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 titrates 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, which 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.

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). 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
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 ts (triangles) 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. S1A), 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. 1E), coinciding with the complete disruption of myofilament structure detected by MYO-3::GFP localization (Fig. 1B). 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. 1A 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. S3A) (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. S3B) (30). By day 2 of adulthood, 50% of the dynamin(ts)-expressing animals failed to endocytose ssGFP (Fig. 2C). The rapid age-dependent decline in dynamin(ts) tissue-specific function coincided with mislocalization of dynamin(ts) (Fig. 2D and Fig. S3 C and D), similar to that observed with animals maintained at the restrictive condition. 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(kc21), unc-63(sx26), 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.

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. 4A). At day 4 and 7 of adulthood, the induction of these genes was dampened by 3- to 8-fold (Fig. 4B). 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. 4B), whereas the levels of the constitutively expressed hsc70 (hsp-1) was unaffected (Fig. 4C). 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
and DAF-16? Activation of these stress response factors by downregulation of age-1 (Fig. S5A) suppressed the ts phenotypes by 4-fold relative to control RNAi (Fig. S5A). Consistent with this observation, knockdown of either hsf-1 or daf-16 (Fig. S5A)

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 ± SEM, >45 animals per data point.

Fig. 4. Different stress responses are compromised early in adulthood. mRNA levels of (A) hsp70(C12CB1), hsp70(P44E5.4), 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.

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 dynamins(ts) animals overexpressing hsf-1 (triangles) [hsf-1(1-8), strain AM586] or daf-16 (squares) [daf-16(1-8), 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 dynamins(ts) animals overexpressing daf-16 (triangles) [daf-16(1-8), strain AM586] or hsf-1 (triangles) [hsf-1(1-8), strain AM707] were scored for percentage of animals showing Osrm 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% Osrm phenotype on day 9 of adulthood of rol-6 or WT animals, respectively).

and further exacerbated the loss of function of paramyosin(ts), and dynamin(ts) (Fig. 5A) and ras(ts) (Fig. 5B, 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. 5B). 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. 5A) was suppressed by HSF-1 or DAF-16 overexpression up to day 12 and 10 of adulthood, respectively (Fig. 5C). Likewise, the age-dependent increase in ras(ts) osm phenotype (as in Fig. 3A) was suppressed by HSF-1 or DAF-16 (Fig. 5D). 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.

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
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 aging (41). This is further supported by the finding that the loss of proteostasis in early adulthood corresponds to the same aging (41). This is further supported by the finding that the loss of proteostasis in early adulthood corresponds to the same aging (41). This is further supported by the finding that the loss of proteostasis in early adulthood corresponds to the same aging (41).

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 feeding were assayed as previously described (2, 20). 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 examined using a Zeiss Confocor 3/510 META confocal microscope through a 63 × 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 [S-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 and 1C and fragment 1C for myosin; see Fig. 1C) 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 OD600 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).
ACKNOWLEDGMENTS. We thank Dr. Ilya Ruvinsky and members of the Morimoto lab for critical discussions and reading of the manuscript. We thank the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources for some of the nematode strains. Antibodies 5-6 and 5-23 were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. These studies were supported by grants from the National Institutes of Health (National Institute of General Medical Sciences and National Institute on Aging).