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CHIP Deficiency Decreases Longevity, with Accelerated Aging Phenotypes Accompanied by Altered Protein Quality Control[▽]

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During the course of biological aging, there is a gradual accumulation of damaged proteins and a concomitant functional decline in the protein degradation system. Protein quality control is normally ensured by the coordinated actions of molecular chaperones and the protein degradation system that collectively help to maintain protein homeostasis. The *c*arboxyl terminus of *H*sp70-*i*nteracting *p*rotein (CHIP), a ubiquitin ligase/cochaperone, participates in protein quality control by targeting a broad range of chaperone substrates for proteasome degradation via the ubiquitin-proteasome system, demonstrating a broad involvement of CHIP in maintaining cytoplasmic protein quality control. In the present study, we have investigated the influence that protein quality control exerts on the aging process by using CHIP$^{-/-}$ mice. CHIP deficiency in mice leads to a markedly reduced life span, along with accelerated age-related pathophysiological phenotypes. These features were accompanied by indications of accelerated cellular senescence and increased indices of oxidative stress. In addition, CHIP$^{-/-}$ mice exhibit a deregulation of protein quality control, as indicated by elevated levels of toxic oligomer proteins and a decline in proteasome activity. Taken together, these data reveal that impaired protein quality control contributes to cellular senescence and implicates CHIP-dependent quality control mechanisms in the regulation of mammalian longevity in vivo.

Maintaining protein folding homeostasis is essential for optimum protein performance and normal cellular function. Molecular chaperones are the group of proteins that perform this essential housekeeping task. Molecular chaperones and their cochaperones ensure the proper folding of nascent proteins, the refolding of damaged/denatured proteins, and the inhibition of protein aggregates (7, 18). When proteins are misfolded, molecular chaperones target these damaged/misfolded proteins for degradation, primarily through the ubiquitin-proteasome system (UPS), which comprises the major protein degradation pathway in eukaryotic cells (17).

The *c*arboxyl terminus of *H*sp70-*i*nteracting *p*rotein (CHIP) possesses U-box-dependent ubiquitin ligase activity as well as cochaperone/chaperone activity (1, 37) and plays an essential role in protein quality control by integrating the molecular chaperone machinery with the UPS (10). CHIP recognizes, ubiquitinates, and degrades unfolded chaperone substrates (9, 30, 34). CHIP is also a key modulator of the stress response, controlling the transcriptional activity of heat shock factor 1 (and thereby regulating the expression of heat shock protein) (11) as well as ubiquitinating and degrading Hsp70 once the cellular stress response has abated (35). In addition, we have shown that mice deficient in CHIP have increased sensitivities to stress associated with hyperthermia and ischemia/reperfusion injury (11, 48). Collectively, these studies indicate that CHIP plays a key role in regulating protein folding homeostasis in vivo during proteotoxic stress conditions.

Recently, the dysregulation of protein quality control has been implicated in the pathogenesis of multiple human degenerative diseases (2). Impaired protein quality control is linked to so-called protein conformational diseases such as Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease, and Huntington's disease, all of which are characterized by excessive protein aggregation and altered UPS function (36). Interestingly, CHIP serves a protective function in protein conformational diseases of the nervous system through its control of homeostasis of disease-related proteins (13, 32). The accumulation of damaged proteins and concomitant UPS dysfunction has also been linked to the degenerative processes associated with biological aging in a variety of systems, including human, rat, mouse, and *Drosophila* (16, 19), although whether this represents a cause or a consequence of aging has not yet been clearly established. Collectively, these observations indicate that the ability of the molecular chaperone system and UPS to maintain protein folding homeostasis has a direct impact on the development of protein conformational diseases and potentially on biological aging as well.

Although studies using invertebrate organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, have implicated molecular chaperones in organismal longevity (22, 44), it is not clear whether the effects of chaperones on aging are due to the suppression of protein misfolding or to other cellular functions of chaperones. In any event, there is no direct evidence for the regulation of mammalian longevity by protein quality control mechanisms. Given the central role of CHIP in coordinating the various arms of the protein quality control machinery, mice lacking CHIP provide an excellent model to

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test the consequences of impaired protein quality control on longevity. Here we show that the loss of CHIP reduces longevity and is associated with an accelerated aging phenotype. Furthermore, we show that CHIP$^{-/-}$ mice accumulate damaged proteins and exhibit a decline in proteasome activity that may be secondary to the accumulation of misfolded proteins. Our results clarify the involvement of protein quality control mechanisms in longevity and demonstrate for the first time that CHIP, through the regulation of protein quality control at multiple points, is an essential regulator of mammalian longevity.

MATERIALS AND METHODS

Animals and pathophysiology. The generation of the CHIP$^{-/-}$ mice used in this study has previously been described (11). Both the CHIP$^{+/+}$ and CHIP$^{-/-}$ mice were maintained on a mixed genetic background of C57BL/6 and 129SvEv. The body fat percentile and bone mineral density were measured using dual-energy X-ray absorptiometry (DEXA) scans and subsequently analyzed with a Lunar PIXImus densitometer (GE-Lunar Corp., Madison, WI). Kyphosis was evaluated by measuring the Cobb's angle on whole-body radiographs of mice of each genotype (42). For histology, tissues were fixed in a 10% formalin solution overnight, processed for paraffin embedding, and subsequently sectioned. All animal husbandry and experiments were approved by the institutional care and use committee for animal research at the University of North Carolina.

MEF culture and 3T9 assays. Murine embryo fibroblasts (MEF) were generated from embryonic day 13.5 embryos according to standard protocols and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), and 2-mercaptoethanol (50 μM). For the 3T9 assay, MEFs were plated at a density of 9×10^5 cells in 100-mm dishes and replated after cell counts every third day to maintain the same cell density. Population doublings were determined by the following formula: $\log_2$ (number of cells harvested/number of cells seeded) (40).

Measuring SA-β-Gal activity and lipid oxidation. Senescence-associated β-galactosidase (SA-β-Gal) activity in MEFs and kidneys was measured by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining using a standard, previously described protocol (14, 31). Lipid oxidation was measured via the detection of 8-isoprostane levels in tissue lysates. Preparation of the tissue lysates and the subsequent assay were performed by using the 8-isoprostane enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol.

Measuring 26S proteasome activity using fluorogenic substrates. The 26S proteasome activity assay was performed as described previously (28). Briefly, tissues were lysed in lysis buffer (250 mM sucrose, 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM dithiothreitol) and nucleic acids were digested with Benzonase (50 U/ml on ice for 15 min). Ten micrograms of cytosolic protein was added to the proteasome reaction buffer (50 mM Tris, pH 7.5, 40 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM ATP, and 0.05 mg/ml bovine serum albumin) that contained fluorogenic substrates (Suc-LLVY-AMC; 75 μM final concentration). Fluorescence was measured every 1 or 2 min for 60 to 90 min at 37°C using a Wallace Victor2 spectrofluorometer (the excitation wavelength was 355 nm, and the emission wavelength was 460 nm). To remove nonspecific substrate hydrolysis, proteins were preincubated with the proteasome inhibitor epoxomycin (50 μM) for 30 min at 37°C and fluorescence units were subtracted from each measurement.

Dot blot analysis. Soluble proteins from brain tissue were prepared in phosphate-buffered saline with protease inhibitor under conditions that avoided protein denaturation and protein misfolding (24). Twenty micrograms of soluble protein was loaded onto nitrocellulose membrane and blotted using antioligomer antibodies obtained either from Biosource (A11) or directly as a generous gift from Charles G. Glabe, University of California, Irvine, CA. Positive and negative controls for antioligomer immunoblotting were carried out using recombinant proteins for mutant (R120G) and wild-type α-B-crystallin, respectively (38) (a generous gift from Atsushi Sanbe, National Research Institute for Child Health and Development, Tokyo, Japan). In cases where the blot was subsequently probed for actin, protein samples were boiled to expose epitopes for the actin antibody before being loaded onto nitrocellulose membrane.

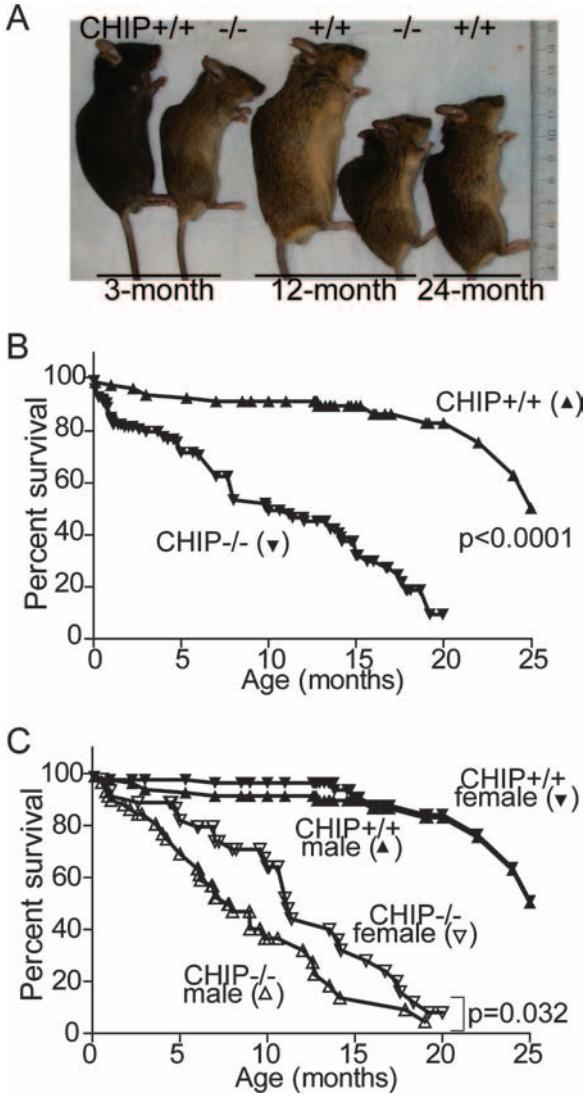

FIG. 1. CHIP$^{-/-}$ mice have shortened life spans compared to those of CHIP$^{+/+}$ mice. (A) Representative photographs of CHIP$^{+/+}$ and CHIP$^{-/-}$ mice in indicated age groups, showing that CHIP$^{-/-}$ mice demonstrated small body sizes and enhanced kyphosis relative to age-matched CHIP$^{+/+}$ mice. (B) Kaplan-Meier survival analyses in a cohort (male and female) of CHIP$^{+/+}$ ($n = 82$) and CHIP$^{-/-}$ ($n = 128$) mice. CHIP$^{-/-}$ mice have significantly shortened life spans compared with those of CHIP$^{+/+}$ mice (log rank test, $P < 0.0001$). (C) Kaplan-Meier survival analyses in CHIP$^{+/+}$ male ($n = 82$), CHIP$^{+/+}$ female ($n = 84$), CHIP$^{-/-}$ male ($n = 58$), and CHIP$^{-/-}$ female ($n = 45$) mouse groups. CHIP$^{-/-}$ male mice showed reduced longevity compared with that of CHIP$^{-/-}$ female mice (log rank test, $P = 0.0324$).

RESULTS

Longevity in mice lacking CHIP. CHIP$^{-/-}$ mice were generated in our laboratory to understand the physiologic consequences of impaired cytoplasmic protein quality control in a mammalian system (11). CHIP$^{-/-}$ mice exhibit partial perinatal lethality that is thought to reflect impaired adaptation to the stress of parturition (11). Further observation of these mice indicated that both male and female CHIP$^{-/-}$ mice displayed smaller body sizes that became more obvious as the mice aged (Fig. 1A). To

TABLE 1. Ratio of tissue weight to body weight for CHIP$^{+/+}$ and CHIP$^{-/-}$ mice at different ages[a]

Tissue type	Tissue weight-to-body weight ratio (mg/g) for indicated age and genotype				
	3 mo		12 mo		>24 mo, CHIP$^{+/+}$
	CHIP$^{+/+}$	CHIP$^{-/-}$	CHIP$^{+/+}$	CHIP$^{-/-}$	
Testis	4.10 ± 0.16	3.88 ± 0.07	3.49 ± 0.06	2.91 ± 0.24*	3.00 ± 0.20
Thymus	1.08 ± 0.02	1.03 ± 0.07	0.89 ± 0.06	0.65 ± 0.02**	0.53 ± 0.03
Gastrocnemius	7.10 ± 0.71	5.07 ± 0.28*	5.52 ± 0.83	3.30 ± 0.42*	4.37 ± 0.14
Quadriceps	7.59 ± 0.17	6.02 ± 0.22**	6.24 ± 0.33	4.63 ± 0.30**	5.02 ± 0.13
Heart	5.62 ± 0.25	6.03 ± 0.67	7.20 ± 0.69	9.59 ± 0.67*	9.65 ± 0.54

[a] Asterisks indicate significant differences between CHIP$^{+/+}$ and CHIP$^{-/-}$ in the same age group (four to eight per group; *, $P < 0.05$; **, $P < 0.01$ [Student's t test]). Data shown are means ± standard errors of the means. Body weights were 31.88 ± 1.46 (CHIP$^{+/+}$) and 22.04 ± 1.32* (CHIP$^{-/-}$) for 3-month-old mice, 34.25 ± 1.39 (CHIP$^{+/+}$) and 24.01 ± 1.06* (CHIP$^{-/-}$) for 12-month-old mice, and 31.71 ± 0.79 for CHIP$^{+/+}$ mice more than 24 months of age.

investigate whether CHIP has an impact on longevity or life span, CHIP$^{+/+}$ and CHIP$^{-/-}$ mice were monitored over a 2-year period. Kaplan-Meier survival analysis indicated that CHIP$^{-/-}$ mice show significantly reduced longevity compared to CHIP$^{+/+}$ mice (log rank test, $P < 0.0001$) (Fig. 1B). Median survival for CHIP$^{+/+}$ mice was 25 months, whereas for CHIP$^{-/-}$ mice it was 10 months, representing a 60% decrease in longevity in the CHIP$^{-/-}$ mice. The longevity of CHIP$^{+/-}$ mice, in contrast, was not different from that of wild-type mice (data not shown). The effect of CHIP on longevity was markedly influenced by gender (log rank test, $P = 0.0324$) (Fig. 1C). The median survival time for CHIP$^{-/-}$ female mice was 11.2 months, whereas the survival time for CHIP$^{-/-}$ male was only 7.8 months, representing a 31% gender-specific decrease in longevity. In contrast, CHIP$^{+/+}$ male and female mice showed no difference in life span (log rank test, $P = 0.5167$). Exhaustive necropsy analyses failed to identify any consistent morbid pathology in CHIP$^{-/-}$ mice that could account for the decreased longevity. Overall, these data indicate that the loss of CHIP results in a significantly shortened life span. In addition, the increased mortality rate in CHIP$^{-/-}$ mice is considerably higher in male mice than in female mice, suggesting that CHIP has a differential effect on gender-related determinants of longevity.

Accelerated anatomical aging in CHIP$^{-/-}$ mice. To test whether CHIP$^{-/-}$ mice exhibit a premature aging phenotype, we compared age-related pathophysiological phenotypes in CHIP$^{+/+}$ and CHIP$^{-/-}$ mice at both 3 months (young adults) and 12 months (aged adults) of age. In addition, 24-month-old CHIP$^{+/+}$ mice were included as a normal aging wild-type control that exceeded the life span of CHIP$^{-/-}$ mice. Body weights were significantly decreased in both 3- and 12-month-old CHIP$^{-/-}$ mice compared to weights in age-matched CHIP$^{+/+}$ mice (Table 1). Atrophy (calculated by the ratio of tissue weight [in milligrams] to body weight [in grams]) was accelerated in skeletal muscles (gastrocnemius and quadriceps), thymus, and testes from CHIP$^{-/-}$ mice at 12 months of age (Table 1), consistent with age-dependent atrophy in these organs (5, 43). In contrast, hearts from CHIP$^{-/-}$ mice at 12 months of age were larger than those from CHIP $^{+/+}$ mice at the same age (Table 1), indicative of acceleration of aging-induced cardiac hypertrophy (29). Because reduced fat stores and dermal atrophy, together with decreased subcutaneous adipose and dermis layers, are sensitive markers of organismal aging (23), we performed histological analysis of skin and quantitative analyses of dermal thickness (Fig. 2A and B). The dermis was significantly thinner, with loss of subcutaneous fat layers, in both 3- and 12-month-old CHIP$^{-/-}$ mice than in the age-matched controls. In addition, whole-body fat stores (measured via a DEXA scan) indicated that CHIP$^{-/-}$ mice had decreased whole-body fat content levels at 12 months of age compared to those of CHIP$^{+/+}$ mice of the same age (Fig. 2C). Lastly, we monitored age-related skeletal changes in these mice by measuring bone mineral density using DEXA scans as well as the degree of kyphosis via the Cobb's angle on radiographic projection of spin. CHIP$^{-/-}$ mice had signs of osteoporosis, as indicated by a decrease in bone mineral density (Fig. 2D), as well as severe kyphosis by 12 months of age (Fig. 2E and F). Collectively, these observations demonstrate accelerated organismal aging in CHIP$^{-/-}$ mice.

Biochemical markers of aging in CHIP$^{-/-}$ mice. To determine whether cellular senescence (8) accompanied the anatomic changes associated with premature aging in CHIP$^{-/-}$ mice, we examined whether CHIP$^{-/-}$ mice display an increased activity of SA-β-Gal, a molecular marker for aging, in vivo and in vitro. Frozen sections of kidneys from CHIP$^{-/-}$ mice had more prominent SA-β-Gal staining in the cortex at both 3 and 12 months of age, whereas age-matched CHIP$^{+/+}$ mice had minimal staining for SA-β-Gal activity (Fig. 3A). Consistent with prior reports (27), there was strong positive SA-β-Gal staining in the 24-month-old CHIP$^{+/+}$ mice that were examined as aged positive controls. Primary MEFs from early and late passages (passage 3 [P3] and P8, respectively) were also examined for SA-β-Gal activity (Fig. 3B). CHIP$^{-/-}$ MEFs displayed a two- to threefold increase in SA-β-Gal-positive cells at all passages examined (7.8% versus 15.6% and 20.3% versus 56.1% in P3 and P8 MEFs, respectively) compared to the value for CHIP$^{+/+}$ MEFs (Fig. 3C). We also performed 3T9 proliferation assays as a marker for cellular replicative senescence in CHIP$^{+/+}$ and CHIP$^{-/-}$ MEFs (Fig. 3D). Consistent with the increased SA-β-Gal activity in CHIP$^{-/-}$ MEFs, the rate of proliferation was lower than, and passages to replicative senescence were reduced compared to, that of CHIP$^{+/+}$ MEFs. As has previously been suggested by others (12), these data suggest that misfolded proteins can activate the senescence tumor suppressor mechanism.

Increased free radical production and enhanced oxidative damage are additional cellular phenomena that are closely associated with the aging process (4), and correlations between oxidative stress and chaperone expression have previously been identified in invertebrate models of aging (49). To deter-

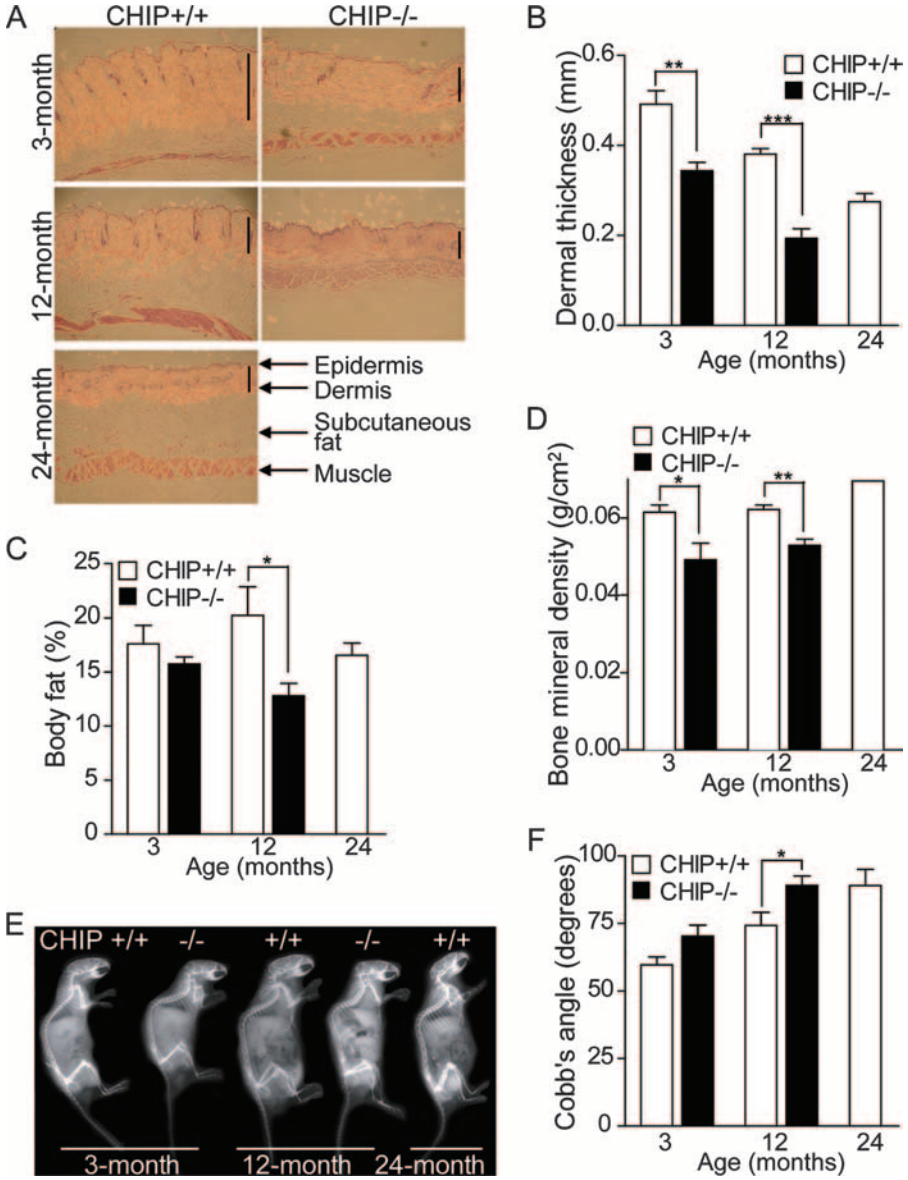

FIG. 2. CHIP$^{-/-}$ mice exhibit a premature aging phenotype. (A) Representative histological sections of skin from CHIP$^{-/-}$ and CHIP$^{+/+}$ mice at indicated age time points (original magnification, ×200). The black bar indicates the dermis layer within the skin section. (B) Quantification of dermal thickness from dorsal skin in indicated genotypes and age groups (three or four mice per group). (C and D) Quantification of body fat stores (C) and bone mineral density (D) measured by DEXA scan in indicated genotypes and age groups (four to eight mice per group). (E) Representative whole-body radiographs show the degree of kyphosis in CHIP$^{-/-}$ and CHIP$^{+/+}$ mice in indicated age groups. (F) Quantification of Cobb's angle, which represents kyphosis, in indicated genotypes and age groups (three to five per group). Data shown are means ± standard errors of the means. Error bars indicate standard deviations. Asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ [Student's t test]).

mine whether the deletion of CHIP predisposed mice to impaired neutralization of aging-related oxidative species, we compared the free radical activity in plasma and tissues from CHIP$^{+/+}$ and CHIP$^{-/-}$ mice by measuring 8-isoprostane levels as an indicator of lipid oxidation (Table 2). Evidence of increased oxidative damage was widespread in CHIP$^{-/-}$ mice, with significant increases observed in the livers at 3 months and in plasma, brains, livers, and lungs at 12 months of age compared to the levels seen for tissues from age-matched CHIP$^{+/+}$ mice. There is debate as to whether increased oxidative stress is a cause or consequence of biological aging, and it should be noted that the accelerated aging seen in CHIP$^{-/-}$ is far more severe than that seen in animals lacking the enzymes (e.g., superoxide dismutase and catalase) for metabolizing oxygen radicals (4). Nonetheless, our observation of premature oxidative damage provides an additional biochemical marker of accelerated aging in CHIP$^{-/-}$ mice, further supporting the contention that protein quality control mechanisms mediated by CHIP are required to retard the onset of aging-related phenotypes in mammals.

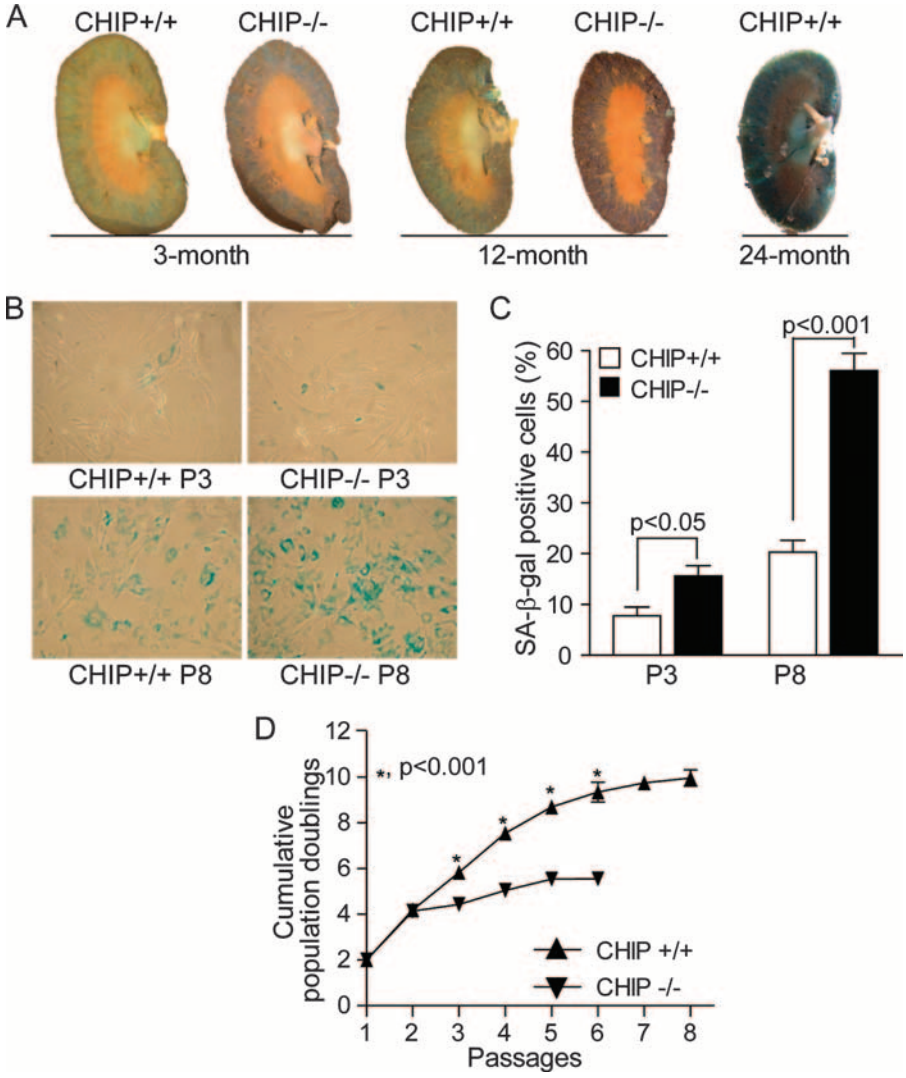

FIG. 3. Deficiency of CHIP leads to increased SA-β-Gal activity and replicative senescence. (A) Photographs of SA-β-Gal staining in kidneys from CHIP$^{-/-}$ and CHIP$^{+/+}$ mice in indicated age groups. SA-β-Gal-positive staining (blue) in the renal cortex was apparent in kidneys from CHIP$^{-/-}$ mice at 3 and 12 months of age as well as in kidneys from 24-month-old CHIP$^{+/+}$ mice. (B) Representative images of SA-β-Gal staining in CHIP$^{-/-}$ and CHIP$^{+/+}$ MEFs at P3 and P8 (original magnification, ×200). (C) Quantitative analysis of the percentage of SA-β-Gal-positive cells from CHIP$^{-/-}$ and CHIP$^{+/+}$ MEFs at indicated passages. Data shown are means ± standard errors of the means. Error bars indicate standard deviations. *P* values were determined by Student's *t* test. (D) 3T9 proliferation analysis in CHIP$^{-/-}$ and CHIP$^{+/+}$ primary MEFs from different passages. Data shown are mean ± standard deviations. *P* values were determined by Student's *t* test.

Age-dependent defects in protein homeostasis in CHIP$^{-/-}$ mice. Previous studies support the idea that CHIP is influential in protecting against the pathogenesis of neurodegenerative diseases by controlling the folding and degradation of disease-related proteins in vivo (13, 32). Therefore, we speculated that CHIP, as a ubiquitin ligase/cochaperone, could also regulate protein quality control and the acceleration of biological aging. To test whether CHIP$^{-/-}$ mice exhibit defects in protein quality control, we assessed the accumulation of damaged proteins in CHIP$^{-/-}$ mice. As an indicator of protein damage and protein folding defects, we used a recently developed antibody called antioligomer (A11) that detects soluble aberrantly folded proteins in the brain (24). Antioligomer antibody can recognize oligomer proteins (Aβ, polyQ, prion, and insulin) in protein conformational diseases, including Alzheimer's disease, prion disease, and desmin-related cardiomyopathy (24, 28, 38). Since this antibody has been used successfully in the study of neurodegenerative diseases, we isolated cortical tissue from CHIP$^{+/+}$ and CHIP$^{-/-}$ mice and tested for antioligomer staining via dot blot analysis. Aberrantly folded proteins detected by this antibody were present in brain lysates of 3-month-old CHIP$^{-/-}$ mice and were even more abundant in the brains of 12-month-old CHIP$^{-/-}$ mice, but were present at much lower levels in CHIP$^{+/+}$ mice of either age (Fig. 4A), only accumulating in appreciable levels in wild-type mice after 24 months (data not shown). The specificity of this antibody for misfolded proteins was confirmed by using R120G α-B-crystallin as a positive control (38) (Fig. 4B). Quantitative analysis indicated that levels of aberrantly folded proteins were threefold higher in brains of CHIP$^{-/-}$ mice at both 3 and 12 months

TABLE 2. Oxidative damage in tissues as measured by 8-isoprostane levels[a]

Tissue type	Mean 8-isoprostane level (pg/ml) ± SEM for indicated age and genotype				
	3 mo		12 mo		>24 mo, $CHIP^{+/+}$
	$CHIP^{+/+}$	$CHIP^{-/-}$	$CHIP^{+/+}$	$CHIP^{-/-}$	
Plasma	31.33 ± 4.01	32.50 ± 3.18	53.20 ± 10.05	75.00 ± 6.72*	80.20 ± 13.82
Brain	1,373.59 ± 209.28	1,459.41 ± 80.71	1,819.20 ± 183.09	2,863.07 ± 119.87**	2,015.18 ± 188.90
Heart	2,404.50 ± 100.50	2,698.00 ± 354.27	2,754.40 ± 676.62	3,339.67 ± 579.18*	3,103.32 ± 456.36
Liver	3,287.66 ± 806.74	4,664.57 ± 444.64**	4,631.00 ± 205.97	6,247.53 ± 541.58*	2,866.39 ± 745.34
Lung	630.38 ± 131.51	674.10 ± 196.30	954.15 ± 226.03	1,563.05 ± 141.59*	1,849.89 ± 329.65

[a] Asterisks indicate significant differences of 8-isoprostane levels in $CHIP^{-/-}$ tissues compared to $CHIP^{+/+}$ tissues of the same age group (*, $P < 0.05$; **, $P < 0.01$ [Student's t test]).

of age than the levels in brains from wild-type mice (Fig. 4C). These analyses provide an in vivo correlate for the protein quality control mechanism ascribed to CHIP in in vitro studies (9, 30). Importantly, the accumulation of misfolded proteins in $CHIP^{-/-}$ mice preceded the manifestations of most of the aging-related biochemical and anatomic phenotypes in these mice, which invites the consideration of a causative role for accumulating levels of toxic misfolded proteins in premature aging.

Protein aggregates, damaged proteins, and toxic oligomers are known to have inhibitory effects on proteasome activity (3). The UPS (along with molecular chaperones) is an essential component in the maintenance of proper protein quality control via its removal of damaged proteins. The inhibition of proteasome activity further enhances cellular injury elicited by aberrant protein conformations, and the measurement of proteasome activity can serve as a cellular marker for the toxic effects of misfolded proteins in the setting of impaired protein quality control. Therefore, we investigated whether CHIP deficiency would alter 26S proteasome activity in tissues from $CHIP^{-/-}$ mice. Skeletal muscle, liver, and lung lysates from $CHIP^{-/-}$ and age-matched controls were analyzed for 26S proteasome activity by using a fluorogenic substrate. In 3-month-old $CHIP^{-/-}$ mice and age-matched $CHIP^{+/+}$ mice, no differences in proteasome activity were evident in any of the tissues assayed (Fig. 5A). However, skeletal muscle and liver from $CHIP^{-/-}$ mice demonstrated a significant reduction in proteasome activity by 12 months of age compared to tissues from age-matched $CHIP^{+/+}$ mice (Fig. 5B). Taken together, these data indicate that $CHIP^{-/-}$ mice exhibited an accumulation of damaged proteins and a decrease in proteasome ac-

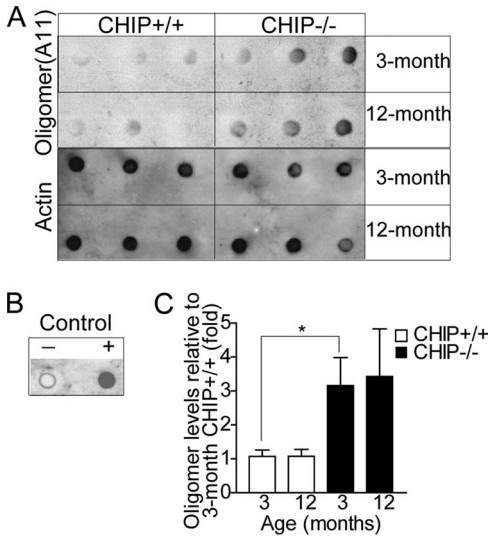

FIG. 4. Accumulation of misfolded proteins in $CHIP^{-/-}$ mice. (A) Expression of misfolded oligomer proteins in brain tissues obtained from indicated genotypes and age groups, as detected by a dot blot analysis using antioligomer (A11) antibody. Actin was used as a loading control. (B) Dot blot analysis showing positive and negative controls for the antioligomer antibody (see Materials and Methods). (C) Quantification of oligomer expression in brain tissue from the dot blot shown in panel A. Expression of oligomer was measured relative to the oligomer level in brain tissues of 3-month-old $CHIP^{+/+}$ mice. Data shown are means ± standard errors of the means. Error bars indicate standard deviations. *, $P < 0.05$ (Student's t test).

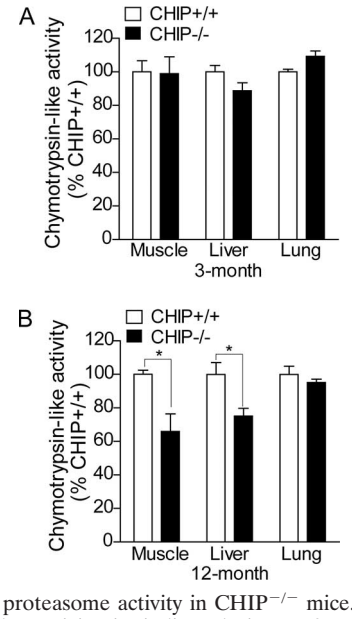

FIG. 5. 26S proteasome activity in $CHIP^{-/-}$ mice. Comparison of chymotrypsin-like activity in indicated tissues from $CHIP^{-/-}$ and $CHIP^{+/+}$ mice at 3-month (A) and 12-month (B) age groups (three mice per group). Chymotrypsin-like activity was measured as described in Materials and Methods by using a fluorogenic substrate (Suc-LLVY-AMC) specific for chymotrypsin-like activity. The percentile of 26S proteasome activity was measured relative to the activity in $CHIP^{+/+}$ mice from each age group. Data shown are means ± standard errors of the means. Error bars indicate standard deviations. *, $P < 0.05$ (Student's t test).

tivity compared to CHIP$^{+/+}$ mice, suggesting that CHIP deficiency accelerates the aging process, at least in part, by disturbing protein quality control and accelerating cellular senescence.

DISCUSSION

The fundamental role of molecular chaperones is to assist in the folding of newly synthesized polypeptides, to build functionally active proteins, and to prevent protein misfolding and aggregation (7, 18). The importance of molecular chaperones is clearly recognized in stress conditions, biological aging, and conformational diseases that are known to provoke protein misfolding and aggregation (2). In terms of the biological aging process, studies of *C. elegans* and *D. melanogaster* have suggested that molecular chaperones directly control longevity. For example, Hsf1 knockdown in *C. elegans* reduces longevity and mtHsp70 knockdown promotes a progeria-like phenotype (22, 26). In addition, numerous heat shock proteins (including Hsp16, mtHsp70, Hsp27, and Hsp70) have also been shown to be directly related to life span in *C. elegans* and *D. melanogaster* (44, 47). In mammalian systems, molecular chaperones preserve protein homeostasis during the response to stress and disease conditions. Studies involving *Hsf1*$^{-/-}$ mice suggest that *Hsf1* is required to protect against demyelination and gliosis that can occur during aging (21), whereas mutations in molecular chaperone genes, such as *Hsf4*, *sHsp*, and α-*B-crystallin*, are known to associate with age-related conformational diseases, such as cataract formation, desmin-related myopathy, and distal motor neuropathy (6, 15, 45). Despite the above-mentioned studies, there is no direct evidence indicating a correlation between molecular chaperones and mammalian longevity in vivo. The data presented here demonstrate a comprehensive age-associated phenotype in various organs and cell types of mice deficient in the molecular chaperone CHIP (Fig. 2 and 3; Tables 1 and 2). Accelerated aging phenotypes were exhibited in CHIP$^{-/-}$ mice as early as 3 months of age, and the shortened life spans were associated with a 60% reduction in median survival (Fig. 1), suggesting that CHIP deficiency leads to accelerated aging phenotypes, which result in shortened life spans. From these results, we have established the first in vivo evidence that molecular chaperones can directly regulate mammalian aging and contend that CHIP is an essential regulator of mammalian longevity.

Protein quality control involves ensuring protein folding homeostasis under normal as well as stressful conditions (17). In addition to maintaining proper protein folding via the molecular chaperone machinery mentioned above, the protein degradation system (primarily the UPS) assists in protein quality control by removing damaged, unfolded, and potentially toxic proteins (3). UPS function has been shown to decrease with age in studies using rodent models as well as in humans (16, 19). UPS function has also been linked to conformational diseases that present, in part, as an accumulation of damaged/misfolded proteins (3, 17, 19). The functional ineffectiveness of the UPS increases damaged or misfolded proteins, resulting in an overall impairment in protein quality control in disease conditions as well as during aging (17). In this study, we examined protein quality control by measuring the accumulation of damaged proteins and the decline of UPS function. Our data suggest that CHIP deficiency disturbs the protein quality control mechanisms in mice. We saw an increase in toxic oligomer proteins in brain tissue and a decrease in UPS function in CHIP$^{-/-}$ mice compared to wild-type controls (Fig. 4 and 5). We speculate that without CHIP, there is an overload of damaged proteins, along with impaired proteasome activity. This results in an imbalance in protein quality control, which in turn could represent a principal mechanism in the determination of longevity.

It is clear from other studies using animal models that numerous biological factors (including the redox system, DNA damage, insulin signaling, and functional defects in stem cells) are affected by the biological aging process (4, 25, 39). From our studies, the mechanistic basis for premature aging in CHIP$^{-/-}$ is not clear, and we cannot disregard the possibility that biological systems other than protein quality control may be affected by CHIP deficiency. CHIP deficiency seems to increase oxidative damage, especially in lipid oxidation (Table 2). However, the lack of CHIP did not affect the expression of FOXOs (forkhead-related transcription factors) or the NAD-dependent histone deacetylase and homologue of the yeast (*Saccharomyces cerevisiae*) sirtuin SIRT (data not shown), both of which are known to contribute to changes in mammalian longevity (39). In CHIP$^{-/-}$ mice, as in several other mammalian models (14, 20, 27, 31), aging closely correlated with the expression of biomarkers of cellular senescence, consistent with a possible causal role of senescence in aging (41). Nevertheless, it remains to be determined whether other age-related signaling pathways may be involved in the premature aging phenotype seen in CHIP$^{-/-}$ mice. In addition, it will be interesting to test whether caloric restriction and exercise, which are the known ways to improve maximal life span in mammals (33, 46), can modify the aging phenotype and longevity in CHIP$^{-/-}$ mice. The observations in this report indicate that CHIP is required for longevity and, as such, this study is the first demonstration of a direct connection between molecular chaperones and mammalian longevity in vivo. Indeed, we propose that protein quality control is one of causal mechanisms that prevent cellular senescence, aging, and longevity in mammalian systems.

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