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A Novel mouse model of enhanced proteostasis: Full-length human heat shock factor 1 transgenic mice

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ABSTRACT

The heat shock response (HSR) is controlled by the master transcriptional regulator heat shock factor 1 (HSF1). HSF1 maintains proteostasis and resistance to stress through production of heat shock proteins (HSPs). No transgenic model exists that overexpresses HSF1 in tissues of the central nervous system (CNS). We generated a transgenic mouse overexpressing full-length non-mutant HSF1 and observed a 2–4-fold increase in HSF1 mRNA and protein expression in all tissues studied of HSF1 transgenic ($HSF1^{+/0}$) mice compared to wild type (WT) littermates, including several regions of the CNS. Basal expression of HSP70 and 90 showed only mild tissue-specific changes; however, in response to forced exercise, the skeletal muscle HSR was more elevated in $HSF1^{+/0}$ mice compared to WT littermates and in fibroblasts following heat shock, as indicated by levels of inducible HSP70 mRNA and protein. $HSF1^{+/0}$ cells elicited a significantly more robust HSR in response to expression of the 82 repeat polyglutamine-YFP fusion construct (Q82YFP) and maintained proteasome-dependent processing of Q82YFP compared to WT fibroblasts. Overexpression of HSF1 was associated with fewer, but larger Q82YFP aggregates resembling aggresomes in $HSF1^{+/0}$ cells, and increased viability. Therefore, our data demonstrate that tissues and cells from mice overexpressing full-length non-mutant HSF1 exhibit enhanced proteostasis.

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1. Introduction

The master transcriptional regulator of chaperones is heat shock factor 1 (HSF1), and its activation is governed by multiple levels of regulation such as stress, unfolded proteins [1], post-translational modifications [2–4], and exercise [5]. Activation of HSF1 can lead to upregulation of multiple cytoprotective chaperones, and initiation of a protective response known as the heat shock response (HSR). The HSR is central in providing protection from proteotoxic stressors encountered in neurodegenerative diseases and aging [6]. Chaperones, or heat shock proteins (HSPs) ensure that proteins remain either properly folded or are directed to appropriate degradative pathways such as the proteasome [7] or lysosome [8,9]. The HSR and HSF1 may also exhibit tissue-specific differences in activation threshold as tissues of the CNS have been reported to have a high threshold for HSF1 induction [10]. HSF1 is maintained as a cytosolic monomer in a negatively regulated state by interaction with a multi-chaperone complex composed of HSP90 and immunophillins [11]. Misfolded proteins generated during heat or oxidative stress compete with HSF1 for HSP90 and thus, lead to HSF1 oligomerization and activation [12]. Following its activation, cytosolic HSF1 monomers trimerize and translocate to the nucleus where they can bind to target genes via conserved heat shock elements (HSEs) [13].

Because HSPs are cytoprotective and have shown to protect against a variety of cellular insults, there is substantial interest in utilizing HSPs in treating diseases/pathologies that involve protein misfolding/aggregation. However, because individual HSPs may not reconstitute the complex and beneficial effects of the chaperone networks they form, it has been argued that modulating the HSF1 transcriptome through overexpression of HSF1or enhancing its activity is a better approach to treating proteotoxic insults rather than overexpression of a single HSP [14]. Overexpression of full-length HSF1 was shown to protect against polyglutamine containing proteins in worms [15] and extend their longevity [15,16]; Importance of HSF1 in protection from neurodegenerative disease is underlined by observations that mice lacking HSF1 have

Abbreviations: HSF1, heat shock factor 1; HSPs, heat shock proteins; CNS, central nervous system; WT, wild type; HSR, heat shock response; YFP, yellow fluorescent protein; BAC, bacterial artificial chromosome.

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a neurodegenerative phenotype [17], have decreased neurogenesis [18], and increased prenatal lethality [19]. Mice overexpressing a truncated constitutively active HSF1 have been generated and protect from polyglutamine when expressed in cells [14], however, these mice fail to overexpress HSF1 in the CNS. Many neurodegenerative diseases commonly involve expression of proteotoxic proteins and deposition of protein aggregates in tissues of the CNS, and thus, overexpression of HSF1 would provide a means to test the role of HSF1 in proteostasis in these tissues. In this study we have generated and characterized the first transgenic mouse model overexpressing full-length non-mutant HSF1 in all tissues. Cells and tissues of these transgenic mice show increased proteostasis in response to a variety of stressors.

2. Materials and methods

2.1. Animals

$HSF1^{+/0}$ mice were generated and maintained on a C57Bl/6 J background, and used at the ages indicated. All procedures for handling animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the Institutional Animal Care and Use Committee at the Audie L. Murphy Memorial Veterans Hospital, San Antonio, Texas.

2.2. Preparation of human HSF1 fragment

The BAC clone 2274G4 containing the entire human HSF1 gene was obtained from Invitrogen (Carlsbad, CA). Supercoiled BAC DNA was restriction digested with *Sgr*AI in order to generate a 50.8 Kb fragment including 26 Kb upstream and 11.7 Kb downstream of the 13.1 Kb human HSF1 gene. Transgenic mice were developed on a C57Bl/6 background, and six primers were used that span the human HSF1 gene to screen for potential founders according to the sequences given in Supplementary Table S1.

2.3. RNA isolation and qRTPCR

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed with the RetroScript Kit (Biorad, Hercules, CA) using Oligo dT and random decamer first strand primers. Quantitative real-time PCR was performed using Jumpstart SYBR green master mix (Sigma Aldrich) on an Applied Biosystems 7900HT Fast Real-Time PCR System. PCR efficiency for each primer set was determined with serially diluted cDNA, and relative mRNA quantities were determined using the efficiency-corrected ΔCt method and normalized by GAPDH. Sequences of primers used are given in Supplementary Table S1.

2.4. Immunoblotting

Tissues were homogenized in 250 mM mannitol, 75 mM sucrose, 500 μM EGTA, 100 μM EDTA, and 10 mM HEPES pH 7.4 containing a cocktail of protease inhibitors (Calbiochem) using a glass Teflon homogenizer in order to isolate a cytosolic fraction following centrifugation at 100,000*g* for 1 h at 4 °C. For *in vitro* experiments, cells were washed once with phosphate buffered saline and scraped and sonicated in RIPA buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100, 2% SDS, 0.5% deoxycholic acid, pH 7.6. Protein concentration was determined with the bicinchoninic acid method (Thermo, Rockford, IL), and protein was diluted in reducing Laemmli buffer and 30 μg of protein was loaded per lane and separated by 12% SDS PAGE as described [20]. Primary antibodies against HSF1 (Stressgen SPA-901), inducible HSP70 (Stressgen SPA-810), HSP90 (Stressgen SPA-846), HSP25 (Stressgen SPA-801), β-Actin (Sigma A-5060), GAPDH (Sigma G-9545), GFP (Chemicon AB3080) were used according to instructions. Following washing with TBST containing 0.2% milk, antibodies were developed by incubating with secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Sc-2054), Alexa 647, Alexa 680 (Invitrogen A21446, A21000, respectively), or IRdye 800CW (LiCOR Lincoln, NE #3221). Alexa 647 and horseradish peroxidase (HRP) conjugated antibodies were visualized by a Typhoon 9410 variable mode imager (GE Healthcare). Alexa 680 and IRdye 800CW antibodies were detected with an Odyssey Imaging System (LiCOR).

2.5. Cell culture

Primary fibroblast cultures were obtained from neonatal mouse tails and skin 1–3 days of age as described [21]. Cells were washed and trypsinized with 0.05%Trypsin/EDTA and aliquotted for cryopreservation. One cell passage was defined as treatment of confluent flasks with trypsin and reseeding in T150 flasks at 1×10^6 cells. Experiments were performed with passage 1–4 cells in F12 medium, while seeder flasks were maintained in DMEM/F12 50/50.

2.6. Immunofluorescence microscopy

Cells were seeded onto glass coverslips at 0.25×10^6/well in 6-well dishes and after 24 h were washed and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) following heat shock treatments or transfection with polyQ19-YFP or Q82-YFP constructs (a kind gift from Dr. Richard Morimoto) using Fugene 6 (Roche). For HSF1 nuclear localization experiments cells were incubated with rabbit anti-HSF1 (Stressgen SPA-901) at 1:50 in humidified 100×15 mm petri dishes for 1hr. Antibody signals were detected using secondary goat anti-rabbit Alexa 488 (Invitrogen) at 1:100 followed by mounting in Vectashield mounting medium with DAPI (Vectorlabs, Burlingame, CA). Transfected cells were fixed and immediately mounted. Cells were visualized with a Zeiss Axioscope Axiovert 200 M and images were acquired using a AxioCam MRm camera and AxioVision software version 4.8. Post-processing including uniform contrast and brightness adjustments were performed equally across all comparative images with Adobe Photoshop 7.0. Aggregates associated with YFP fluorescence were quantitated using Image J version 1.41o.

2.7. Statistical analysis

Statistical significance was determined using GraphPad Prism 5.01 software (GraphPad, San Diego, CA, USA). Two-tailed unpaired students *t* test or two way ANOVA was used to determine statistical significance between groups at the $p < 0.05$ level using Dunnet's post hoc test to determine significant differences from baseline measurements.

3. Results and discussion

3.1. Mice overexpressing human HSF1 exhibit elevated HSF1 and altered HSP expression

In order to generate mice overexpressing full-length human HSF1, a bacterial artificial chromosome (BAC) clone transgenic approach was adopted to generate transgenic mice on a C57Bl/6 background. BAC clone 2274G4 was digested with *Sgr*AI in order to generate a 50.8 Kb fragment containing the 13.1 Kb human HSF1 gene, removing 13 of the 16 3′ exons of the block of proliferation 1 (BOP1) gene and promoter and first exon of diacylglycerol O-acyltransferase homolog 1 (DGAT1) (Fig. 1A). In total, six transgenic founders were identified, four males and two females

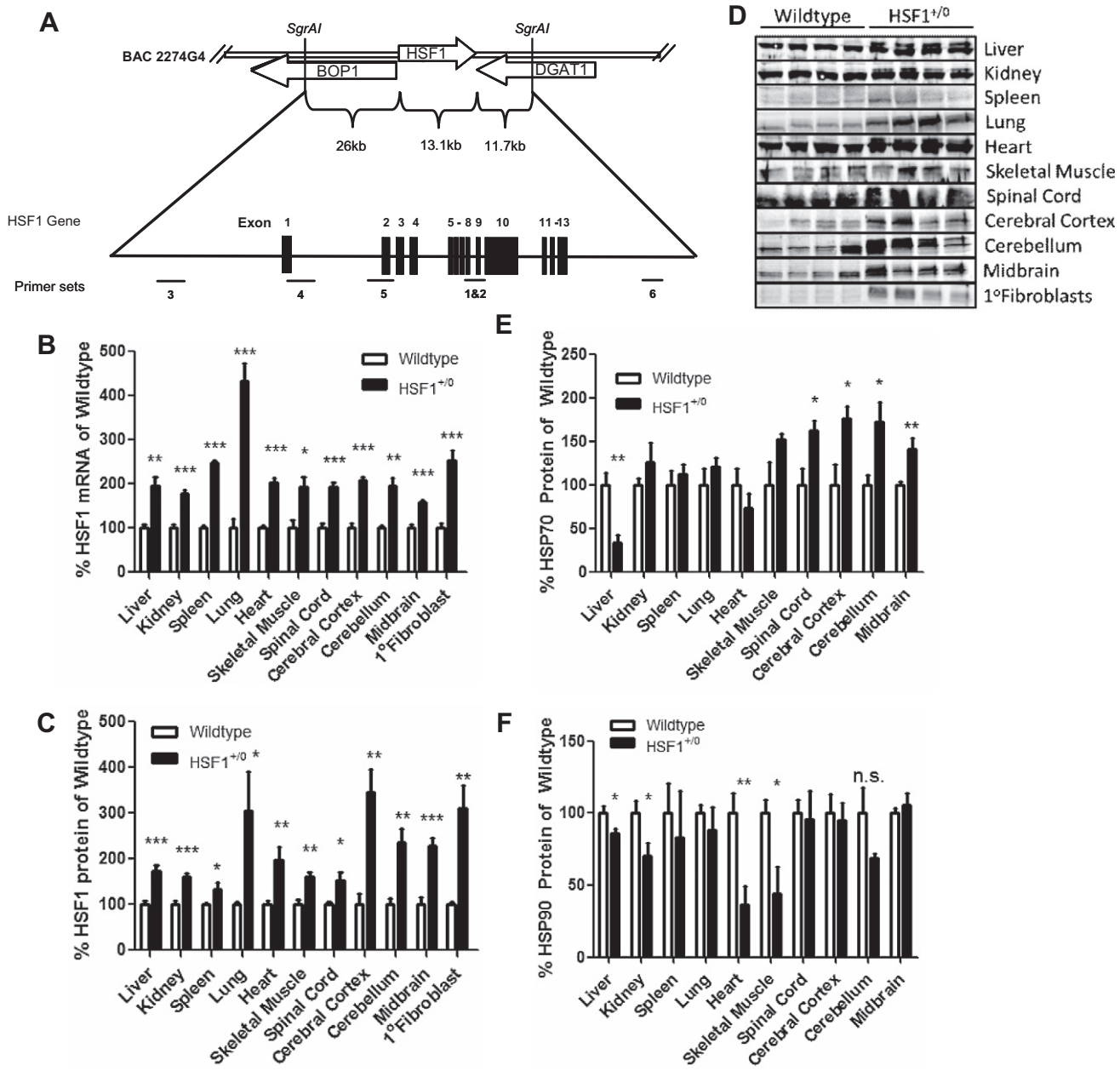

Fig. 1. Human HSF1 transgene fragment and tissue HSF1 and HSP levels. (A) BAC clone 2274G4 and strategy for screening founders using a set of PCR primer pairs. (B) Quantitative PCR of total HSF1 mRNA in tissues from 12- to 14-month-old male HSF1$^{+/0}$ (closed bars) and WT littermates (open bars). (C) Immunoblot quantification of total cytosolic HSF1 in tissues and primary fibroblasts (1°Fibroblast). (D) Immunoblot of total cytosolic HSF1 levels in tissues. (E) Quantitation of HSP70 immunoblot from 12- to 14-month-old male HSF1$^{+/0}$ (closed bars) and WT littermates (open bars). (F) Quantitation of HSP90 immunoblot. Bars represent average of four mice/group +/- SEM. $^{*}p = 0.05$, $^{**}p = 0.01$, $^{***}p = 0.001$.

(Supplementary Fig. S1A). HSF1$^{+/0}$ mice and their offspring showed no gross abnormalities or defects in fertility, and exhibited normal body weight and normalized tissue weights from 4 to 12–14 months of age (data not shown). In order to determine whether HSF1mRNA and protein are overexpressed in HSF1$^{+/0}$ mice, tissues from offspring (line 128) were collected from 12–14 month old mice and total HSF1 mRNA was quantitated by quantitative real-time PCR (qRTPCR). HSF1 mRNA was overexpressed in all tissues examined, with transgenic mice overexpressing HSF1 mRNA 2-fold higher than WT littermates on average across all tissues examined, with lung as high as 4-fold (Fig. 1B). In order to determine whether suppression of other HSFs occurred as a result of HSF1 overexpression, HSF2 and HSF4 mRNA were also measured across the same tissues and were unchanged in most tissues (Supplementary Fig. S2). Cytosolic protein was isolated from the same tissues and immunoblotted for HSF1 in order to determine the degree of HSF1 protein overexpression (Fig. 1C and D) and in general, we found a 2-fold increase in most tissues. Importantly, transgenic mice had 2 to 3-fold higher HSF1 in all areas of the CNS examined, including spinal cord, cerebellum, cerebral cortex, and midbrain. Similar results were also obtained from the 127 line (data not shown). To determine if the overexpression of HSF1 has an impact on basal levels of heat shock proteins, we measured the levels of HSP70 as well as HSP90 (because of its role in negative regulation of HSF1) in tissues of the HSF1$^{+/0}$ and WT mice. Basal HSP70 levels were increased ~2-fold in CNS tissues; however, in other tissues there was no significant difference in HSP70 levels except for liver, which showed a 66% decrease (Fig. 1E&F). Basal levels of HSP90

levels were either unchanged or reduced in the HSF1$^{+/0}$ mice; the decrease was greatest in heart (63%), and skeletal muscle (56%).

3.2. HSF1$^{+/0}$ mice have an enhanced heat shock response at the tissue and cellular level

It is known that exercise leads to induction of HSP70 in skeletal muscle; therefore, we subjected HSF1$^{+/0}$ and WT mice to forced exercise, and measured HSP70 levels by immunoblot in the gastrocnemius muscle after a 24 h recovery (Fig. 2A). WT and HSF1$^{+/0}$ mice had similar levels of HSP70 prior to exercise. HSP70 levels were induced 60% in WT mice after a bout of forced exercise; HSP70 levels were induced 90% in HSF1$^{+/0}$ mice. To determine whether cells from HSF1$^{+/0}$ mice exhibited an enhanced HSR, we measured the HSR in primary cultures of fibroblasts from HSF1$^{+/0}$ and WT mice. Cells were heat shocked for 30 min at 42 °C and HSP70 mRNA levels were measured after initiation of the heat shock at the indicated times as shown in Fig. 2B. HSP70 mRNA levels were found to be induced more rapidly, were more robust, and were induced for a longer duration in fibroblasts from HSF1$^{+/0}$ mice compared to fibroblasts from WT mice. HSF1$^{+/0}$ fibroblasts had significantly increased HSP70 mRNA from WT as early as 15 min following initiation of the 30 min heat shock, and levels were highest 2 h following initiation compared to cells from WT mice. HSP70 mRNA levels remained elevated in cells from HSF1$^{+/0}$ after 180 min compared to cells from WT mice that had returned to baseline. HSP70 protein levels were significantly elevated in HSF1$^{+/0}$ cells as early as 1 h after heat shock compared to 3 h for cells from WT mice (Fig. 2C&D). In order to determine whether the dynamics of HSF1 subcellular localization were changed in response to stress, HSF1 localization was examined by immunofluorescence microscopy in fibroblasts from HSF1$^{+/0}$ and WT mice after initiation of the heat shock at various times (Supplementary Fig. S3). HSF1 nuclear localization was elevated significantly above WT cells even prior to heat shock, indicating that HSF1$^{+/0}$ cells may be primed for activation of the HSR due to increased HSF1 in the nucleus. Increased nuclear HSF1 could prime cells in an epigenetic manner by enhancing the chromatin remodeling activities of HSF1 at the HSP70 promoter [22]. Considering that nuclear levels of HSF1 may also be elevated in tissues of HSF1$^{+/0}$ mice, the total level of HSF1 may be higher than that observed in the cytosolic fraction alone. These data, when taken together with the absence of HSP90 upregulation observed in tissues, may together explain the more rapid, robust, and prolonged heat shock response observed in HSF1$^{+/0}$ fibroblasts.

3.3. HSF1$^{+/0}$ fibroblasts have an elevated HSR in response to polyglutamine

To determine if HSF1 overexpression in the HSF1$^{+/0}$ mice would have a functional benefit in the context of expression of a proteotoxic stressor, we measured the HSR response in fibroblasts from HSF1$^{+/0}$ and WT mice transfected with an 82 repeat polyglutamine yellow fluorescent protein fusion protein (Q82YFP), or the non-aggregating Q19YFP as a negative control. As shown in Fig. 3A, HSP70 was found to be induced more rapidly and robustly in HSF1$^{+/0}$ fibroblasts in response to expression of Q82YFP. HSP70 expression was induced as early as 24 h in fibroblasts from HSF1$^{+/0}$ cells compared to WT, which remained significantly elevated over cells obtained from WT mice for 72 h. HSP90 levels

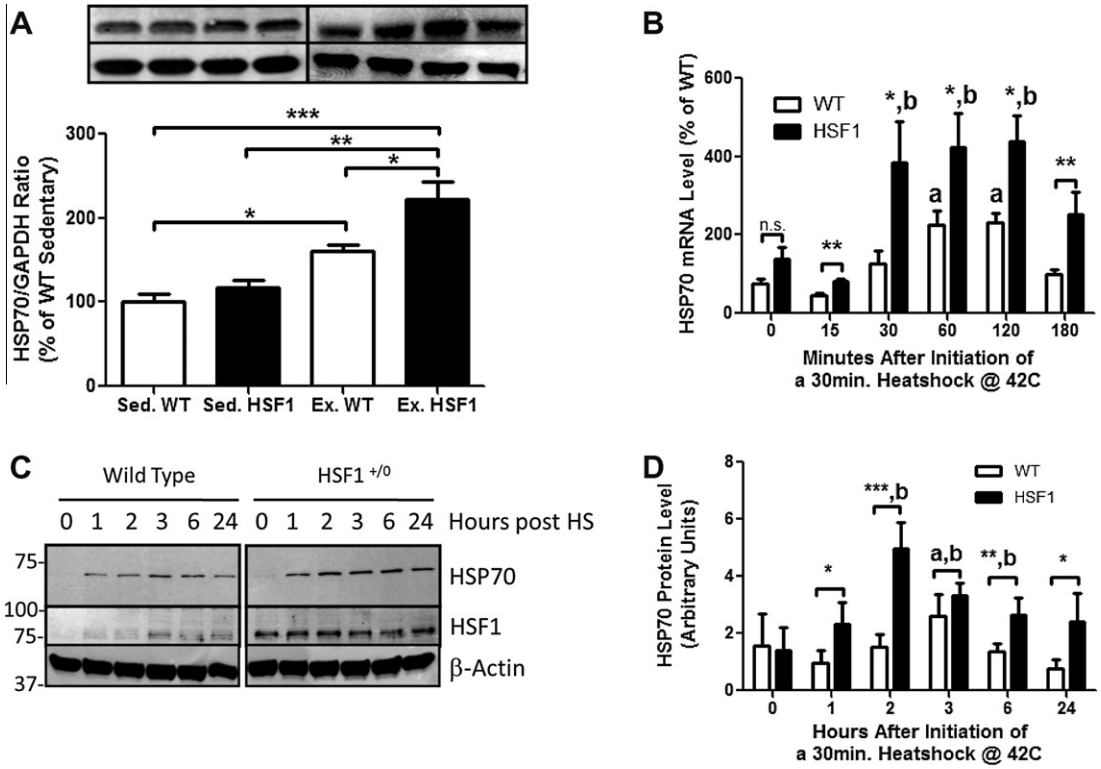

Fig. 2. Induction of HSP70 mRNA and protein following forced exercise or heat shock (A) Ten-month old male HSF1$^{+/0}$ and WT littermates were subjected to forced treadmill exercise (Exer-6, Columbus Instruments, Columbus, OH) to exhaustion for 2 h as described[28]. After 24 h of recovery, gastrocnemius muscle from sedentary (Sed.) and exercised (Ex.) HSF1$^{+/0}$ (closed bars) and WT littermates (open bars) were immunoblotted for HSP70 and GAPDH. B) Primary cultures of fibroblasts from WT (open bars) and HSF1$^{+/0}$ (closed bars) mice were heat shocked at 42 °C for 30 min. Total RNA was isolated from cells after initiation of the heat shock at the times indicated and HSP70 mRNA was quantitated by qRTPCR and normalized by GAPDH. C) Protein was isolated from heat shocked fibroblasts and immunoblotted for HSP70, HSF1, and GAPDH. D) Densitometric analysis of Immunoblot data. Bars represent the mean of six individual cultures from six mice/group +/− SEM. $^{*}p = 0.05$, $^{**}p = 0.01$, $^{***}p = 0.001$ or n.s. (non-significant). Significant difference from unstressed WT or HSF1$^{+/0}$ controls are indicated by a, and b, respectively.

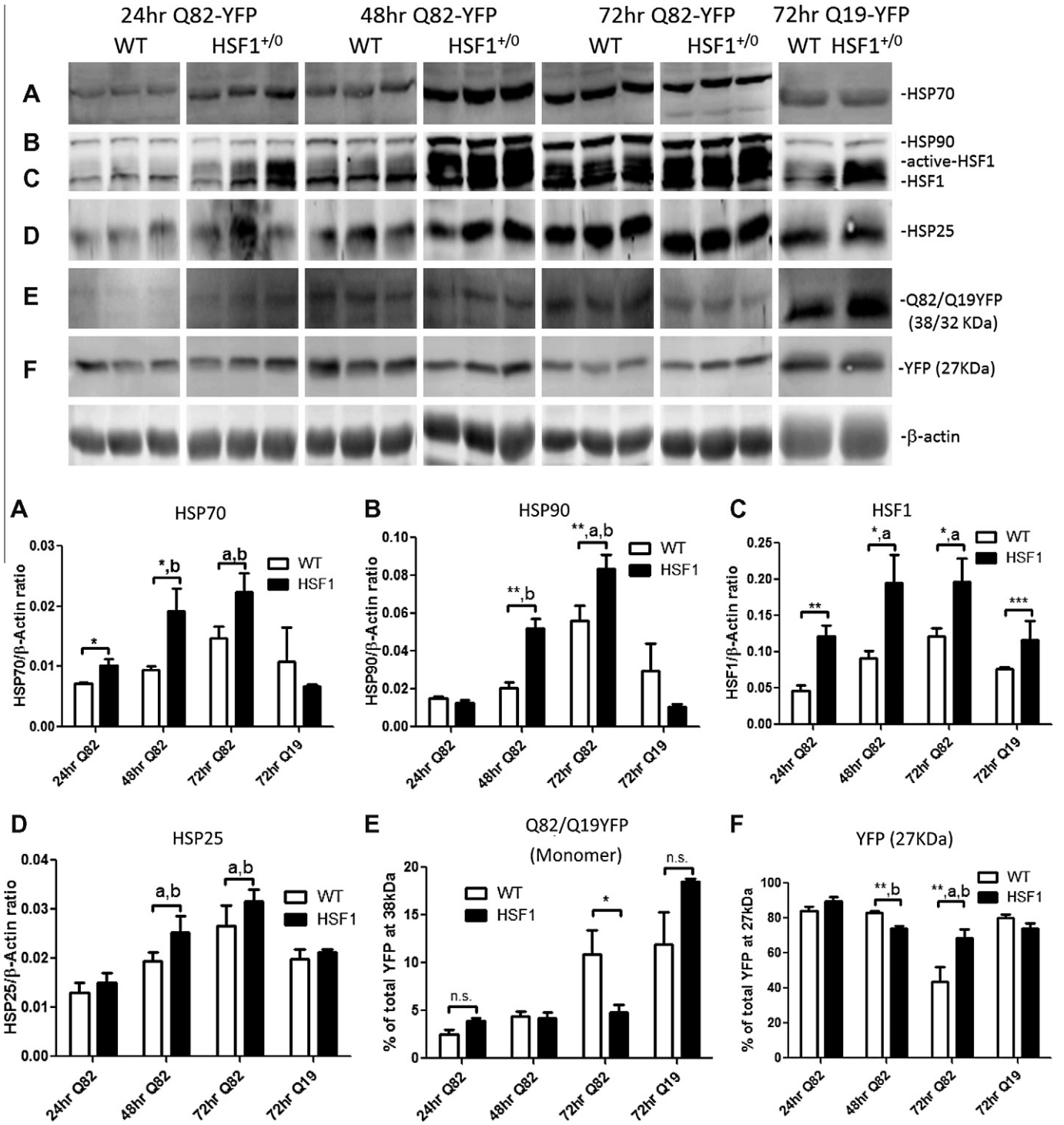

Fig. 3. HSR of HSF1$^{+/0}$ cells transfected with Q82YFP Fibroblasts were transfected for the times indicated with Q82YFP and immunoblotted for HSPs. Fibroblasts from WT mice did not show a significant increase in HSP70 expression until 72 h (A). HSP90 (B), total HSF1 (C) or HSP25 (D) were also quantitated. (E) Fibroblasts were transfected for the times indicated with Q82YFP and immunoblotted for YFP. Bands corresponding to monomeric Q82YFP or (F) YFP monomer are shown. Expression of Q19YFP had no effect on HSP70 levels in either cell type. Bars represent the mean of six individual cultures from six mice/group +/− SEM. $^{*}p = 0.05$, $^{**}p = 0.01$. Significant difference from 24 h WT or HSF1$^{+/0}$ are indicated by a, and b, respectively.

were also induced more rapidly in cells from HSF1$^{+/0}$ mice in response to Q82YFP compared to cells from WT mice (Fig. 3B). HSF1$^{+/0}$ cells maintained significantly elevated expression of HSF1 throughout the experiment compared to time-matched WT cells (Fig. 3C). However, HSP25 was induced to the same degree in both WT and HSF1$^{+/0}$ cells in response to Q82-YFP, and increased incrementally with time (Fig. 3D). Therefore, the HSR in HSF1$^{+/0}$ fibroblasts is induced more rapidly, and is more robust than that exhibited by fibroblasts from WT cells when stressed with a proteotoxic stressor such as Q82-YFP. Fibroblasts from HSF1$^{+/0}$ and WT mice transfected with Q19YFP showed no induction on HSP70 levels.

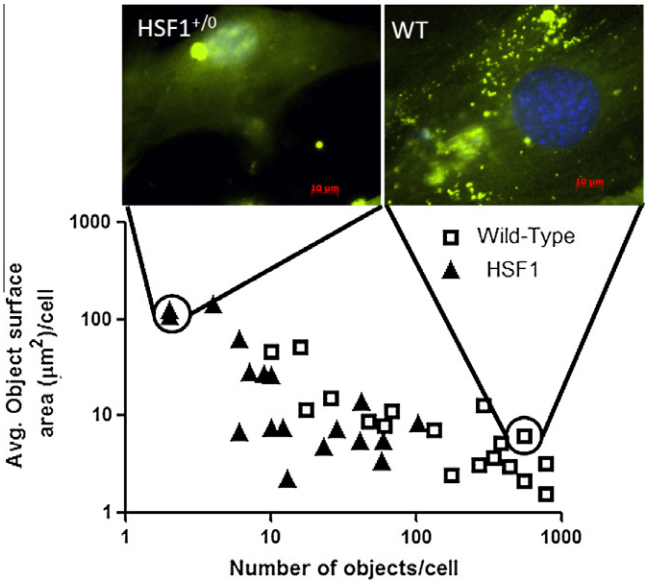

Fig. 4. Alteration in Q82YFP aggregate size and number in HSF1$^{+/0}$ cells. Fibroblasts from WT (open squares) and HSF1$^{+/0}$ (filled triangles) mice were transfected for 72 h with Q82YFP (Green) and stained with DAPI (blue) for nuclei and imaged by fluorescence microscopy. Aggregates were counted by Image J to determine total number of aggregates per cell (number of objects/cell) and their average size as measured in μm^2. Symbols represent the average of 1–3 cells per section, 2–3 sections were quantitated per individual culture. HSF1 overexpression reduced the average number of YFP positive punctae/cell in HSF1$^{+/0}$ cells by 11-fold (273.1 +/− 60.8 vs. 24.1 +/− 6.3 objects/cell $p = 0.0003$) and increased the average size of YFP positive punctae/cell 2.9-fold (11.4+/− 3.4 vs. 32.5+/− 10.5 μm^2 $p = 0.0351$).

3.4. HSF1$^{+/0}$ fibroblasts display enhanced processing of Q82YFP aggregates

Q82-YFP monomers form aggregates and aggresomes in cells and low molecular weight variants of the fusion protein corresponding to a YFP monomeric species are also produced by cleavage of the polyglutamine linker by the proteasome which is inhibitable by MG132[23]. Therefore, we measured monomeric Q82-YFP and its cleavage products in cell extracts from Q82-YFP transfected cells to assess the ability of HSF1$^{+/0}$ fibroblasts to process Q82-YFP. Q82-YFP transfected WT cells were observed to accumulate a YFP positive band corresponding to the molecular weight of the Q82-YFP monomer over time following transfection with Q82-YFP, and also exhibited a progressive decline in the proteasome-dependent cleavage product. However, HSF1$^{+/0}$ cells showed a significant reduction in the accumulation of the Q82-YFP monomeric band after 72hr, suggesting an active processing of monomeric Q82-YFP by either the proteasome or an active aggregation mechanism (Fig. 3E). Meanwhile, the ability of HSF1$^{+/0}$ cells to produce the YFP monomeric cleavage product of Q82-YFP was significantly higher after 72hr compared to WT, suggesting better maintenance of proteasome function and proteostasis in HSF1$^{+/0}$ cells (Fig. 3F). Since we observed an apparent alteration in processing of Q82-YFP by HSF1$^{+/0}$ cells, we next determined whether HSF1$^{+/0}$ cells were capable of altering polyglutamine aggregation.

One response to proteotoxic stressors that cells possess involves the active formation of aggregates into a large juxtanuclear aggregate or aggresome. Protein aggregates can be actively transported to a juxtanuclear site and are termed aggresomes [24]. Aggresomes comprised of polyglutamine repeat containing and other aggregation-prone proteins have been shown to be cytoprotective [25,26]. Therefore, the number and size of aggregates were quantitated by fluorescence microscopy in fibroblasts from WT and HSF1$^{+/0}$ mice after 72 h of culture. As shown in Fig. 4, HSF1 overexpression reduced the average number of YFP positive punctae/cell in HSF1$^{+/0}$ cells by 11-fold and increased the average size of YFP positive punctae/cell 2.9-fold. Aggregates in HSF1$^{+/0}$ were more frequently juxtanuclear and resembled aggresomes. This alteration in aggregate size and number was accompanied by a protection from polyglutamine associated toxicity in HSF1$^{+/0}$ cells, preventing a significant decrease in viability observed in WT Q82YFP transfected cells (Supplementary Fig. S4). In studies using cells transfected with constitutively active mutant HSF1, Fujimoto et al. [14] showed an overall reduction in poly Q aggregate number, but no information on aggresome formation or increased size of aggregates was given. However, in a separate study, it was reported that overexpression of mutant, constitutively active HSF1 suppressed polyglutamine Ataxin-1 aggregates, and in a surprising reference to data not shown, the authors note a perplexing tendency for cells overexpressing WT HSF1 (but not constitutively active mutants) to exhibit increases in larger (0.2–3.14 μm^2) polyglutamine Ataxin-1 aggregates[27]. Thus, there may be functional differences in the processing of aggregates offered by full-length non-mutant HSF1 versus constitutively active or mutant HSF1.

In conclusion, our data demonstrate that overexpression of full-length non-mutant HSF1 in mice enhanced proteostasis at the whole-animal and cellular level. This mouse model will be a useful model of enhanced proteostasis, facilitating our ability to test the role of the heat shock response in maintenance of proteostasis in a variety of disease conditions, especially those of the CNS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.111.

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