

**Figure 1** ASC-J9 selectively promotes AR degradation by disrupting the interaction between AR and AR coregulators. (a) Coimmunoprecipitation results showing that ASC-J9 disrupted the AR-ARA70 complex in LNCaP cells pretreated with the proteasomal inhibitor MG132 (5  $\mu$ M). (b) DHT-induced AR transactivation in COS-1 and PC12/AR-10Q cells was suppressed after ASC-J9 treatment, as assessed by (ARE)4-LUC reporter assay. (c) The steady-state level of AR protein in LNCaP and PC12/AR-10Q cells from western blot assays indicated that the AR signal decreased substantially (in a dose-dependent manner) after treatment with ASC-J9. (d) ASC-J9 selectively decreased the steady-state level of AR protein, in the absence and the presence of ligand. In contrast, 17-AAG treatment decreased the steady-state level of all four ligand-activated nuclear receptors (AR, ER $\alpha$ , GR and RXR $\alpha$ ), both in the absence and in the presence of the specific ligand.

Moreover, the SBMA mice showed normal sexual activity and improved fertility, suggesting that this strategy might provide a better approach to treating SBMA in men.

## RESULTS

### ASC-J9 selectively promotes AR degradation

To test our hypothesis that disrupting the interaction between AR and AR-associated proteins is an improved strategy for battling SBMA, we first used a coimmunoprecipitation assay in cultured prostate cancer LNCaP cells, which demonstrated that ASC-J9 promotes the dissociation between AR and its coregulator ARA70 (Fig. 1a). We then found that, in COS-1 cells, ASC-J9-induced dissociation between AR and ARA70 led to suppression of AR transactivation (Fig. 1b). Similar suppression effects also occurred when we replaced COS-1 cells with PC12/AR-10Q cells (Fig. 1b).

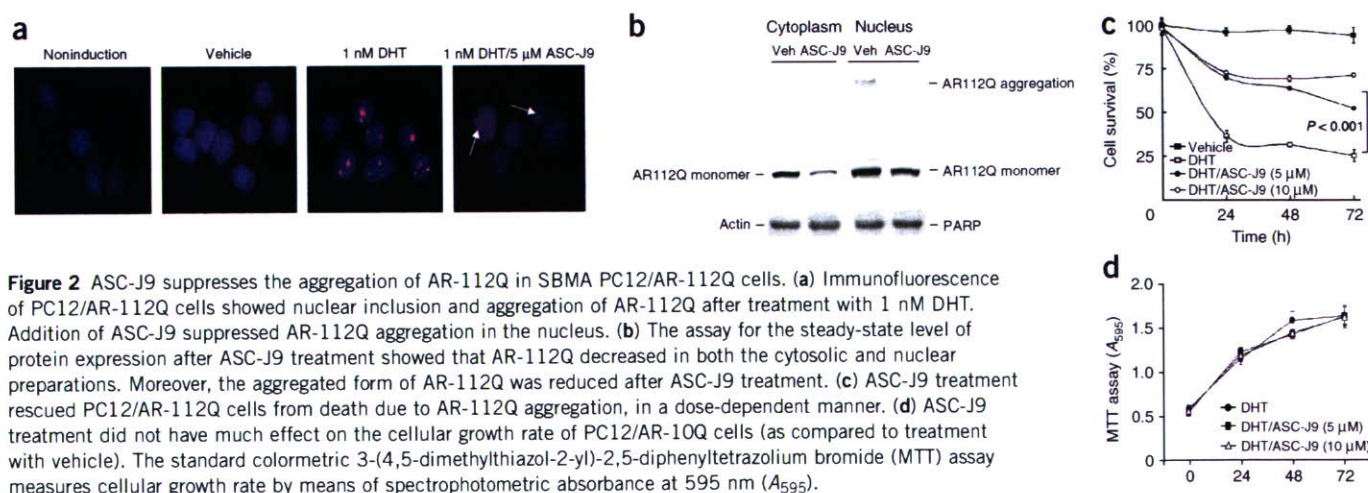
To determine whether AR degrades more rapidly when freed of its association with coregulators, we measured the steady-state level of AR protein after the administration of different doses of ASC-J9 in LNCaP and PC12/AR-10Q cells. ASC-J9 treatment decreased the steady-state level of AR protein in the absence and presence of the hormone dihydrotestosterone (DHT), suggesting that ASC-J9 might promote AR protein degradation by disrupting the interaction of AR with AR coregulators (Fig. 1c). As the interaction between AR and ARA70 is

relatively selective<sup>9</sup>, we expected that the degradation of AR by ASC-J9 would also be selective. Whereas ASC-J9 promoted the degradation of AR, it had little effect on other members of the family of ligand-activated nuclear receptors, such as glucocorticoid receptor (GR), estrogen receptor- $\alpha$  (ER $\alpha$ ) and retinoid X receptor- $\alpha$  RXR $\alpha$  (Fig. 1d). In contrast, the hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), which uncouples the interaction between hsp90 and members of this family, unselectively promoted the degradation of AR as well as GR, ER $\alpha$  and RXR $\alpha$ .

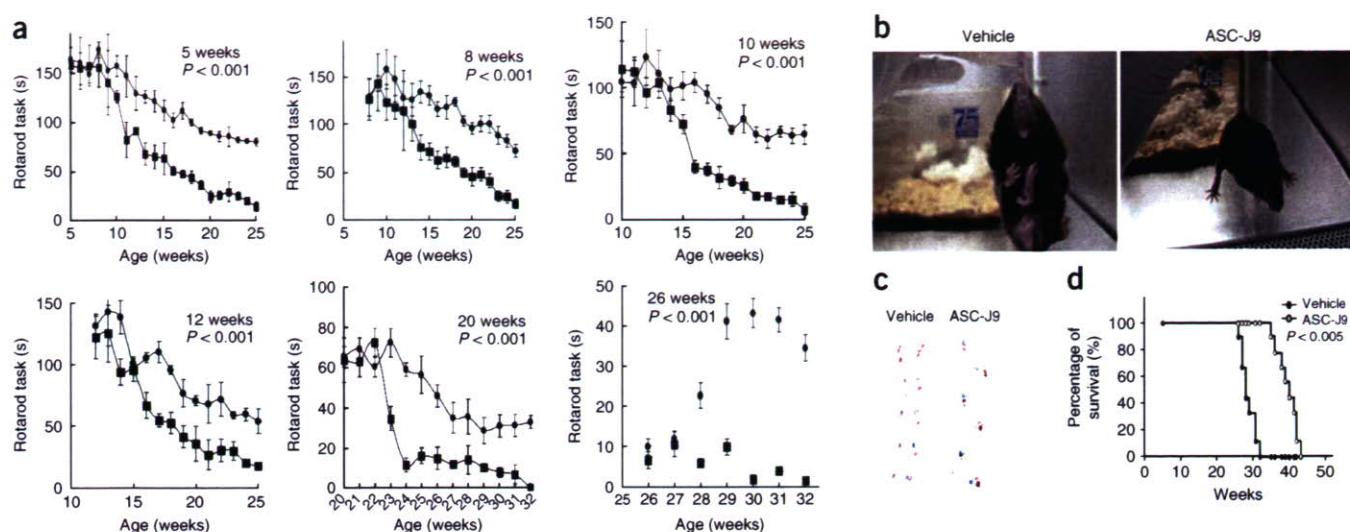
Collectively, the results demonstrated that ASC-J9, but not 17-AAG, classic antiandrogen hydroxylflutamide (HF) or curcumin (Fig. 1d and **Supplementary Fig. 1** online), can selectively promote AR degradation, which might be secondary to disrupting the interaction between AR and AR coregulators, which results in the suppression of AR transactivation.

### ASC-J9 reduces the AR aggregated AR-112Q in cells

The aggregation of AR-polyQ in the nucleus, which is toxic to motor neurons, has been linked to the pathogenesis of SBMA (ref. 11). We tested PC12 cells stably transfected with inducible AR-112Q (PC12/AR-112Q) in a model that mimics the nuclear aggregation present in SBMA (ref. 23) to see whether ASC-J9 can reduce the pathogenesis of SBMA. We found that AR-112Q localized in the cytoplasm in the



**Figure 2** ASC-J9 suppresses the aggregation of AR-112Q in SBMA PC12/AR-112Q cells. (a) Immunofluorescence of PC12/AR-112Q cells showed nuclear inclusion and aggregation of AR-112Q after treatment with 1 nM DHT. Addition of ASC-J9 suppressed AR-112Q aggregation in the nucleus. (b) The assay for the steady-state level of protein expression after ASC-J9 treatment showed that AR-112Q decreased in both the cytosolic and nuclear preparations. Moreover, the aggregated form of AR-112Q was reduced after ASC-J9 treatment. (c) ASC-J9 treatment rescued PC12/AR-112Q cells from death due to AR-112Q aggregation, in a dose-dependent manner. (d) ASC-J9 treatment did not have much effect on the cellular growth rate of PC12/AR-10Q cells (as compared to treatment with vehicle). The standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay measures cellular growth rate by means of spectrophotometric absorbance at 595 nm ( $A_{595}$ ).



**Figure 3** Effects of ASC-J9 (50 mg/kg every 48 h) on SBMA symptoms in male SBMA mice. (a) Performance of mice of different ages on the rotarod tasks ( $n = 6$  in each group). Mice treated with ASC-J9 showed noticeable improvement even when the treatment started as late as 26 weeks after the onset of SBMA phenotype. (b–d) SBMA mice were treated with vehicle or ASC-J9 starting at 5 weeks, and were examined at 15 weeks. With ASC-J9, phenotypic clasping behavior was ameliorated (b), footprint patterns were normal (c) and the survival rate had improved (d) ( $n = 9$  mice in each group). In c, front paws are in blue and hind paws in red.

absence of DHT, and addition of 1 nM DHT resulted in the translocation of AR-112Q into the nucleus and subsequent formation of aggregates (Fig. 2a). Notably, addition of 5  $\mu$ M ASC-J9 substantially suppressed the aggregate formation, with little AR-112Q detected in the nucleus (Fig. 2a). Western blotting analysis also showed that ASC-J9 treatment promoted the degradation and reduced the amount of aggregated AR-112Q protein in the nucleus (Fig. 2b).

In addition, PC12 cell death induced by AR-112Q nuclear aggregation was rescued by the addition of ASC-J9 in a dose-dependent manner (Fig. 2c), with little influence on the proliferation of PC12/AR-10Q cells (Fig. 2d). Collectively, these results demonstrated that ASC-J9 might reduce cytotoxicity by suppressing the aggregation of AR-112Q in the nucleus and increasing its degradation in the PC12/AR-112Q cells, with little effect on the PC12/AR-10Q cells.

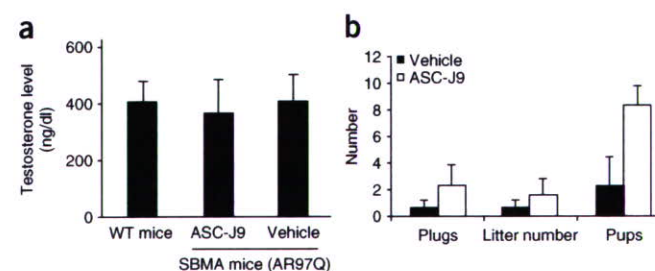
#### ASC-J9 rescues the SBMA symptoms in AR-97Q mice

We further examined the *in vivo* effects of ASC-J9 in SBMA mice with transgenic AR-97Q (ref. 5). Every other day, we injected male SBMA mice intraperitoneally with ASC-J9 in corn oil at the effective dose of 50 mg per kg body weight, and assessed their motor impairment by testing rotarod activity weekly. We found that the motor impairments were substantially improved in SBMA mice treated with ASC-J9, regardless of whether treatment was started early (at 5 weeks of age) or later (at 26 weeks) (Fig. 3a). This observation suggests that ASC-J9 treatment substantially delays the onset and symptomatic progression of motor impairment, which usually appears at 10 weeks. We also found that cage activity increased in SBMA mice treated with ASC-J9 (Supplementary Video 1 online). The SBMA symptoms of gait disturbance, including severe dragging of hind limbs and erratic footprinting patterns, markedly declined in ASC-J9-treated SBMA mice, suggesting that ASC-J9 treatment can substantially ameliorate the SBMA symptoms in these mice (Fig. 3b,c). Notably, ASC-J9 treatment prolonged the lives of these mice, from an average of 28 weeks to 39 weeks (Fig. 3d).

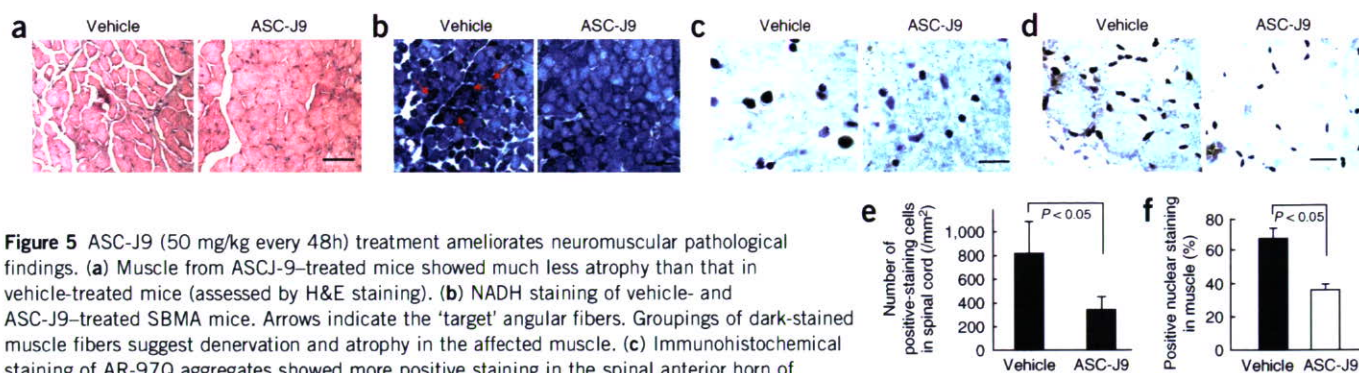
#### Improved sexual functions in SBMA mice treated with ASC-J9

SBMA patients might be reluctant to undergo the helpful but aggressive treatment of surgical or chemical castration because such treatments suppress serum testosterone levels, leading to a loss of normal sexual genital functions and fertility. Notably, we found that ASC-J9-treated SBMA mice had relatively normal serum testosterone concentrations (Fig. 4a). Sexual activity, as judged by vaginal plug numbers in female mice caged with the treated males, and fertility, as judged by both pup numbers and litter numbers, were substantially improved during 4 weeks of fertility tests (Fig. 4b).

These results demonstrated that there was little adverse influence on serum testosterone and that sexual genital functions and fertility were in fact improved in SBMA mice treated with ASC-J9.



**Figure 4** Effect of ASC-J9 (50 mg/kg every 48h) on the fertility and testosterone level of male SBMA mice. We began treatment of SBMA mice at 5 weeks of age. (a) We took blood samples from wild-type (WT) and vehicle- or ASC-J9-treated SBMA mice at 20 weeks of age ( $n = 9$  mice in each group), and measured serum testosterone levels using an ELISA kit. We found little difference between vehicle- and ASC-J9-treated mice in terms of serum testosterone levels. (b) Fertility tests performed on 13-week-old SBMA mice ( $n = 4$  mice in each group) showed increased sexual activity following ASC-J9 treatment (compared to vehicle treatment).



**Figure 5** ASC-J9 (50 mg/kg every 48h) treatment ameliorates neuromuscular pathological findings. (a) Muscle from ASC-J9-treated mice showed much less atrophy than that in vehicle-treated mice (assessed by H&E staining). (b) NADH staining of vehicle- and ASC-J9-treated SBMA mice. Arrows indicate the 'target' angular fibers. Groupings of dark-stained muscle fibers suggest denervation and atrophy in the affected muscle. (c) Immunohistochemical staining of AR-97Q aggregates showed more positive staining in the spinal anterior horn of vehicle-treated mice than in ASC-J9-treated mice. (d) Muscle fibers of SBMA mice treated with ASC-J9 showed much less aggregation of AR-112Q than the muscle fibers from vehicle-treated SBMA mice. (e,f) There was approximately 50% reduction in AR-aggregated positive-staining cells in the spinal cord and the muscle fibers after ASC-J9 treatment compared with vehicle treatment. Scale bars: 100  $\mu$ m in a and b, 20  $\mu$ m in c and d.

### ASC-J9 reverses muscular atrophy and restores VEGF expression

Hematoxylin and eosin staining showed that ASC-J9 treatment substantially reduced muscular atrophy compared to vehicle treatment (Fig. 5a). By using nicotinamide adenine dinucleotide (NADH) to stain muscle, we also found that the groupings of muscle fibers were markedly altered in the ASC-J9-treated mice (Fig. 5b). There were more groupings of ankylated muscle fibers, suggesting the denervation and atrophy of muscle fibers, in vehicle-treated mice than in ASC-J9-treated mice (Fig. 5b). Immunohistochemical staining using an antibody to AR, N20, showed the intranuclear aggregation of AR-97Q in spinal cord motor neurons and skeletal muscle cells (Fig. 5c,d). Also, the intranuclear AR-97Q aggregation in spinal cord neurons and muscle cells was significantly lower (by almost 50%) in the ASC-J9-treated mice than in the vehicle-treated mice (Fig. 5e,f).

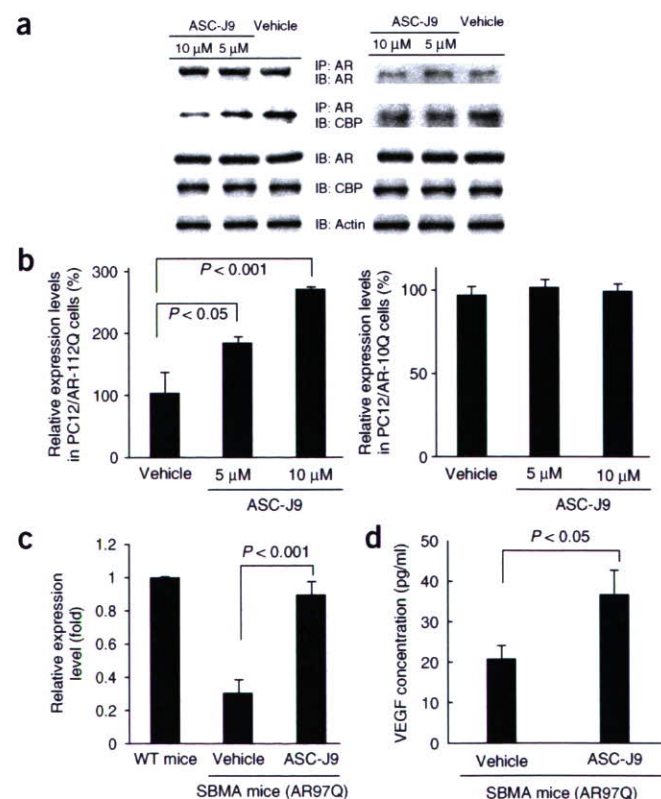
Motor neuron survival and proper function requires VEGF164 expression. Previous studies have suggested that aggregated AR-97Q might associate abnormally with CBP, resulting in the disruption of CBP-mediated VEGF164 expression<sup>24</sup>. By examining coimmunoprecipitation in PC12/AR-112Q and PC12/AR-Q10 cells, we found that ASC-J9 dose-dependently disrupted the interaction between AR-112Q and CBP in PC12/AR-112Q cells but had little influence in PC12/AR-10Q cells (Fig. 6a). Releasing CBP from its interaction with AR-112Q in PC12/AR-112Q cells was associated with the induction of VEGF164 expression (Fig. 6b).

We further confirmed this finding by showing a significant increase (from 20% to 90%) in the expression of mRNA for VEGF164 in the spinal cord of SBMA mice following ASC-J9 treatment (Fig. 6c). Moreover, the ELISA assay to detect the levels of VEGF protein in the spinal cord of these mice also demonstrated a significant increase of VEGF expression after ASC-J9 treatment (Fig. 6d). These results do not formally prove that ASC-J9 ameliorates SBMA symptoms by restoring VEGF164 expression, but they are consistent with this idea.

**Figure 6** Molecular mechanisms of the effect of ASC-J9 on the SBMA phenotypes. (a) Treatment with 5  $\mu$ M or 10  $\mu$ M ASC-J9 showed dose-dependent disruption of the interaction between CBP and AR-112Q or AR-10Q in PC12 cells pretreated with the proteasomal inhibitor MG132 (5  $\mu$ M). (b) ASC-J9 treatment increased VEGF164 expression in PC12/AR-112Q cells but had little influence in PC12/AR-10Q cells pretreated with MG132. (c) ASC-J9 treatment had a dose-dependent effect on increasing mRNA for VEGF164 in the spinal cord. (d) Amounts of VEGF protein from homogenized L5 spinal cords were increased after ASC-J9 treatment.

### DISCUSSION

It has been shown that one of hsp90's associated proteins, AR, can be degraded when the interaction between hsp90 and its associated proteins is disrupted by using the hsp90 inhibitor 17-AAG. This degradation reduces the longer AR-97Q nuclear aggregates in SBMA mice<sup>25</sup>. However, 17-AAG also disrupts the interaction between hsp90 and many other proteins, including GR, ER $\alpha$  and RXR $\alpha$  (Fig. 1d), and Her2, Her3 receptor tyrosine kinase, Erk1/2 and Rb (refs. 25–30), rendering them more susceptible to degradation. Recent reports have clearly documented that common adverse reactions to 17-AAG include anorexia, diarrhea, nausea, fatigue and vomiting, along with the reversible elevation of liver enzymes (in 29.5% of subjects)<sup>31</sup>. This inhibitor also enhances bone metastasis and osteolytic lesions, which



lead to increased osteolysis and incidence of skeletal tumors<sup>32</sup>. The nonspecific disruption by 17-AAG of the interaction between hsp90 and most of its associated proteins leads to extensive adverse and unwanted side effects, and therefore limits the applicability of 17-AAG in the treatment of SBMA. In contrast, ASC-J9 (at a dose of 50 mg/kg every 48 h for more than 20 weeks) had no obvious toxic effects and did not result in a loss of body weight in mice. Moreover, the sexual genital functions and fertility in these SBMA mice were improved markedly. Together, these positive results of ASC-J9 treatment demonstrate that this new approach—involving the selective disruption of interactions between AR-polyQ and AR coregulators, such as CBP—might offer improved treatment for SBMA. Additional dosage studies of ASC-J9 or its derivatives to investigate how SBMA symptoms may be effectively ameliorated, without toxicity, might lead to treatments that could substantially improve the quality of life of SBMA patients.

## METHODS

**Therapeutic agent and administration protocol.** ASC-J9 from AndroScience was synthesized as described previously<sup>22</sup>. We dissolved it in corn oil and injected it intraperitoneally into mice (50 mg/kg every other day) at various ages until the end of the study. Control mice received DMSO in corn oil only.

**ASC-J9 characterization.** For the AR degradation study, we treated LNCaP cells with vehicle and 1 nM DHT with or without 5  $\mu$ M ASC-J9, in RPMI supplemented with 10% charcoal dextran–stripped (CDS) FBS. At selected time intervals, we harvested cells and analyzed AR protein levels by western blotting, quantitated the results by Bio-Rad PDQuest Image software, and normalized densitometric values to actin. We purchased the antibodies for AR (N20), CBP (C-1), ER $\alpha$  (HC-20), GR (P-20), RXR $\alpha$  (D-20), CBP, PARP and actin from Santa Cruz Biotechnology and generated ARA70 antibody as previously described<sup>33</sup>.

For the AR-112Q protein steady-state assay, we cultured PC12/AR-112Q cells as described previously<sup>23</sup>, in the presence of 10  $\mu$ g/ml doxycycline for 24 h, and then treated cells with or without 1 nM DHT or 1 nM DHT and 5  $\mu$ M ASC-J9 for 3 d. We performed the cytosolic and nuclear extraction by FractionPREP™ Cell Fraction System (BioVision) and analyzed the AR-112Q by western blotting.

We performed the protein steady-state assay of AR, GR, ER $\alpha$  and RXR $\alpha$  in response to 5  $\mu$ M ASC-J9 or 360 nM 17-AAG, on LNCaP cells (AR and RXR $\alpha$ ), MCF-7 cells (ER $\alpha$ ) and PC3 cells (GR), in the presence or absence of ligand (1 nM DHT for AR, 1 nM E2 for ER $\alpha$ , 1 nM dexamethasone for GR and 1  $\mu$ M 9-cis-RA for RXR $\alpha$ ). We determined the amounts of AR, GR, ER $\alpha$  and RXR $\alpha$  proteins by western blotting and analyzed the interactions between AR-ARA70 complex and AR-CBP complex by coimmunoprecipitation<sup>24,33</sup>. We assayed AR transactivation activity as described previously<sup>34</sup>.

**Immunofluorescence staining and cell survival.** We cultured PC12/AR-112Q cells in two-well Chamber slides (Nalge Nunc) supplemented by DMEM, 10% CDS horse serum and 100  $\mu$ g/ml nerve growth factor (BD Biosciences), and induced AR-112Q by 10  $\mu$ g/ml doxycycline (Sigma) for 4 h. Then we treated cells with vehicle, 1 nM DHT, or 1 nM DHT and 5  $\mu$ M ASC-J9 for 3 d. We stained AR-112Q with N20 antibody and Texas Red–conjugated streptavidin (Vector Laboratories), mounted slides in fluorescent mounting medium containing 4',6-diamidino-2-phenylindole (DAPI), and observed fluorescent staining using an Olympus fluorescent microscope.

For the cell survival assay, we cultured PC12/AR-112Q and PC12/AR-10Q cells as described previously<sup>23</sup> and incubated cells in the presence of 10  $\mu$ g/ml doxycycline for 24 h. Then we treated cells with vehicle, 5  $\mu$ M ASC-J9 or 10  $\mu$ M ASC-J9, along with 1 nM DHT, and determined cell viability using Trypan blue staining at specific time intervals.

**SBMA mouse model generation, maintenance, genotyping and motor activity assessment.** We generated the AR-97Q SBMA mice as described previously<sup>5</sup>. We performed all animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and with approval from the Department of Laboratory Animal

Medicine at the University of Rochester. We assessed rotarod performance weekly using an Economex Rotarod (Colombus Instruments) as described<sup>35</sup>, and observed footprints for ASC-J9– or vehicle-treated SBMA mice by dipping their forepaws in water-soluble red paint and hind paws in blue paint. The mice then walked through a narrow tunnel, leaving footprints on a strip of white paper<sup>21</sup>.

**Serum testosterone and male fertility.** We killed SBMA mice receiving ASC-J9 or vehicle treatment at 20 weeks of age, drew 1 ml of blood by cardiocentesis, and assayed serum testosterone with the Coat-A-Count Total Testosterone radioimmunoassay (Diagnostic Products) according to the manufacturer's protocol. We observed the reproductive capacities of ASC-J9 and vehicle-treated SBMA mice by mating one male mouse with two B6 female mice for 1 week checking female mice for vaginal plugs each morning and recording litter sizes on delivery after four successive matings.

**Histology and immunohistochemistry.** We fixed tissues by 4% paraformaldehyde and embedded them in paraffin. For general histologic inspection, we treated tissue sections with H&E or NADH, and then used an ABC kit (Vector Laboratories) to detect AR immunostaining by an N20 antibody to AR. We performed the assessment of cells with intranuclear aggregated AR-polyQ in the ventral horn of the spinal cord as described previously<sup>36,37</sup>. We expressed the populations of AR-positive cells as the number per square millimeter. We counted AR-positive cells in randomly selected areas from more than 500 muscle fibers and expressed AR-positive cells as the number per 100 muscle fibers.

**Quantitative real time RT-PCR.** We harvested L5 spinal cords of mice treated with vehicle or ASC-J9 (50 mg/kg every 48 h), extracted total RNA using TRIZOL and reverse transcribed. We subjected 1  $\mu$ g of total RNA to reverse transcription using Superscript II (Invitrogen), with the primer probe sequences and PCR conditions for VEGF164 as described previously<sup>24</sup>. We performed amplification, detection, and data analysis using a Bio-Rad iCycler system.

**ELISA.** We obtained total protein lysates by homogenizing tissues in an extraction buffer as described previously<sup>24</sup>. After centrifugation, we analyzed the amount of VEGF protein in the supernatant using ELISA kit (R&D Systems).

**Statistical analysis.** We analyzed the results by unpaired *t*-tests and log-rank tests for survival rate using Sigmaplot software. *P*-values less than 0.05 were considered to be statistically significant.

*Note: Supplementary information is available on the Nature Medicine website.*

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## COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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## Dorfin-CHIP chimeric proteins potently ubiquitylate and degrade familial ALS-related mutant SOD1 proteins and reduce their cellular toxicity

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The ubiquitin–proteasome system (UPS) is involved in the pathogenic mechanisms of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS). Dorfin is a ubiquitin ligase (E3) that degrades mutant SOD1 proteins, which are responsible for familial ALS. Although Dorfin has potential as an anti-ALS molecule, its life in cells is short. To improve its stability and enhance its E3 activity, we developed chimeric proteins containing the substrate-binding hydrophobic portion of Dorfin and the U-box domain of the carboxyl terminus of Hsc70-interacting protein (CHIP), which has strong E3 activity through the U-box domain. All the Dorfin-CHIP chimeric proteins were more stable in cells than was wild-type Dorfin (Dorfin<sup>WT</sup>). One of the Dorfin-CHIP chimeric proteins, Dorfin-CHIP<sup>L</sup>, ubiquitylated mutant SOD1 more effectively than did Dorfin<sup>WT</sup> and CHIP *in vivo*, and degraded mutant SOD1 protein more rapidly than Dorfin<sup>WT</sup> does. Furthermore, Dorfin-CHIP<sup>L</sup> rescued neuronal cells from mutant SOD1-associated toxicity and reduced the aggresome formation induced by mutant SOD1 more effectively than did Dorfin<sup>WT</sup>.

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**Keywords:** Dorfin; ALS; SOD1; CHIP; Neurodegeneration; Ubiquitin–proteasome system

**Abbreviations:** ALS, amyotrophic lateral sclerosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHIP, carboxyl terminus of Hsc70-interacting protein; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; FCS, fetal calf serum; IP, immunoprecipitation; LB, Lewy body; PD, Parkinson's disease; RING-IBR, in-between-ring-finger; SCF, Skp1-Cullin-F box complex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD1, Cu/Zn super oxide dismutase; UPS, ubiquitin–proteasome system.

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Amyotrophic lateral sclerosis (ALS), one of the most common neurodegenerative disorders, is characterized by selective motor neuron degeneration in the spinal cord, brainstem, and cortex. About 10% of ALS cases are familial; of these, 10%–20% are caused by Cu/Zn superoxide dismutase (SOD1) gene mutations (Rosen et al., 1993; Cudkowicz et al., 1997). However, the precise mechanism that causes motor neuron death in ALS is still unknown, although many have been proposed: oxidative toxicity, glutamate receptor abnormality, ubiquitin proteasome dysfunction, inflammatory and cytokine activation, neurotrophic factor deficiency, mitochondrial damage, cytoskeletal abnormalities, and activation of the apoptosis pathway (Julien, 2001; Rowland and Shneider, 2001).

Misfolded protein accumulation, one probable cause of neurodegenerative disorders, including ALS, can cause the deterioration of various cellular functions, leading to neuronal cell death (Julien, 2001; Ciechanover and Brundin, 2003). Recent findings indicate that the ubiquitin–proteasome system (UPS), a cellular function that recognizes and catalyzes misfolded or impaired cellular proteins (Jungmann et al., 1993; Lee et al., 1996; Bercovich et al., 1997), is involved in the pathogenesis of various neurodegenerative diseases, among them ALS, Parkinson's disease (PD), Alzheimer's disease, polyglutamine disease, and prion disease (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). The ubiquitin ligase (E3), a key molecule for the UPS, can specifically recognize misfolded substrates and convey them to proteasomal degradation (Scheffner et al., 1995; Glickman and Ciechanover, 2002; Tanaka et al., 2004).

Dorfin, an E3 protein, contains an in-between-ring-finger (RING-IBR) domain at its N-terminus. The C-terminus of Dorfin can recognize mutant SOD1 proteins, which cause familial ALS (Niwa et al., 2001; Ishigaki et al., 2002b; Niwa et al., 2002). In cultured cells, Dorfin colocalized with aggresomes and ubiquitin-positive inclusions, which are pathological hallmarks of neurodegenerative diseases (Hishikawa et al., 2003; Ito et al., 2003). Dorfin also interacted with VCP/p97 in ubiquitin-positive inclusions in

ALS and PD (Ishigaki et al., 2004). Moreover, formation of this complex was found to be necessary for the E3 activity of Dorfin against mutant SOD1. These findings suggest that Dorfin is involved in the quality-control system for the abnormal proteins that accumulate in the affected neurons in neurodegenerative disorders.

Dorfin degrades mutant SOD1s and attenuates mutant SOD1-associated toxicity in cultured cells (Niwa et al., 2002). However, in Dorfin/mutant SOD1 double transgenic mice, we found only a modest beneficial effect on mutant SOD1-induced survival and motor dysfunction (unpublished data). These findings, combined with the short half-life of Dorfin protein, led us to hypothesize that the limiting effect of the Dorfin transgene may be a consequence of autodegradation of Dorfin, since Dorfin can execute autoubiquitination *in vivo* (Niwa et al., 2001).

Carboxyl terminus of Hsc70-interacting protein (CHIP) is also an E3 protein; it has a TPR domain in the N terminus and a U-box domain in the C terminus. The U-box domain of CHIP is responsible for its strong E3 activity, whereas the TPR domain recruits heat shock proteins harboring misfolded client proteins such as cystic fibrosis transmembrane conductance regulator (CFTR), denatured luciferase, and tau (Meacham et al., 2001; Murata et al., 2001, 2003; Hatakeyama et al., 2004; Shimura et al., 2004).

To prolong the protein lifetime of Dorfin and thereby obtain more potent ubiquitination and degradation activity against mutant SOD1s than is provided by Dorfin or CHIP alone, we generated chimeric proteins containing the substrate-binding domain of Dorfin and the UPR domain of CHIP substitute for RING/IBR of Dorfin. We developed 12 candidate constructs that encode Dorfin-CHIP chimeric proteins and analyzed them for their E3 activities and degradation abilities against mutant SOD1 protein in cultured cells.

## Experimental procedures

### Plasmids and antibodies

We designed constructs expressing Dorfin-CHIP chimeric protein. In these constructs, different-length fragments of the C-terminus portion of Dorfin, including the hydrophobic substrate-binding domain (amino acids 333–838, 333–700, and 333–454) and the C-terminus UPR domain of CHIP with amino acids 128–303 or without amino acids 201–303, a charged region was fused in various combinations as shown in Fig. 2C. Briefly, Dorfin-CHIP<sup>A, B, C, G, H,</sup> and <sup>I</sup> had the C-terminus portion of Dorfin in their N-terminus and the U-box of CHIP in their C-terminus; Dorfin-CHIP<sup>D, E, F, J, K,</sup> and <sup>L</sup> had the U-box of CHIP in their N-terminus and the C-terminus portion of Dorfin in their C-terminus.

We prepared a pCMV2/FLAG-Dorfin-CHIP chimeric vector (Dorfin-CHIP) by polymerase chain reaction (PCR) using the appropriate design of PCR primers with restriction sites (*Clal*, *KpnI*, and *XbaI* or *EcoRI*, *Clal*, and *KpnI*). The PCR products were digested and inserted into the *Clal*–*KpnI* site in pCMV2 vector (Sigma, St. Louis, MO). These vectors have been described previously: pFLAG-Dorfin<sup>WT</sup> (Dorfin<sup>WT</sup>), FLAG-Dorfin<sup>C132S/C135S</sup> (Dorfin<sup>C132S/C135S</sup>), pFLAG-CHIP (CHIP), pFLAG-Mock (Mock), pcDNA3.1/Myc-SOD1<sup>WT</sup> (SOD1<sup>WT</sup>), pcDNA3.1/Myc-SOD1<sup>G93A</sup> (SOD1<sup>G93A</sup>), pcDNA3.1/Myc-SOD1<sup>G85R</sup> (SOD1<sup>G85R</sup>), pcDNA3.1/Myc-SOD1<sup>H46R</sup> (SOD1<sup>H46R</sup>), pcDNA3.1/Myc-SOD1<sup>G37R</sup> (SOD1<sup>G37R</sup>), pEGFP/SOD1<sup>WT</sup> (SOD1<sup>WT</sup>-GFP), and pEGFP/SOD1<sup>G85R</sup> (SOD1<sup>G85R</sup>-GFP) (Ishi-

gaki et al., 2004). We used monoclonal anti-FLAG antibody (M2; Sigma), monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-HA antibody (12CA5; Roche, Basel, Switzerland), and polyclonal anti-SOD1 (SOD-100; Stressgen, San Diego, CA).

### Cell culture and transfection

We grew HEK293 cells and neuro2a (N2a) cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin, and 50 µg/ml streptomycin. At subconfluence, we transfected these cells with the indicated plasmids, using Effectene reagent (Qiagen, Valencia, CA) for HEK293 cells and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for N2a cells. After overnight posttransfection, we treated the cells with 1 µM MG132 (Z-Leu-Leu-Leu-al; Sigma) for 16 h to inhibit cellular proteasome activity. We analyzed the cells 24–48 h after transfection. To differentiate N2a cells, cells were treated for 48 h with 15 µM of retinoic acid in 2% serum medium.

### Immunological analysis

At 24–48 h after transfection, we lysed cells ( $4 \times 10^5$  in 6-cm dishes) with 500 µl of lysis buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, and 1 mM ethylenediaminetetraacetic acid (EDTA), as well as a protease inhibitor cocktail (Complete Mini, Roche). The lysate was then centrifuged at  $10,000 \times g$  for 10 min at 4°C to remove debris. We used a 10% volume of the supernatants as the lysate for SDS-PAGE. When immunoprecipitated, the supernatants were precleared with protein A/G agarose (Santa-Cruz). A specific antibody, either anti-FLAG (M2) or anti-Myc (9E10), was then added. We incubated the immune complexes, first at 4°C with rotation and with protein A/G agarose (Roche) for 3 h, after which they were collected by centrifugation and washed four times with the lysis buffer. For protein analysis, immune complexes were dissociated by heating in SDS-PAGE sample buffer and loaded onto SDS-PAGE. We separated the samples by SDS-PAGE (15% gel or 5%–20% gradient gel) and transferred them onto polyvinylidene difluoride membranes. We then immunoblotted samples with specific antibodies.

### Immunohistochemistry

We fixed differentiated N2a cells grown in plastic dishes in 4% paraformaldehyde in PBS for 15 min. The cells were then blocked for 30 min with 5% (vol/vol) normal goat serum in PBS, incubated overnight at 4°C with anti-FLAG antibody (M2), washed with PBS, and incubated for 30 min with Alexa 496 nm anti-mouse antibodies (Molecular Probes, Eugene, OR). We mounted the cells on slides and obtained images using a fluorescence microscope (IX71; Olympus, Tokyo, Japan) equipped with a cooled charge-coupled device camera (DP70; Olympus). Photographs were taken using DP Controller software (Olympus).

### Analysis of protein stability

We assayed the stability of proteins by pulse-chase analysis using [<sup>35</sup>S] followed by immunoprecipitation. Metabolic labeling was performed as described previously (Yoshida et al., 2003). Briefly, in the pulse-chase analysis of Dorfin proteins, HEK293 cells in 6-cm dishes were transiently transfected with 1 µg of

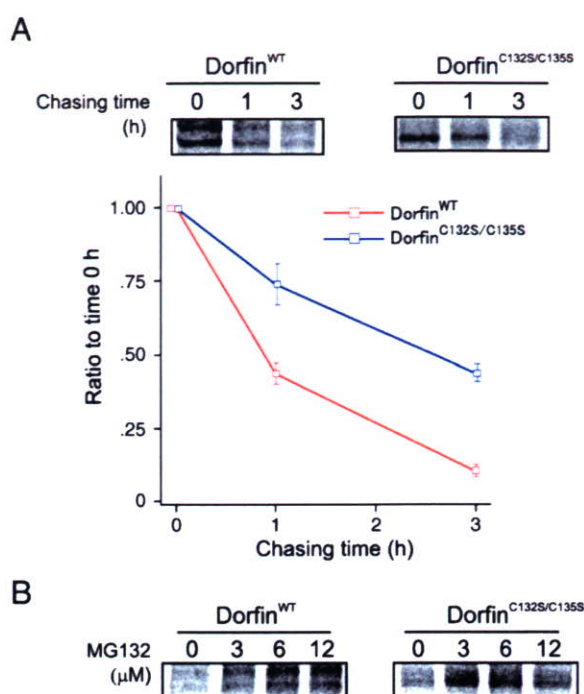


Fig. 1. Pulse-chase analysis of Dorfin<sup>WT</sup> and Dorfin<sup>C132S/C135S</sup>. (A) Dorfin<sup>WT</sup> or Dorfin<sup>C132S/C135S</sup> was overexpressed in HEK293 cells. After overnight incubation, [<sup>35</sup>S]-labeled Met/Cys pulse-chase analysis was performed. Cells were harvested and analyzed at 0, 1, or 3 h after labeling and immunoprecipitation by anti-FLAG antibody (upper panels). To determine serial changes in the amount of Dorfin<sup>WT</sup> or Dorfin<sup>C132S/C135S</sup>, four independent experiments were performed and the amounts of Dorfin<sup>WT</sup> and Dorfin<sup>C132S/C135S</sup> were plotted. The differences between the amounts of Dorfin<sup>WT</sup> and Dorfin<sup>C132S/C135S</sup> were significant at 1 h ( $p < 0.01$ ) and 3 h after labeling ( $p < 0.001$ ) (lower panels). Values are the means  $\pm$  SE,  $n = 4$ . Statistics were done using an unpaired  $t$ -test. (B) Cells overexpressing Dorfin<sup>WT</sup> or Dorfin<sup>C132S/C135S</sup> were treated with different concentrations of MG132 for 3 h after labeling.

FLAG-Dorfin<sup>WT</sup> or FLAG-Dorfin<sup>C132S/C135S</sup>. In pulse-chase experiments using SOD1<sup>G85R</sup>, N2a cells in 6-cm dishes were transiently transfected with 1  $\mu$ g of SOD1<sup>G85R</sup>-Myc or SOD1<sup>G93A</sup>-Myc and FLAG-Mock, FLAG-Dorfin, or FLAG-Dorfin-CHIP<sup>L</sup>. FLAG-Mock was used as a negative control. After starving the cells for 60 min in methionine- and cysteine-free DMEM with 10% FCS, we labeled them for 60 min with 150  $\mu$ Ci/ml of Pro-Mix L-[<sup>35</sup>S] *in vitro* cell-labeling mix (Amersham Biosciences). Cells were chased for different lengths of time at 37°C. In experiments with proteasomal inhibition, we added different amounts of MG132 in medium during the chase period. We performed immunoprecipitation using protein A/G agarose, mouse monoclonal anti-FLAG (M2), and anti-Myc (9E10). The intensity of the bands was quantified by ImageGauge software (Fuji Film, Tokyo, Japan).

#### MTS assay

We transfected N2a cells (5000 cells per well) in 96-well collagen-coated plates with 0.15  $\mu$ g of SOD1<sup>G85R</sup>-GFP and 0.05  $\mu$ g of Dorfin, CHIP, Dorfin-CHIP<sup>L</sup>, or pCMV2 vector (Mock) using Effecten reagent (Qiagen). Then we performed 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assays using Cell Titer 96

(Promega) at 48 h after incubation. This procedure has previously been described (Ishigaki et al., 2002a).

#### Aggregation assay

We transfected N2a cells in 6-cm dishes with 1.0  $\mu$ g of SOD1<sup>G85R</sup>-GFP and 1.0  $\mu$ g of FLAG-Mock, FLAG-Dorfin, FLAG-CHIP, or FLAG-Dorfin-CHIP<sup>L</sup>. After overnight incubation, we changed the medium to 2% FCS containing medium with 15  $\mu$ M retinoic acid (RA) for differentiation. In the MG132 (+) group, 1  $\mu$ M of MG132 was added after 24 h of differentiation stimuli. After 48 h of differentiation stimuli, we examined the cells in their living condition by fluorescence microscopy. The transfection ratio was equivalent (75%) among all groups. Visually observable macro aggregation-harboring cells were counted as “aggregation positive” cells (Fig. 7C). All cells were counted in fields selected at random from the four different quadrants of the culture dish. Counting was done by an investigator who was blind to the experimental condition.

## Results

### Dorfin degradation by the UPS *in vivo*

We analyzed the degradation speed of FLAG-Dorfin by the pulse-chase method using [<sup>35</sup>S] labeling, finding that more than half of wild-type Dorfin (Dorfin<sup>WT</sup>) was degraded within 1 h (Fig. 1A). This degradation was dose-dependently inhibited by MG132, a proteasome inhibitor (Fig. 1B). On the other hand, the RING mutant form of Dorfin (Dorfin<sup>C132S/C135S</sup>), which lacks E3 activity (Ishigaki et al., 2004), degraded significantly more slowly than did Dorfin<sup>WT</sup> (Fig. 1A and Table 1). As shown in Fig. 1A, Dorfin<sup>WT</sup> showed two bands, whereas Dorfin<sup>C132S/C135S</sup> had a single band. This was also seen in our previous study (Ishigaki et al., 2004) and may represent posttranslational modification.

### Construction of Dorfin-CHIP chimeric proteins

It is known that the C-terminus portion of Dorfin can bind to substrates such as mutant SOD1 proteins or Synphilin-1 (Niwa et al., 2002; Ito et al., 2003). We attempted to identify the domain of Dorfin that interacts with substrates. Although there was no obvious known motif in the C-terminus of Dorfin (amino acids 333–838), its first quarter contained rich hydrophobic amino acids (amino acids 333–454) (Fig. 2A). Immunoprecipitation analysis revealed that the hydrophobic region of Dorfin (amino acids 333–454) was able to bind to SOD1<sup>G85R</sup>, indicating that this hydrophobic region is responsible for recruiting mutant SOD1 in Dorfin protein (Fig. 2B).

To establish more effective and more stable E3 ubiquitin ligase molecules that can recognize and degrade mutant SOD1s, we

Table 1

Serial changes in the amounts of Dorfin<sup>WT</sup>, Dorfin<sup>C132S/C135S</sup>, and Dorfin-CHIP<sup>L</sup>

	0 h (%)	1 h (%)	3 h (%)
Dorfin <sup>WT</sup>	100	43.7 $\pm$ 7.0	10.3 $\pm$ 4.4
Dorfin <sup>C132S/C135S</sup>	100	73.9 $\pm$ 13.8	43.7 $\pm$ 1.9
Dorfin-CHIP <sup>L</sup>	100	89.0 $\pm$ 5.7	47.5 $\pm$ 5.3

Values are the mean and SD of four independent experiments.



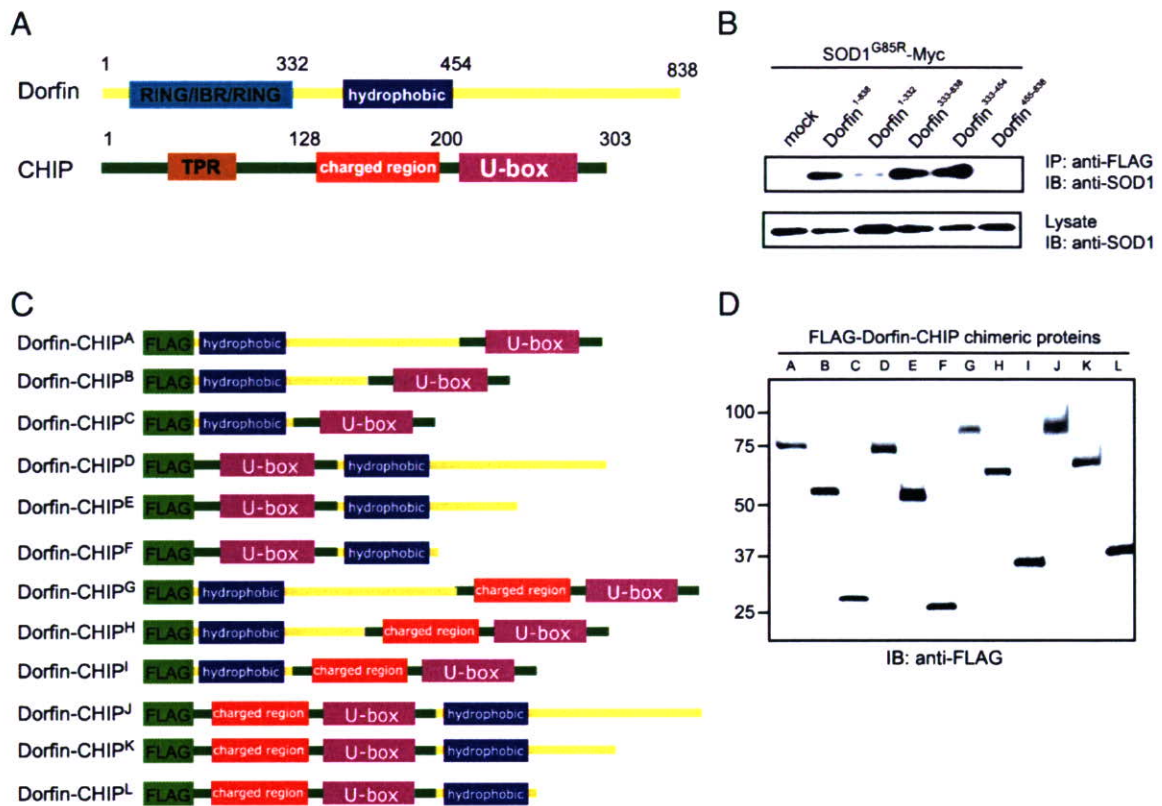


Fig. 2. Construction of Dorfin-CHIP chimeric proteins. (A) Dorfin has a RING/IBR domain in its N-terminus and a substrate-binding portion in the C-terminus. CHIP contains a TPR domain that binds to heat-shock proteins at the N-terminus; its C-terminal U-box domain has strong E3 ubiquitin ligase activity. (B) SOD1<sup>G85R</sup>-Myc and FLAG-Dorfin derivatives were overexpressed in HEK 293 cells. Cell lysates were immunoprecipitated with anti-myc antibody. Immunoblotting showed that FLAG-Dorfin derivatives containing Dorfin<sup>333-454</sup> bound to SOD1<sup>G85R</sup>-Myc, indicating that the hydrophobic region of Dorfin (Dorfin<sup>333-454</sup>) is essential for interaction with mutant SOD1 *in vivo*. (C) Scheme of engineered Dorfin-CHIP chimeric proteins. Three different lengths of C-terminal Dorfin containing the hydrophobic region of Dorfin (Dorfin<sup>333-454</sup>) and the U-box domain of CHIP with or without the charged region were fused. (D) Dorfin-CHIP chimeric proteins were overexpressed in HEK293 cells. Harvested cells were lysed and analyzed by immunoblotting using anti-FLAG antibody.

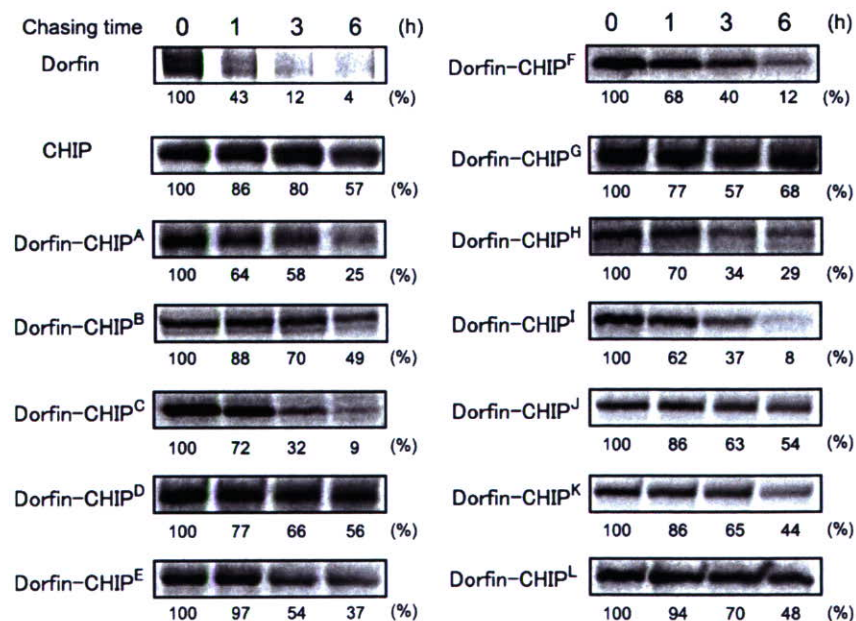


Fig. 3. The stability of Dorfin-CHIP chimeric proteins. Pulse-chase analysis using [<sup>35</sup>S]-Met/Cys was performed. Dorfin, CHIP, and all the Dorfin-CHIP chimeric proteins were overexpressed in HEK293 cells and labeled with [<sup>35</sup>S]-Met/Cys. Immunoprecipitation using anti-FLAG antibody and SOD-PAGE analysis revealed the degradation speed of FLAG-Dorfin-CHIP chimeric proteins. The amount of each Dorfin-CHIP chimeric protein was measured by quantifying the band using ImageGauge software.

designed Dorfin-CHIP chimeric proteins containing both the hydrophobic substrate-binding domain of Dorfin and the U-box domain of CHIP, which has strong E3 activity (Fig. 2C). We verified that all of the 12 candidate chimeric proteins were expressed in HEK293 cells (Fig. 2D).

*Expression of Dorfin-CHIP chimeric proteins in cells*

The half lives of all the Dorfin-CHIP chimeric proteins were more than 1 h. In some of these proteins, such as Dorfin-CHIP<sup>D, G, J, and L</sup>, moderate amounts of protein still remained at 6 h after labeling, indicating that they were degraded much more slowly than was Dorfin<sup>WT</sup> (Fig. 3). Repetitive experiments using Dorfin-CHIP<sup>L</sup>

yielded a significant difference between the amount of Dorfin<sup>WT</sup> and Dorfin-CHIP<sup>L</sup> at 1 h and 3 h (Table 1).

*E3 activity of Dorfin-CHIP chimeric proteins against mutant SOD1*

Immunoprecipitation analysis demonstrated that Dorfin and CHIP bound to mutant SOD1<sup>G85R</sup> in equivalent amounts and that all of the Dorfin-CHIP chimeric proteins interacted with mutant SOD1<sup>G85R</sup> *in vivo*. Dorfin-CHIP<sup>A, D, E, F, J, K, and L</sup> bound to the same or greater amounts of SOD1<sup>G85R</sup> than did Dorfin, whereas Dorfin-CHIP<sup>B, C, G, H, and I</sup> did not (Fig. 4A, upper panel). None of the Dorfin-CHIP chimeric proteins bound to SOD1<sup>WT</sup> *in vivo*

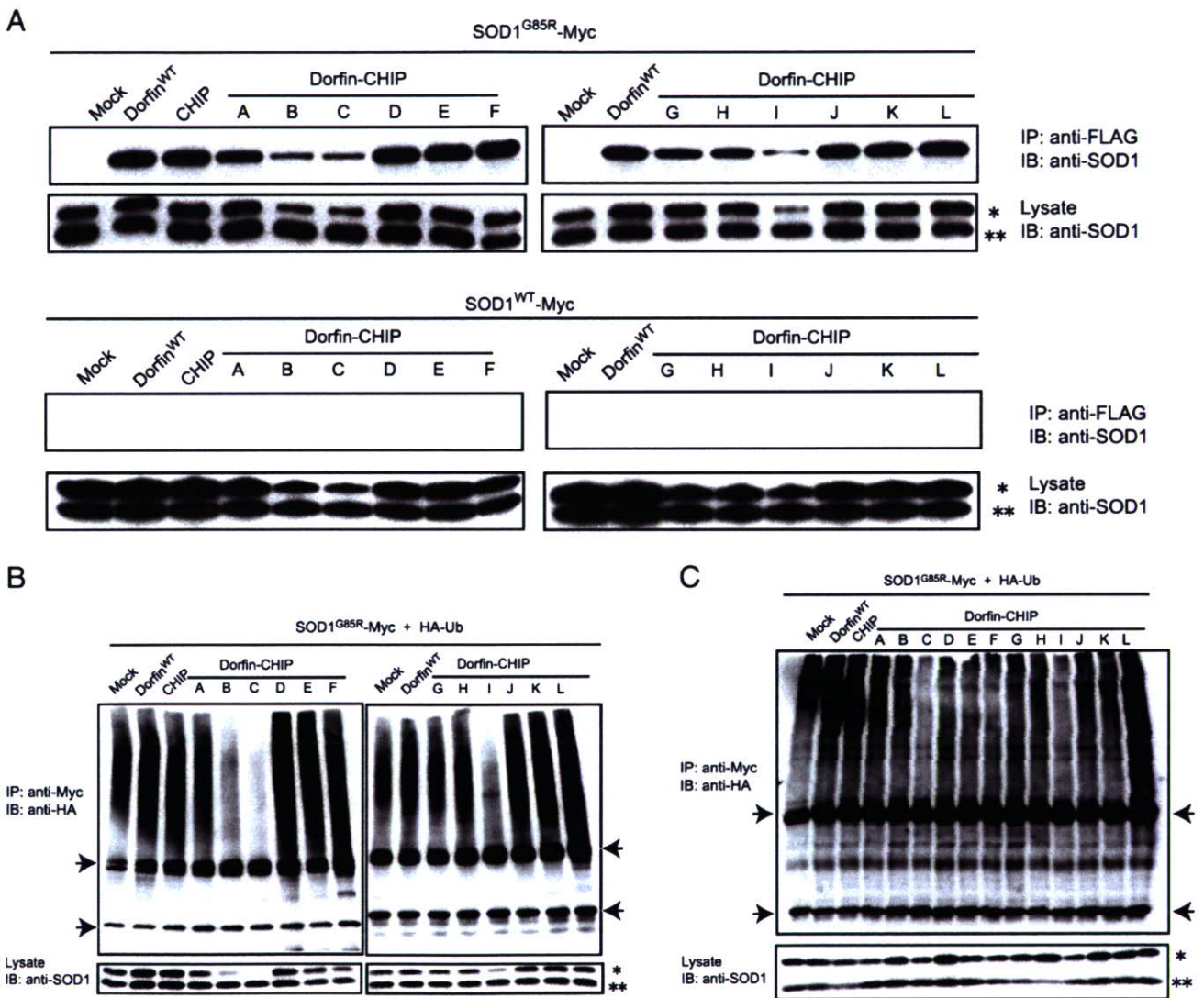


Fig. 4. The E3 activity of Dorfin-CHIP chimeric proteins on mutant SOD1 *in vivo*. (A) *In vivo* binding assay with both wild-type and mutant SOD1s. SOD1<sup>G85R</sup>- or SOD1<sup>WT</sup>-Myc and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in HEK293 cells. Immunoprecipitation was done using anti-Myc antibody. Immunoblotting with anti-FLAG antibody revealed that all the Dorfin-CHIP chimeric proteins bound *in vivo* to SOD1<sup>G85R</sup>-Myc but not to SOD1<sup>WT</sup>-Myc. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively. (B) *In vivo* ubiquitylation assay in HEK293 cells. SOD1<sup>G85R</sup>-Myc, HA-Ub, and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in HEK293 cells. Immunoblotting with anti-HA antibody demonstrated the ubiquitylation level of SOD1<sup>G85R</sup>-Myc by FLAG derivatives of Dorfin-CHIP chimeric proteins *in vivo*. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed SOD1 and mouse endogenous SOD1, respectively. (C) *In vivo* ubiquitylation assay in N2a cells. SOD1<sup>G85R</sup>-Myc, HA-Ub, and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in N2a cells. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively.

(Fig. 4A, lower panel). Some Dorfin-CHIP chimeric proteins, such as Dorfin-CHIP<sup>B, C, and L</sup>, had lower amounts of both SOD1<sup>WT</sup> and SOD1<sup>G85R</sup> in the lysates. We performed quantitative RT-PCR using specific primers for SOD1-Myc, finding that coexpression of Dorfin-CHIP<sup>B, C, or L</sup> suppressed the mRNA expression of overexpressed SOD1 gene (Supplementary Fig. 1). Considering the possibility that these Dorfin-CHIP chimeric proteins might have unpredicted toxicity for cells by affecting gene transcription via unknown mechanisms, we excluded them from further analysis. Other Dorfin-CHIP proteins did not affect SOD1-Myc gene expression, which validated the comparison among IPs and ubiquitylated mutant SOD1 in Figs. 4A–C.

To assess the effectiveness of the E3 activity of Dorfin-CHIP chimeric proteins, we did an *in-vivo* ubiquitylation analysis by coexpression of SOD1<sup>G85R</sup>-Myc, HA-Ub, and Dorfin-CHIP chimeric proteins in HEK293 cells. We found that Dorfin and CHIP enhanced the ubiquitylation of SOD1<sup>G85R</sup> protein and that the ubiquitylation levels of these two E3 ligases were almost equivalent. Moreover, Dorfin-CHIP<sup>D, E, F, J, K, and L</sup> ubiquitylated SOD1<sup>G85R</sup> more effectively than did Dorfin or CHIP (Fig. 4B).

Performing the same *in-vivo* ubiquitylation assay using N2a cells, we observed that the levels of ubiquitylation of SOD1<sup>G85R</sup> by Dorfin and CHIP were equivalent, as they were in HEK293 cells. Among Dorfin-CHIP chimeric proteins, only Dorfin-CHIP<sup>L</sup>

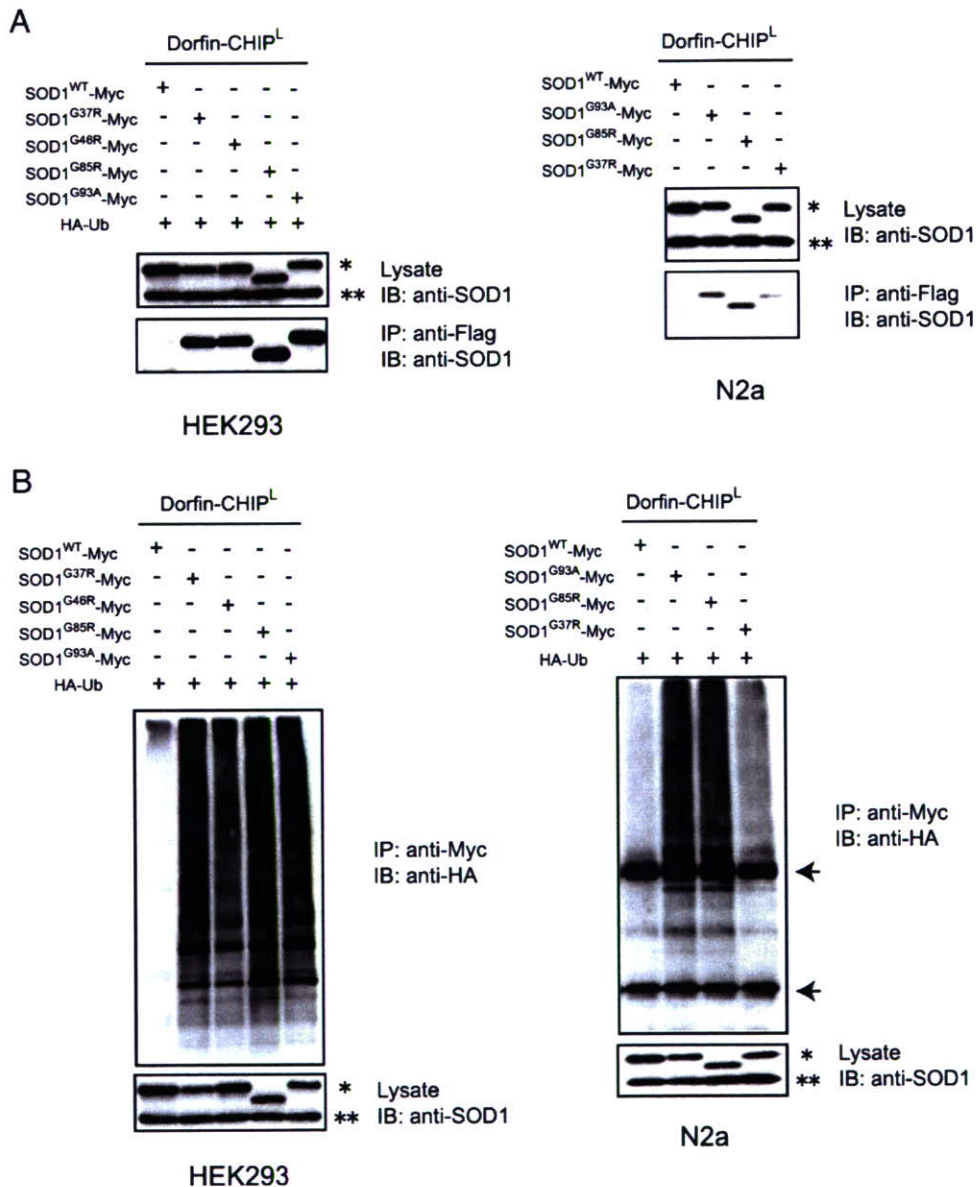


Fig. 5. Dorfin-CHIP<sup>L</sup> specifically ubiquitylates mutant SOD1s *in vivo*. (A) *In vivo* binding assay with various mutant SOD1s. SOD1<sup>WT</sup>-Myc, SOD1<sup>G93A</sup>-Myc, SOD1<sup>G85R</sup>-Myc, SOD1<sup>H46R</sup>-Myc or SOD1<sup>G37R</sup>-Myc, and FLAG-Dorfin-CHIP<sup>L</sup> were coexpressed in HEK293 (left) and N2a cells (right). Immunoprecipitation was done using anti-Myc antibody. Immunoblotting with anti-FLAG antibody showed that both chimeric proteins specifically bound to mutant SOD1s *in vivo*. Single and double asterisks indicate overexpressed SOD1 and mouse endogenous SOD1, respectively. (B) *In vivo* ubiquitylation assay. SOD1<sup>WT</sup>-Myc, SOD1<sup>G93A</sup>-Myc, SOD1<sup>G85R</sup>-Myc, SOD1<sup>H46R</sup>-Myc or SOD1<sup>G37R</sup>-Myc, as well as FLAG-Dorfin-CHIP<sup>L</sup> and HA-Ub, was coexpressed in HEK293 (left) and N2a cells (right). Immunoblotting with anti-HA antibody showed the specific ubiquitylation of mutant SOD1-Myc by FLAG-Dorfin-CHIP<sup>L</sup> *in vivo*. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively.

ubiquitylated SOD1<sup>G85R</sup> more effectively than did Dorfin or CHIP, while Dorfin-CHIP<sup>D</sup>, <sup>E</sup>, <sup>F</sup>, <sup>J</sup>, and <sup>K</sup> did not (Fig. 4C). Thus, Dorfin-CHIP<sup>L</sup> was the most potent candidate of the chimeric proteins.

#### Ubiquitylation of mutant SOD1 by Dorfin-CHIP<sup>L</sup>

Dorfin specifically ubiquitylated mutant SOD1 proteins, but not SOD1<sup>WT</sup> protein (Niwa et al., 2002; Ishigaki et al., 2004). Similarly, Dorfin-CHIP<sup>L</sup> interacted with SOD1<sup>G93A</sup>, SOD1<sup>G85R</sup>,

SOD1<sup>H46R</sup>, and SOD1<sup>G37R</sup>, but not SOD1<sup>WT</sup>, in HEK293 cells. This was confirmed in N2a cells (Fig. 5A). In both HEK293 and N2a cells, Dorfin-CHIP<sup>L</sup> also ubiquitylated mutant SOD1 proteins but not SOD1<sup>WT</sup> (Fig. 5B).

#### Degradation of mutant SOD1 by Dorfin-CHIP chimeric proteins

To assess the degradation activity of Dorfin-CHIP<sup>L</sup> against mutant SOD1s, we performed the pulse-chase analysis on N2a

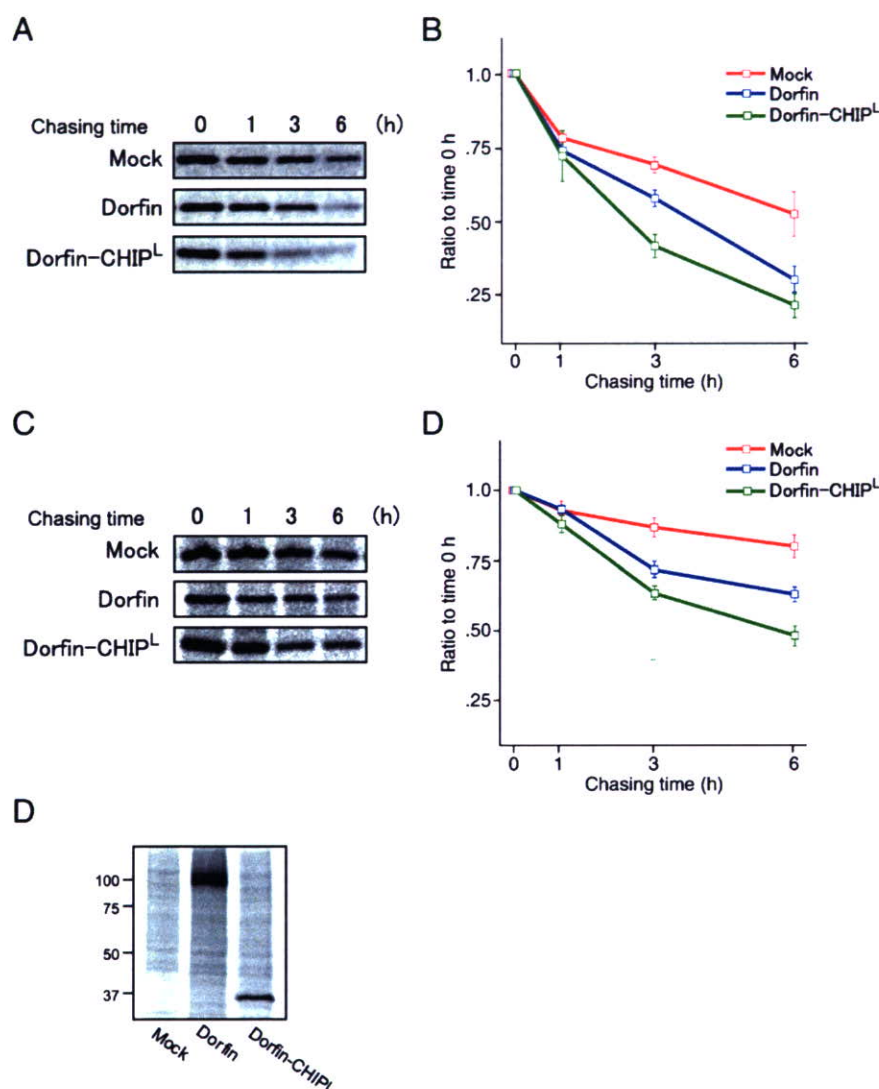


Fig. 6. Degradation of mutant SOD1 proteins with Dorfin-CHIP<sup>L</sup>. (A) Pulse-chase analysis of SOD1<sup>G85R</sup> with Dorfin-CHIP<sup>L</sup>. N2a cells were coexpressed with SOD1<sup>G85R</sup>-Myc and Mock, Dorfin, and Dorfin-CHIP<sup>L</sup>. Pulse-chase experiments using [<sup>35</sup>S]-Met/Cys were done. Immunoprecipitation using anti-Myc antibody and SOD-PAGE analysis revealed the degradation speed of SOD1<sup>G85R</sup>-Myc. (B) Serial changes in the amount of SOD1<sup>G85R</sup> coexpressed with Mock, Dorfin, or Dorfin-CHIP<sup>L</sup>. Four independent experiments were performed and the amounts of SOD1<sup>G85R</sup> were plotted. There were significant differences between Mock and Dorfin ( $p < 0.005$ ), Mock and Dorfin-CHIP<sup>L</sup> ( $p < 0.005$ ), and Dorfin and Dorfin-CHIP<sup>L</sup> ( $p < 0.05$ ) at 3 h, as well as between Mock and Dorfin ( $p < 0.05$ ), and Mock and Dorfin-CHIP<sup>L</sup> ( $p < 0.05$ ) at 6 h after labeling. Values are the means  $\pm$  SE,  $n = 4$ . Statistical analysis was done by one-way ANOVA. (C) Pulse-chase analysis of SOD1<sup>G93A</sup> with Dorfin-CHIP<sup>L</sup>. N2a cells were coexpressed with SOD1<sup>G93A</sup>-Myc and Mock, Dorfin, and Dorfin-CHIP<sup>L</sup> as in panel A. (D) Serial changes in the amount of SOD1<sup>G93A</sup> coexpressed with Mock, Dorfin, or Dorfin-CHIP<sup>L</sup>. Four independent experiments were performed and the amounts of SOD1<sup>G93A</sup> were plotted. There were significant differences between Mock and Dorfin ( $p < 0.05$ ) and Mock and Dorfin-CHIP<sup>L</sup> ( $p < 0.01$ ) at 3 h, as well as between Mock and Dorfin ( $p < 0.05$ ), Mock and Dorfin-CHIP<sup>L</sup> ( $p < 0.01$ ), and Dorfin and Dorfin-CHIP<sup>L</sup> ( $p < 0.05$ ) at 6 h after labeling. Values are the means  $\pm$  SE,  $n = 4$ . Statistics were done by one-way ANOVA. (E) The equivalent protein expression levels of Dorfin and Dorfin-CHIP<sup>L</sup>. Half of the volume of samples used in the pulse-chase analysis of panel C at 0 h was used for immunoprecipitation using anti-Flag M2 antibody. The following SOD-PAGE analysis revealed the amounts of Dorfin and Dorfin-CHIP<sup>L</sup> in the experiment shown in panel C.

cells, using [ $^{35}$ S] labeled Met/Cys. The protein levels of SOD1<sup>G85R</sup> and SOD1<sup>G93A</sup> declined more rapidly with Dorfin coexpression. Dorfin-CHIP<sup>L</sup> remarkably declined in both SOD1<sup>G85R</sup> and SOD1<sup>G93A</sup> (Figs. 6A, C). Dorfin and Dorfin-CHIP<sup>L</sup> had similar expression levels at 0 h of this experiment (Fig. 6E). As compared to Mock, Dorfin showed significant declines of both SOD1<sup>G85R</sup> at 3 h ( $p < 0.001$ ) and 6 h ( $p < 0.05$ ) after labeling, as shown in a previous study (Niwa et al., 2002). Dorfin-CHIP<sup>L</sup> also significantly accelerated the decline of SOD1<sup>G85R</sup> at 3 h ( $p < 0.001$ ) and 6 h ( $p < 0.05$ ) after labeling again as compared to Mock. At 3 h after labeling, a significant difference between Dorfin-CHIP<sup>L</sup> and Dorfin was present with respect to SOD1<sup>G85R</sup> degradation ( $p < 0.05$ ). As compared to Dorfin, Dorfin-CHIP<sup>L</sup> also tended toward accelerated SOD1<sup>G85R</sup> degradation at 6 h after labeling (Fig. 6B). Similarly, Dorfin showed significant declines of SOD1<sup>G93A</sup> at 3 h ( $p < 0.05$ ) and 6 h ( $p < 0.05$ ) after labeling, and Dorfin-CHIP<sup>L</sup> significantly accelerated the declines of SOD1<sup>G93A</sup> at 3 h ( $p < 0.01$ ) and 6 h ( $p < 0.01$ ) after labeling as compared to Mock. A significant difference between Dorfin-CHIP<sup>L</sup> and Dorfin was present at 6 h in SOD1<sup>G93A</sup> degradation ( $p < 0.05$ ) (Fig. 6D).

*Attenuation of the toxicity of mutant SOD1 and decrease in the formation of visible aggregations of mutant SOD1 in cultured neuronal culture cells*

The ability of Dorfin-CHIP chimeric proteins to attenuate mutant SOD1-related toxicity was analyzed by MTS assay using N2a cells. The expression of SOD1<sup>G85R</sup>, as compared to that of SOD1<sup>WT</sup>, decreased the viability of cells. Overexpression of Dorfin reversed the toxic effect of SOD1<sup>G85R</sup>, whereas overexpression of CHIP did not. Dorfin-CHIP<sup>L</sup> had a significantly greater rescue effect on SOD1<sup>G85R</sup>-related cell toxicity than did Dorfin (Fig. 7A). We also measured the cell viability of N2a cells overexpressing Mock, Dorfin, and Dorfin-CHIP<sup>L</sup> with various amounts of constructs, and found no difference in toxicity among them (Supplementary Fig. 2).

A structure that Johnston et al. (1998) called aggresome is formed when the capacity of a cell to degrade misfolded proteins is exceeded. The accumulation of mutant SOD1 induces visible macroaggregation, which is considered to be 'aggresome' in N2a cells. We examined the subcellular localizations of Dorfin, CHIP, and Dorfin-CHIP<sup>L</sup> by immunostaining N2a cells expressing SOD1<sup>G85R</sup>-GFP. Dorfin was localized in aggresomes with substrate proteins, as in our previous studies. Dorfin-CHIP<sup>L</sup> was also seen in aggresomes, whereas the staining of CHIP was diffusely observed in the cytosol (Fig. 7B). We counted these visible aggregations with or without MG132 treatment. Dorfin decreased the number of aggregation-containing cells, as has been reported (Niwa et al., 2002), but Dorfin-CHIP<sup>L</sup> did so more

effectively. These effects were inhibited by the treatment of MG132 (Fig. 7C).

## Discussion

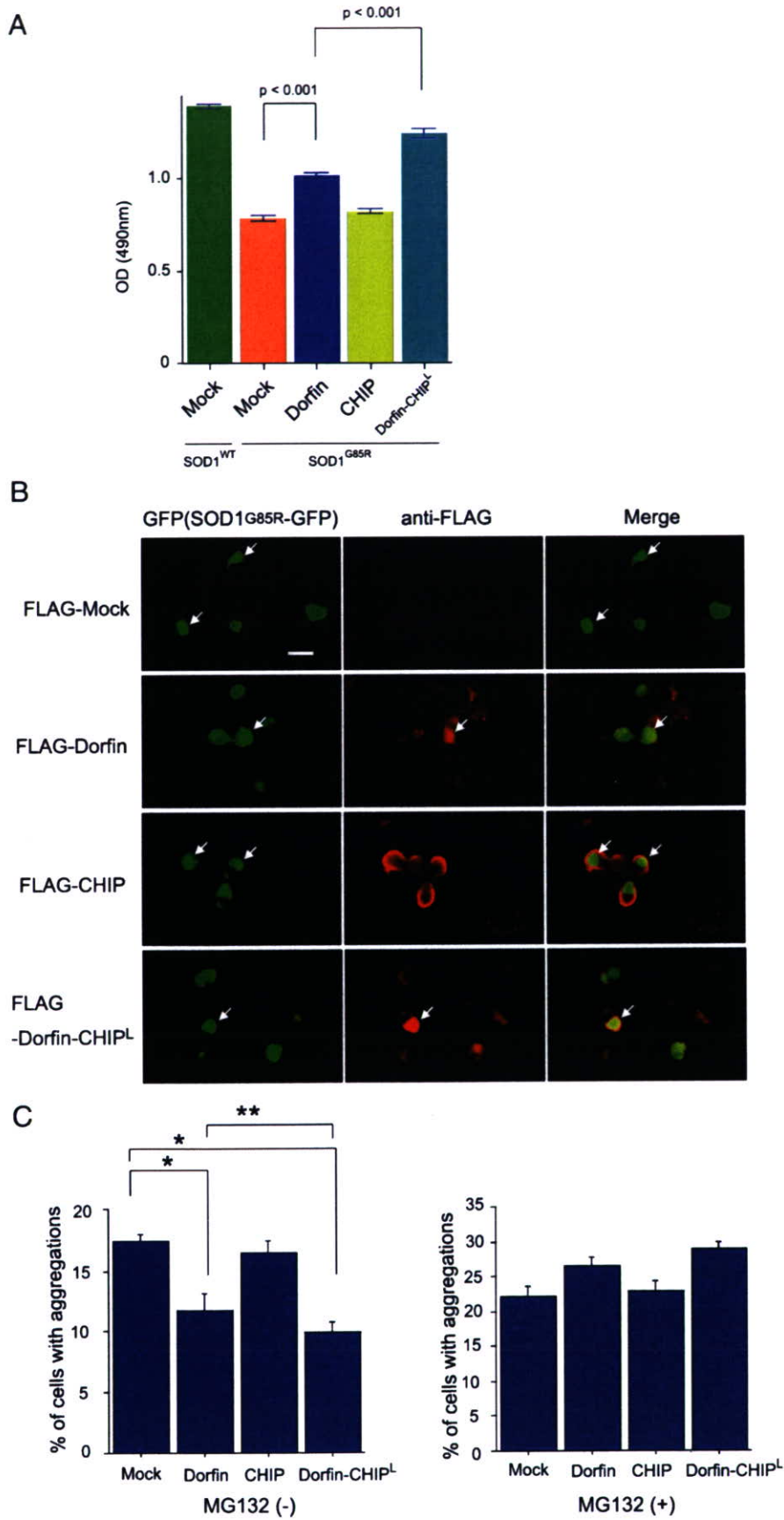
E3 proteins can specifically recognize and degrade accumulating aberrant proteins, which are deeply involved in the pathogenesis of neurodegenerative disorders, including ALS (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). For this reason, E3 proteins are candidate molecules for use in developing therapeutic technology for neurodegenerative diseases. Dorfin is the first E3 molecule that has been found specifically to ubiquitlylate mutant SOD1 proteins as well as to attenuate mutant SOD-associated toxicity in cultured neuronal cells (Niwa et al., 2002).

NEDL1, a HECT type E3 ligase, has also been reported to be a mutant SOD1-specific E3 ligase and to interact with TRAP $\delta$  and dvl1 (Miyazaki et al., 2004). It has also been reported that ubiquitylation of mutant SOD1-associated complex was enhanced by CHIP and Hsp70 *in vivo* (Urushitani et al., 2004). CHIP ubiquitylated Hsp70-holding SOD1 complexes and degraded mutant SOD1, but did not directly interact with mutant SOD1 (Urushitani et al., 2004). Among these E3 molecules, Dorfin seems to be the most potentially beneficial E3 protein for use in ALS therapy since it is the only one that has been demonstrated to reverse mutant SOD1-associated toxicity (Niwa et al., 2002). Furthermore, Dorfin has been localized in various ubiquitin-positive inclusions such as Lewy bodies (LB) in PD, as well as LB-like inclusions in sporadic ALS and glial cell bodies in multiple-system atrophy. These findings indicate that Dorfin may be involved in the pathogenesis of a broad spectrum of neurodegenerative disorders other than familial ALS (Hishikawa et al., 2003; Ito et al., 2003; Ishigaki et al., 2004).

The half-life of Dorfin<sup>WT</sup> is, however, less than 1 h (Fig. 1, Table 1). The amount of Dorfin is increased in the presence of MG132, a proteasome inhibitor, indicating that Dorfin is immediately degraded in the UPS. Since the nonfunctional RING mutant form of Dorfin, Dorfin<sup>C132S/C135S</sup>, degraded more slowly than did Dorfin<sup>WT</sup>, Dorfin seemed to be degraded by auto-ubiquitylation. The degradation of Dorfin<sup>C132S/C135S</sup> is also inhibited by MG132, suggesting that it is degraded by endogenous Dorfin or other E3s. This immediate degradation of Dorfin is a serious problem for its therapeutic application against neurodegenerative diseases.

Several reports have shown that engineered chimera E3s are able to degrade certain substrates with high efficiency. Protac, a chimeric protein-targeting molecule, was designed to target methionine aminopeptidase-2 to Skp1-Cullin-F box complex (SCF) ubiquitin ligase complex for ubiquitylation and degradation (Sakamoto et al.,

Fig. 7. Dorfin-CHIP chimeric proteins can attenuate toxicity induced by mutant SOD1 and decrease the formation of visible aggregation of mutant SOD1 in N2a cells. (A) N2a cells were grown in 96 collagen-coated wells (5000 cells per well) and transfected with 0.15  $\mu$ g of SOD1<sup>WT</sup> and 0.05  $\mu$ g of Mock or 0.15  $\mu$ g of SOD1<sup>G85R</sup> and 0.05  $\mu$ g of Mock, Dorfin, CHIP, or Dorfin-CHIP<sup>L</sup>. After the medium was changed, MTS assays were done at 48 h of incubation. Viability was measured as the level of absorbance (490 nm). Values are the means  $\pm$  SE,  $n = 6$ . Statistics were carried out by one-way ANOVA. There were significant differences between SOD1<sup>G85R</sup>-expressing cells coexpressed with Mock and SOD1<sup>G85R</sup>-expressing cells coexpressed with Dorfin ( $p < 0.001$ ), as well as between SOD1<sup>G85R</sup>-expressing cells coexpressed with Dorfin and SOD1<sup>G85R</sup>-expressing cells coexpressed with Dorfin-CHIP<sup>L</sup> ( $p < 0.001$ ). (B) N2a cells were transiently expressed with SOD1<sup>G85R</sup>-GFP and Mock, Dorfin, CHIP, or Dorfin-CHIP<sup>L</sup>. Immunostaining with anti-FLAG antibody revealed that Dorfin, CHIP, and Dorfin-CHIP<sup>L</sup> were localized with SOD1<sup>G85R</sup>-GFP in macroaggresomes (arrows). Scale bar = 20  $\mu$ m (C) The visible macroaggregations in N2a cells expressing both SOD1<sup>G85R</sup>-GFP and Mock, Dorfin, CHIP, or Dorfin-CHIP<sup>L</sup> with or without MG132 treatment were counted and the ratio of cells with aggregations to those with GFP signals was calculated. Values are the means  $\pm$  SE,  $n = 4$ . Statistics were done by one-way ANOVA. \* $p < 0.01$  denotes a significant difference between cells with Mock and Dorfin or Dorfin-CHIP<sup>L</sup>. \*\* $p < 0.05$  denotes a significant difference between cells with Dorfin and Dorfin-CHIP<sup>L</sup>.



2001, 2003). Oyake et al. (2002) developed double RING ubiquitin ligases containing the RING finger domains of both BRCA and BARD1 linked to a substrate recognition site PCNA. Recently, Hatakeyama et al. developed a fusion protein composed of Max, which forms a heterodimer with c-Myc, and the U-box of CHIP. This fusion protein physically interacted with c-Myc and promoted the ubiquitylation of c-Myc. It also reduced the stability of c-Myc, resulting in the suppression of transcriptional activity dependent on c-Myc and the inhibition of tumorigenesis (Hatakeyama et al., 2005). This indicated that the U-box portion of CHIP is able to add an effective E3 function to a U-box-containing client protein.

We postulated that engineered forms of Dorfin could be stable and still function as specific E3s for mutant SOD1s. Dorfin has a RING/IBR domain in the N-terminal portion (amino acids 1–332), but has no obvious motif in the rest of the C-terminus (amino acids 333–838). In this study, we have demonstrated that the hydrophobic domain of Dorfin (amino acids 333–454) is both necessary and sufficient for substrate recruiting (Fig. 2B). In our engineered proteins, the RING/IBR motif of N-terminal Dorfin was replaced by the UPR domain of CHIP, which had strong E3 activity (Murata et al., 2001). Some of the engineered Dorfin-chimeric proteins, such as Dorfin-CHIP<sup>D</sup>, <sup>G</sup>, <sup>J</sup>, and <sup>L</sup>, were degraded *in vivo* far more slowly than was wild-type Dorfin, indicating that they were capable of being stably presented *in vivo* (Fig. 3). However, Dorfin-CHIP<sup>G</sup> failed to show strong ubiquitylation activity against SOD1<sup>G85R</sup> in HEK293 cells. Since Dorfin-CHIP<sup>D</sup>, <sup>J</sup>, and <sup>L</sup> were able to bind to SOD1<sup>G85R</sup> more strongly than did Dorfin-CHIP<sup>G</sup>, the binding activity was more important for the E3 activity than for the protein stability.

We next showed that although all of the Dorfin-CHIP chimeric proteins bound to mutant SOD1 *in vivo*, some of them, such as Dorfin-CHIP<sup>B</sup>, <sup>C</sup>, and <sup>I</sup>, bound less than others (Fig. 4A). In HEK293 cells, Dorfin-CHIP<sup>D</sup>, <sup>E</sup>, <sup>F</sup>, <sup>J</sup>, <sup>K</sup>, and <sup>L</sup> ubiquitylated SOD1<sup>G85R</sup> more effectively than did Dorfin or CHIP; however, in N2a cells only Dorfin-CHIP<sup>L</sup> had more effective E3 activity than did Dorfin or CHIP. This discrepancy may be due to differences between HEK 293 and N2a cells which could provide slight different environment for the E3 machinery. Therefore, Dorfin-CHIP<sup>L</sup> was the most potent of the candidate chimeric proteins in degrading mutant SOD1 in the UPS in neuronal cells. We also showed that Dorfin-CHIP<sup>L</sup> could specifically bind to and ubiquitylate mutant SOD1s but not SOD1<sup>WT</sup> *in vivo*, as Dorfin had done (Niwa et al., 2002; Ishigaki et al., 2004) (Fig. 5). This observation confirmed that the hydrophobic domain of Dorfin (amino acids 333–454) is responsible for mutant SOD1 recruiting.

Pulse-chase analysis using N2a cells showed that Dorfin-CHIP<sup>L</sup> degraded SOD1<sup>G85R</sup> and SOD1<sup>G93A</sup> more effectively than did Dorfin (Fig. 6). This is compatible with the finding that Dorfin-CHIP<sup>L</sup> had a greater effect than Dorfin did on the ubiquitylation against mutant SOD1. The cycloheximide assay verified that the degradation ability of Dorfin-CHIP<sup>L</sup> against SOD1<sup>G85R</sup> was stronger than that of Dorfin or CHIP in HEK293 cells (data not shown).

Dorfin-CHIP<sup>L</sup> also reversed SOD1<sup>G85R</sup>-associated toxicity in N2a cells more effectively than did Dorfin (Fig. 7). This therapeutic effect of Dorfin-CHIP<sup>L</sup> was expected from its strong E3 activity and degradation ability against SOD1<sup>G85R</sup>. Visible protein aggregations have been considered to be hallmarks of neurodegeneration. Increased understanding of the pathway involved in protein aggregation may demonstrate that visible macroaggregates represent the end-stage of a molecular cascade of

steps rather than a direct toxic insult (Ross and Poirier, 2004). Two facts that Dorfin-CHIP<sup>L</sup> decreased aggregation formation of SOD1<sup>G85R</sup> and that this effect was inhibited by a proteasome inhibitor should reflect the ability of Dorfin-CHIP<sup>L</sup> to degrade mutant SOD1 in the UPS of cells.

Based on our present observations, Dorfin-CHIP<sup>L</sup>, an engineered chimeric molecule with the hydrophobic substrate-binding domain of Dorfin and the U-box domain of CHIP, had stronger E3 activity against mutant SOD1 than did Dorfin or CHIP. Indeed, it not only degraded mutant SOD1 more effectively than did Dorfin or CHIP but, as compared to Dorfin, produced marked attenuation of mutant SOD1-associated toxicity in N2a cells. This protective effect of Dorfin-CHIP<sup>L</sup> against mutant SOD1 has potential applications to gene therapy for mutant SOD1 transgenic mice because this protein has a long enough life to allow the constant removal of mutant SOD1 from neurons. Since Dorfin was originally identified as a sporadic ALS-associated molecule (Ishigaki et al., 2002b) and is located in the ubiquitin-positive inclusions of various neurodegenerative diseases (Hishikawa et al., 2003), this molecule is an appropriate candidate for future use in gene therapy not only for familial ALS, but also for sporadic ALS and other neurodegenerative disorders.

So far, most reports on engineered chimera E3s have targeted cancer-promoting proteins. Dorfin-CHIP chimeric proteins are the first chimera E3s to be intended for the treatment of neurodegenerative diseases. Since the accumulation of ubiquitylated proteins in neurons is a pathological hallmark of various neurodegenerative diseases, development of chimera E3s like Dorfin-CHIP<sup>L</sup>, which can remove unnecessary proteins, is a new therapeutic concept. Further analysis, including transgenic over-expression and vector delivery of Dorfin-CHIP chimeric proteins using ALS animal models will increase our understanding of the potential utility of Dorfin-CHIP chimeric proteins as therapeutic tools.

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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.nbd.2006.09.017.

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## Altered venous capacitance as a cause of postprandial hypotension in multiple system atrophy

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**Abstract** Patients with multiple system atrophy (MSA) often have clinically significant postprandial hypotension (PPH). To elucidate the cause of insufficient cardiac preload augmentation that underlies PPH, we recorded calf venous capacitance (CVC) by strain-gauge plethysmography, in 17 MSA patients and eight healthy controls before and after oral glucose ingestion. Among 17 MSA patients, nine who showed a decrease in systolic blood pressure exceeding 20 mmHg and were diagnosed with PPH. MSA patients without PPH showed a significant decrease in CVC and a significant increase in cardiac output after oral glucose ingestion, as did controls; those with MSA exhibiting PPH showed a significant increase in CVC and no significant change in cardiac output. The change in CVC correlated positively with the de-

crease in systolic and diastolic blood pressure after glucose ingestion, and also correlated negatively with the increase in cardiac output. Physiologically, PPH is prevented by a decrease in venous capacitance, which increases circulating blood volume and cardiac output. In some MSA patients, failure of venous capacitance to decrease may induce PPH.

**Key words** postprandial · hypotension · multiple system atrophy · venous capacitance · autonomic nervous system

### Introduction

Postprandial hypotension (PPH) was first reported as a clinical problem in a patient with parkinsonism [20], although a change in blood pressure provoked by eating already had been recognized [5, 21]. Currently PPH is known to occur frequently among patients with autonomic failure caused by multiple system atrophy (MSA), pure autonomic failure (PAF)

[16, 17], Parkinson's disease [18, 25], and diabetes mellitus [13]; PPH even may occur in hypertensive patients and healthy elderly subjects [14]. Like orthostatic hypotension (OH), PPH can be a major clinical problem because of various alarming, sometimes serious symptoms such as dizziness, nausea, lightheadedness, weakness, syncope, falls, angina pectoris, and cerebral ischemia [13, 26]. Various reported studies have sought to clarify the mechanism of PPH. Food ingestion increases portal blood flow

and may cause hypotension [19, 24]. In normal healthy subjects, cardiac output increases postprandially while blood pressure does not decrease; in patients with PPH, cardiac output fails to increase [10, 16]. Cardiac output can be increased by increasing preload (venous return), decreasing afterload (systemic vascular resistance), and/or increasing myocardial contractility ( $\beta_1$  sympathetic stimulation). In a previous study, we found decreased afterload (vascular resistance) in PPH despite unchanged cardiac output, while healthy controls showed a postprandial increase in cardiac output in the absence of a significant increase in heart rate (HR) [6, 10]. If myocardial contractility augmentation by  $\beta_1$  sympathetic excitation were a major contribution to the normal postprandial increase in cardiac output, HR would be expected to increase more. Based on these observations, we hypothesized that the preloading effect of venous compliance is a crucial physiologic factor preventing PPH. However, no reported studies or anecdotal accounts have described venous compliance in patients with PPH. Venous compliance is pressure dependent value, so the measurement of venous compliance needs frequent examinations and takes some times [7, 23], and standard methods have not been established. PPH is a phenomenon which changes in minutes, so the measurement of venous compliance is difficult in PPH. We therefore substituted calf venous capacitance (CVC) for venous compliance [3], assessing its involvement in the pathophysiology of PPH.

## Methods

### ■ Subjects

The 17 consecutive probable MSA patients participating in this study included nine men and eight women (mean age at examination  $\pm$  SD,  $59.8 \pm 7.9$  years, range, 46–73; mean illness duration,  $4.2 \pm 2.6$  years, range, 2–10). Probable MSA was diagnosed according to the criteria established by a consensus statement concerning the diagnosis of MSA [4]. Criteria for exclusion from this study were presence of cardiovascular disease, diabetes mellitus, peripheral neuropathy, and severely impaired motor function defined as a Modified Rankin Scale score exceeding 4 (inability to walk independently with or without a crutch). Eight normal healthy control subjects also were studied (Table 1). The ethics committee of the Nagoya University School of Medicine approved this study in full. We obtained informed consent from all subjects prior to study participation.

### ■ Glucose loading test

#### Protocol

The test began at 9:00 AM in our laboratory, at an ambient temperature of 25°C. All medications and oral intake were withheld after the night before the study. Subjects lay supine on a

**Table 1** Clinical features and autonomic function parameters in MSA patients with PPH, MSA patients without PPH, and controls

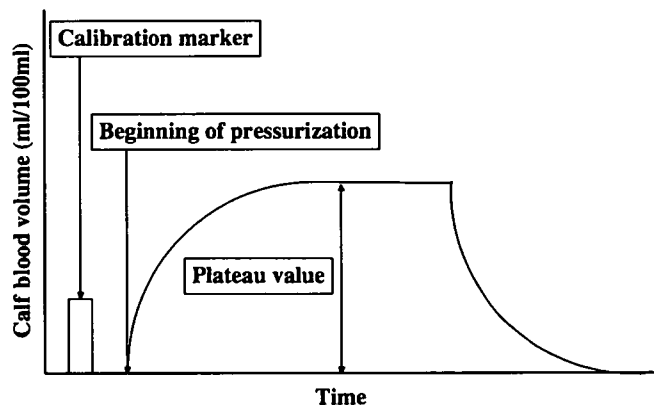
	MSA with PPH (n = 9)	MSA without PPH (n = 8)	Controls (n = 8)
Age (years)	59.6 (9.0)	60.5 (8.3)	48.6 (10.2)
Gender (M:F)	4:5	5:3	7:1
Disease duration (years)	3.4 (1.6)	4.6 (3.4)	
<b>Baseline</b>			
SBP (mmHg)	147 (18) <sup>5</sup>	125 (12)	121 (15)
DBP (mmHg)	82 (9)	78 (6)	70 (11)
HR (bpm)	74 (12)	71 (9)	71 (10)
Clinical PPH	2/9 (22%)	0/8 (0%)	0/8 (0%)
<b>Change after glucose ingestion</b>			
• SBP (mmHg)	-35 (12) <sup>†</sup>	-2 (5)	0 (7)
• DBP (mmHg)	-21 (7) <sup>†</sup>	-6 (6)	1 (6)
• HR (bpm)	4 (6)	1 (4)	-1 (5)
OH	9/9 (100%)*	6/8 (75%)**	0/8 (0%)
Clinical OH	7/9 (78%) <sup>‡</sup>	1/8 (13%)	0/8 (0%)
<b>Change after 60° head up</b>			
• SBP (mmHg)	-47 (20)*	-29 (22)***	-3 (9)
• DBP (mmHg)	-25 (11)*	-12 (21)	1 (6)
NE supersensitivity	8/9 (89%)	4/8 (50%)	n.e.
<b>Change after NE infusion</b>			
• SBP (mmHg)	31 (6)	23 (15)	n.e.
• DBP (mmHg)	19 (10)	13 (17)	n.e.
NE (pmol/l)	992 (610)	1520 (654)	1778 (824)
AVP (pg/ml)	1.83 (1.57)	0.98 (0.64)	1.22 (0.70)

\* $p < 0.001$  vs. controls. \*\* $p < 0.01$  vs. controls. \*\*\* $p < 0.05$  vs. controls. <sup>†</sup> $p < 0.001$  vs. both MSA without PPH and controls. <sup>‡</sup> $p < 0.05$  vs. MSA without PPH,  $< 0.001$  vs. controls. <sup>5</sup> $p < 0.05$  vs. MSA without PPH,  $< 0.01$  vs. controls. MSA, multiple system atrophy; PPH, postprandial hypotension; OH, orthostatic hypotension; NE, norepinephrine; AVP, arginine vasopressin; n.e., not evaluated

bed and were monitored at rest for 30 min (baseline period), and then for another 30 min after oral ingestion of 75 g of glucose in 225 ml of water. Systolic blood pressure (SBP), diastolic blood pressure (DBP), HR, cardiac output, and arterial blood flow in the lower legs (LBF) were measured continuously. CVC was measured twice, at baseline and at 30 min after glucose ingestion. We defined PPH as a decrease in SBP following glucose administration that exceeded 20 mmHg [13]. Clinical PPH was defined as sensation of dizziness, visual disturbance, or a gradual fading of consciousness after food ingestion during the study or previously.

### ■ Hemodynamic measurements

SBP, DBP, and HR were measured in the right radial artery at the wrist by tonometry (BP-508; COLIN, Aichi, Japan), which noninvasively determined the subject's beat-to-beat blood pressure. The electrocardiogram (ECG) was monitored. Cardiac output and LBF were monitored by impedance plethysmograph (4134; NEC San-ei, Tokyo, Japan). CVC was measured using a mercury-filled silastic strain-gauge plethysmograph (EC-5R; Hokanson Inc., Washington, USA). The strain gauge was positioned around the midpoint of the right calf to measure change in lower limb volume. After a blood pressure cuff at the right ankle had been inflated to 200 mmHg and had rendered the sole of the foot temporarily ischemic, a thigh blood pressure cuff was inflated additionally to 40 mmHg for 2 min, while the change in lower limb volume was recorded. CVC was defined as volume increase (ml/100 ml) [2, 3, 12]. CVC was



**Fig. 1** Illustration of limb volume curve. Calf volume changes following applied thigh blood cuff pressurization. Calf venous capacitance was calculated from plateau value divided by the height of the calibration marker. This marker represents a 1% change in the electrical resistance of the conductor inside the gauge, which is equal to a 1% limb volume

calculated by measuring the plateau value of limb volume curve after thigh blood pressure cuff was inflated to 40 mmHg and dividing this value by the height of calibration marker; this marker represents a 1% change in the electrical resistance of the conductor inside the gauge, which is equal to a 1% change in limb volume (Fig. 1). The change of blood pressure can influence the plateau time, but it does not influence the plateau value except when SBP goes down to less than 40 mmHg. Cardiac output and LBF were presented here as percentage variation from the baseline value, because the measurement of absolute value was not possible by impedance method.

#### Head-up tilting

On a different day than the hemodynamic tests, head-up tilting was carried out with the subject in a supine position on a tilt table with a foot-plate support. The table was tilted in a stepwise manner (20°, 40°, and 60° for 5 min each). We diagnosed OH when the decrease of SBP during head-up tilting exceeded 20 mmHg, or the decrease of DBP during head-up tilting exceeded 10 mmHg [15]. Clinical OH was defined as sensation of dizziness, visual disturbance, or a gradual fading of consciousness after standing during the study or previously.

#### Denervation supersensitivity to norepinephrine (NE)

To assess sensitivity to NE, we administered an intravenous NE infusion at a rate of 3 µg/min for 3 min. A diagnosis of the denervation supersensitivity was made when SBP increased more than 25 mmHg [9].

#### Plasma norepinephrine (NE) and arginine vasopressin (AVP)

To determine plasma NE and AVP, venous blood was sampled after rest in the supine position for at least 30 min.

Both NE infusion tests and examinations of plasma NE and AVP were performed on the same day as head-up tilting. NE infusion tests were not performed in the healthy controls.

#### Statistical analysis

The Fisher's exact probability test was used to compare the usage of vasopressor between the group with MSA inducing PPH and with MSA without PPH. The Mann-Whitney *U*-test was used to assess differences for continuous variables between the group with MSA inducing PPH and with MSA without PPH. Scheffé's *F*-test was used for post hoc testing between three groups (MSA with PPH, MSA without PPH, and controls). Wilcoxon signed-ranks tests were used to assess differences in cardiac output and CVC between determinations at baseline and after glucose ingestion. Relationships between change in CVC and decrease in SBP and DBP, between change in CVC and increase in cardiac output, were analyzed using Spearman's correlation coefficient by rank. Calculations were performed using the StatView statistical software package (version 5.0; Abacus Concepts, Berkeley, CA). Statistical significance was defined by  $p < 0.05$ . Values are presented as the means  $\pm$  SD.

## Results

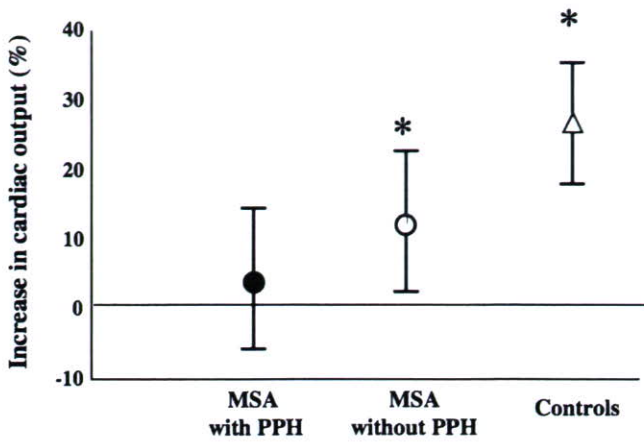
Clinical features and autonomic functions in each group are summarized in Table 1. Of 17 MSA patients, nine were diagnosed with PPH positive, while the other eight were PPH negative. All eight controls were PPH negative.

#### Clinical features

No significant difference was evident in illness duration or age at examination between the MSA group with PPH and the MSA group without PPH. Controls were slightly younger than subjects in the other two groups, but the age difference was not significant. Four out of nine MSA patients with PPH took vasopressor before the examination. One took droxidopa, one amezium, one took both droxidopa and midodrine, and another both droxidopa and amezium. Only one out of eight MSA patients without PPH took vasopressor, both droxidopa and midodrine before the examination. More patients with PPH used vasopressor than patients without PPH, although the difference was not significant.

#### Response to glucose loading test

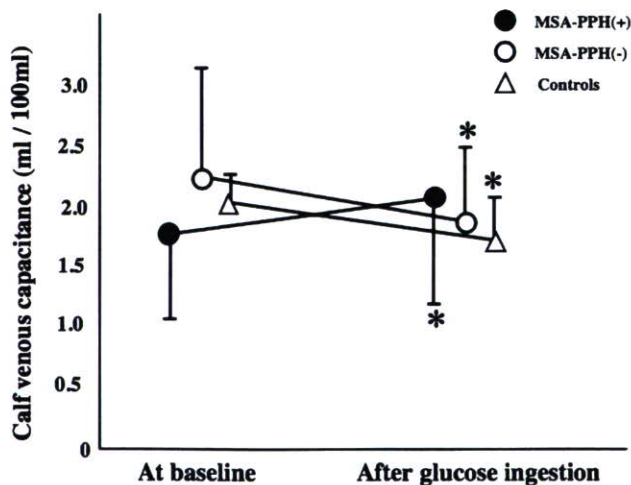
SBP at baseline in MSA with PPH was significantly higher than in MSA without PPH or in controls ( $p < 0.05$  and  $p < 0.01$ , respectively). DBP or HR at baseline was not significantly different between MSA with PPH, MSA without PPH, and controls. The change in SBP after glucose ingestion in MSA with PPH was significantly greater than in MSA without PPH or in controls (both  $p < 0.001$ ). The change in DBP after glucose ingestion in MSA with PPH was



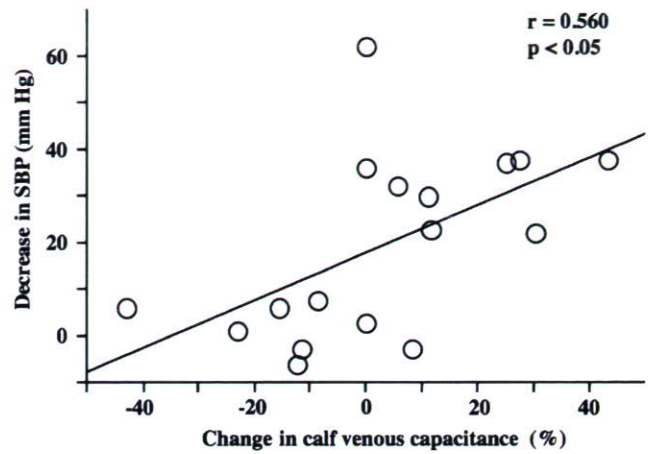
**Fig. 2** Increase in cardiac output after glucose ingestion in multiple system atrophy (MSA) patients with postprandial hypotension (PPH), MSA patients without PPH, and normal control subjects. Values are means, with error bars indicating SD. \* $p < 0.05$  vs. baseline

significantly greater than in MSA without PPH or in controls (both  $p < 0.001$ ). The change in HR after glucose ingestion was not significantly different between the three groups (Table 1).

Cardiac output increased significantly in MSA without PPH ( $13 \pm 10\%$ ,  $p < 0.05$ ) and in controls ( $26 \pm 9\%$ ,  $p < 0.05$ ), while output did not change significantly in MSA with PPH ( $4 \pm 10\%$ ; Fig. 2). After glucose ingestion, CVC decreased significantly in MSA without PPH (from  $2.20 \pm 0.88\%$  to  $1.84 \pm 0.60\%$ ,  $p < 0.05$ ) and in controls (from  $2.04 \pm 0.24\%$  to  $1.72 \pm 0.36\%$ ,  $p < 0.05$ ), but increased significantly in MSA with PPH (from  $1.80 \pm 0.72\%$  to  $2.08 \pm 0.88\%$ ,  $p < 0.05$ ; Fig. 3). The change in CVC correlated positively with the decrease



**Fig. 3** Change in calf venous capacitance after glucose ingestion in multiple system atrophy (MSA) patients with postprandial hypotension (PPH), MSA patients without PPH, and normal control subjects. Values are means, with error bars indicating SD. \* $p < 0.05$  vs. baseline

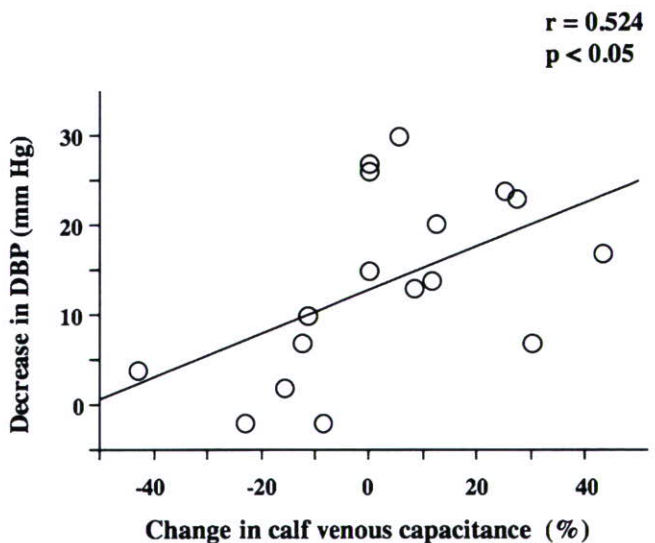


**Fig. 4** Change in calf venous capacitance showed significant positive correlation with decrease in systolic blood pressure (SBP) after glucose ingestion across the whole MSA population ( $r = 0.560$ ,  $p < 0.05$ )

in SBP ( $r = 0.560$ ,  $p < 0.05$ ; Fig. 4), and with the decrease in DBP ( $r = 0.524$ ,  $p < 0.05$ ; Fig. 5) after glucose ingestion, and correlated negatively with the increase in cardiac output ( $r = -0.583$ ,  $p < 0.05$ ; Fig. 6). The increases of LBF after glucose ingestion were  $1 \pm 19\%$  in MSA with PPH,  $0 \pm 37\%$  in MSA without PPH, and  $3 \pm 9\%$  in controls, and there were no significant differences among three groups.

**Other autonomic function tests**

All nine MSA patients with PPH demonstrated OH. Frequency of OH in MSA with PPH (100%) and in MSA without PPH (75%) was significantly higher than in controls (0%); ( $p < 0.001$ ,  $p < 0.01$ , respectively).



**Fig. 5** Change in calf venous capacitance showed significant positive correlation with decrease in diastolic blood pressure (DBP) after glucose ingestion across the whole MSA population ( $r = 0.524$ ,  $p < 0.05$ )