *daf-2(−)* backgrounds. Each experiment was repeated independently with similar effects. Each strain was scored on three independent plates. Error bars represent SEM for repeat plates within the experiment. Asterisks mark Student *t* test values of *P* < 0.01.

ternative ER stress response pathways involving *pek-1* or *atf-6* may promote ER homeostasis in *daf-2* mutants. However, this was not the case, as null mutations in *pek-1* or *atf-6* genes did not affect *daf-2* mutants' resistance to tunicamycin (Fig. 3). We also considered the possibility that translation is reduced in *daf-2* mutants, slowing the flow of proteins into the ER and thereby reducing the need for XBP-1 and IRE-1. However, translation rates in the *daf-2(e1370)* mutant have been measured under these growth conditions, and they appear normal (17). Finally, we considered the general possibility that the physiology of *daf-2* mutants reduced the level of unfolded proteins, and hence placed a lower demand on the ER. However, if *daf-2* mutants simply had fewer unfolded proteins, then they would be more resistant to tunicamycin even in the absence of *ire-1* and *xbp-1*, but they were not. Instead, mutations in *ire-1* or *xbp-1* genes completely abrogated the enhanced ER stress resistance of *daf-2* mutants, such that *daf-2(−)* mutants had no advantage over *daf-2(+)* animals (Fig. 3). This finding was significant, as it indicated that the *ire-1/xbp-1* branch of the UPR played an essential, integral role in conferring enhanced ER stress resistance to *daf-2* mutants.

How could the *ire-1/xbp-1* pathway confer enhanced longevity to *daf-2* mutants, and yet be set at a lower level than in wild type? We hypothesized that, instead of simply raising the level of spliced *xbp-1* and its known target genes, lowering insulin/IGF-1 signaling may be affecting the very nature of the IRE-1/XBP-1 pathway, for example, by changing the transcriptional outputs of the XBP-1 transcription factor. How could this be achieved? One possible model was that some of the multiple stress-protective transcription factors that are switched on in *daf-2* mutants (9–14) act in a combinatorial fashion with XBP-1 to activate the expression of new longevity and stress-resistance genes. In such a case, these new XBP-1 target genes could specifically enhance the *daf-2* mutant's longevity and stress resistance in a manner that is completely dependent on *ire-1* and *xbp-1* without necessarily increasing the level of spliced XBP-1.

xbp-1 and *daf-16* Collaborate to Express *dox-1*, a New Longevity Gene, in *daf-2* Mutants.

To identify genes that function downstream of XBP-1 in this pathway, we used microarray analysis. We compared the transcription profiles of two *daf-2* mutants (*e1370* and *e1368*) versus those of the corresponding *daf-2(−) xbp-1(−)* double mutants on the first day of adulthood. We identified genes whose expression changed in *daf-2* mutants upon inactivation of *xbp-1* in a highly consistent fashion, regardless of the degree to which their expression was changed Table S2). We then carried out a functional analysis of these genes using RNA interference to identify those that significantly affected lifespan.

Using this approach, we identified a gene, *Y52B11A.9* (which we named *dox-1* for "downstream of *xbp-1*") that encodes an evolutionarily conserved Zn-finger protein. Based on its orthologues

and conserved protein domains, *dox-1* is predicted to function in RNA splicing or processing. Inactivation of *dox-1* had a substantial effect on the lifespan of *daf-2* mutants without affecting their apparent health. In four of six trials, *dox-1* RNAi shortened the lifespan of *daf-2* animals substantially more than it shortened wild-type lifespan (Fig. 4*A* and Table S1). Thus, *dox-1* contributes significantly to the longevity of *daf-2* mutants.

Next, we investigated the regulation of *dox-1* in more detail. *dox-1* was identified in our microarray analysis as a gene whose expression in *daf-2* mutants was significantly dependent on *xbp-1*. By comparing the levels of the *dox-1* transcripts in microarrays profiling *daf-2* and wild-type animals (Table S3), we found that the levels of *dox-1* transcripts were higher in *daf-2* mutants than in wild type. [The levels of *dox-1* transcript were increased by a factor of 1.37 in *daf-2* mutants (*t* test, *P* = 0.028).] We validated the microarray results by analyzing a GFP reporter fused to the *dox-1* promoter. We found that a significantly higher fraction of transgenic *daf-2* mutants expressed high levels of *Pdox-1::GFP* than did their wild-type counterparts and that loss of *xbp-1* significantly reduced the proportion of animals expressing high levels of *Pdox-1::GFP* (Fig. 4*C*). In addition, we found that loss of *daf-16* also significantly reduced the proportion of *daf-2* animals expressing high levels of *Pdox-1::GFP* (Fig. 4*C*). Thus, consistent with our model, in *daf-2* mutants, the expression of a powerful new longevity gene, *dox-1*, is up-regulated in a manner that requires both the transcription factor
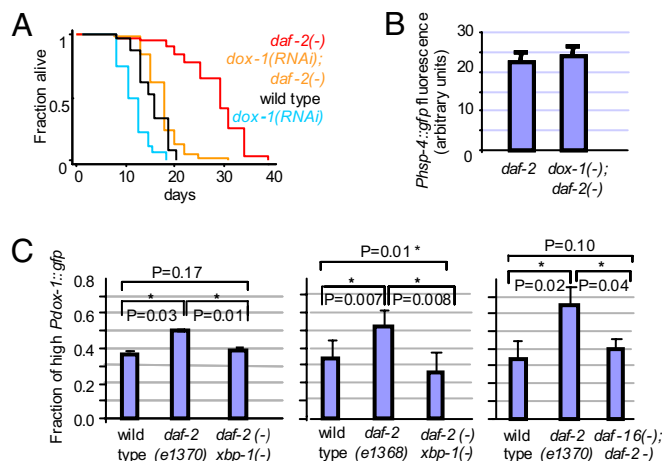
Fig. 4. *dox-1* promotes longevity, but not ER stress resistance, in *daf-2* mutants. (*A*) Reduction of *dox-1* activity by RNAi shortened the lifespan of *daf-2(mu150)* mutants more than it shortened the lifespan of *daf-2(+)* animals. Animals were in a *fer-15(b26); fem-1(hc17)* background to prevent reproduction. Similar results were seen in *daf-2(e1370)* mutants (Table S1). (*B*) Reduction of *dox-1* did not increase the set-point of the *ire-1/xbp-1* branch of the UPR in *daf-2* mutants. Bar graph showing the mean intensity of day-2 adults expressing a *Phsp-4::gfp* transgene. *daf-2(e1370)*, *n* = 13, relative mean fluorescence intensity (m) = 21.9 ± 2.2; *dox-1(RNAi); daf-2(e1370)*, *n* = 17, m = 23.3 ± 2.3, *P* = 0.677. See Fig. S4 for representative micrographs and wild-type data. (*C*) *dox-1* expression was up-regulated in *daf-2* mutants. Bar graph showing the fraction of animals expressing high levels of *Pdox-1::gfp*. Day-1 adults of each strain were classified as expressing high or low GFP levels (Fig. S4). The fraction of animals expressing high levels of *Pdox-1::gfp* was 1.35 fold higher in transgenic *daf-2(e1370)* mutants than in wild-type animals (*P* = 0.037), but was not significantly increased in *daf-2(e1370) xbp-1(tm2457)* double mutants. The fraction of animals expressing high levels of *Pdox-1::gfp* was 1.52 fold higher in transgenic *daf-2(e1368)* mutants than in wild-type animals (*P* = 0.007), but was not increased in *daf-2(e1368) xbp-1(tm2457)* double mutants. The fraction of animals expressing high levels of *Pdox-1::gfp* was higher by 1.8 fold in transgenic *daf-2(e1370)* mutants than in wild-type animals (*P* = 0.026), but was not significantly increased in *daf-16(mu86); daf-2 (e1370)* double mutants. Error bars represent SEM of at least two independent trials. Asterisks mark Student *t* test values of *P* < 0.05.

XBP-1 and the stress-protective transcription factor DAF-16, which is activated when insulin/IGF-1 signaling is low.

We asked whether inactivation of *dox-1*, which was sufficient to affect longevity, also affected ER homeostasis in *daf-2* mutants. To test this, we used the reduced level of the ER chaperone reporter *Phsp-4::gfp* as a reflection of the improved ER homeostasis in *daf-2* mutants. Unexpectedly, we found that inactivation of *dox-1* did not elevate the levels of this reporter (Fig. 4B). Thus, even though *dox-1* expression is dependent on the ER stress–related transcription factor *xbp-1*, *dox-1* appears to contribute specifically to *daf-2*'s longevity but not to the improved ER homeostasis of *daf-2* mutants.

daf-16 Promotes the Enhanced ER Stress Response in daf-2 Mutants.
How is ER homeostasis improved in insulin/IGF-1 mutants? In *daf-2* mutants, multiple stress-protective transcription factors, such as DAF-16/FOXO, HSF-1, ELT-3, and SKN-1, switch on downstream genes that promote longevity (9–14). We wondered if any of these *daf-2*–related transcription factors acted in a combinatorial fashion with *xbp-1*, as in the case of the *dox-1* gene, to turn on new ER stress–response genes that are essential to the enhanced ER stress response in *daf-2* mutants. As outlined earlier, if this were the case, the enhanced ER stress resistance of *daf-2* mutants would be completely *xbp-1*–dependent (as the expression of these genes would require *xbp-1*), and yet specific to *daf-2* mutants (as the expression of these genes would also require a transcription factor that is activated only when insulin/IGF-1 signaling is reduced). Moreover, if one or more of these transcription factors contributed to the expression of new ER stress response genes in *daf-2* mutants, its inactivation should compromise ER homeostasis in *daf-2* mutants. This reduced ER homeostasis, in turn, would activate the classical *ire-1/xbp-1* ER stress response pathway, raising the set-point of XBP-1 and its normal downstream targets. We found that these predictions were met for DAF-16. Using the reduced level of *Phsp-4::gfp* as a reflection of the improved ER homeostasis in *daf-2* mutants, we asked whether inactivation of any of these transcription factors would reduce ER homeostasis (i.e., elevate the expression of *Phsp-4::gfp*) in *daf-2* mutants. Inactivation of the transcription factors *hsf-1*, *skn-1*, and *elt-3* by RNAi had a weak and inconsistent effect on the level of *Phsp-4::gfp* expression. In contrast, the level of this reporter was consistently increased in *daf-2* mutants upon inactivation of *daf-16* (Fig. 5A). Consistent with this finding, by using PAGE analysis of previously published *daf-2* and *daf-16*; *daf-2* microarray data (18), we found that a group of 17 genes (including *hsp-4*) that previously had been found to be induced in an *xbp-1*–dependent fashion upon tunicamycin treatment (3, 5) was significantly up-regulated in *daf-16*; *daf-2* double mutants compared with *daf-2* single mutants (P = 2.328E-06; Table S4). Finally, by using RT-PCR, we found that the level of spliced *xbp-1* mRNA was consistently higher in *daf-2* mutants upon inactivation of *daf-16* (Fig. 2A and Fig. S2).

As loss of *daf-16* activates this UPR pathway in *daf-2* mutants, these findings may bring to mind the possibility that *daf-16* is simply a direct repressor of the *ire-1/xbp-1* pathway. However, this possibility is inconsistent with the enhanced (and *xbp-1*–dependent) ER stress resistance of *daf-2* mutants. Instead, this finding is consistent with DAF-16's being a factor that collaborates with XBP-1 to induce new stress-protective gene expression in *daf-2* mutants. One prediction of this model would be that *daf-16* contributes to the ER stress resistance of *daf-2* mutants. We found that this was the case, as inactivation of *daf-16* in *daf-2* mutants significantly compromised the ability of *daf-2* mutants to develop into adults when treated with tunicamycin (Fig. 5B). This finding, too, argues against the possibility that *daf-16* simply represses the *ire-1/xbp-1* pathway: if this were the case, loss of *daf-16* should increase, not decrease, ER stress resistance.

Together these findings support the model that DAF-16 collaborates with XBP-1 to up-regulate the expression of genes that
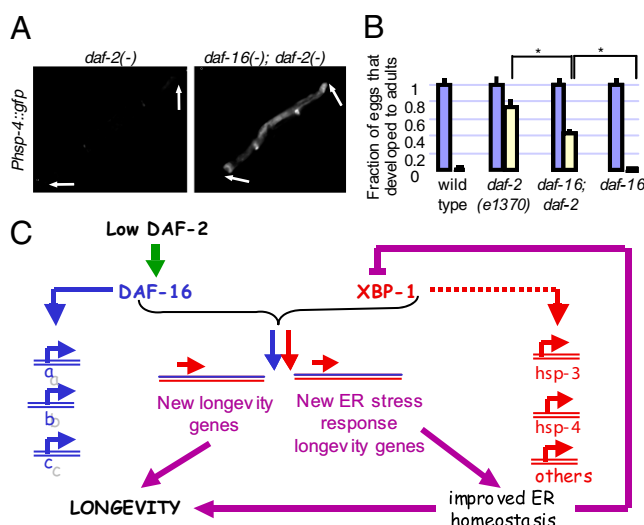
Fig. 5. *daf-16* contributes to the ER stress resistance in *daf-2* mutants. (A) Representative fluorescence micrographs of day-1 adults harboring an integrated *Phsp-4::gfp* transgene in a *daf-2(e1370)* or *daf-16(mu86); daf-2(e1370)* background. *daf-16* deletion (and inactivation of *daf-16* by RNAi) increased the level of *Phsp-4::gfp* in *daf-2(–)* mutants. *daf-2(e1370)*, n = 20, relative mean fluorescence intensity (*m*) = 1 ± 0.06; *daf-16(mu86); daf-2(e1370)*, n = 28, *m* = 2.70 ± 0.2, P < 0.0001. Error bars represent SEM. Arrows point at anterior and posterior ends of the animals. (B) Tunicamycin resistance developmental assay. Eggs from wild-type, *daf-2(e1370)*, and *daf-16(mu86); daf-2(e1370)* animals were grown in the presence of 0 or 5 μg/mL tunicamycin (corresponding to the blue and off-white bars in the graph). Percentage of eggs that developed into mature adults was scored. Student *t* test values were calculated relative to *daf-16(–); daf-2(–)*. Asterisks mark Student *t* test values of P < 0.05. This experiment was repeated independently with similar effects. (C) Model for how XBP-1 extends lifespan, increases ER stress resistance, and also lowers XBP-1 levels when insulin/IGF-1 signaling is reduced. In *daf-2* mutants, multiple transcription factors, such as DAF-16/FOXO, HSF-1, ELT-3, and SKN-1 are activated. These can act in a combinatorial fashion with XBP-1 to turn on new longevity genes and new ER stress response genes (both in purple), which, unlike the "normal" ER stress–protective genes (in red), cannot be induced by XBP-1 alone. This combinatorial control results in a wider array of longevity and ER stress–protective genes, both of which contribute to the longevity of *daf-2* mutants. In addition, as part of a negative feedback loop, the wider array of ER stress protective genes expressed in *daf-2* mutants by *xbp-1* can better reduce the level of unfolded proteins in the ER and improve ER homeostasis. Improved ER homeostasis would, in turn, dictate a lower basal activity level of the *ire-1/xbp-1* pathway. In this model, the enhanced ER stress resistance of *daf-2* mutants is completely *xbp-1*–dependent, and yet, because the ER stress response is more efficient, the set point of XBP-1 and its normal downstream targets is lower than in wild type.

promote ER homeostasis in *daf-2* mutants (Fig. 5C). In this model, loss of *daf-16* results in loss of ER stress protection in *daf-2(−)* mutants, compromising ER function and homeostasis and eliciting ER stress, which in turn increases the splicing of *xbp-1* as well as the expression of XBP-1's "normal" ER stress response genes. We note that the ER stress resistance of *daf-2* mutants was not completely dependent on *daf-16* (Fig. 5B). This implies that DAF-16 is one of several factors, rather than the only factor, that collaborates with XBP-1 to positively regulate the expression of genes that promote ER homeostasis in *daf-2* mutants.

Discussion
Reduced insulin/IGF-1 signaling, which happens naturally in response to stressful environmental conditions, is thought to extend lifespan by catalyzing a physiological shift toward pathways that promote cell maintenance and protection. Insulin/IGF-1 pathway mutants are resistant to a variety of environmental stressors, in-

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cluding heat, paraquat, hydrogen peroxide, DNA damaging agents, hypoxia, pathogens, and xenobiotic agents. Many genes encoding proteins predicted to increase environmental stress resistance are expressed at higher levels when insulin/IGF-1 signaling is reduced. These genes encode chaperones, innate-immunity proteins, metabolic genes, proteins that detoxify reactive oxygen species, and even proteins that protect the chromatin of the germline (16, 19). In general, the loss of any one of these stress-related genes shortens the lifespan of *daf-2* mutants more than it shortens the lifespan of wild-type animals (15, 16). Thus it was not surprising to find that *daf-2* mutants also exhibited increased ER stress resistance and that loss of the ER stress response genes *ire-1* or *xbp-1* shortened the lifespan of *daf-2* mutants substantially.

This finding suggested a very simple model: namely that, as with other stress-protection pathways, the IRE-1/XBP-1 pathway was constitutively active in *daf-2* mutants, and its enhanced ability to reduce ER stress prevented age-related imbalances in protein homeostasis that accelerate aging. However, this simple model was incorrect. By using RT-PCR to measure the level of spliced (i.e., active) *xbp-1* mRNA and an in vivo GFP reporter to assay the expression of a representative ER stress–response gene activated by XBP-1, we found that this pathway was not constitutively activated in *daf-2* mutants. Instead, its activity was actually lowered.

To explain these paradoxical findings, we considered the model that *daf-2* mutants reduce the load on their ER by activating ER stress–protective pathways that are independent of XBP-1 and IRE-1. A reduced load on the ER would improve ER homeostasis and increase ER stress resistance while also lowering the set point of the *ire-1/xbp-1* pathway. However, this model predicts that the improved ER homeostasis of *daf-2* animals should be, at least in part, independent of the *ire-1/xbp-1* pathway. However, all the increased ER stress resistance in these animals was XBP-1–dependent. Thus, this model was flawed too.

To resolve this conundrum, we devised a model that reconciles all these findings. In this model, XBP-1 collaborates with one or more of the transcription factors that are activated when insulin/IGF-1 signaling is reduced to stimulate the expression of new cell-protective genes. As the expression of these genes requires *xbp-1*, this model accounts for why the increased longevity and ER stress resistance require *xbp-1*. Likewise, as the expression of these genes also requires a transcription factor that is activated only when insulin/IGF-1 signaling is reduced, this model accounts for the fact that *xbp-1* promotes a greater degree of ER stress resistance and longevity in *daf-2* mutants than it does in wild type. Finally, the expression of new ER stress–response genes in *daf-2* mutants would increase ER homeostasis, and thus could account for the lower set-point of XBP-1 and its normal downstream targets.

By looking for such genes by means of microarrays, we tested and validated this model for the role of XBP-1 in longevity. We identified *dox-1* as a lifespan-extending gene that is up-regulated in *daf-2* mutants by both XBP-1 and a transcription factor activated in *daf-2* mutants, DAF-16/FOXO. Thus, directly or indirectly, XBP-1 and DAF-16 collaborate to activate *dox-1* expression. Within the first 300 bp upstream of the *dox-1* coding sequence, we identified two potential DAF-16/FOXO binding sites (20, 21) and a potential XBP-1 ETS-like binding site (22). Thus, it is possible that both of these proteins bind to the *dox-1* promoter to activate *dox-1* expression. Alternatively, one or both transcription factors could act on intermediate components of a more complex regulatory pathway.

Loss of *dox-1* reduces the lifespan of *daf-2* mutants substantially, much more than does the loss of most of the DAF-16 target genes that have been examined. The sequence of *dox-1* suggests that it encodes a regulatory protein; specifically, a Zn-finger protein whose orthologues in other species are implicated in RNA splicing or processing. The fact that *dox-1* could potentially influence multiple downstream targets is consistent with its having a relatively large effect on lifespan.

The finding that *ire-1* and *xbp-1* promote both ER stress resistance and longevity in *daf-2* mutants suggested that they extend lifespan by enhancing the ER stress response, thereby improving protein homeostasis. Thus it was surprising to find that loss of *dox-1* activity influenced lifespan but not ER homeostasis. This finding indicates that, when insulin/IGF-1 signaling is low, IRE-1 and XBP-1 can engage in processes that extend lifespan without affecting ER homeostasis.

Loss of *daf-16* does reduce the ER stress resistance of *daf-2* mutants, suggesting that XBP-1 and DAF-16 together may control the expression of other genes that do affect ER stress resistance. We propose that genes regulated in this combinatorial fashion may allow *daf-2* mutants to achieve better ER homeostasis under normal conditions, and to restore ER homeostasis more effectively when confronted with ER stress. In principle, these two transcription factors could regulate one or a few potent ER stress–response genes, or they could affect the expression of many ER stress–response genes that have small effects singly but together produce large increases in ER homeostasis in *daf-2* mutants. Recently, Kapahi and coworkers showed that IRE-1 and XBP-1 contribute to the longevity of animals with altered levels of the oxygen sensor hypoxia-inducible factor–1 (23). The mechanism here may be different, as this longevity pathway does not require DAF-16. It will be interesting to learn whether, and to what extent, these pathways may converge on similar targets.

It will also be interesting to learn whether this pathway has been conserved in higher organisms. This seems possible, as many insulin/IGF-1 pathway mutations extend lifespan in mice, and the DAF-16 orthologue FOXO3A has been linked to exceptional longevity in humans (24).

In summary, our data suggest that, in *C. elegans*, the transcription factors responsible for the global, cell-protective physiological shift that takes place in *daf-2* mutants collaborate with classical ER stress response proteins to promote longevity and ER stress resistance. We hope these findings will stimulate additional studies in higher organisms, as these may form the basis of new therapeutic strategies to combat diseases that are associated with protein misfolding in the ER.

Materials and Methods

Strains and Lifespan Analysis. Strain maintenance and lifespan analysis were performed as described previously (25) and are detailed further in *SI Materials and Methods*.

RNAi. The identity of RNAi clones was verified by sequencing. RNAi clones were obtained from the Ahringer genomic RNAi library. For all RNAi experiments, animals were grown from hatching on bacteria expressing dsRNA, unless indicated otherwise.

SI Materials and Methods includes details about the systematic RNAi lifespan screen, which was carried out using RNAi clones corresponding to the approximately 600 candidate genes identified in the microarray study.

RT-PCR. Total RNA isolation and reverse transcription using random hexamer primers of synchronized day-1 adult *C. elegans* RNA were carried out as described (26). Experiments were performed on serial dilutions of the cDNA from three independent biological replicates per strain, within the linear range of the PCR. See *SI Materials and Methods* for *xbp-1* primer design.

ER Stress Resistance. Eggs of different genetic backgrounds were placed onto plates containing 0.1% DMSO and 0, 2, or 5 μg/mL tunicamycin (Sigma) or 0, 2, or 5 mM DTT. The number of eggs was noted and compared with the number of animals that reached the L4/adult stage within 72 to 96 h. For each strain, typically 50 to 200 eggs were placed on each of three plates.

Microscopy and Quantification. Young adults expressing *Phsp-4::GFP* were immobilized on agarose pads containing 2 mM levamisole. Whole worm images were captured using a Retiga EXi Fast1394 CCD digital camera (QImaging) attached to an Axioplan 2 compound microscope (Zeiss), using the OpenLab program (version 4.0.2). For each individual trial, exposure time was calibrated to avoid saturated pixels for the set of animals. OpenLab 4.0.2 software was used to measure and quantify fluorescence intensity within the

entire animal. Total fluorescence was calculated by the OpenLab program as measured by intensity of each pixel in the selected area of the worm.

Microarray Analysis. Standard RNA purification, labeling using the Agilent QuickAmp Kit, and hybridization on Agilent 4 × 44K *C. elegans* arrays using the Hi-RPM hybridization kit were performed by the University Health Network Microarray Centre (Toronto, ON, Canada). See *SI Materials and Methods* for analysis and sample description.

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