Extended survival of misfolded G85R SOD1-linked ALS mice by transgenic expression of chaperone Hsp110

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Recent studies have indicated that mammalian cells contain a cytosolic protein disaggregation machinery comprised of Hsc70, DnaJ homologs, and Hsp110 proteins, the last of which acts to accelerate a rate-limiting step of nucleotide exchange of Hsc70. We tested the ability of transgenic overexpression of a Thy1 promoter-driven human Hsp110 protein, HspA4L (Apg1), in neuronal cells of a transgenic G85R SOD1YFP ALS mouse strain to improve survival. Notably, G85R is a mutant version of Cu/Zn superoxide dismutase 1 (SOD1) that is unable to reach native form and that is prone to aggregation, with prominent YFP-fluorescent aggregates observed in the motor neurons of the transgenic mice as early as 1 mo of age. The several-fold overexpression of Hsp110 in motor neurons of these mice was associated with an increased median survival from ∼5.5 to 7.5 mo and increased maximum survival from 6.5 to 12 mo. Improvement of survival was also observed for a G93A mutant SOD1 ALS strain. We conclude that neurodegeneration associated with cytosolic misfolding and aggregation can be ameliorated by overexpression of Hsp110, likely enhancing the function of a cytosolic disaggregation machinery.

ALS | chaperone | Hsp110 | SOD1 | survival

A number of neurodegenerative diseases are associated with misfolding and aggregation of specific proteins in the cytosol of particular neuronal cell types, including α-synuclein in the striatum in Parkinson's disease (1) and Cu/Zn superoxide dismutase 1 (SOD1) in motor neurons in ∼2% of ALS cases (2). The cytosol is a surprising location in which to find protein misfolding, even of these abundant proteins, because the cytosol is replete with molecular chaperones that, under normal conditions, prevent or reverse misfolding and aggregation of both nascent and preexisting proteins as an essential function. Notably, however, it appears that differentiated neurons fail to exhibit a "heat shock response"—that is, they appear unable to induce expression of chaperones to counteract misfolding under stress. For example, motor neurons facing a misfolded mutant G85R SOD1 in a transgenic G85R SOD1YFP mouse strain fail to exhibit significant induction of chaperone RNAs, with the exception of an approximately twofold induction of one of the cytosolic Hsp110 chaperones (HspH1) (3). Similar lack of induction has been observed for neurons in culture, where undifferentiated neuronal precursors exhibit induction of the major inducible cytosolic chaperone, Hsp70, upon stress exposure, but, once differentiated, fail to do so (4). The lack of chaperone induction in these contexts leaves open the question of whether, for example, forced overexpression of molecular chaperones could prevent or reverse misfolding of such abundant cytosolic proteins as SOD1 in expressing motor neurons.

Recent studies have suggested that mammalian cells contain a cytosolic disaggregase machinery (5) that performs an action homologous to the Hsp104 or ClpB AAA+ hexameric ring assemblies found in the cytoplasm of yeast/plants and bacteria (6), respectively, but which are lacking from the mammalian cytosol. In vitro, this machinery, comprised of a trio of chaperone components including Hsc70, DnaJA or DnaJB class chaperones, and an Hsp110, appears to be able to dissociate amorphous aggregates of luciferase (7) and even amyloid fibrils of α-synuclein (8). Hsc70, notably, is the most abundant molecular chaperone in the motor neuron cytosol and is constitutively expressed, whereas Hsp70 proteins are at least fivefold less abundant. DnaJ proteins are also present at low levels relative to Hsc70, but, as observed recently, both the DnaJA and DnaJB class proteins play a cooperating role in disaggregation (7). Similarly, the three mammalian Hsp110 proteins (HspA4, HspA4L, and HspH1) are present at relatively low levels, but, as noted above, HspH1 was the only chaperone found to be induced in G85R mutant SOD1YFP-expressing motor neurons in vivo (3). Hsp110s have an overall structure that resembles that of an Hsp70 class protein, but their function has been indicated to be that of a nucleotide exchange factor (9, 10), proffering an ATP binding pocket directly to that of Hsc70 (11), releasing ADP from substrate protein-bound Hsc70, and enabling ATP to enter the Hsc70 nucleotide pocket to discharge bound substrate protein. Although nucleotide exchange activity is well-established for Hsp110 proteins, it remains unclear whether they can also directly bind substrate proteins in vivo, as has been observed in vitro in several studies (12, 13). Regardless of whether Hsp110 proteins directly bind substrate proteins in vivo, ADP/ATP exchange appears to be the rate-limiting step in the Hsc70 protein folding cycle, suggesting a critical role for Hsp110 (5).

When mutant misfolded G85R SOD1YFP was immune-captured (through its folded YFP moiety) from the spinal cord of transgenic ALS mice, it brought down Hsc70, DnaJA1, and all three mammalian Hsp110 proteins (14). Thus, this disaggregase complex appears to recognize the mutant misfolded protein, but it is insufficient, even with the twofold induction of endogenous

Significance

Amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease) is a progressive paralyzing condition affecting ∼1:1,000 adults, associated with death of motor neurons, for which there is no effective treatment. ALS is inherited in 10% of cases, whereas the remainder are "sporadic," yet all behave very similarly. In mice, the condition has been modeled by transgenesis with mutant versions of superoxide dismutase 1 (SOD1) (∼2% of human cases). Mutant SOD1s misfold and form aggregates inside the cytosol of motor neurons in the spinal cord. Recently, a disaggregating machinery has been described consisting of three chaperones, one of which, Hsp110, plays a rate limiting role. Here, we have transgenically overexpressed Hsp110 in motor neurons of a mutant SOD1 strain and observed extended survival.

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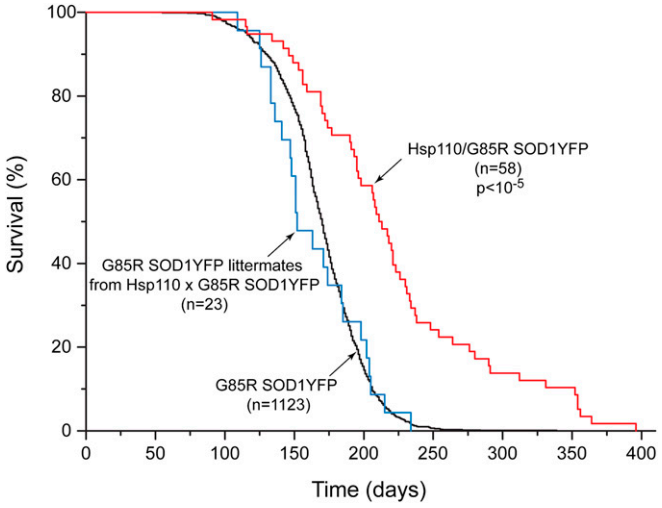

Fig. 1. Survival curves of homozygous G85R SOD1YFP mouse strain (black trace) and backcross progeny containing homozygous G85R SOD1YFP and hemizygous 106 copy Thy1-Hsp110 transgene, showing increased survival of transgenic mice with Thy1-Hsp110 and G85R SOD1YFP (red trace) compared with littermates with only G85R SOD1YFP (blue trace). Median survival was extended by 62 d relative to littermates and by 42 d relative to the entire G85R SOD1YFP cohort. *n*, number of animals of the designated genotype. *P* value, significance of difference between survival of transgenic Thy1-Hsp110/G85R SOD1YFP (red) and G85R SOD1YFP littermates (blue), determined by log-rank test (see *Methods, Survival Curves*).

HspH1, to forestall progression to motor deficit and paralysis. Here, we observe that providing additional Hsp110 from a transgene does, in fact, forestall progression of motor disease, indicating that, for this misfolding condition, the supply of additional amounts of a molecular chaperone that affects the presumed rate-limiting step of the disaggregase can have a clinical benefit.

Results

Thy1-Hsp110 Transgene Introduced into G85R SOD1YFP ALS Strain Produces Extended Survival. A strain of G85R SOD1YFP homozygous transgenic mice with transgene copy numbers between 210 and 320, on a B6/SJL background, has been followed over several years, observing that all of these mice develop ALS, reaching end-stage lower extremity paralysis by 6.5 mo of age (Fig. 1, black line). We sought to modify the timing of this end-stage phenotype by crossing in various transgenes, initially expressed in B6/SJL, then back-crossing to produce hemizygosity of the modifier and homozygosity of G85R SOD1YFP (210–320 copy number of the latter, ascertained in all mice by real-time PCR). Transgenes including Tet-regulated PGC1α cDNA, Thy1-driven mitochondrial-targeted human catalase cDNA, and Thy1-driven bovine Hsc70 cDNA did not significantly affect the time of survival. In contrast, a transgene composed of the Thy1 promoter driving human Hsp110 cDNA (HspA4L; Apg1) produced a substantial extension of survival (Fig. 1*A*, red line), with a median survival ∼2 mo longer relative to littermates lacking the Hsp110 transgene (blue line) and ∼1.5 mo longer relative to the cohort of parental G85R SOD1YFP animals (black line). There was a maximum extension of survival of ∼6 mo, approximately doubling the usual life span. A second transgenic line with a lower Thy1-Hsp110 copy number (36 vs. 106 copies) produced almost the same extension of survival (Fig. S1). A third line with both the 106 copy Thy1-Hsp110 transgene and a Thy1-Hsc70 transgene exhibited improvement in survival similar to that of the 106 copy Hsp110 line (Fig. S2).

We stratified the survival curve of the 106 copy Thy1-Hsp110/G85R SOD1YFP line in relation to the copy number of G85R SOD1YFP, observing that mice with a copy number of 270 or greater did not achieve improved survival with the Hsp110 transgene compared with the G85R SOD1YFP mice with a copy number of 210–270 (Fig. 2). This difference of effect on survival would suggest that Hsp110 transgene expression (over and above that of endogenous mouse Hsp110s) and participation in the

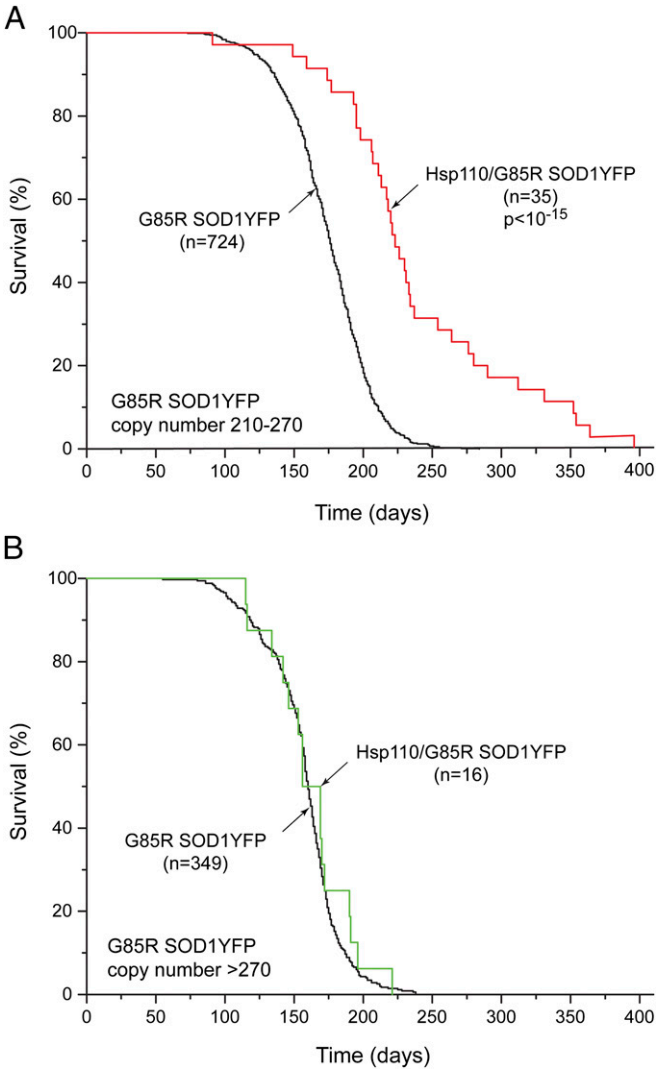

Fig. 2. Stratification of survival of Thy1-Hsp110/G85R SOD1YFP transgenic mice relative to copy number of G85R SOD1YFP. (*A*) The survival curve of Thy1-Hsp110/G85R SOD1YFP mice with a G85R SOD1YFP copy number between 210 and 270 (red trace) compared with the survival curve of G85R SOD1YFP mice with a copy number between 210 and 270 (black). There is substantial prolongation of survival, similar to that observed in Fig. 1 (e.g., 56 d median survival here vs. 62 d in Fig. 1). (*B*) The survival curve of Thy1-Hsp110/G85R SOD1YFP transgenic mice with a G85R SOD1YFP copy number greater than 270 (green trace) compared with the survival curve of mice of the G85R SOD1YFP strain with a copy number greater than 270 (black trace). Note that the survival curves are virtually superposable, indicating no beneficial effect of transgenic Thy1-Hsp110 on survival of mice with G85R SOD1YFP copy numbers greater than 270. Note also that the survival curve of the G85R SOD1YFP strain with copy number greater than 270 is slightly shifted to the left relative to the curve of this strain with 210–270 copies as shown in *A*, indicating that the survival of animals with the larger copy number is decreased (median survival reduced by ∼15 d). *n* specifies number of mice of the designated genotype, and *P* values were determined as in Fig. 1.

Hsc70/DnaJ/Hsp110 disaggregase system cannot exert a significant effect on mutant G85R SOD1YFP when the mutant protein is expressed beyond the level produced by 270 copies.

The 106 copy Thy1-Hsp110 transgene was also crossed into G93A SOD1 (hemizygous transgenic) ALS mice, also carried in a B6/SJL background [JAX Tg(SOD1-G93A)1Gur/J]. Here, we examined five litters, comparing survival of G93A progeny with littermates carrying both G93A and Hsp110 transgenes (Fig. S3, blue and red plots, respectively, with survival of the individual mice from each litter represented by specifically colored X's). As with G85R SOD1YFP, the presence of the Hsp110 transgene was associated with an extension of survival (Fig. S3). The G93A SOD1 progeny ($n = 9$) developed paralysis by 97–120 d of age, whereas the Thy1-Hsp110/G93A SOD1 mice ($n = 10$) paralyzed at 128–140 d of age. Median survival of the 106 copy Thy1-Hsp110/G93A SOD1 mice was lengthened by 29 d (105 vs. 134 d).

Transcription and Translation of Hsp110 Transgene in Motor Neurons. Expression of the 106 copy Thy1-Hsp110 transgene was examined at both the RNA and protein level in motor neurons. At the RNA level, quantitative RT-PCR (qRT-PCR) of RNA isolated from laser-captured motor neurons of the 106 copy Hsp110/G85R SOD1YFP line was carried out, and the amount of human HspA4L RNA was compared with endogenous mouse Hsp110 (HspA4L; Apg1) RNA (Fig. 3*A*). In three mice examined, the level of transgenic human Hsp110 RNA was 7- to 12-fold greater than that of the endogenous mouse Hsp110 RNA (Fig. 3*A*).

To exclude the possibility that transgenic human Hsp110 expression could be improving survival simply by reducing the level of G85R SOD1YFP RNA expression in motor neurons, the Thy1-Hsp110/G85R SOD1YFP motor neurons were also analyzed for G85R SOD1YFP mRNA levels and compared with motor neurons from G85R SOD1YFP (lacking the Hsp110 transgene), measuring transgene RNA levels in both cases relative to an internal reference of endogenous mouse SOD1 RNA (Fig. 3*B*). The level of SOD1YFP RNA was unaffected by the presence of the Hsp110 transgene: In both strains, the level of transgenic G85R SOD1YFP RNA was ∼70-fold greater than that of endogenous mouse SOD1 RNA (Fig. 3*B*).

At the protein level, motor neurons were directly inspected for Hsp110 protein by immunostaining with a polyclonal antibody (Santa Cruz Biotechnology) raised against a peptide from the C-terminal region of Hsp110 (also see Fig. S4 for Western blot of total spinal cord). We observed strong anti-Hsp110 immunostaining of motor neurons in Hsp110/G85R SOD1YFP cord cross-sections (Fig. 4, *Right*, red channel), whereas G85R SOD1YFP animals lacking the Hsp110 transgene showed only a faint signal in their motor neurons (Fig. 4, *Left*, red channel). Considering that the antibody was produced against a peptide that is identical in human and mouse Hsp110 (HspA4L; Apg1), we conclude that there is considerably more total Hsp110 in the motor neurons of the Thy1-Hsp110/G85R SOD1YFP mice, compared with the endogenous mouse Hsp110 in the G85R SOD1YFP strain. Although Western blot analysis of total spinal cord lysate showed that the Thy1-Hsp110/G85R SOD1YFP mice exhibited ∼2.5-fold more Hsp110 than G85R SOD1YFP mice (Fig. S4), immunostaining suggested that the level of Hsp110 present specifically in spinal cord motor neurons was substantially greater.

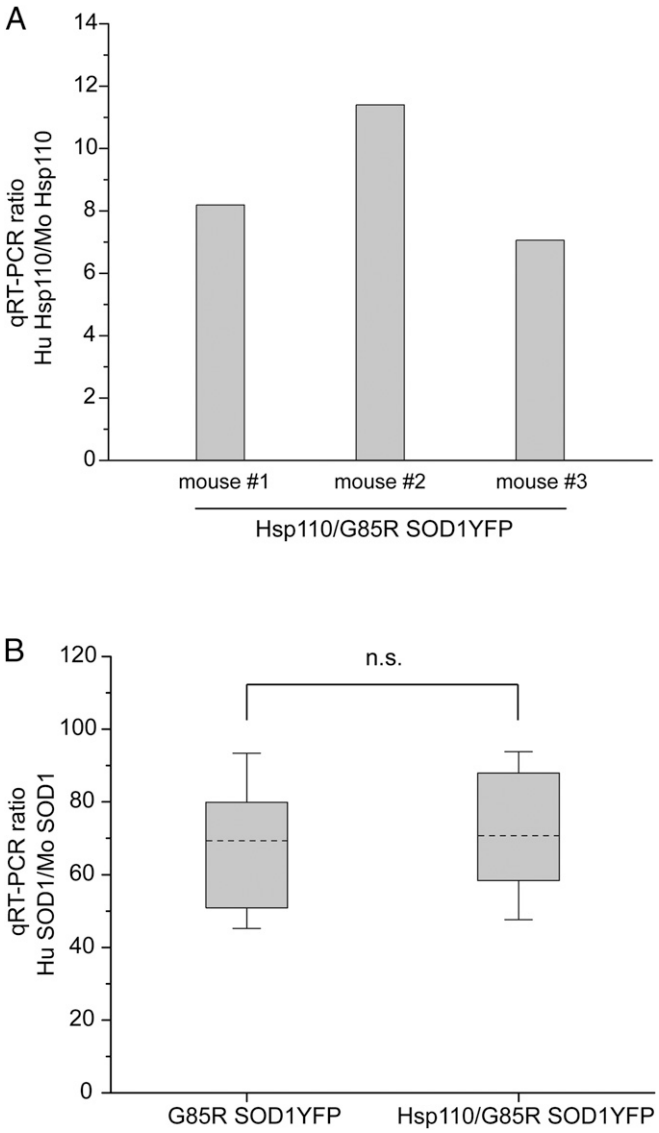

Fig. 3. qRT-PCR analysis of RNA levels of transgenic Thy1-Hsp110 and G85R SOD1YFP from laser-captured spinal cord motor neurons, measured as ratios of transgene-derived RNA to RNA from corresponding endogenous mouse gene. *A* shows the ratio of Hsp110 (HspA4L/Apg1) from the human transgene relative to endogenous mouse Hsp110 (HspA4L/Apg1) from motor neuron RNA of three different mice transgenic for Thy1-Hsp110/G85R SOD1YFP. The human transgene-derived Hsp110 RNA was ∼10-fold more abundant than endogenous mouse Hsp110 RNA (see *Methods* for details). *B* shows the ratio of transgenic G85R SOD1YFP to endogenous mouse SOD1 for either the G85R SOD1YFP strain or the Thy1-Hsp110/G85R SOD1YFP for three mice of each strain. The data are plotted as a whisker plot, showing no significant difference in the level of G85R SOD1YFP RNA between the G85R SOD1YFP strain and the Hsp110/G85R SOD1YFP strain ($P > 0.9$ by two-sample *t* test in Origin). Dashed lines are the means, top and bottom of the boxes are 75th and 25th percentile and whiskers comprise 1.5 SD above and below the mean.

Discussion

It seems likely that the action of transgenic Hsp110 to improve the survival of both G85R SOD1YFP and G93A SOD1 mice is attributable to an additional supply, beyond the endogenous level, of a molecular chaperone component that functions as part of a disaggregation machinery in the metazoan cytosol, composed of Hsc70, DnaJ, and Hsp110. This machinery has been shown in a number of cases in vitro to dismantle soluble aggregates (7) and recently, also in vitro, to dissociate amyloid fibrils composed of α-synuclein (8). The currently understood action of Hsp110 is to catalyze nucleotide exchange of Hsc70 (removing bound ADP from substrate-bound Hsc70, enabling ATP binding and substrate protein release). Here, the favorable action of providing additional Hsp110 would suggest that this component may be rate-limiting in vivo in the disaggregation of misfolded G85R SOD1YFP inside motor neurons. Notably, G85R mutant forms of SOD1 appear incapable of reaching the native state. In particular, G85R SOD1 subunits fail

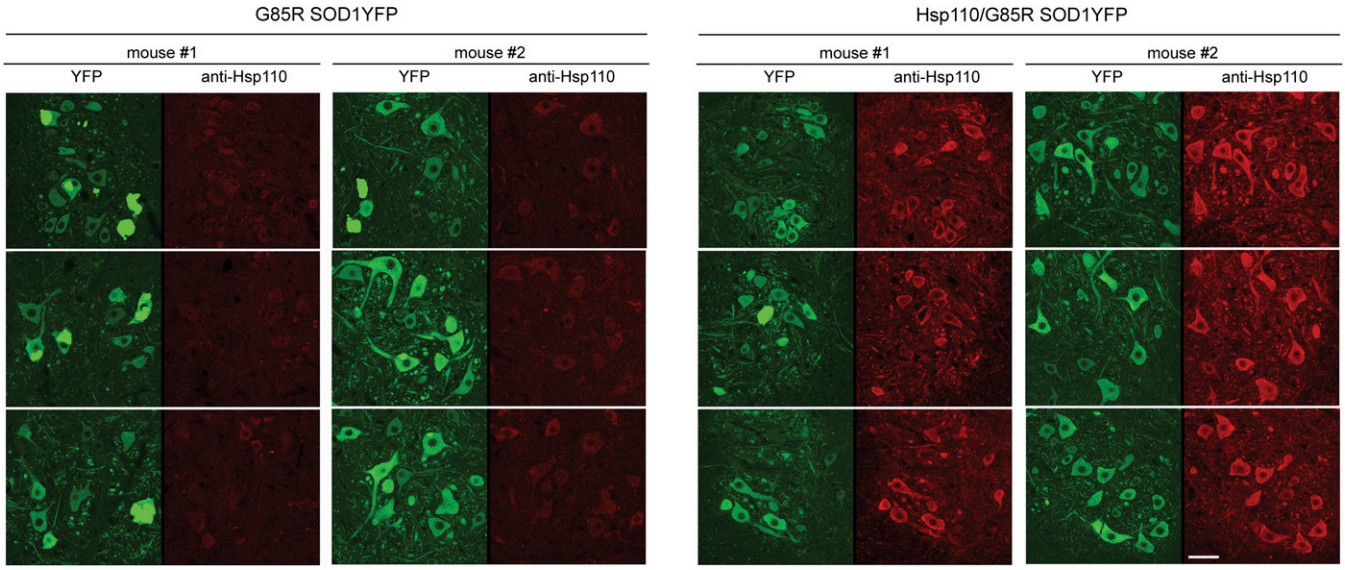

Fig. 4. Expression of Hsp110 protein in motor neurons of Thy1-Hsp110/G85R SOD1YFP strain determined by immunostaining. Twenty-micrometer transverse lumbar sections of fixed spinal cords were prepared from 3-mo-old mice of the G85R SOD1YFP strain or of Thy1-Hsp110/G85R SOD1YFP strain and immunostained with a polyclonal antibody directed against a peptide from the C-terminal region of human Hsp110 (Santa Cruz Biotechnology), as described in *SI Methods*. (*Left*) YFP-fluorescent large motor neurons observed in the ventral horn. (*Right*) Fluorescence of the motor neurons in the red channel from anti-Hsp110 immunostaining, showing weak signals from endogenous mouse Hsp110 in sections from the G85R SOD1YFP strain and strong signals in sections from the Thy1-Hsp110/G85R SOD1YFP strain. This observation is consistent with the presence of additional Hsp110 protein in the motor neurons of the double transgenic mice. See text for details. (Scale bar: 50 μm.)

to be metallated, fail to form the normal disulfide bond, fail to homodimerize, and have no enzymatic activity (15–17). The abundant misfolded states are evidently prone to aggregation in vivo, considering the large fluorescent aggregates observed in motor neurons of G85R SOD1YFP mice as early as weaning age, and, as such, provide a substrate for the Hsc70/DnaJ/Hsp110 disaggregation machinery. In contrast, G93A SOD1 substantially populates the native active state, but it also populates nonnative, aggregation-prone forms (15, 18). This latter mutant form was also affected by the presence of the Hsp110 transgene, judging from the increased survival data, albeit survival was not extended as much as for G85R SOD1YFP.

The potentially more efficient disaggregation system provided by higher levels of Hsp110 from a transgene makes the prediction that formation of visible YFP fluorescent aggregates by G85R SOD1YFP should be reduced in the motor neurons of the double transgenic mice. This prediction was difficult to evaluate because analysis of spinal cord morphology of any given animal precludes measuring its long-term survival. Thus, we were unable to directly correlate the reduction or absence of aggregates at 3 mo of age with the greatest prolongation of survival. [Note that the presence of aggregates in ventral horn motor neurons of G85R SOD1YFP mice is prominent between 1 and 3 mo of age but then is substantially reduced thereafter (19)]. However, our observation from six double transgenic animals killed at 2.5 mo of age for morphology analysis was that two animals of the six exhibited strikingly low levels of aggregate formation. In contrast, we have not observed such absence of aggregation in large numbers of 2- to 3-mo-old G85R SOD1YFP mice. In such animals, we routinely observe prominent aggregate formation affecting many of the ∼20 large motor neurons in each ventral horn of any given 20-μm section of spinal cord. We can only speculate that the double transgenic mice lacking aggregates would have been longest-term survivors.

Concerning the longest-term Hsp110 transgenic survivors, 1 y in the case of the 106 copy transgene, it is possible that the long survival in part relates to polymorphism in the B6/SJL background. That is, whereas transgenic mice were produced by injection into heterozygous zygotes, subsequent breeding might produce population heterogeneity at any given locus for B6 and SJL alleles, which, if particular alleles have influence on disease initiation and progression, could further modify the survival (see, e.g., ref. 20). Arguing against a strong effect of heterogeneity in the Hsp110 transgenic strains, however, is the stratification data, which seem to point toward a strict effect of transgenic Hsp110, such that when the amount of the G85R SOD1YFP mutant substrate exceeds the apparent capacity of Hsp110 to enhance survival (>270 copies), there is complete collapse of the survival curve to that of the G85R SOD1YFP strain (Fig. 2). It remains possible, however, that the longest-surviving Hsp110 transgenic animals might have background polymorphisms that, for example, enhance Hsp110 production.

The loss of beneficial effect of Hsp110 when G85R SOD1YFP exceeds 270 copies also argues that the principal action of the Hsc70/DnaJ/Hsp110 system is upon G85R SOD1YFP as its substrate, rather than other known actions of the chaperone system, e.g., in facilitating clathrin uncoating in the vesicle recycling system. In support of direct action on the mutant protein, in an earlier study (14), we observed physical association of all three mouse Hsp110 proteins, as well as DnaJA1 and Hsc70, with G85R SOD1YFP in coimmunoprecipitation of spinal cord lysates with anti-YFP antisera. This observation thus supports a primary action of Hsp110 on the mutant misfolded protein, most likely mediated via association of Hsp110 with substrate-bound Hsc70.

Recent in vitro studies of Hsp110 in heterologous systems also support a role in detoxifying misfolded protein species. Addition of G85R SOD1YFP to squid axoplasm inhibits anterograde axonal vesicle transport, but purified human Hsp110 (HspA4L) prevents this effect even when added at substoichiometric amounts relative to the misfolded protein, suggesting that it cooperates with endogenous Hsc70 and DnaJ homologs to dissociate toxic oligomeric forms (21). In studies in *Drosophila* eye, transgenic expression of either *Drosophila* Hsp110 or human

Hsp110 (HspA4L; APG1) was able to rescue toxicity from coexpressed polyQ-expanded proteins (22, 23). As mentioned, in motor neurons of the G85R SOD1YFP line, one of three endogenous mouse Hsp110 RNAs, HspH1, is induced approximately twofold compared with wtSOD1YFP motor neurons, one of the few RNAs observed to be altered (3). This endogenous induction falls well short of the 10-fold Hsp110 (HspA4L) RNA induction observed in the 106 copy Thy1-Hsp110/G85R SOD1YFP line. It remains to be seen whether small molecule inducers of Hsp110 could be identified that might be used therapeutically.

Methods

Mouse Strains. Mouse studies were carried out under a protocol approved by the Yale University Animal Care and Use Committee. The transgenic G85R SOD1YFP strain (containing greater than 210 copies of the transgene via homozygosity, referred to as the 737 line) has been described (14). By 3–4 mo of age, hind-limb clenching and rotarod dysfunction is observed (19), and lower extremity paralysis develops by 6.5 mo of age. The strain transgenic for Thy1-human Hsp110 (HspA4L) was produced by injecting B6/SJL zygotes (Yale Transgenic Mouse Service) with a construct described in *SI Methods*. Mice hemizygous for the Thy1-Hsp110 transgene were crossed with homozygous G85R SOD1YFP mice, and offspring hemizygous for both Thy1-Hsp110 and G85R SOD1YFP were crossed with G85R SOD1YFP homozygous mice to produce mice hemizygous for Thy1-Hsp110 and homozygous for G85R SOD1YFP. Mice from the second cross that were homozygous for G85R SOD1YFP but lacked the Thy1-Hsp110 transgene were followed as littermate controls for the survival curve of Fig. 1. Two Thy1-Hsp110 strains were examined, one designated "106 copy" (Fig. 1), the other "36 copy" (Fig. S1), based on the copy number of the respective founder mice. Similar copy number was ascertained in progeny mice by real-time PCR. A "bichaperone" strain was produced by crossing (106 copy)Thy1-Hsp110$^{+/-}$/G85R SOD1YFP$^{+/+}$ mice with Thy1-Hsc70$^{+/-}$/G85R SOD1YFP$^{+/+}$ mice that had been similarly produced and selecting the Thy1-Hsc70$^{+/-}$/Thy1-Hsp110$^{+/-}$/G85R SOD1YFP$^{+/+}$ offspring. The Thy1-Hsp110/G93A mice were produced by crossing hemizygous 106 copy Thy1-Hsp110 female mice with (hemizygous) G93A male mice in the B6/SJL background [the latter the B6SJL-Tg(SOD1-G93A)1Gur/J, 002726, strain from Jackson Laboratory]. Offspring that were double transgenic or contained only the G93A transgene were compared for survival until paralysis. Note that 106 copy Thy1-Hsp110 transgenic mice, either hemizygous or homozygous, are clinically normal and exhibit long-term survival (>18 mo currently).

All mice were genotyped by real-time PCR by using genomic DNA prepared from tail biopsies (DNeasy Blood & Tissue Kit; Qiagen). Mouse ApoB was used as the reference gene. Primer sets for mouse ApoB and human SOD1 were suggested by Jackson Laboratory on their website (https://www.jax.org). Primer sets for the chaperone transgenes consisted of a forward primer in the Thy1.2 sequence and a reverse primer near the beginning of the chaperone coding sequence. Each of these primers was selected manually and confirmed by its efficiency in quantitative PCR, its lack of amplification when template DNA did not contain the respective transgene, and by sequencing the PCR product. To help ensure a consistent ALS phenotype, only G85R SOD1YFP mice with a homozygous copy number >210 were used in the reported experiments.

Survival Curves. Survival curves (paralysis and euthanasia as end-point) and statistical significance (χ^2 and P values) between the survival functions for different strains were generated in OriginPro by using the Kaplan–Meier Estimator function for the log-rank, Breslow, and Tarone–Ware tests. Each gave similar results, and the log-rank test results are reported.

Transgenic constructs, RNA preparation and qRT-PCR, Hsp110 immunostaining, and Western blotting are detailed in *SI Methods*.

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