Collapse of proteostasis represents an early molecular event in *Caenorhabditis elegans* aging

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Protein damage contributes prominently to cellular aging. To address whether this occurs at a specific period during aging or accumulates gradually, we monitored the biochemical, cellular, and physiological properties of folding sensors expressed in different tissues of *C. elegans*. We observed the age-dependent misfolding and loss of function of diverse proteins harboring temperature-sensitive missense mutations in all somatic tissues at the permissive condition. This widespread failure in proteostasis occurs rapidly at an early stage of adulthood, and coincides with a severely reduced activation of the cytoprotective heat shock response and the unfolded protein response. Enhancing stress responsive factors HSF-1 or DAF-16 suppresses misfolding of these metastable folding sensors and restores the ability of the cell to maintain a functional proteome. This suggests that a compromise in the regulation of proteostatic stress responses occurs early in adulthood and tips the balance between the load of damaged proteins and the proteostasis machinery. We propose that the collapse of proteostasis represents an early molecular event of aging that amplifies protein damage in age-associated diseases of protein conformation.

daf-16 | folding sensors | hsf-1 | protein misfolding | stress response

The stability of the proteome is challenged by conditions that cause proteotoxic stress including errors during protein synthesis, oxidant-induced covalent modifications, inherited polymorphisms, and misfolding (1–3). Consequently, all cells have highly conserved stress-inducible pathways that detect, prevent, and resolve such damage (4, 5). Accumulation of misfolded proteins titrate the negative regulation of chaperones from HSF-1 (4, 6, 7). Likewise, an E3 ubiquitin ligase was shown to regulate the levels of DAF-16, also indicating a link between stress response activation to the balance between proteostasis capacity and the load of damaged proteins (8). The activation of cellular stress responses limits the accumulation of damaged proteins by suppression of misfolding and enhancing chaperone-mediated folding, promoting the enzymatic removal of covalent modifications, and stimulating clearance by ubiquitin-mediated, proteosomal, and autophagic processes (1, 4, 5, 9, 10). The dynamics of these processes during aging, however, are poorly understood.

Aging can be characterized by the loss of cellular function and increased vulnerability to environmental and physiological stress that results in enhanced susceptibility to disease. Many age-dependent changes have been detected at mid-to-late lifespan of *C. elegans*, including the accumulation of damaged macromolecules, cell and organellar degeneration, and physiological decline (11–17). Likewise, aggregation and toxicity of disease-associated aggregation-prone proteins, such as huntingtin and Aβ peptide, is enhanced during aging in *C. elegans* and other model systems (18–21).

The regulation of aging and the maintenance of proteostasis has been recently established in *C. elegans.* Downregulation of the insulin-like signaling (ILS) pathway enhances lifespan and stress resistance, and suppresses aggregation of polyglutamine expansion proteins and Aβ toxicity (20–22). This requires the activities of the stress responsive factors HSF-1 and DAF-16, and the elevated expression of molecular chaperones (20, 21, 23–26). These observations have led to the hypothesis that there is an age-dependent failure in protein-folding quality control. Whether damaged proteins are functionally sequestered and neutral to cellular processes, toxic, or cleared more rapidly at different stages of development and adulthood is likely to have profound implications for the functionality of biological systems when challenged by aging and disease.

To examine the stability of the proteome during aging, we monitored the activities of folding sensors that harbor missense mutations with temperature-sensitive (ts) phenotypes. We selected diverse, well-characterized ts mutations expressed in different tissues that are fully functional under physiological permissive (15 °C) conditions and exhibit a complete loss of function at the restrictive (25 °C) conditions. Here we show that each folding sensor misfolds at an early stage of adulthood during a period that coincides with a sharp decline in the ability to activate cellular stress responses. This collapse of proteostasis can be restored by the stress responsive factors HSF-1 and DAF-16, thus prolonging the health of the organism. Dysregulation of proteostasis at the transition to adulthood offers an explanation for the age-dependent onset observed for many heritable diseases.

Results

The effects of aging on proteostasis in *C. elegans* muscle cells were examined using ts mutations in the thick filament proteins paramyosin (UNC-15) and myosin heavy chain B (UNC-54). Animals expressing paramyosin(ts) [*unc-15*(*e1402*)] or myosin(ts) [*unc-54*(*e1301*)] at the restrictive condition exhibit severe movement defects (uncoordination) and have disrupted thick filaments that result from misfolding of paramyosin(ts) or myosin(ts) (2, 27, 28). At the permissive temperature, as expected, paramyosin(ts)- or myosin(ts)-expressing animals show wild-type (WT) phenotypes through day ≈6 of adulthood (Fig. 1 *A* and *G*). However, at day ≈7 of adulthood and thereafter, animals expressing either ts-mutant protein exhibited significant uncoordination, whereas the motility of WT animals was unaffected until day 10 of adulthood, when aging-associated decline in motility was observed (Fig. 1 *A* and *G*) (11).

To determine whether the decline in motility, observed for animals expressing muscle cell ts-mutant proteins, is due to disruption of myofilaments, we used cellular and biochemical assays to monitor the subcellular localization of paramyosin(ts) and myosin(ts). WT animals expressing myosin heavy chain A tagged with green fluorescence protein (GFP) (MYO-3::GFP) provided a complementary assay to monitor the state of myofilament structure. Animals expressing either ts-mutant protein

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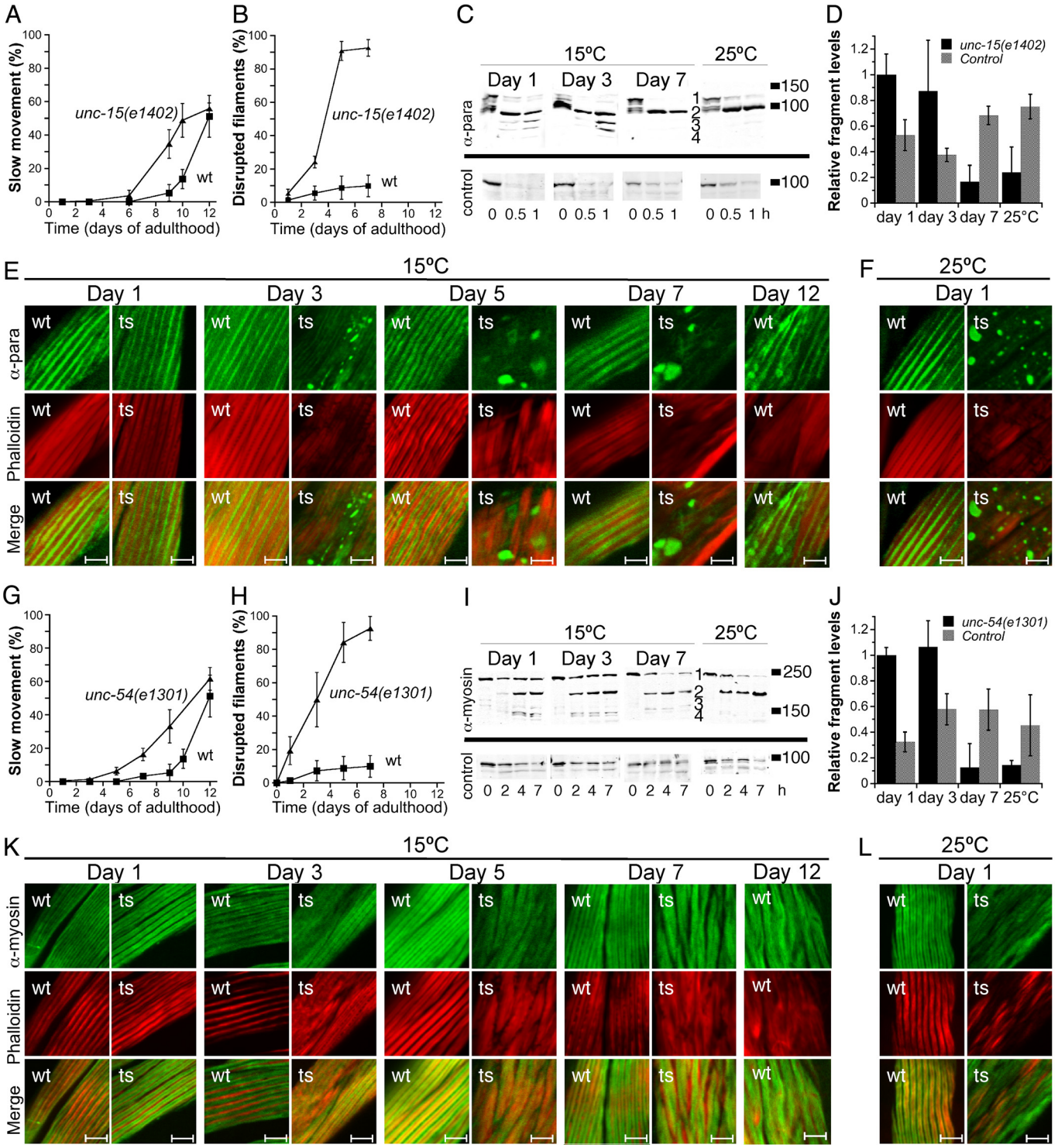

Fig. 1. The folding and assembly of metastable proteins in muscle cells are perturbed in early adulthood. (*A*) Percentage of slow-moving, age-synchronized animals expressing WT (squares) or ts (triangles) paramyosin (*unc-15*) at 15 °C. Data are mean ± SD, >95 animals per data point. (*B*) Percent of cells with disrupted myofilaments, quantified from confocal images of MYO-3::GFP fluorescence of animals expressing WT (squares) or ts (triangles) paramyosin at 15 °C. Data are mean ± SD, >210 cells per data point. (*C*) Differential sensitivity of paramyosin to chymotrypsin proteolysis. Total protein extracts of age-synchronized animals were treated for indicated times, and gels were incubated with paramyosin (*Top*) or dynamin (as an internal control) (*Bottom*) antibodies. Proteolysis experiments were performed on 3 independent biological samples. Images are from a representative proteolysis experiment. (*D*) Quantification of proteolyzed paramyosin (fragment 3 + 4) (black) and dynamin (gray) after 1 h of treatment normalized to day 1. (*E* and *F*) Confocal images of body-wall muscles. Age-synchronized animals expressing WT or ts paramyosin grown at 15 °C (*E*) or 25 °C (*F*) were stained with anti-paramyosin and phalloidin. (Scale bar: 5 μm.) (*G*) Percentage of slow movement of animals expressing WT (squares) or ts (triangles) myosin (*unc-54*) as in (*A*). Data are mean ± SD, >110 animals per data point. (*H*) Percent of cells with disrupted myofilaments expressing WT (squares) or (triangles) ts myosin as in (*B*). Data are mean ± SD, >190 cells per data point. (*I*) Differential sensitivity of myosin(ts) protein to enterokinase digestion as in (*C*). Gels were exposed to myosin (*Top*) or dynamin (*Bottom*) antibodies. (*J*) Quantification of digested myosin (fragment 1 levels after 7 h digest) (black) or dynamin (gray) from (*I*) normalized to day 1. (*K* and *L*) Confocal images of animals expressing WT or ts myosin grown at 15 °C (*K*) or 25 °C (*L*) as in (*E*). Labels: wt, wild type (WT); ts, time-sensitive.

showed a rapid deterioration of myofilament structure detected initially at day 2–3 of adulthood (Fig. 1 *B* and *H* and Fig. S1*A*), whereas the myofilament structure in animals expressing WT muscle cell proteins was maintained through day 12 of adulthood, at which point age-dependent sarcopenia was observed (Fig. S1) (11). These observations were further supported by immunostaining assays, which show the mislocalization of paramyosin(ts) to paracrystalline-like aggregate structures at day 3 of adulthood, similar to that observed at the restrictive condition (Fig. 1 *E* and *F*) (2, 27). By day 5 of adulthood, paramyosin(ts) was mislocalized in nearly all muscle cells (Fig. 1*E*), coinciding with the complete disruption of myofilament structure detected by MYO-3::GFP localization (Fig. 1*B*). Likewise, myosin(ts) showed an age-dependent mislocalization similar to restrictive conditions (Fig. 1 *K* and *L*). Consistent with the onset of age-dependent sarcopenia (Fig. 1*A* and Fig. S1 *B* and *C*), the subcellular localization of WT paramyosin and myosin was disrupted at day 12 of adulthood, which phenocopies paramyosin(ts) and myosin(ts) mislocalization at day 3 of adulthood (Fig. 1 *E* and *K*). This rapid, age-dependent increase in paramyosin(ts) phenotypes is not due to reduced levels of RNA or protein expression or effects on lifespan (Fig. S2). Rather, the exposure of ts phenotypes is initiated by the misfolding of paramyosin(ts) or myosin(ts); this was further demonstrated using protease sensitivity as a tool to monitor conformational change (2). The proteolytic fragmentation patterns for paramyosin(ts) at day 7 of adulthood have distinct characteristics compared with day 1 of adulthood and are consistent with paramyosin(ts) fragmentation pattern at the restrictive condition (Fig. 1 *C* and *D*). Similar results were also observed for myosin(ts) proteolytic sensitivity during aging (Fig. 1 *I* and *J*), but not for a control WT protein (Fig. 1 *C*, *D*, *I*, and *J*). Taken together, these findings show that the early and rapid loss of function observed for metastable proteins during aging is due to their misfolding and cellular dysfunction that results in early onset of sarcopenia with deleterious physiological consequences at the organismal level.

We next addressed whether the unexpected sensitivity of ts proteins during aging in muscle cells extends to other proteins and tissues by examining dynamin (DYN-1) that is expressed in both neuronal cells and coelomocytes (29, 30). The expression of a ts mutation in dynamin [*dyn-1*(*ky51*)] leads to the rapid loss of endocytosis and synaptic vesicle recycling at the restrictive condition (29, 31). Consequently, dynamin(ts) function in neurons can be monitored by localization of the synaptic protein, synaptobrevin-1, tagged with GFP (SNB-1::GFP) (Fig. S3*A*) (32). By day 3 of adulthood, SNB-1::GFP was mislocalized in 50% of animals expressing mutant dynamin(ts), leading to decreased motility with no effects observed in animals expressing WT dynamin (Fig. 2 *A* and *B*). Likewise, dynamin(ts) function in coelomocytes was monitored by the uptake of GFP secreted into the body cavity fluid (ssGFP) (Fig. S3*B*) (30). By day ≈2 of adulthood, 50% of the dynamin(ts)-expressing animals failed to endocytose ssGFP (Fig. 2*C*). The rapid age-dependent decline in dynamin(ts) tissue-specific function coincided with mislocalization of dynamin(ts) (Fig. 2*D* and Fig. S3 *C* and *D*), similar to that observed with animals maintained at the restrictive conditions. These findings show that the loss of function observed for dynamin(ts) in neurons and coelomocytes occurs very early (day 2) in adulthood of animals maintained at the permissive condition and therefore is not specific to a particular tissue.

To investigate this further, we examined 4 additional ts-proteins: ras(ts), gas-1(ts), acetylcholine receptor(ts), and perlecan(ts) [*let-60*(*ga89*), *gas-1*(*fc21*), *unc-63*(*x26*), and *unc-52*(*e669*, *su250*), respectively] that vary in expression over 4 orders of magnitude (33, 34). These proteins are expressed in the intestinal and hypodermal cells (ras and perlecan), neurons (ras, gas-1, and acetylcholine receptor), and muscle cells (ras, gas-1, acetylcholine receptor, and perlecan) (35–37). For the phenotypes associated with each of these ts-mutant protein, we observed the rapid loss of function between days 4 and 6 of adulthood in animals maintained at the permissive condition, whereas the corresponding WT proteins were unaffected (Fig. 3). These findings establish that diverse and unrelated proteins that vary in concentration and are expressed in diverse tissues are nevertheless equally at risk for misfolding early in adulthood.

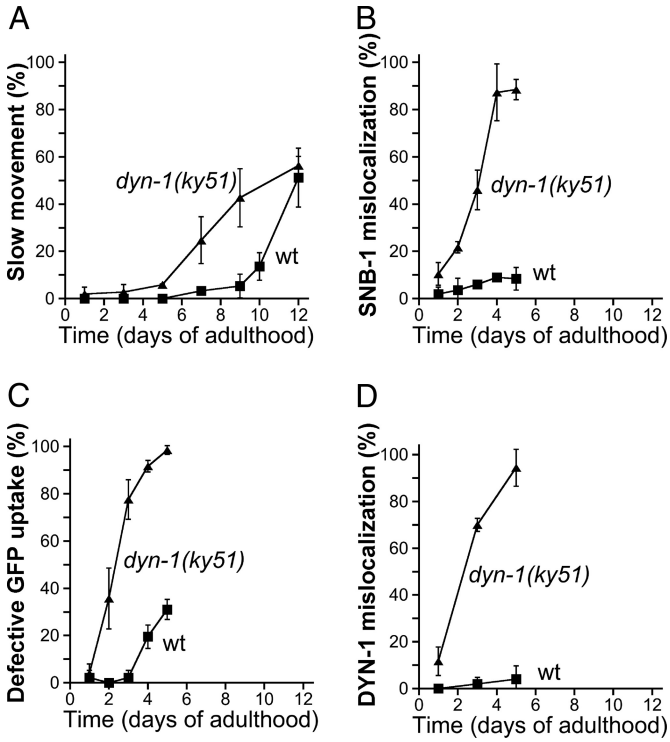

Fig. 2. Dynamin(ts) function in different tissues declines with age. Percentage of age-synchronized animals, expressing WT (squares) or ts (triangles) dynamin (*dyn-1*) showing (*A*) slow movement, (*B*) SNB-1::GFP mislocalization in neurons, (*C*) defective GFP uptake in coelomocytes, and (*D*) dynamin mislocalization. Data are mean ± SD, >45 animals per data point.

The consistency of these observations, that proteins harboring a destabilizing ts mutation rapidly lose function very early in adulthood of *C. elegans*, suggests that critical cellular components that protect the stability of the proteome become deficient during aging. We asked if the proteostatic stress response becomes limiting during adulthood, by testing the ability of WT animals to respond to proteotoxic challenges that induce the heat shock (HS) response and the unfolded protein response (UPR) that protect proteins in the cytoplasm and lumen of the cell. When young (day 1) WT adults were challenged with HS (30 min at 32 °C), expression of the HS genes, *hsp70* and *hsp16*, was strongly induced (Fig. 4*A*). At day 4 and 7 of adulthood, the induction of these genes was dampened by 3- to 8-fold (Fig. 4*A*). This was supported by an age-dependent decline in survival after HS (Fig. S4). Likewise, the ability of WT animals to induce the UPR by measuring the induction of BIP (*hsp-4*) was also significantly reduced (2-fold) during early adulthood (Fig. 4*B*), whereas the levels of the constitutively expressed *hsc70* (*hsp-1*) was unaffected (Fig. 4*C*). These findings suggest that the regulation of proteostasis changes early in adulthood, coinciding with the sensitivity uncovered by ts-protein folding sensors.

If stress responses such as the HS response become limiting during early adulthood, can this be reversed or stabilized by enhancing the activities of the stress transcription factors HSF-1

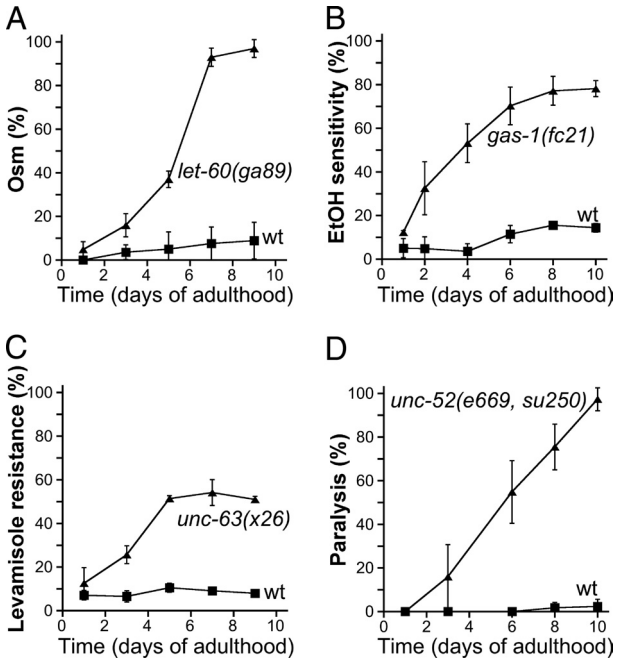

Fig. 3. Diverse ts-mutant proteins expressed in multiple tissues lose functionality with age. Percentage of age-synchronized animals expressing WT (squares) or ts (triangles): (*A*) ras (*let-60*), (*B*) *gas-1*, (*C*) acetylcholine receptor (*unc-63*), or (*D*) perlecan (*unc-52*) showing (*A*) osmoregulation (Osm), (*B*) EtOH sensitivity, (*C*) levamisole resistance, or (*D*) paralysis phenotypes. Data are mean ± SD, >45 animals per data point.

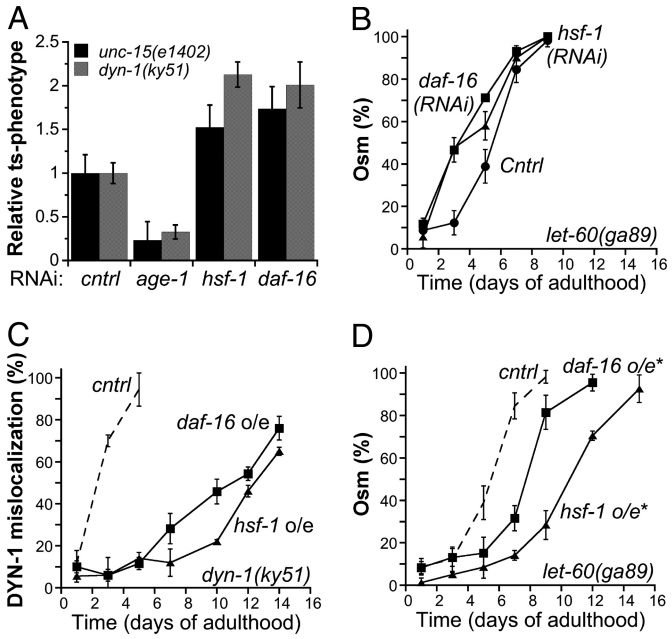

Fig. 5. HSF-1 or DAF-16 modulate the age-dependent misfolding of multiple metastable proteins. (*A*) Age-synchronized (L4) animals expressing paramyosin(ts) [*unc-15(e1402)*] or dynamin(ts) [*dyn-1(ky51)*] were transferred to control (L4440), *hsf-1*, *daf-16*, or *age-1* RNAi-expressing bacteria and scored for slow movement (black) or defective ssGFP uptake (gray) phenotypes respectively. Data are mean ± SD, >92 per RNAi treatment. (*B*) Age-synchronized (L4) animals expressing ras(ts) [*let-60(ga89)*] were transferred to control (circles), *daf-16* (squares), or *hsf-1* (triangles) RNAi-expressing bacteria and scored for percentage of Osm phenotype with age. Data are mean ± SD, >46 per data point. (*C*) Age-synchronized dynamin(ts) animals overexpressing *hsf-1* (triangles) (*hsf-1 o/e*, strain AM586) or *daf-16* (squares) (*daf-16 o/e*, strain AM707) were scored for percentage of animals showing dynamin mislocalization with age. Dashed line is dynamin(ts) animals for reference. Data are mean ± SD, >45 animals per data point. (*D*) Age-synchronized ras(ts) animals overexpressing *hsf-1* (triangles) (*hsf-1 o/e**, strain AM708) or *daf-16* (squares) (*daf-16 o/e**, strain AM558) were scored for percentage of animals showing Osm phenotype with age. Dashed line is ras(ts) for reference. Data are mean ± SD, >65 animals per data point. The roller phenotype (*rol-6*) did not affect the behavior scored (we observed 4.5 ± 4.8 compared to 8.9 ± 8.4% Osm phenotype on day 9 of adulthood of *rol-6* or WT animals, respectively).

and DAF-16? Activation of these stress response factors by downregulation of *age-1* (Fig. S5*A*) suppressed the ts phenotypes by 4-fold relative to control RNAi (Fig. 5*A*). Consistent with this observation, knockdown of either *hsf-1* or *daf-16* (Fig. S5*A*) further exacerbated the loss of function of paramyosin(ts), and dynamin(ts) (Fig. 5*A*) and ras(ts) (Fig. 5*B*, day 3 and 5 of adulthood) by 1.5- to 3.5-fold relative to control RNAi, resulting in an even earlier functional decline (Fig. 5*B*). We then examined whether enhancing the levels of *hsf-1* and *daf-16* could suppress this proteostasis failure. Overexpression (o/e) of HSF-1 or DAF-16 was protective on paramyosin(ts) and dynamin(ts). Dynamin(ts) mislocalization in 50% of the animals (as in Fig. S3*B*) was suppressed by HSF-1 or DAF-16 overexpression up to day 12 and 10 of adulthood, respectively (Fig. 5*C*). Likewise, the age-dependent increase in ras(ts) osm phenotype (as in Fig. 3*A*) was suppressed by HSF-1 or DAF-16 (Fig. 5*D*). It is noteworthy that the expression of components of the proteostasis network, many of which are regulated by HSF-1 or DAF-16 (Fig. S5 *B* and *C*), changes during early adulthood (38, 39). These findings suggest that direct modulation of HSF-1 or DAF-16 protects the stability of diverse metastable proteins and can restore the age-dependent collapse of proteostasis.

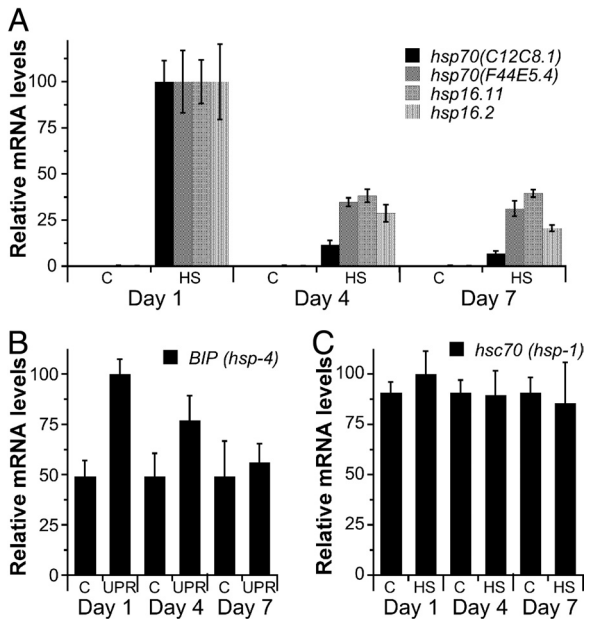

Fig. 4. Different stress responses are compromised early in adulthood. mRNA levels of (*A*) *hsp70*(*C12C8.1*), *hsp70*(*F44E5.4*), *hsp16.11*, and *hsp16.2*, (*B*) *BIP*(*hsp-4*), and (*C*) *hsc70*(*hsp-1*) from age-synchronized WT adults untreated or treated with (*A* and *C*) HS (32 °C; 30 min) or (*B*) UPR (3 mM DTT; 7 h). Data are relative to untreated animals and normalized to day 1 of adulthood-treated animals. Data are mean ± SE, >3 independent biological samples.

Discussion

Our use of diverse metastable proteins harboring missense mutations as sensors of protein homeostasis reveals that aging is associated with a general reduction in proteostasis capacity that

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occurs early in adulthood. Relative to other biological markers of aging that have identified changes at the midpoint of lifespan of *C. elegans*, at days 8–14 of adulthood, the loss of proteostasis occurs at a much earlier stage in multiple cell types, including neurons and muscles (11–17). How does an early event in adulthood affect cellular pathology and loss of physiological function during aging? Our findings show that aging-associated sarcopenia, a general biomarker of aging, corresponds to misfolding of various WT muscle proteins. Given that muscle is a multiprotein complex that requires both specialized and general components of the proteostasis machinery for folding and assembly, it is possible that the decline in proteostasis capacity decreases muscle filaments stability (40). This interpretation is supported by the "disposable soma" theory of aging, which suggests that an early event of maintenance dysregulation can have profound downstream consequences on multiple cellular pathways and physiological functions later in life, as the accumulation of misfolded and damaged proteins is amplified during aging (41). This is further supported by the finding that the loss of proteostasis in early adulthood corresponds to the same period during adult aging when polyglutamine-expansion proteins exhibit age-dependent aggregation and toxicity in *C. elegans* (20). It is interesting to note that the lifespan for WT *C. elegans* under laboratory conditions is 2–3 weeks, whereas in soil conditions this was reduced by nearly an order of magnitude (42). This would suggest that the maintenance of proteostasis is essential throughout development and extends only through the reproductive phase of adulthood, when there is an evolutionary advantage to allocate resources to proteostasis maintenance (41).

The ability of HSF-1 and DAF-16 to restore proteostasis supports the central roles of these stress-responsive transcription factors in more general aspects of biology to prevent global instability of the proteome. Enhancing the activities of these master regulators during development and early in adulthood could affect the levels of proteostasis machinery, preventing proteostasis collapse (43, 44). This is supported by their central role in delaying aging-associated cellular dysfunction and age-related diseases (20, 21, 23, 24). Moreover, because both HSF-1 and DAF-16 are regulated by the NAD-dependent sirtruin, SIRT1, and the ILS-pathway, it is reasonable to suggest that stress biology and proteostasis are intimately linked to each other and to metabolic control and lifespan (20, 23, 24, 45–47). In *C. elegans*, ILS-dependent life extension and the activation of the HS response are regulated by active neuronal signaling and cell nonautonomous control (48, 49). We therefore suggest that the transition between development and adulthood is critical at the cellular and organismal levels to the regulation of proteostasis. Regulatory override of the switch by enhancing HSF-1 and DAF-16 could prolong the "youthfulness" of the proteostatic machineries and ensure that the regulation of proteostasis remains robust, thus reducing the rate of aging, and extending lifespan.

Our experiments, by their design, provide a semiquantitative analysis of specific phenotypes associated with each folding sensor. It seems reasonable to extend our observations to the effects of aging on the stability of other coexpressed metastable proteins, many of which are tissue specific and may lose function as the proteostasis network becomes compromised during aging. By extrapolation to the entire proteome, missense mutations are not rare, as 60,000 single nucleotide substitutions are estimated to occur in human coding exons (an average of 2 sites per gene) (50), and 25%–30% of these are predicted to affect protein stability or folding (51). Moreover, missense mutations represent only one source of damage that could affect the stability of the proteome. For example, misincorporation during translation is estimated to result in one missense substitution in 18% of expressed proteins, with additional opportunities to express damaged proteins from errors in transcription and splicing, posttranslational modifications, and targeting (3). The spectrum of affected proteins would therefore be predicted to contribute significantly to the high degree of variability in onset and progression of aging-associated decline (11). The exposure of missense mutations during aging, which are normally masked by the proteostasis network, offers an explanation for the increased risk observed for many heritable age-dependent diseases, such as familial ALS, Parkinson's disease, and Huntington's disease (52–56).

Materials and Methods

Nematode Strains and Maintenance. For a list of strains used in this work and name abbreviations see Tables S1 and S2. Nematodes were grown on NGM plates seeded with *Escherichia coli* OP50 strain at 15 °C. Animals were picked as L4 larvae and scored at the indicated times during adulthood. All animals were scored at the same chronological age early in adulthood. Animals were moved every 2–3 days during the reproductive period to avoid progeny contamination, and were discarded after scoring. Experiments were repeated at least 3 times. All assays were performed blind.

Assays for Specific Temperature-Sensitive Phenotypes. For the Osm phenotype, slow movement (Unc), paralysis, and defective ssGFP uptake assays, animals were scored as previously described (2, 30). For sensitivity to EtOH or levamisole, animals were picked into 0.4 M EtOH or 50 μM levamisole solution respectively, equilibrated for 5 min, and scored for motility. For MYO-3::GFP and SNB-1::GFP mislocalization, synchronized adults expressing the transgene were fixed (2) and GFP fluorescence was monitored. Animals were imaged using a Zeiss ConfoCor 3/510 META confocal microscope through a 63 $\times$ 1.0 numerical aperture objective with a 488 nm line for excitation.

Immunostaining. Immunofluorescence studies were performed as previously described (2). Animals were stained with rhodamine phalloidin (Molecular Probes) and with either anti-paramyosin [5-23] (57), anti-myosin heavy chain B [28.2] (57), anti-myosin heavy chain A [5-6] (57) or anti-dynamin-1 antibodies (29). Secondary antibodies were IgG-FITC goat anti-mouse (Sigma) or Rhodamine Red goat anti-rabbit (Invitrogen). Animals were imaged as described using 488- and 543-nm lines for excitation.

Proteolysis. Nematodes were lysed, and digests were analyzed as previously described (2). Total protein of 120–200 μg was digested at RT with chymotrypsin (Sigma) or enterokinase (Roche) at enzyme-to-protein ratio 1:1,200 and 1:33, respectively. Aliquots were removed at the indicated times. Each digest was repeated at least 3 times using independent biological samples. Protein levels were determined using ImageJ software. To quantify the change in fragmentation, we identified differences in the fragmentation pattern of animals grown at 15 °C or 25 °C (fragments 3 and 4 for paramyosin(ts) [see Fig. 1*C*] and fragment 1 for myosin(ts) [see Fig. 1*J*]) and examined the levels of these fragments after a digest of 1 h for paramyosin or 7 h for myosin, relative to undigested proteins of animals collected at indicated times during adulthood. Values were normalized to day 1 of adulthood.

RNA Interference (RNAi). Between 15 and 30 L4 larvae were placed on *E. coli* strain HT115(DE3) transformed with indicated RNAi vectors (J. Ahringer, University of Cambridge, Cambridge, U.K.) as previously described (58).

RNA Levels. Total RNA was extracted using TRIzol reagent. For cDNA synthesis, mRNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed as previously described (49). See Table S3 for primer sequences.

Stress Induction. A total of 15–20 age-synchronized WT animals were used for each assay. For HS, animals were moved to new plates, sealed, and placed at 32 °C for 30 min. For induction of UPR, animals were shifted to 24-well plates and grown for 7 h in M9 buffer supplemented with bacteria (final OD_{600} 0.1) with or without 3 mM DTT. Approximately 15 animals were collected for each condition and frozen immediately following the stress.

Thermotolerance Assay. Animals were heat shocked at 35 °C for 6 h, and animals' survival was examined by touch-induced movement (49).

Lifespan. Assays were performed at 15 °C as previously described (24). As was previously reported for other cultivation temperatures, overexpression of HSF-1 or DAF-16 increased the lifespan of animals grown at 15 °C by 15%–40% (24, 59).

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