Heat shock factor 2 is required for maintaining proteostasis against febrile-range thermal stress and polyglutamine aggregation

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ABSTRACT **Heat shock response is characterized by the induction of heat shock proteins (HSPs), which facilitate protein folding, and non-HSP proteins with diverse functions, including protein degradation, and is regulated by heat shock factors (HSFs). HSF1 is a master regulator of HSP expression during heat shock in mammals, as is HSF3 in avians. HSF2 plays roles in development of the brain and reproductive organs. However, the fundamental roles of HSF2 in vertebrate cells have not been identified. Here we find that vertebrate HSF2 is activated during heat shock in the physiological range. HSF2 deficiency reduces threshold for chicken HSF3 or mouse HSF1 activation, resulting in increased HSP expression during mild heat shock. HSF2-null cells are more sensitive to sustained mild heat shock than wild-type cells, associated with the accumulation of ubiquitylated misfolded proteins. Furthermore, loss of HSF2 function increases the accumulation of aggregated polyglutamine protein and shortens the lifespan of R6/2 Huntington's disease mice, partly through αB-crystallin expression. These results identify HSF2 as a major regulator of proteostasis capacity against febrile-range thermal stress and suggest that HSF2 could be a promising therapeutic target for protein-misfolding diseases.**

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INTRODUCTION

All living cells maintain balance among the synthesis, folding, clearance, binding interaction, and location of individual proteins to maintain the proper conformation and physiological concentration of proteins, which is called protein homeostasis or proteostasis (Balch *et al.*, 2008). Not only metabolic/environmental stress but also the expression of an inherited misfolding-prone protein can cause the perturbation of proteostasis network pathways, including chaperone-mediated folding and proteasome-associated degradation. Loss of proteostasis causes dysregulation of a wide range of cellular pathways, resulting in many systemic and neurodegenerative disorders, known as protein misfolding diseases, such as Alzheimer's disease, Parkinson's disease, and polyglutamine diseases (Powers *et al.*, 2009). Physiological aging is also associated with a reduction in proteostasis capacity, which causes widespread protein aggregation (Ben-Zvi *et al.*, 2009; David *et al.*, 2010). To deal with inherited misfolding proteins, aging, and or metabolic/environmental stress, cells have highly sophisticated mechanisms, including the heat shock response and endoplasmic reticulum unfolded protein response, that regulate proteostasis network capacity (Ron and Walter, 2007; Morimoto, 2008).

When cells are exposed to environmental stress, such as high temperature, they induce the expression of many proteins, including a limited number of heat shock proteins (HSPs) that facilitate protein folding and maintain proteostasis (Lindquist, 1986; Richter *et al.*, 2010). This process, called the heat shock response, is an adaptive response to proteotoxic stress and is regulated mainly at

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Abbreviations used: GFP, green fluorescent protein; HSE, heat shock element; HSF, heat shock factor; HSP, heat shock protein; MEF, mouse embryonic fibroblast; polyQ, polyglutamine.

the level of transcription by heat shock factor (HSF; Wu, 1995; Akerfelt *et al.*, 2010). In vertebrates, four HSF family members (HSF1, HSF2, HSF3, and HSF4) bind to the heat shock element (HSE), which is composed of inverted repeats of a consensus nGAAn sequence. Among them, HSF1 is the master regulator of HSP expression in mammalian cells, as is HSF3 in avian cells, suggesting that *HSF1* and *HSF3* genes evolved differently in mammalian and avian cells (Fujimoto and Nakai, 2010). Nevertheless, both HSF1 and HSF3 acquire DNA-binding activity and are translocated to the nucleus during heat shock, whereas only a small amount HSF2 does (Sarge *et al.*, 1993; Baler *et al.*, 1993; Sheldon and Kingston, 1993; Nakai *et al.*, 1995; Alastalo *et al.*, 2003; Fujimoto *et al.*, 2010). Furthermore, mammalian HSF1 or avian HSF3, but not HSF2, is required and sufficient for the HSP expression (Tanabe *et al.*, 1998; McMillan *et al.*, 1998, 2002; Nakai and Ishikawa, 2001). Consistently, HSF1 significantly suppresses the progression of mouse models of protein misfolding diseases such as Huntington's disease and prion disease (Fujimoto *et al.*, 2005; Steele *et al.*, 2008; Hayashida *et al.*, 2010), like a single HSF in *Caenorhabditis elegans* (Hsu *et al.*, 2003; Morley and Morimoto, 2004). Because HSF2 is expressed abundantly and ubiquitously but is dispensable for HSP expression in vertebrate cells, its developmental roles have been extensively analyzed (Abane and Mezger, 2010). It was found that HSF2 is important for development of the brain and reproductive organs (Kallio *et al.*, 2002; Wang *et al.*, 2003; Chang *et al.*, 2006); however, the fundamental roles of HSF2 in individual vertebrate cells have not been identified.

HSF2 is a short-lived protein that is tightly regulated by the ubiquitin proteasome pathway (Mathew *et al.*, 1998, 2001; Ahlskog *et al.*, 2010; Xing *et al.*, 2010). The blockade of proteasome activates HSF2 as well as HSF1; nevertheless, HSF1, but not HSF2, is essential and sufficient for the up-regulation of HSP expression during proteasome inhibition (Pirkkala *et al.*, 2000). Therefore HSF2 is unlikely to be a major regulator of HSP expression. Rather, HSF2 contributes to the inducible expression of HSPs to some extent by interacting directly with HSF1 (Alastalo *et al.*, 2003; He *et al.*, 2003; Ostling *et al.*, 2007; Sandqvist *et al.*, 2009) or by bookmarking the *HSP* gene during mitosis (Xing *et al.*, 2005; Björk and Sistonen, 2010). Recently we found that two vertebrate heat-responsive factors, HSF1 and HSF3, can protect cells from heat shock irrespective of their ability to regulate HSP expression (Inouye *et al.*, 2003; Fujimoto *et al.*, 2010). Remarkably, mammalian HSF1 regulates the expression of not only HSPs, but also of non-HSP proteins that suppress protein aggregation by controlling protein degradation (Hayashida *et al.*, 2010, 2011). These results raised the possibility that HSF family members may have a conserved role in the regulation of proteostasis capacity during evolution. We generated chicken B lymphocyte DT40 cells deficient in HSF2 and showed that chicken HSF2 significantly protects cells from elevated temperatures within the physiological febrile range and suppresses the accumulation of misfolded proteins upon proteotoxic stress. Furthermore, mouse HSF2 had the same function, and its deficiency accelerated disease progression in Huntington's disease model mice, suggesting that HSF2, as well as HSF1, could be a possible therapeutic target for protein misfolding diseases.

RESULTS

Vertebrate HSF2 is activated during mild heat shock

Biochemical characterization of mammalian HSF2 upon heat shock at nonphysiological extreme temperatures such as 42°C has been difficult because it became insoluble in buffers consisting of nonionic detergents (Mathew *et al.*, 2001; Sistonen *et al.*, 1994). However, if HSF2 is involved in the regulation of proteostasis capacity, it could be activated at least during mild heat shock at physiological temperatures. The body temperature in avians is around 41°C (Mills and Heath, 1970; Aschoff *et al.*, 1973), but it is <38°C in mammals, including humans (Mackowiak, 1998). Therefore chicken DT40 cells and mouse fibroblasts maintained at 37°C were treated with mild heat shock at 41 and 40°C, respectively.

We first examined the stability of chicken HSF2, as well as HSF1 and HSF3, upon heat shock for 1 h at 41°C (mild), 43°C (intermediate), and 45°C (extreme) in chicken DT40 cells in which HSF4 protein and DNA-binding activity were undetectable (Nakai and Ishikawa, 2001). Consistently, HSF2 was unstable at an extreme temperature of 45°C, whereas HSF3, a master regulator of chicken *HSP* genes, was stable (Figure 1A; Nakai *et al.*, 1995; Nakai and Ishikawa, 2001). Because HSF2 was stable at a physiological temperature of 41°C, we examined the acquisition of DNA-binding activity of HSF2 and its translocation to the nucleus during heat shock at 41°C. We found that HSF2, which is localized in both the cytoplasm and nucleus of DT40 cells under unstressed condition, accumulates to the nucleus during heat shock (Figure 1, B and C). Chicken HSF2 existed as a trimer that can bind to DNA and a small dimer in control DT40 cells and predominantly as a trimer upon heat shock (unpublished data). In spite of the slight change of the oligomeric state, the DNA-binding activity of HSF2 in the nucleus and its occupancy on the HSP70 promoter in vivo were increased during heat shock (Figure 1, D and E).

We next examined the activation of HSF2 in mouse NIH cells and found that mouse HSF2 was stable during mild heat shock at a physiological temperature of 40°C until 1 h (Supplemental Figure S1A). Mouse HSF2 was present as a dimer and a trimer in control NIH3T3 cells (Sistonen *et al.*, 1994) and was converted predominantly to a trimer upon heat shock at 40°C for 1 h, whereas only a small amount of HSF1 was converted (Supplemental Figure S1B). Heat shock and other stresses induce nuclear translocation of at least a part of mammalian HSF2 (Sheldon and Kingston, 1993; Mathew *et al.*, 2001). As a result, the DNA-binding activity of HSF2 in the nucleus and its occupancy on the HSP70 promoter in vivo were markedly increased during mild heat shock (Supplemental Figure S1, C and D). We conclude that vertebrate HSF2 is activated during mild heat shock in the physiological range, suggesting that HSF2 may be involved in resistance to mild heat shock and other proteotoxic stress.

Increased HSP expression in HSF2-null chicken cells during mild heat shock

To analyze the roles of chicken HSF2, we generated HSF2-null DT40 cells (HSF2−/−) by sequential transfection of targeting construct and drug selection (Figure 2A and Supplemental Figure S2; Nakai and Ishikawa, 2001). Furthermore, drug-resistant genes flanked by mutant loxP sites were removed by overexpressing green fluorescent protein (GFP)–Cre fusion protein (HSF2−/−ΔneoΔpuro) (Arakawa *et al.*, 2001), and an expression vector for cHSF2 was stably transfected into HSF2-null cells by neomycin selection (HSF2−/−/cHSF2). Levels of HSF2 were reduced or absent in these cells, except in HSF2−/−/cHSF2 cells (Figure 2B). In all of the cells, levels of HSF1 and HSF3, as well as those of major HSPs, including HSP110, HSP90, HSP70, and HSP40, were constant under unstressed condition (Figure 2B). First, we examined the induction of HSP70 mRNA during heat shock at an intermediate temperature of 43°C. Consistent with the fact that mouse HSF2 contributes to the inducible expression of HSP70 through the direct interaction with HSF1 (He *et al.*, 2003; Ostling *et al.*, 2007; Sandqvist *et al.*, 2009), the induction of HSP70 mRNA was reduced until 20 min in the

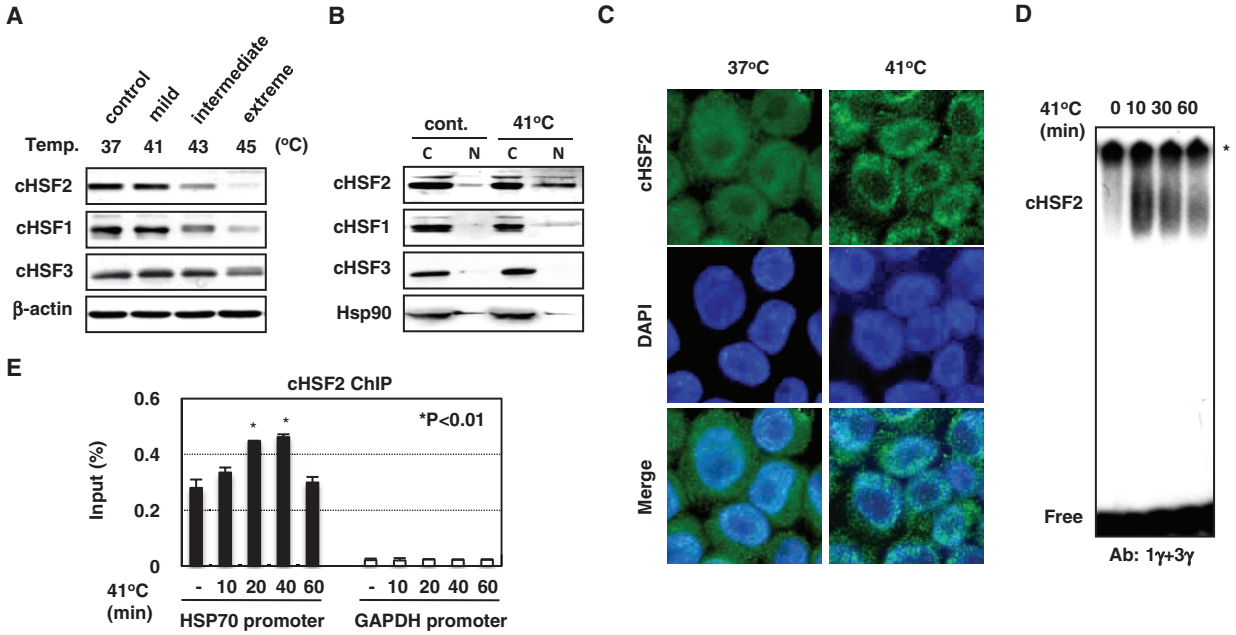

FIGURE 1: Chicken HSF2 is activated during mild heat shock. (A) Expression levels of HSF2, HSF1, and HSF3 in DT40 cells. Cell cultures maintained at 37°C were submerged in a water bath at 41°C (mild), 43°C (intermediate), or 45°C (extreme) for 1 h. Extracts were prepared in NP-40 lysis buffer and were subjected to Western blotting. HSF4 cannot be detected in DT40 cells. (B) Localization of HSF2 during heat shock. DT40 cells were heat shocked at 41°C for 45 min. The cytoplasmic (C) and nuclear (N) extracts were prepared (Nakai *et al.*, 1995) and subjected to Western blotting. (C) DT40 cells were heat shocked at 41°C for 45 min, and indirect immunofluorescence analysis using anti-cHSF2 serum was performed. The nuclei, which contain large nucleoli, were stained with 4′,6-diamidino-2-phenylindole. (D) Analysis of HSF2 DNA-binging activity. Nuclear extracts were isolated from control and heat-shocked (41°C for 10, 30, and 60 min) cells and subjected to gel shift assay in the presence of antisera for HSF1 (1γ) and HSF3 (3γ) (right). HSF1 and HSF3 DNA-binding activities were retarded on top of the gel (asterisk) (Nakai and Ishikawa, 2001). Complexes of HSF2 and a ^{32}P-labeled HSE probe (HSF2) and a free probe (Free) are indicated. (E) HSF2 occupancy on HSP70 promoter. DT40 cells were heat shocked at 41°C for the indicated periods, and ChIP-real time PCR was performed. HSF2 occupancy on GAPDH promoter was analyzed as a control (mean ± SD, n = 3). Significance at $p < 0.01$ by Student's *t* test.

absence of chicken HSF2 (Supplemental Figure S3A); however, the induced mRNA and protein levels in HSF2-null cells were similar to those in wild-type cells at later time points, such as 40 min (Supplemental Figure S3, A and B). This was not due to incomplete HSF3 activation in the absence of HSF2. Indeed, chicken HSF3 was fully activated in both wild-type and HSF2-null cells during extreme heat shock (Supplemental Figure S3C; Nakai *et al.*, 1995), and its binding to HSP70 promoter in vivo was rather increased in the absence of chicken HSF1 or HSF2 before and after the extreme heat shock (Supplemental Figure S3D).

Next, we examined the expression of HSPs during sustained exposure to mild heat shock at 41°C until 6 h. Unexpectedly, we found that the expression of HSP70 and HSP40 in HSF2-null cells was higher than that in wild-type or cHSF2-restored cells (Figure 2, C and D). Although chicken HSF3 was hardly activated during mild heat shock at 41°C in wild-type cells, it was converted to a trimer, acquired DNA-binding activity, and bound to HSP70 promoter in vivo in HSF2-null cells (Figure 2, E–G). Chicken HSF3 was also more activated by treatment with sodium arsenite and a proteasome inhibitor MG132 at low concentrations in HSF2-null cells than in wild-type cells (Supplemental Figure S4). These results demonstrate that the threshold for chicken HSF3 activation is reduced in the absence of HSF2, although it is unclear whether HSF2 affects HSF3 activity directly or indirectly, and they imply unanticipated roles of chicken HSF2, in addition to the role that mammalian HSF2 plays in contributing to the inducible expression of HSPs (He *et al.*, 2003; Ostling *et al.*, 2007).

HSF2-null chicken cells are highly sensitive to sustained heat shock

Previously we showed that wild-type DT40 cells grow exponentially even at 43°C until 72 h, but the growth rate of HSF1-null cells is more reduced than that of wild-type cells, which is associated with an increase in the proportion of the sub-G_1 fraction consisting of dead cells, independent of the HSP expression (Nakai and Ishikawa, 2001; Inouye *et al.*, 2003). Therefore we examined the growth properties of HSF2-null DT40 cells. To our surprise, HSF2-null cells did not grow at all at 43°C until 96 h, and the proportion of the sub-G_1 fraction significantly increased (Figure 3, A and B, and Supplemental Figure S5, A and B). Clonal effects were excluded by the examination of several cell clones in which cHSF2 was reexpressed (Supplemental Figure S5C). HSF2-null cells were also more sensitive to treatment with sodium arsenite or MG132 than wild-type cells or cells reexpressing cHSF2 (Figure 3C). Remarkably, among the cells deficient in one of three chicken HSFs, the sensitivity to heat shock was highest in cells deficient in HSF2, irrespective of the HSP expression (Figure 3, D and E, and Supplemental Figure S5D). As was expected from the fact that HSPs are accumulated in HSF2-null cells at 43°C at the same level as in wild-type cells, HSF2-null cells acquired induced thermotolerance, as did chicken HSF1-null cells (Figure 3F; Inouye *et al.*, 2003). These results demonstrate that chicken HSF2 is required for resistance to sustained heat shock independent of HSP expression.

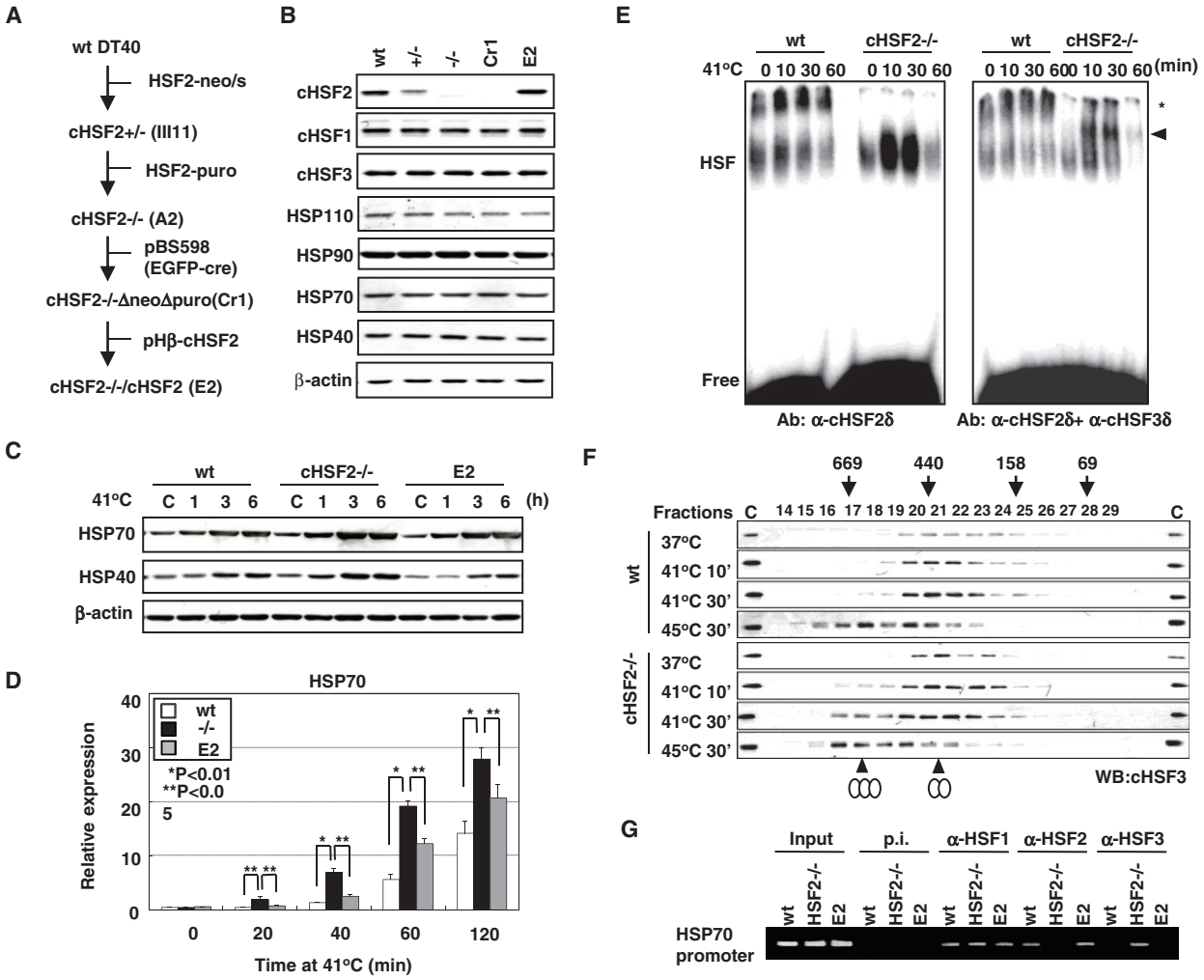

FIGURE 2: Increased expression of HSPs in HSF2-null DT40 cells during mild heat shock. (A) Outline of transfection procedure to isolate DT40 cells possessing mutated *HSF2* genes. Wild-type (wt), HSF2-hetero (cHSF2+/−, clone III11), HSF2-null (cHSF2−/−, clone A2), HSF2-null cells removing drug-resistant and *HSV-tk* genes (cHSF2−/−ΔneoΔpuro, clone Cr1), and HSF2-restored cells (cHSF2−/−/cHSF2, clone E2) are shown. (B) Expression of HSFs and HSPs in unstressed cells. Cell extracts were prepared from the indicated cells in NP-40 lysis buffer and were subjected to Western blotting using each specific antiserum. (C) Expression of HSPs during mild heat shock. Cell cultures were moved to an incubator at 41°C for the indicated periods, and extracts were prepared in NP-40 lysis buffer and subjected to Western blotting. (D) Expression of HSP mRNAs during mild heat shock. The cells were exposed to heat shock at 41°C for the indicated periods. mRNA levels of HSPs were measured by real-time PCR (mean ± SD, n = 3). Significance at $p < 0.05$ by Student's *t* test. (E) Reduced threshold temperature for chicken HSF3 activation in HSF2-null DT40 cells. Whole-cell extracts were prepared from wild-type (wt) and HSF2-null (cHSF2−/−) cells treated with heat shock at 41°C for the indicated periods. Gel shift assay was performed in the presence of a specific antiserum for HSF2 or specific antisera for HSF2 and HSF3. Arrow indicates supershifted complexes consisting of chicken HSF3 and anti-HSF3 antibody. HSF2 DNA-binding activity was retarded on top of the gel (asterisk). (F) Oligomeric states of chicken HSF3. Wild-type and HSF2-null cells were heat shocked at 41°C for 10 or 30 min or at 45°C for 30 min. Whole-cell extracts isolated from the cells were subjected to gel filtration. The elution position of the HSF3 trimer or dimer is shown at the bottom. (G) ChIP of wild-type (wt), HSF2-null (cHSF2−/−), and cHSF2−/−/cHSF2 (E2) cells treated with or without heat shock at 41°C for 30 min was performed using a preimmune serum (p.i.) or each specific antiserum. A DNA fragment of HSP70 promoter (−340 to −17) was amplified by PCR.

Accumulation of polyglutamine aggregation and misfolded cellular proteins in HSF2-knockdown chicken cells

Reduced resistance to heat shock in HSF1-null mouse embryonic fibroblast (MEF) cells is accompanied by increased aggregate formation of aggregation-prone proteins, such as a pathological length (81) of polyglutamine (polyQ) fused to GFP (polyQ81-GFP; Hayashida *et al.*, 2010), and overexpression of mouse HSF3 or chicken HSF1 in cells not only can restore resistance to heat shock, but it can also reduce polyQ aggregate formation (Fujimoto *et al.*, 2010). Therefore we examined polyQ aggregate formation in chicken embryonic fibroblasts (CEFs) and found that the number of cells forming polyQ inclusions and the amounts of NP-40–insoluble polyQ81-GFP are increased in HSF2-knockdown CEF cells (Supplemental Figure 6, A and B). Furthermore, the level of ubiquitylated cellular proteins, which is a hallmark of misfolded proteins, was markedly increased in HSF2-knockdown cells, indicating impairment

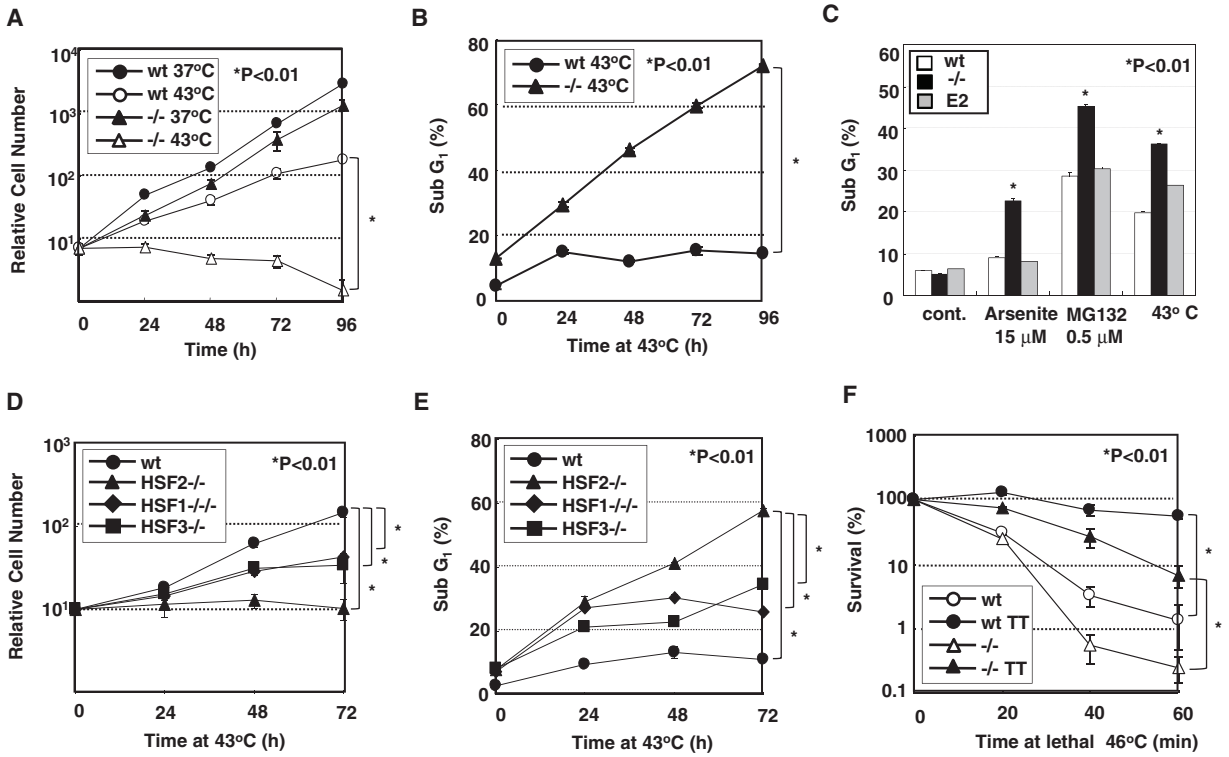

FIGURE 3: HSF2-null DT40 cells are highly sensitive to high temperatures. (A) Growth curves of wild-type (wt) and HSF2-null (−/−) cells at 37 and 43°C. Aliquots of 1×10^4 cells were inoculated into 35-mm dishes, and the surviving cells were counted after trypan blue staining until 96 h (mean ± SD, n = 3). Significance at $p < 0.01$ by ANOVA. (B) Cell death at a high temperature. Cells were incubated at 43°C until 96 h, and the proportions of sub-G_1 cells are shown (mean ± SD, n = 3). Significance at $p < 0.01$ by ANOVA. (C) Wild-type (wt), HSF2-null (−/−), and HSF2-restored (E2) cells were treated without (Cont.) or with sodium arsenite (15 μM for 24 h), MG132 (2.0 μM for 36 h), or heat shock (43°C for 48 h), and the proportions of sub-G_1 cells are shown (mean ± SD, n = 3). Significance at $p < 0.01$ by Student's *t* test. (D) Growth curves of wild-type (wt), HSF2-null (HSF2−/−), HSF1-null (HSF1−/−/−, clone 59), and HSF3-null (HSF3−/−, clone 21) cells at 43°C. Aliquots of 1×10^4 cells were inoculated into 35-mm dishes, and the surviving cells were counted after trypan blue staining until 72 h (mean ± SD, n = 3). Significance at $p < 0.01$ by ANOVA. (E) Cells were incubated at 43°C until 72 h, and the proportions of sub-G_1 cells are shown (mean ± SD, n = 3). Significance at $p < 0.01$ by ANOVA. (F) HSF2-null cells acquire induced thermotolerance. Wild-type (wt) and HSF2-null (−/−) cells grown at 37°C were incubated at a lethal 46°C for the indicated periods. The surviving cells were counted by colony formation assay, and percentage survival is shown. Some cells were pretreated with sublethal 43°C exposure for 30 min and then allowed to recover for 2 h before the lethal heat shock (wt TT, −/− TT) (mean ± SD, n = 3). Significance at $p < 0.01$ by ANOVA.

of the ubiquitin-proteasome system (den Engelsman *et al.*, 2003; Bennett *et al.*, 2007). Conversely, overexpression of cHSF2 in CEF cells reduced the numbers of cells with polyQ inclusions, the amount of NP-40–insoluble polyQ81-GFP, and the accumulation of insoluble ubiquitylated cellular proteins (Supplemental Figure S6, C and D). These results indicate that chicken HSF2 plays a significant role in inhibiting the accumulation of polyQ aggregates and misfolded cellular proteins and suggest that it is involved in the maintenance of proteostasis capacity.

Mouse HSF2 reduces the accumulation of misfolded proteins on proteotoxic stress condition

Amino acid sequences of each member of the HSF family are highly conserved among vertebrate species, but the functions of *HSF1* and *HSF3* genes evolved differently in mammalian and avian species (Fujimoto and Nakai, 2010). Therefore it is necessary to analyze whether the mouse *HSF2* gene is functionally an orthologue of the chicken *HSF2* gene.

We examined the resistance of wild-type and HSF2-null MEF cells against heat shock. In contrast to HSF1 deficiency (McMillan *et al.*, 1998; Zhang *et al.*, 2002; Inouye *et al.*, 2003), deficiency of mouse HSF2 had no effect on the survival rate during exposure to an extreme temperature of 42°C until 12 h or on the induced thermotolerance (Supplemental Figure S7, A and B). Growth rates of wild-type and HSF2-null MEF cells were similar at 37°C, and that of wild-type cells was slightly suppressed at a physiological temperature of 40°C until 96 h (Figure 4A). Remarkably, we found that the growth rate of HSF2-null cells at 40°C was much more suppressed than that of wild-type cells, which was associated with the significant increase in the proportion of sub-G_1 fraction (Figure 4, A and B, and Supplemental Figure S7C). These results indicate that mouse HSF2 is required for resistance to sustained mild heat shock.

In mammalian cells, misfolded proteins during heat shock are mainly degraded by the proteasome (Parag *et al.*, 1987). Therefore the level of ubiquitylated cellular proteins would be increased in HSF-deficient cells during heat shock due to the accumulation of excessive misfolded proteins (Salomons *et al.*, 2009). Consistent with the reduced survival rates described previously, the ubiquitylated protein level was more increased in HSF2-null MEF cells than in wild-type cells during mild heat shock at 40°C but not in

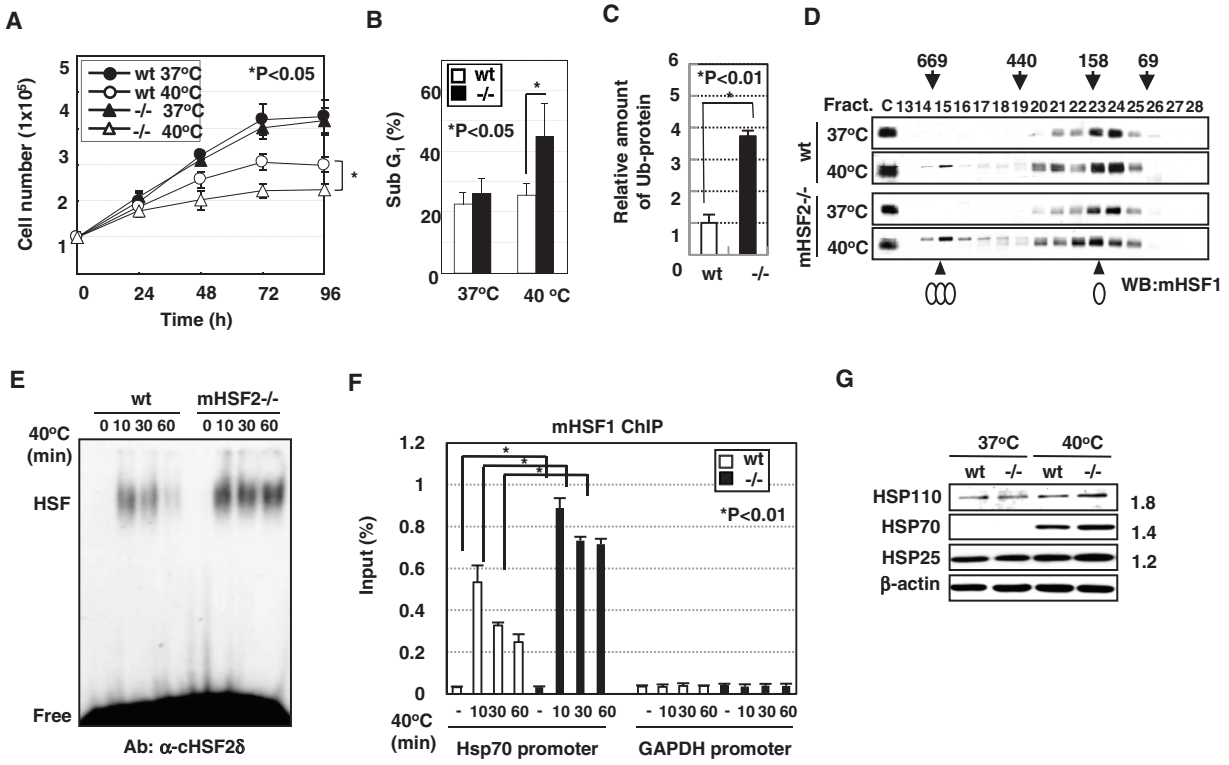

FIGURE 4: Increased accumulation of misfolded proteins in HSF2-null MEF cells during sustained heat shock. (A) HSF2-null cells are sensitive to mild heat shock. Growth curves of wild-type (wt) and HSF2-null (−/−) primary MEF cells from three different embryos at 37 and 40°C. Aliquots of 1×10^5 cells were inoculated into 60-mm dishes, and the surviving cells were counted until 96 h (mean ± SD, n = 3). Significance at $p < 0.05$ by ANOVA. (B) Cell death in mild heat shock condition. Wild-type (wt) and HSF2-null (−/−) primary MEF cells were incubated at 37 or 40°C for 72 h, and the proportions of sub-G_1 cells are shown (mean ± SD, n = 3). Significance at $p < 0.05$ by Student's *t* test. (C) Wild-type (wt) and HSF2-null (−/−) primary MEF cells were infected with Ad-HA-Ub for 2 h, maintained with normal medium at 37°C for 22 h, and then incubated at 40°C for 48 h. Accumulation of insoluble ubiquitylated proteins was quantified (mean ± SD, n = 3) (den Engelsman *et al.*, 2003). Significance at $p < 0.01$ by Student's *t* test. (D) Whole-cell extracts were prepared from wild-type and HSF2-null primary MEF cells treated with heat shock at 40°C for 1 h. Gel filtration was performed, and fractions were subjected to Western blotting. The elution positions of the HSF1 trimer and monomer are shown at the bottom. (E) Whole-cell extracts were prepared from wild-type and HSF2-null primary MEF cells treated with heat shock at 40°C for the indicated periods. Gel shift assay was performed in the presence of antiserum for HSF2 (α-cHSF2δ). (F) HSF1 occupancy on HSP70-1 promoter. Wild-type and HSF2-null primary MEF cells were treated with heat shock at 40°C for indicated periods, and ChIP-real time PCR was performed. HSF1 occupancy on GAPDH promoter was analyzed as a control (mean ± SD, n = 3). Significance at $p < 0.01$ by Student's *t* test. (G) Expression of HSPs during mild heat shock. Extracts from wild-type and HSF2-null primary MEF cells exposed to heat shock at 40°C for 12 h were prepared in NP-40 lysis buffer and were subjected to Western blotting. Fold changes of HSP levels at 40°C in HSF2-null cells compared with those in wild-type cells are shown at the right.

HSF2-null cells during extreme heat shock at 42°C (Figure 4C and Supplemental Figure S8, A and B). Simultaneously, mouse HSF1, a master regulator of HSPs, acquired DNA-binding activity, bound to HSP70 promoter in vivo, and induced HSPs, including HSP70 and HSP110, much more in HSF2-null MEF cells than in wild-type cells (Figure 4, D–G). These results demonstrate that misfolded proteins accumulate much more in mouse HSF2-null cells than in wild-type cells during sustained mild heat shock.

We next examined polyQ aggregate formation in MEF cells and found that the number of cells forming polyQ inclusions and the amount of NP-40–insoluble polyQ81-GFP are increased in HSF2-null MEF cells, and in addition the level of ubiquitylated cellular proteins is markedly increased in HSF2-null cells (Figure 5, D–F, and Supplemental Figure S9, A and B). We generated expression vectors for human HSF2 mutants, hHSF2R63G and hHSF2R63A, in which a putative DNA contact site was substituted with other amino acids, and hHSF2ΔAB, in which the oligomerization domain was deleted (Figure 5A; Inouye *et al.*, 2003, 2007). Overexpression of hHSF2 or cHSF2 restored all of the defects in HSF2-null MEF cells, whereas overexpression of hHSF2 mutants that cannot bind to DNA did not (Figure 5, B–F, Supplemental Figure S9B). Taken together the results indicate that mouse HSF2, as well as chicken HSF2, reduces the accumulation of misfolded proteins under proteotoxic stress condition, probably through the regulation of target gene expression.

Shortening of lifespan in HSF2-null Huntington's disease mice

We investigated the impact of HSF2 on polyQ disease in vivo by using an R6/2 mouse model of Huntington's disease (Mangiarini *et al.*, 1996), which is transgenic for the human *huntingtin* gene exon 1 carrying fewer CAG repeats (94- to 97-fold; Hayashida *et al.*,

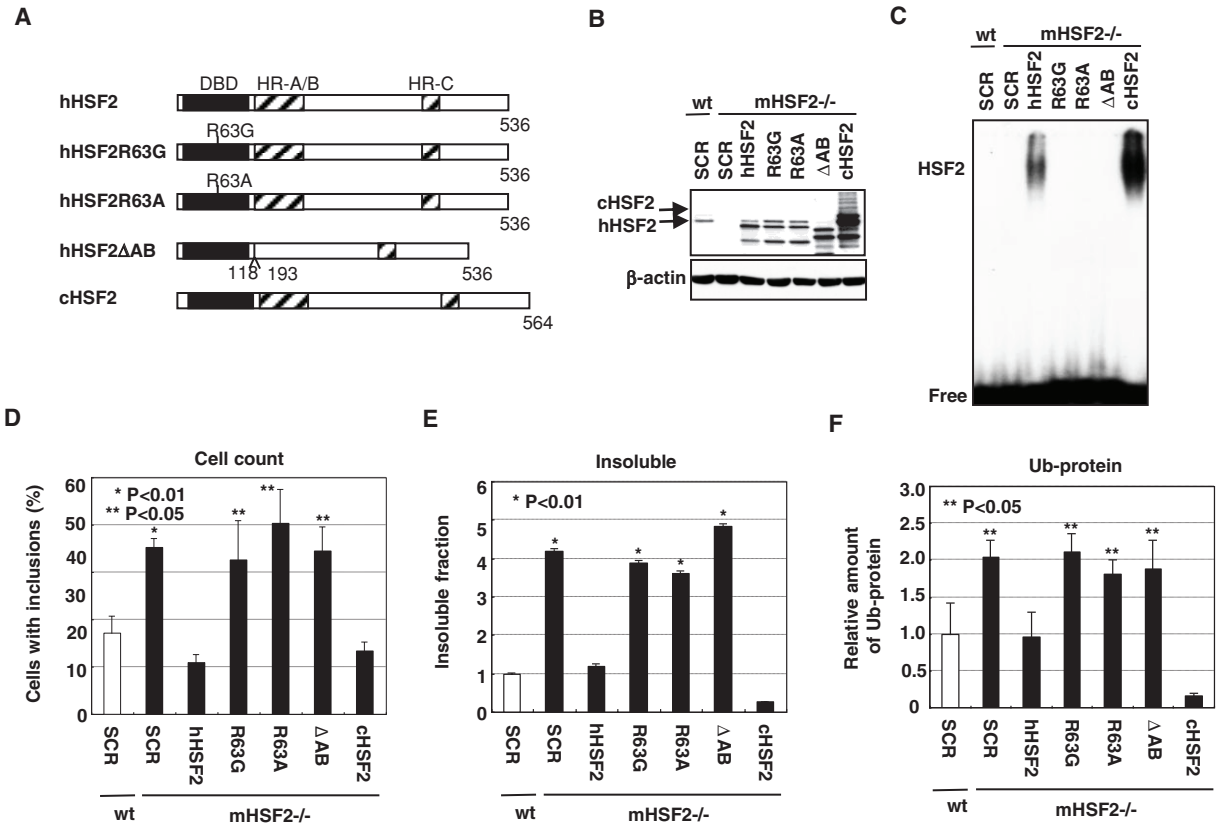

FIGURE 5: DNA-binding activity is necessary for mammalian HSF2 to suppress polyQ aggregation. (A) Schematic representation of hHSF2, hHSF2 mutants, and cHSF2. (B) Wild-type (+/+) and HSF2-null (HSF2−/−) primary MEF cells were infected with adenovirus expressing SCR, hHSF2, hHSF2 mutants, and cHSF2. Cell extracts in NP-40 lysis buffer were prepared and subjected to Western blotting. Arrows indicate positions of endogenous hHSF2 and cHSF2. (C) DNA-binding activity of overexpressed HSF2 and HSF2 mutants. Whole-cell extracts were prepared from cells infected with adenovirus as described earlier, and gel shift assay was performed. Complexes of HSF2 and a ^{32}P-labeled HSE probe (HSF2) and a free probe (Free) are indicated. (D) Wild-type (+/+) and HSF2-null (HSF2−/−) primary MEF cells were infected with Ad-SCR, Ad-hHSF2, Ad-cHSF2, or adenovirus expressing each hHSF2 mutant for 2 h and maintained with normal medium for 22 h. The cells were then infected with Ad-polyQ81-GFP and Ad-HA-Ub for 2 h and maintained with normal medium for 22 h. Cells having inclusions were counted (mean ± SD, n = 3). Significance at $p < 0.01$ or 0.05 by Student's *t* test. (E) Accumulation of insoluble polyQ protein in the cells shown in D was quantified (mean ± SD, n = 3). (F) Accumulation of insoluble ubiquitylated protein in the cells shown in D was quantified (mean ± SD, n = 3).

2010). We generated HSF2-null R6/2 (CAG94-97) mice with a C57BL/6 background. As was shown previously, a single nuclear polyQ–huntingtin aggregate per cell was observed in the striatum at 28 wk (Figure 6, I and J), whereas several small aggregates were observed in the nucleus at 8 wk (Figure 6, A–D). Remarkably, the number of cells having aggregates in the striatum was increased and each aggregate in the nucleus was more evident in both HSF2-hetero and HSF2-null R6/2 mice at 8 wk than in wild-type R6/2 mice (Figure 6, E–H, K, Supplemental Figure S10A). Furthermore, the formation of highly insoluble polyQ–huntingtin aggregates examined by filter trap assay (Figure 6L), as well as the accumulation of polyQ–huntingtin protein (Supplemental Figure S10B), was more increased in the HSF2-hetero and HSF2-null brains than in the wild-type R6/2 brain.

We next examined lifespan. R6/2 mice (CAG94-97) with the C57BL/6 background lived at least for 48 wk and died within 60 wk (Figure 6M). In contrast, HSF2-hetero R6/2 mice lived for 38 wk and died within 49 wk, and HSF2-null mice lived for 29 wk and died within 47 wk. Thus the disruption of one *HSF2* allele and of both alleles shortened the median lifespan of R6/2 mice by 17.2 and 36.2%, respectively. A neurological symptom of clasping appeared earlier in HSF2-hetero and HSF2-null R6/2 mice than in control R6/2 mice (Supplemental Figure S10C). These results demonstrate that the reduction of mouse HSF2 function accelerates the accumulation of misfolded polyQ aggregates in vivo in the brain of R6/2 mice and that mouse HSF2 has a strong impact on disease progression, including the lifespan.

Mouse HSF2 controls proteostasis capacity partly through the expression of αB-crystallin

Because mammalian HSF2 modulates HSF1 activity through direct interaction (He *et al.*, 2003; Ostling *et al.*, 2007; Sandqvist *et al.*, 2009), it is possible that mouse HSF2 is required for the HSF1-mediated pathways that control proteostasis. Among the eight HSF1-target genes (Hayashida *et al.*, 2010), we found that the expression of αB-crystallin is markedly reduced in both unstressed and heat-shocked (40°C) HSF2-null MEF cells as in HSF1-null cells (Figure 7, A and B). To analyze binding of HSF1 and HSF2 to the αB-crystallin promoter in vivo, we treated MEF cells with heat shock at 42°C for 15 min, as in these cells HSF1 binding to the promoter markedly increased (Hayashida *et al.*, 2010) and HSF2 protein level was even stable (data not shown). HSF2 bound to the αB-crystallin promoter

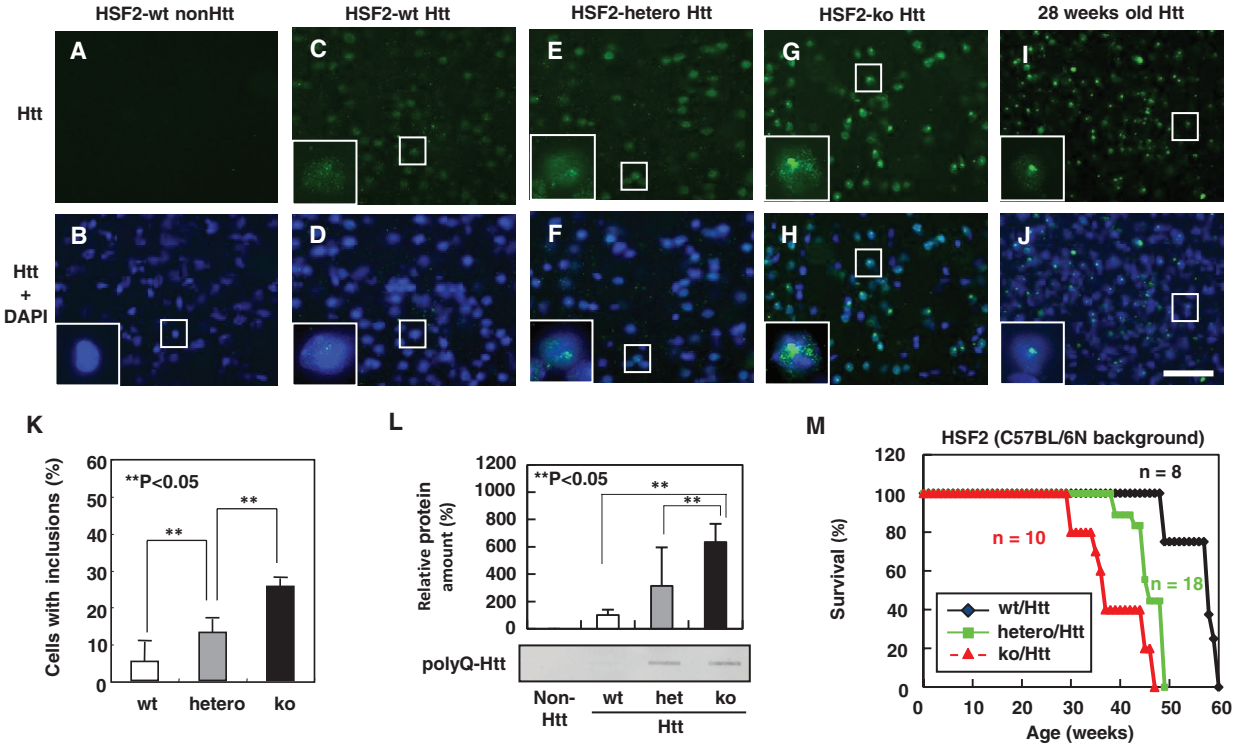

FIGURE 6: Shortening of lifespan in HSF2-null Huntington's disease mice. PolyQ–huntingtin protein aggregates were detected by immunohistochemistry using a goat anti-huntingtin antibody in the striatum of 8-wk-old wild type (A, B) or R6/2 mice (94–97 CAG repeats; Htt) with HSF2+/+ (wt) (C, D), HSF2+/− (hetero) (E, F), or HSF2−/− (ko) (G, H) genotype (C57BL/6N background). Typical inclusions in R6/2 mice at 28 wk old are shown (I, J). Boxed regions are magnified in the insets. Bar, 50 μm. (K) Percentage of cells with inclusions in A–H (mean ± SD, n = 3). Significance at $p < 0.05$ by Student's *t* test. (L) Filter trap assay of polyQ–huntingtin protein in the brain of R6/2 mice (Htt) with HSF2+/+ (wt), HSF2+/− (het), or HSF2−/− (ko) genotype. SDS-insoluble aggregates were trapped on a cellulose acetate membrane, and immunoblotting was performed using a goat anti-huntingtin antibody. Representative blot is shown (bottom). The intensity of the signals was quantified (mean ± SD, n = 3). Significance at $p < 0.05$ by Student's *t* test. (**M**) The lifespan of R6/2 mice (Htt) with HSF2+/+ (wt), HSF2+/− (hetero), or HSF2−/− (ko) genotype. Numbers of mice are indicated.

only in the presence of HSF1, as it does to the HSP70 promoter (Figure 7C; Ostling *et al.*, 2007), which is consistent with the reduced expression of αB-crystallin in HSF1-null cells. Although HSF1 binding to the αB-crystallin promoter was still increased in HSF2-null cells (Figure 7C), the αB-crystallin expression was not increased. These results indicate a unique requirement of cooperativity between HSF1 and HSF2 on the αB-crystallin promoter, which differs from that on the HSP70 promoter. The αB-crystallin expression was also reduced in the brain or skeletal muscle of HSF2-null mice (Figure 7D).

As was expected from the fact that αB-crystallin plays roles in both protein folding and degradation (Hayashida *et al.*, 2010), reexpression of αB-crystallin into HSF2-null MEF cells partially prevented the accumulation of polyQ aggregation and misfolded cellular proteins (Figure 7, E and F), indicating that mouse HSF2 controls proteostasis capacity in part by regulating the expression of αB-crystallin.

DISCUSSION

The heat shock response is an evolutionally conserved adaptive response to high-temperature stress and is regulated mainly by HSF. A single gene encodes HSF in yeast, *C. elegans*, and *Drosophila*, whereas four *HSF* genes exist in vertebrate cells, suggesting that duplication of an ancestral gene and functional diversification of the duplicated genes may have occurred during evolution (Fujimoto and Nakai, 2010). It was clearly shown that HSF1 is required and is sufficient for HSP expression in mammalian cells, as is HSF3 in avian cells (McMillan *et al.*, 1998, 2002; Tanabe *et al.*, 1998; Nakai and Ishikawa, 2001). Although HSF family members play roles in development (Nakai, 2009; Abane and Mezger, 2010), the fundamental roles of the members, including HSF2 and HSF4, in individual vertebrate cells have not been identified (Akerfelt *et al.*, 2010; Björk and Sistonen, 2010; Fujimoto and Nakai, 2010). In contrast to the activation of heat-responsive factors HSF1 and HSF3, activation of HSF2 or HSF4 in response to heat shock has not been shown clearly. Here we unexpectedly found that HSF2 is activated during heat shock, and its deficiency significantly increased the sensitivity of vertebrate cells to heat shock (Figures 3 and 4). Of importance, heat shock induces both apoptotic and necrotic cell death, but the pathways of cell death and factors that are primarily impaired may differ, depending on the temperature and duration of heat shock (Lindquist, 1986). In fact, HSF2 deficiency in MEF cells had little effect on the survival rate during exposure to an extreme temperature or on induced thermotolerance, unlike HSF1 (McMillan *et al.*, 1998; Zhang *et al.*, 2002; Inouye *et al.*, 2003), whereas it significantly reduced the survival rate during heat shock at febrile-range temperatures (Figure 4), which are elicited by exercise, feeding, infection, and disease (Mackowiak, 1998; Leon, 2002). Similarly, the stress-dependent cell death phenotype was shown in HSF2-deficient mouse embryos (Le Masson and Christians, 2011). Thus these results

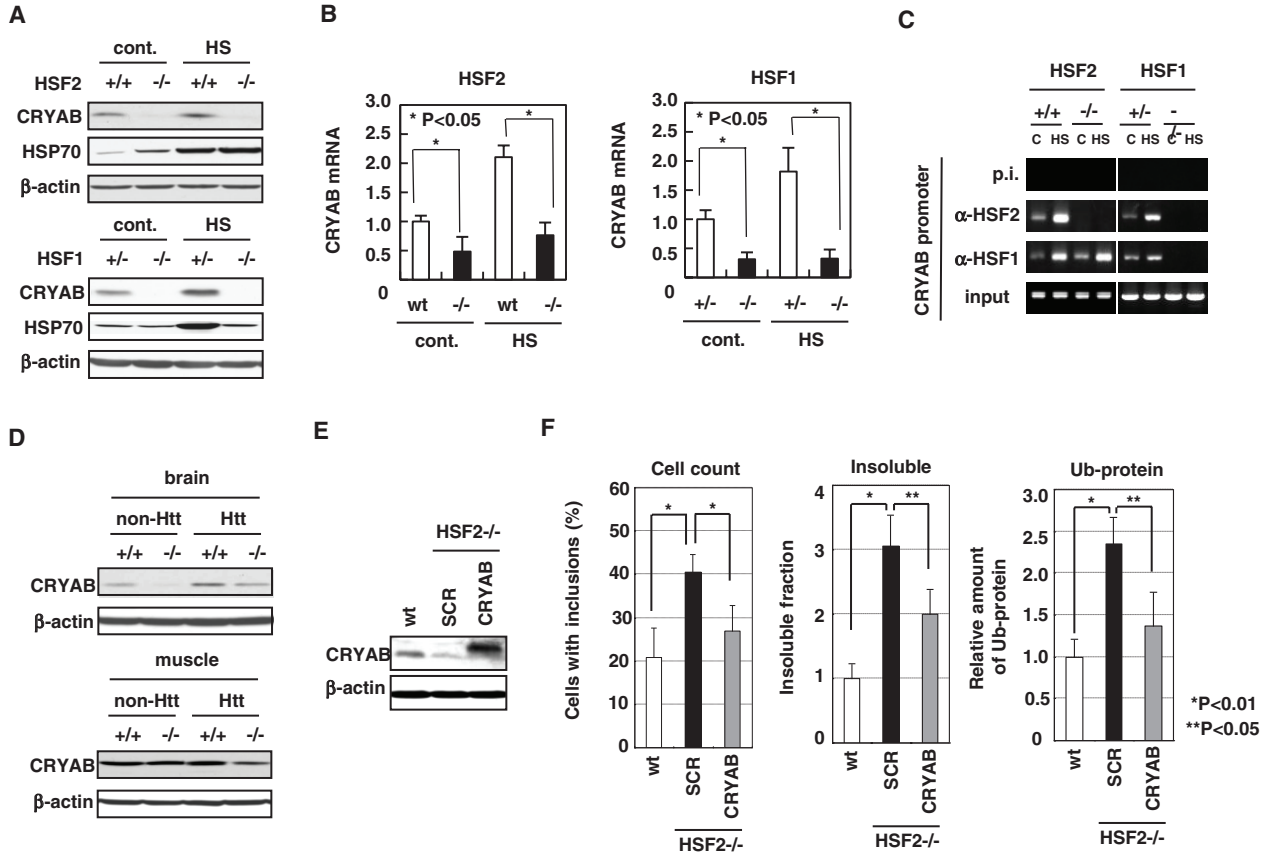

FIGURE 7: HSF2 suppresses the accumulation of misfolded proteins partly through the expression of αB-crystallin. (A) Western blotting of αB-crystallin in primary MEF cells. Wild-type, HSF2-null (top), HSF1-hetero, and HSF1-null (bottom) MEF cells were heat shocked at 40°C for 6 h. (B) Real-time PCR analysis of αB-crystallin mRNA levels in wild-type, HSF2-null (left), HSF1-hetero, and HSF1-null (right) primary MEF cells exposed to heat shock at 40°C for 1 h. The intensity of the signals was quantified (mean ± SD, n = 3). Significance at $p < 0.05$ by Student's *t* test. (C) In vivo binding of HSF2 and HSF1 in αB-crystallin promoter. ChIP of control and heat-shocked (42°C, 15 min) wild-type, HSF2-null, HSF1-hetero, and HSF1-null MEF cells was performed using a preimmune serum (p.i.) and an antiserum for HSF2 and HSF1. DNA fragments of the αB-crystallin promoter (−297 to +1) were amplified by PCR. (D) Expression of αB-crystallin in mouse tissues. Extracts in RIPA buffer were prepared from the brain and skeletal muscle of HSF2+/+ and HSF2−/− R6/2 (Htt) or non-Htt mice and were subjected to Western blotting. Representative data are shown. (E) Overexpression of HA-tagged αB-crystallin in HSF2-null primary MEF cells. Extracts were prepared from wild-type cells and HSF2-null cells infected with Ad-SCR or Ad-αB-crystallin-HA for 24 h and were subjected to Western blotting. (F) The percentages of cells with polyQ inclusions (left), accumulation of insoluble polyQ protein (middle), and accumulation of insoluble ubiquitylated protein (right) were determined as in Figure 6 (mean ± SD, n = 3). Significance at $p < 0.05$ by Student's *t* test.

demonstrate a unique requirement of HSF2 on cell survival, especially under sustained mild heat shock conditions, which is evolutionally conserved in mammalian and avian species. Taken together with divergent cell-protective roles of HSF1 and HSF3 (Inouye *et al.*, 2003; Fujimoto *et al.*, 2010), these results indicate that HSF family members may have a conserved role in adaptation to temperature elevation during vertebrate evolution.

When cells are exposed to heat shock, cellular proteins are misfolded, and misfolded proteins are ubiquitylated for degradation by proteasome (Parag *et al.*, 1987). Prolonged heat shock may exceed cellular proteostasis capacity, resulting in the accumulation of ubiquitylated misfolded proteins (Supplemental Figure S8; Salomons *et al.*, 2009), as in the accumulation of ubiquitylated and misfolded proteins associated with aging and age-related diseases (Bennett *et al.*, 2007; Ben-Zvi *et al.*, 2009; David *et al.*, 2010; Demontis and Perrimon, 2010). HSF2 deficiency increased the accumulation of ubiquitylated cellular proteins during sustained mild heat shock (Figure 4), suggesting that HSF2 suppresses protein misfolding and or promotes the degradation of misfolded proteins. To demonstrate this assumption more directly, we expressed a misfolding-prone polyQ protein in the absence of HSF2. As a result, the accumulation of aggregated polyQ protein was markedly increased in MEF and CEF cells and in vivo in the mouse brain in the absence of HSF2 (Figures 5 and 6 and Supplemental Figure S6). Remarkably, the disruption not only of two *HSF2* alleles, but also of one allele significantly shortened the lifespan of R6/2 mice. These results demonstrate the strong impact of HSF2 and HSF1 on disease progression of Huntington's disease model mice (Fujimoto *et al.*, 2005; Hayashida *et al.*, 2010) and indicate that HSF family members finely tune proteostasis network pathways. Taking these results together, we conclude that HSF2 is required for proper control of proteostasis capacity during proteotoxic stress.

We showed recently the importance of non-HSP proteostasis pathways regulated by HSF1 (Hayashida *et al.*, 2011). Here we revealed one pathway that is regulated by mouse HSF2, based on the fact that mammalian HSF2 modulates HSF1 activity (Ostling *et al.*,

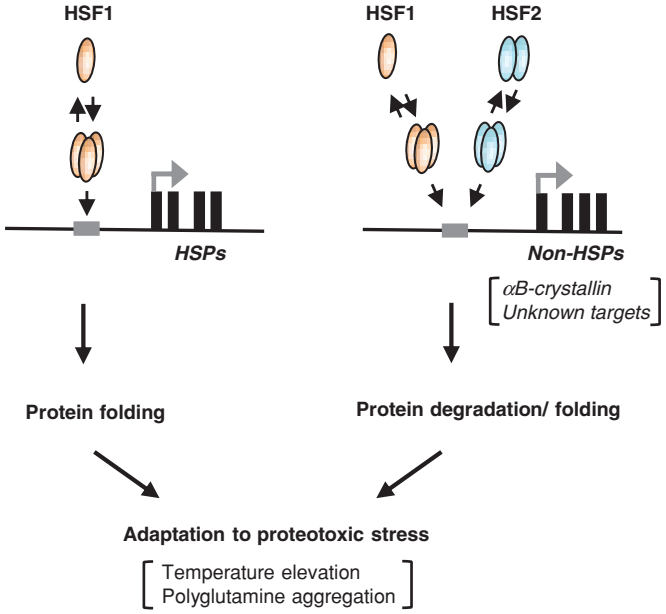

FIGURE 8: HSP and non-HSP proteostasis pathways regulated by HSF1 and HSF2. Schematic models are generated based on the results in mouse fibroblasts. In control condition, HSF1 stays mostly as an inactive monomer and HSF2 as an inactive dimer. During heat shock in febrile ranges (<40°C), HSF2, as well as HSF1, is converted into an active trimer and accumulated into the nucleus. HSF2 is dispensable for adequate HSP expression, although it modulates the expression to some extent (Björk and Sistonen, 2010). In contrast, it is required for the expression of non-HSP proteins such as αB-crystallin that are involved in protein folding and degradation and plays a major role in adaptation to proteotoxic stress.

2007; Sandqvist *et al.*, 2009; Björk and Sistonen, 2010). Among HSF1-target genes that suppress polyQ aggregation (Hayashida *et al.*, 2010), we showed that the expression of mouse HSF2 is required for αB-crystallin under unstressed and heat-shock conditions (Figure 7). It was also reported that human HSF2 activation coincides with the up-regulation of αB-crystallin in glial cells exposed to a high concentration of extracellular potassium (Sadamitsu *et al.*, 2001). αB-Crystallin is not only a molecular chaperone that belongs to a small HSP family, but also a component of the E3 ubiquitin ligase complex (Lin *et al.*, 2006). Thus HSF2 controls both folding and degradation pathways through the expression of αB-crystallin (Figure 8). Recently mouse HSF2 was also shown to regulate the expression of the components of proteasome (Lecomte *et al.*, 2010). Of importance, neither HSF1 nor HSF2 is dispensable for the expression of these genes, indicating cooperative regulation of the target gene expression by HSF1 and HSF2. Cooperative regulation of other targets by HSF family members was also suggested by the fact that mouse HSF4 competes with HSF1 on the expression of fibroblast growth factor 7 in the lens (Fujimoto *et al.*, 2004), and a substantial number of HSF4-binding regions (70%) are cooccupied by HSF1 and HSF2 (Fujimoto *et al.*, 2008). Furthermore, both HSF2 and HSF4 are suggested to regulate the expression of hypoxia-inducible factor-1α in human cells (Chen *et al.*, 2011). Because every member of the HSF family can bind to the HSE consensus sequence, we can state that the HSE-mediated pathways might regulate the expression of HSPs and non-HSP proteins that control proteostasis capacity. The requirement of each HSF for the expression of non-HSP proteins, as well as for HSPs, might differ, depending on the cell type.

Even though HSFs play an evolutionally conserved role in regulating proteostasis network pathways, our observations showed that the expressions of major HSPs, including HSP110, HSP90, HSP70, HSP60, HSP40, and HSP25, and that of other, non-HSP proteins are differentially regulated by vertebrate HSFs (Figure 8). Deficiency of HSF2, which is required for the expression of non-HSP proteins, elevated the expression of HSPs by accelerating the activation of mouse HSF1 or chicken HSF3 during sustained mild heat shock (Figures 2 and 4; Paslaru *et al.*, 2003). Thus mouse HSF1 or chicken HSF3 seems to compensate for the loss of HSF2 function. There is increasing evidence that non-HSP proteins, including components of an ubiquitin proteasome pathway, regulate proteostasis capacity (Nollen *et al.*, 2004; Bilen and Bonini, 2007). Our results strengthen the importance of the roles of the non-HSP proteins that are regulated under the control of the HSE-mediated pathways (Hayashida *et al.*, 2011). Identification and functional analysis of these proteins in the future would reveal more clearly the mechanisms and biological significance of the heat shock response.

One promising therapeutic strategy for aging and protein misfolding disorders such as polyQ diseases is to elevate proteostasis capacity by controlling proteostasis network pathways (Powers *et al.*, 2009). To this end, small molecules that activate HSF1 were identified and shown to ameliorate disease progression in vivo (Westerheide *et al.*, 2004; Neef *et al.*, 2010). However, conversely, the activation of HSF1 at unnecessary levels is detrimental to neuronal and germ cells (Nakai *et al.*, 2000; Hayashida *et al.*, 2006; Dirks *et al.*, 2010). Furthermore, HSF1 may promote tumor initiation and survival (Dai *et al.*, 2007), partly by inducing HSPs that potentiate oncogenesis in various ways, such as influencing the apoptotic pathways (Mosser and Morimoto, 2004). We show here that mouse HSF2 has a strong impact on disease progression, including the lifespan of Huntington's disease R6/2 mice. Our results suggest that pharmacological regulation of HSF2, which does not induce marked expression of HSPs, would be a new therapeutic strategy for protein misfolding diseases.

MATERIALS AND METHODS

Cell culture, transfection, and screening of DT40 cells

Cell culture and transfection of DT40 cells were performed as described previously (Tanabe *et al.*, 1998; Nakai and Ishikawa, 2001). HSF2−/− DT40 cells were generated by sequential transfection of an *HSF2* gene-targeting construct containing neomycin-resistant gene and puromycin-resistant gene flanked by mutant loxP sites (HSF2-neo/s and HSF2-puro; Supplemental Figure S2A; Arakawa *et al.*, 2001). HSF2−/− cells were then transfected with an expression vector of Cre recombinase (pBS598; Addgene, Cambridge, MA) and divided into 96-well plates. At 18 h after transfection, medium containing ganciclovir was added to a final drug concentration of 2 μg/ml. After 14 d, drug-resistant clones (HSF2−/−ΔneoΔpuro) were expanded and genomic DNA was isolated. Recombination of the mutant loxP sites was confirmed by PCR (Supplemental Figure S2B). Expression of cHSF2 was restored by stably transfecting the cells with cHSF2 expression vector pHβ-cHSF2 (Nakai *et al.*, 1995; HSF2−/−/cHSF2).

Immunofluorescence

To examine the localization of chicken HSF2 in cells, wild-type and HSF2-null DT40 cells were stained with anti-cHSF2 serum (α-cHSF2α) as described previously (Nakai *et al.*, 1995). Immunofluorescence analysis of huntingtin was performed as described previously (Hayashida *et al.*, 2010). Sections 10 μm thick were cut using a CM1900 cryostat (Leica Microsystems, Nussloch, Germany).

Immunohistochemistry was carried out using a goat anti-huntingtin polyclonal antibody (N-18; Santa Cruz Biotechnology, Santa Cruz, CA).

Western blot analysis and gel filtration

Western blotting was performed using specific antisera, α-cHSF1x, α-cHSF2α, and α-cHSF3γ, to detect chicken HSFs (Nakai *et al.*, 1995) or specific antisera, α-mHSF1J, α-mHSF2-4, α-mHSF3-1, and α-hHSF4b, to detect mouse HSFs (Fujimoto *et al.*, 2008, 2010). Antisera for HSPs were described previously (Inouye *et al.*, 2003; Fujimoto *et al.*, 2005). Size fractionation of cell extracts by gel filtration was performed as described previously (Nakai *et al.*, 1995). The protein standards were as follows: thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; albumin, 67 kDa.

Gel shift assay

Gel shift assay and the supershift experiment using α-cHSF1γ, α-cHSF2δ, α-cHSF3γ, or α-hHSF4b antiserum were performed using a ^{32}P-labeled ideal HSE probe as described previously (Nakai *et al.*, 1995, 1997). Nuclear extracts from DT40 and NIH3T3 cells were prepared as described previously (Nakai *et al.*, 1995).

Estimation of mRNA levels

Total RNA was isolated from DT40 cells using TRIzol (Invitrogen, Carlsbad, CA) and was reverse transcribed by avian myeloblastosis virus reverse transcriptase and oligo(dT)$_{20}$ according to the manufacturer's instructions (Invitrogen). Real-time PCR analyses of CRYAB (αB-crystallin) mRNA were performed using primers described previously (Hayashida *et al.*, 2010). Real-time PCR analyses were performed using the StepOnePlus system (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Primers used for the real-time PCR are listed in Supplemental Table S1. Relative quantities of HSP70 mRNA were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. All reactions were performed in triplicate with samples derived from three experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP)-enriched DNAs were prepared as described previously (Fujimoto *et al.*, 2008), using a preimmune serum, an antiserum for chicken HSF1 (α-cHSF1c), HSF2 (α-cHSF2α), or HSF3 (α-cHSF3γ). Chicken HSP70 promoter (−340 to −17) was amplified by PCR using primers: cHSP70pro-F, 5′-GAA AAA ACA GGA AGA AGC CCG A-3′; and cHSP70Pro-R, 5′-TGG TCA GTC AGC CAC TCG CTC CGC-3′; and primers for αB-crystallin promoter (−297 to +1) (Hayashida *et al.*, 2010).

Real-time PCR of the ChIP-enriched DNAs for chicken HSP70 promoter (−307 to −242) and GAPDH promoter (−296 to −240) were performed as described above using the primers listed in Supplemental Table S2. Percentage input was determined by comparing the cycle threshold value of each sample to a standard curve generated from a five-point serial dilution of genomic input. All reactions were performed in triplicate with samples derived from three experiments. Mouse HSP70-1 (−235 to −172) and GAPDH (−356 to −291) promoters were similarly analyzed using an antiserum for mouse HSF1 (α-mHSF1J) or HSF2 (α-mHSF2-4) (Fujimoto *et al.*, 2008).

Cell cycle analysis

The cell cycle was analyzed as described previously (Nakai and Ishikawa, 2001). To determine the proportion of sub-G$_1$ cells, fixed cells were stained with propidium iodide and analyzed with a flow cytometer (Cytomics FC 500; Beckman Coulter, Brea, CA).

Analysis of polyQ aggregation

Primary CEF cells were infected with adenovirus expressing short hairpin RNA for cHSF2 (Ad-sh-cHSF2) or scramble RNA (Ad-sh-SCR) (4×10^7 pfu/ml) for 2 h and maintained with normal medium for 22 h. The cells were infected again with Ad-polyQ81-GFP (1×10^7 pfu/ml) and Ad-HA-Ub (5×10^7 pfu/ml) for 2 h and then maintained with normal medium for 46 h. The percentages of cells with polyQ inclusions, accumulation of insoluble polyQ protein, and accumulation of insoluble ubiquitylated protein were estimated as described previously (Hayashida *et al.*, 2010). Briefly, the same volumes of NP-40–insoluble fractions were subjected to Western blotting using antibodies for GFP, hemagglutinin (HA), and β-actin, and signals were quantified using ImageJ software (National Institutes of Health, Bethesda MD). A level in the extract of cells infected with Ad-sh-SCR is set as value 1. To examine the effects of cHSF2 overexpression, cells were infected with Ad-cHSF2 for 2 h and maintained with normal medium for 22 h. The cells were then infected again with Ad-polyQ81-GFP and Ad-HA-Ub and analyzed as described earlier.

Establishment of HSF2-null R6/2 mice

The transgenic mouse line R6/2, which was originally transgenic for the human *huntingtin* gene carrying 154 CAG repeats (Mangiarini *et al.*, 1996), was obtained from Jackson Laboratory (Bar Harbor, ME). Because the impact of HSF1 on the disease progression of R6/2 mice was extremely strong, we analyzed one subline with a transgene carrying shorter (95–97) CAG repeats (Hayashida *et al.*, 2010). R6/2 male mice (CBA × C57BL/6 background) were crossed with HSF2+/− female mice (C57BL/6N background) (Kallio *et al.*, 2002), and HSF2+/− R6/2 mice were generated. HSF2+/− R6/2 male mice were crossed again with HSF2+/− female mice (C57BL/6 background). Thus the resulting mice, with different six genotypes, including HSF2wt/Htt, HSF2-hetero/Htt, and HSF2-ko/Htt, had a mixed background of CBA × C57BL/6 (87.5:12.5%). We examined the life span of all mice having six genotypes. All experimental protocols related with these mice were reviewed by the Committee for Ethics on Animal Experiments of Yamaguchi University Graduate School of Medicine.

Statistical analysis

Data were analyzed with Student's *t* test or analysis of variance (ANOVA). Error bars represent the SD for more than three independent experiments. $p < 0.05$ was considered significant.

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