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RESEARCH ARTICLE

Amyloid β-induced FOXRED2 mediates neuronal cell death via inhibition of proteasome activity

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Abstract Proteasome inhibition has been regarded as one of the mediators of Aβ neurotoxicity. In this study, we found that FOXRED2, a novel endoplasmic reticulum (ER) residential protein, is highly up-regulated by Aβ in rat cortical neurons and SH-SY5Y cells. Over-expression of FOXRED2 inhibits proteasome activity in the microsomal fractions containing ER and interferes with proteasome assembly, as evidenced by gel filtration and native gel electrophoresis analysis. In contrast, reduced expression of FOXRED2 rescues Aβ-induced inhibition of proteasome activity. FOXRED2 is an unstable protein with two degradation boxes and one KEN box, and its N-terminal oxidoreductase domain is required for proteasome inhibition. Ectopic expression of FOXRED2 induces ER stress-mediated cell death via caspase-12, which is inhibited by Salubrinal. Further, down-regulation of FOXRED2 expression attenuates Aβ-induced cell death and the ER stress response. These results suggest that up-regulated FOXRED2 inhibits proteasome activity by interfering with 26S proteasome assembly to contribute to Aβ neurotoxicity via an ER stress response.

Keywords FOXRED2 · Proteasome · Amyloid-beta · ER stress · Cell death

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Introduction

Proteins are targeted for selective degradation by the ubiquitin–proteasome system (UPS) in eukaryotic cells. Short-lived or damaged proteins are tagged by covalent modification of ubiquitin (Ub) using E1, E2, and E3 enzymes [1]. Target proteins labeled with Ub are recognized by large protein complexes known as proteasomes. The 26S proteasome consists of a 20S core particle and a 19S regulatory particle. The 20S core particle is composed of α subunits with a docking domain for the 19S and β subunits with catalytic activity, and the 19S regulatory particle consists of a lid subcomplex with polyubiquitin-binding subunits and a base subcomplex with ATPase activity [2]. The 26S proteasome comprises 33 distinct subunits and requires a regulator for accurate interaction between subunits or subcomplexes. A total of nine proteasome-assembly chaperones, namely, PAC1, PAC2, PAC3, PAC4 (Pba1-4), POMP/Ump1, p27/Nas2, p28/Nas6, PAAF1/Rpn14, and S5b/Hsm3, have been identified to date [3, 4]. There is an increasing body of evidence suggesting that proteasome activity can be modulated by dysregulation of proteasome-assembly chaperones and the modification, phosphorylation, or glycosylation of the subunits [2, 5]. However, regulation of the multimeric 26S proteasome complex is not well understood.

Molecular mechanisms of Aβ-mediated neurotoxicity have been studied with the aim of gaining an understanding of the pathogenesis of Alzheimer's disease (AD). Several hypotheses underlying AD pathogenesis, including amyloid theory and tau aggregation, dysregulated UPS, and endoplasmic reticulum (ER) stress, have been proposed in AD research [1, 6–10]. Malfunction of the UPS is known to accelerate Aβ-induced neuronal loss through the accumulation of cellular proteins; therefore, a dysregulated UPS,

especially proteasome inhibition, has been proposed as a main pathogenesis [1]. The accumulation of Ub-conjugated proteins and inclusion bodies has been reported as a hallmark of AD and neurodegenerative disorders [8]. In an earlier publication, our group reported that Aβ-induced expression of E2-25K/Hip2, an Ub-activating enzyme, is required for Aβ neurotoxicity and caspase-12 activation via proteasome inhibition [9, 10]. Therefore, abnormal regulation of the UPS by gene expression is believed to contribute to the progression of neurodegenerative diseases, making the identification of such regulatory genes that affect proteasome activity a very important objective of research in this field.

The ER is responsible for protein folding, quality control, trafficking, and maintenance of Ca^{2+} homeostasis, and unfolded or misfolded proteins are eliminated via the ER-associated degradation system (ERAD) [11]. The ER stress response can be induced by the accumulation of unfolded or misfolded protein aggregates and dysfunction of the ERAD, triggering an unfolded protein response (UPR) [11]. To prevent further accumulation of unfolded proteins, the UPR inhibits protein translation and promotes degradation of misfolded proteins by the ERAD [11]. However, if cells are not tolerant to stress, ER stress induces cell death via activation of ER-specific caspase and increases the expression of CHOP or apoptotic transcription factor [12]. An up-regulated ER stress response and polymorphism of the ERAD component have been reported in AD patients [13, 14]. Moreover, dysfunction of ERAD increases Aβ generation [15]. Consequently, an understanding of the ER stress response in the context of the pathogenesis of human diseases, including AD, has become increasingly important [12]. FOXRED2 is a FAD-dependent oxidoreductase domain-containing protein that is localized in the ER [16]. It is known to be an ER luminal flavoprotein that functions in the ERAD via interaction with ERAD components [17].

We performed genome-wide functional screening and isolated FOXRED2 as a novel factor in the regulation of UPS. Here, we show that FOXRED2 mediates Aβ-induced neuronal cell death via inhibition of proteasome activity, suggesting that FOXRED2 is one of the key molecules responsible for Aβ neurotoxicity.

Materials and methods

Material

The following antibodies were used: anti-green fluorescent protein (GFP), anti-tubulin, anti-His, anti-actin, anti-GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ub (Santa Cruz Biotechnology in all figures; Chemicon in Fig. 3b), and anti-S4, anti-S2, and anti-20S core (BIOMOL Int, Plymouth Meeting, PA). MG132 was purchased from BIOMOL Int. Ethidium homodimer was purchased from Molecular Probes (Eugene, OR) and Aβ from Sigma (St. Louis, MO). Anti-Aβ (NU-1) antibody was kindly provided by Dr. W. L. Klein (Northwestern University, Evanston, IL).

Cell culture and transfection

SH-SY5Y and HEK293T cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in DMEM containing 10% FBS and penicillin/streptomycin at 37°C under 5% CO_2 (v/v). According to the manufacturer's instructions, DNA was transfected into cells using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) and siRNA using Oligofectamine (Invitrogen).

Genome-wide functional screening

HEK293T cells were plated on a 96-well culture plate and cotransfected with GFP^U and each of 5,500 cDNAs in a mammalian expression vector for 36 h. FOXRED2 was then isolated among the putative positive cDNA clones affecting GFP^U fluorescence under a fluorescence microscope (Olympus, Tokyo, Japan).

Assays for proteasome activities

Cell lysates were prepared by sonication using rectic buffer (30 mM Tris pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM ATP). Proteasome activities were measured by use of fluorogenic substrates (Suc-LLVY-AMC, Bz-VGR-AMC, Ac-GPLD-AMC) (BIOMOL Int) and a fluorometer (FlexStation 3 Microplate Reader; Molecular Devices, Sunnyvale, CA) with excitation at 380 nm and emission at 460 nm.

Reverse transcriptase-PCR

RNA was prepared using TRI reagent (Molecular Research Center, Cincinnati, OH), and cDNA was synthesized by reverse transcriptase (RT; Invitrogen), followed by PCR amplification. According to the manufacturer's instructions, real-time RT-PCR was performed using Taqman technology (SYBR Premix Ex Taq; TaKaRa, Otru, Japan). Primer sequences used for FOXRED2 RT-PCR analysis were as follows: forward 5′-ATG ACC GGT CTG TGG-3′ and reverse 5′-TCA GAG CTC CTC TTT G-3′.

Plasmid construction and site-directed mutagenesis

Primer sequences used for the construction of FOXRED2 deletion mutants were as follows: FOXRED2ΔC(1–245)

(forward: 5′-ATA AGA ATG CGG CCG CTA ATG GGC CTC TCC GCT GC-3′; reverse: 5′-TGC TCT AGA GGA GCG GCT GAG CAT ATG G-3′); His-FOXRED2ΔC(1–245) (forward: 5′-CGC GGA TCC GTG CCC CCG CGC CGG GAC-3′; reverse: 5′-CGC GGA TTC GGA GCG GCT GAG CAT ATG G-3′); His-FOXRED2ΔN(250–684) (forward: 5′-CGC GGA TCC TGG GCC ACC CAC TAC G-3′; reverse: 5′-CGC GGA TCC TCA GAG CTC CTC TTT G-3′). Primer sequences used for site-directed mutagenesis were as follows: FOXRED2(D_1)–R246G/L249V (forward: 5′-CGC TCC GGG GTC CGT GTG TCC TGG GCC-3′; reverse: 5′-GGC CCA GGA CAC ACG GAC CCC GGA GCG-3′); FOXRED2(D_2)–R582G/L585V (forward: 5′-CCT ACC TCT GGG GCG CTT CGT GGA GAA CTG TTT GG-3′; reverse: 5′-CCA AAC AGT TCT CCA CGA AGC GCC CCA GAG GTA GG-3′); FOXRED2(K)–K460A (forward: 5′-GTC ATC CTG TTG GCG GAG AAT TCC ACG-3′; reverse: 5′-CGT GGA ATT CTC CGC CAA CAG GAT GAC-3′). PCR was performed using Pfu polymerase, and PCR products were subcloned into pcDNA3 or pET-28a. PCR products were confirmed by DNA sequencing analysis. FOXRED2 siRNAs (forward 5′-GAC CAA GAG CCA CUA GGU U-3′; reverse: 5′-AAC CUA GUG GCU CUU GGU C-3′) were purchased from Bioneer Corp. (Daejeon, Republic of Korea).

SDS-PAGE and immunoblot analysis

Protein samples were prepared in sample buffer by sonication and centrifuged at 12,000 rpm at 4°C for 10 min. Supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and resolved proteins were transferred onto nitrocellulose membranes using a Bio-Rad semi-dry transfer unit (Bio-Rad, Hercules, CA). Blots were blocked with 3% (w/v) non-fat dry milk in TBS-T solution [25 mM Tris pH 7.5, 150 mM NaCl, and 0.05% (w/v) Tween-20]. After washing with TBS-T, blots were incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected by ECL reagents (GE Healthcare, Little Chalfont, UK).

Primary culture of rat cortical neurons

Rat cortical neurons of embryonic day 17 were dissociated by incubation with 0.01% trypsin/EDTA (Invitrogen) and plated on culture dishes in Neurobasal medium supplemented with 2% B27 (Invitrogen). The plates were coated with poly-L-lysine (0.01% in 100 mM borate buffer, pH 8.5) (Sigma), and the neurons grown in vitro for 5 days were treated with 5 μM $Aβ_{1–42}$ (Invitrogen).

Recombinant protein production

Recombinant His-FOXRED2ΔC and His-FOXRED2ΔN proteins were purified from *Escherichia coli* strain BL21(DE3) cells that had been transformed with pET-28a-FOXRED2ΔC and pET-28a-FOXRED2ΔN plasmids, respectively. Transformed *E. coli* were incubated with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 12 h, at which point the cultures had reached an OD_{600} of 0.6. According to the manufacturer's instructions, His-tagged proteins were purified on Ni–NTA agarose (Qiagen, Hilden, Germany).

In vitro ubiquitination

Purified His-FOXRED2ΔC protein (3 μg) was incubated with 200 μl reaction mixture containing SH-SY5Y cell lysates (250 μg) in rectic buffer (10 mM Tris pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM ATP, 1× ATP regeneration system, 1× protease inhibitor cocktail) at 37°C for the indicated times. Following incubation, cell lysates were separated by SDS-PAGE and subjected to Western blotting or assayed for proteasome activity using Suc-LLVY-AMC.

Subcellular fractionation

Cells were collected, resuspended in buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride), and incubated on ice for 20 min in the presence of 250 mM sucrose. Cell lysates were centrifuged at 1,000 *g* for 5 min at 4°C, and the supernatant was further centrifuged at 8,000 *g* for 20 min at 4°C. The supernatant was once again centrifuged at 100,000 *g* for 3 h at 4°C. The resulting pellet retained the microsomal fraction, whereas the supernatant contained cytosol.

Gel filtration

Cells were lyzed in lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 2 mM ATP), after which the lysates were centrifuged at 12,000 rpm for 30 min at 4°C and the supernatant filtered through a 0.2-μm membrane (Sartorius, Goettingen, Germany). Gel filtration was carried out using a Superose 6 FPLC column (AKTA; GE Healthcare), and 0.25-ml fractions were collected for analysis.

Native-PAGE

Proteasome assembly was examined in a native PAGE, as previously described [18]. Cell lysates were separated by

4% (w/v) native-PAGE at 4°C (80 V × 2.5 h). The gel was overlaid with buffer containing Suc-LLVY-AMC with or without 0.01% SDS, and the fluorescence signal was visualized on a UV trans-illuminator.

Immunoprecipitation

Cell lysates were prepared by sonication using rectic buffer (30 mM Tris pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 0.5 mM DTT, 1 mM ATP) and then centrifuged at 12,000 rpm for 30 min at 4°C. The supernatants were incubated with anti-20S core antibody and pulled down by Protein G Sepharose beads (GE Healthcare).

Results

Aβ exposure increases FOXRED2, leading to inhibition of proteasome activity

We previously reported that treatment of neuronal cells with Aβ42 inhibits proteasome activity [9]. This result led us to the question of whether Aβ42 affects the expression level of FOXRED2 in neuronal cells. Exposing primary cultured rat cortical neurons to Aβ42 led to a highly elevated level of FOXRED2 protein at 24 h, compared to untreated control cells (Fig. 1a, left). A similar induction of FOXRED2 protein was observed in SH-SY5Y neuronal cells after exposure to Aβ42 (Fig. 1a, right). The accumulation of Ub-conjugates in Aβ42-treated cells was consistently observed. Real-time RT-PCR analysis revealed that, compared to untreated cells, the level of FOXRED2 mRNA in Aβ-treated SH-SY5Y cells had increased about twofold (Fig. 1b). On the contrary, H_2O_2 treatment did not affect FOXRED2 mRNA level. These results show that FOXRED2 expression increases in neuronal cells exposed to Aβ42, at least based on increased levels of mRNA.

We employed GFP-degron (GFP-CL1, GFP^U), which is rapidly degraded in cells by 26S proteasome [19], for genome-wide screening of proteasome regulator(s) [20, 38]. GFP^U is not degraded when UPS is dysfunctional or the levels of aggregation-prone substrates are increased [21]. Therefore, we exaggerated the effect of FOXRED2 on proteasome activity. Ectopic expression of FOXRED2 in cells induced the accumulation of unstable GFP^U, but not GFP (Fig. 2a, b), which is more potent than E2-25K/Hip-2. Cellular accumulation patterns of unstable GFP^U showed a correlation with the expression level of FOXRED2 (Fig. 2c). Measurement of enzyme activity in cell extracts using fluorogenic substrates revealed that over-expression of FOXRED2 inhibited proteasome activities, including chymotrypsin, trypsin, and caspase-like activities (Fig. 2d).

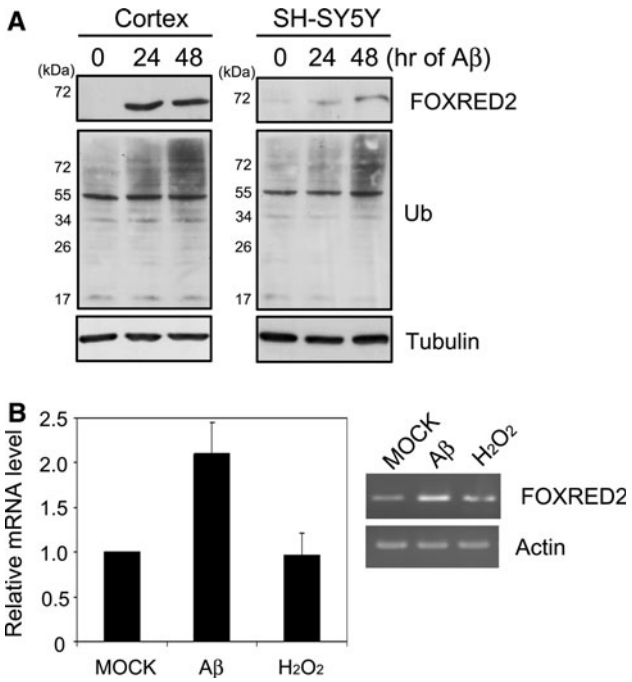


Fig. 1 FOXRED2 is up-regulated in neuronal cells after Aβ42 exposure. **a** Accumulation of FOXRED2 protein by Aβ42. Primary rat cortical neurons and SH-SY5Y cells were treated with 5 μM Aβ for the indicated times and cell extracts were analyzed by Western blotting using anti-FOXRED2, anti-ubiquitin (*Ub*), and anti-tubulin antibodies. **b** Increase of FOXRED2 mRNA by Aβ42. SH-SY5Y cells were left untreated (*Mock*) or exposed to 5 μM Aβ42 or 10 μM hydrogen peroxide (H_2O_2) for 48 h. Total RNA was then isolated using TRIZOL reagent, and FOXRED2 mRNA was analyzed by real-time reverse transcriptase (RT)-PCR (*left*) and quantitative RT-PCR analysis (*right*). Actin served as an internal control. *Bars*: Mean ± standard deviation (SD) ($n > 3$)

Moreover, there was a greater accumulation of Ub-conjugated proteins in cells over-expressing FOXRED2 than in control cells [Fig. 2e; Electronic Supplementary Material (ESM) Fig. 1]. These results indicate that increased expression of FOXRED2 suppresses proteasome activity.

The contribution of FOXRED2 to Aβ-induced proteasome inhibition was then examined by reducing the expression level of FOXRED2. Knockdown of FOXRED2 expression by siRNA resulted in the enhancement of proteasome activity by 140–170% in a dose-dependent manner (Fig. 3a, left) and reduced cellular accumulation of Ub-conjugates (Fig. 3a, right). The knockdown of FOXRED2 expression also abolished both the Aβ42-mediated inhibition of proteasome activity (Fig. 3b, left) and the Aβ42-mediated accumulation of Ub-conjugates (Fig. 3b, right). Conversely, over-expression of FOXRED2 further suppressed proteasome activity and increased accumulation of Ub-conjugates after exposure to Aβ42 (Fig. 3b). These results suggest that FOXRED2 mediates Aβ-induced inhibition of proteasome activity.

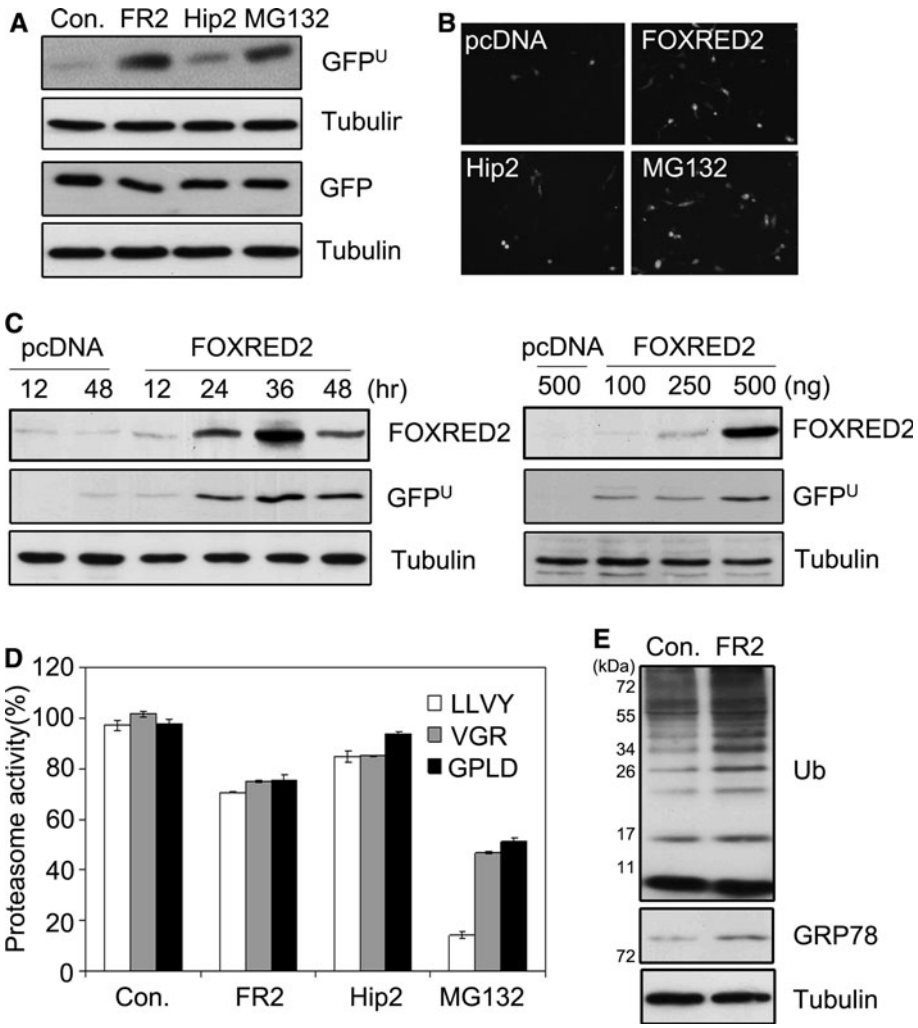


Fig. 2 FOXRED2 over-expression inhibits proteasome activity and induces the accumulation of Ub-conjugates. **a** Accumulation of green fluorescent protein (*GFP*)-degron by FOXRED2 over-expression. HEK293T cells were cotransfected with pGFP^{u}, pEGFP, pcDNA3 (*Con*), pFOXRED2 (*FR2*), and/or pE2-25K/Hip2 (*Hip2*) as indicated for 36 h in the presence or absence of 1 μM MG132. Cell extracts were prepared and analyzed by Western blotting using anti-GFP and anti-tubulin antibodies. **b** Typical photograph showing accumulation of GFP-degron by FOXRED2. SH-SY5Y cells were cotransfected with pGFP^{u} and either pcDNA, pFOXRED2, or pHip2 for 36 h in the presence or absence of 1 μM MG132 and then observed under a fluorescence microscope. **c** Accumulation kinetics of GFP-degron by FOXRED2. SH-SY5Y cells were cotransfected with pGFP^{u} and pFOXRED2 for the indicated times (*left*) or cotransfected with pGFP^{u} and increasing amounts of pFOXRED2 for 36 h (*right*). Western blot analysis was performed as in **a**. **d** Inhibition of proteasome activities by FOXRED2. SH-SY5Y cells were cotransfected with Con, FR2, or Hip2 for 36 h, or exposed to 1 μM MG132 for 12 h. Proteasome activities, including chymotrypsin (Suc-LLVY-AMC), trypsin (Bz-VGR-AMC), and caspase (Ac-GPLD-AMC)-like activities, were measured in cell extracts using fluorogenic substrates at excitation/emission (380 nm/460 nm). *Bars*: Mean ± SD ($n > 3$). **e** Accumulation of Ub-conjugates by FOXRED2. SH-SY5Y cells were transfected with Con or FR2 for 36 h, and cell lysates were analyzed by Western blotting using anti-Ub and anti-GRP78 antibodies

Oxidoreductase domain of FOXRED2 inhibits proteasome activity

From sequence comparison analysis (available at: http://myhits.isb-sib.ch), we found that FOXRED2 has a FAD-dependent oxidoreductase domain (amino acid residues 1–250) at its N-terminus and two degradation boxes (D box; 246–249 and 582–585) and one KEN box (K; 460–462) at its C-terminus (Fig. 4a). Association of the degradation box and KEN box with protein stability has been proposed [22, 23]. In order to dissect the function of FOXRED2, we constructed several deletion and point mutants of FOXRED2 and examined their effects on proteasome activity (Fig. 4a). Ectopic expression of FOXRED2ΔC(1–245) containing only the FAD-dependent oxidoreductase domain induced GFP^{U} accumulation as much as FOXRED2 (Fig. 4b). On the contrary, FOXRED2(1–250) containing one degradation box (D_1) or FOXRE2ΔN(251–684) lacking the N-terminus failed to do so (Fig. 4a and data not shown), indicating that the N-terminal

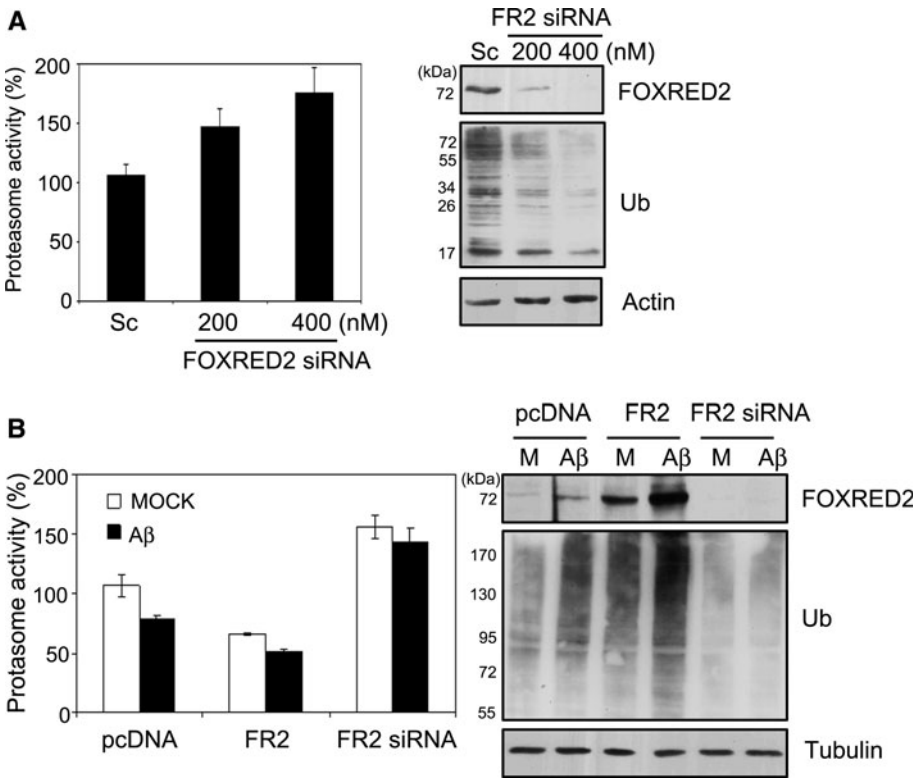


Fig. 3 Reduced expression of FOXRED2 rescues Aβ-induced inhibition of proteasome activity and the accumulation of Ub-conjugates. **a** Increased proteasome activities and accelerated clearance of Ub-conjugates by FOXRED2 down-regulation. SH-SY5Y cells were transfected with scrambled siRNA (*Sc*) or FOXRED2 siRNA for 48 h. Cell lysates were prepared and examined for chymotrypsin-like proteasome activity using a fluorogenic substrate (Suc-LLVY-AMC) (*left*). Cell lysates were also analyzed by Western blotting using anti-FOXRED2 and anti-Ub antibodies (*right*). **b** Rescue of Aβ-induced proteasome inhibition and accumulation of Ub-conjugates in FOXRED2 knockdown cells. SH-SY5Y cells were transfected with pcDNA, FR2, or FOXRED2 siRNA for 24 h and then treated with 5 μM Aβ. After 24 h, cell extracts were prepared and measured for chymotrypsin-like proteasome activity (*left*) or analyzed by Western blotting using anti-FOXRED2 and anti-Ub antibodies (*right*). *Bars*: mean ± SD ($n > 3$)

region of FOXRED2 is responsible for proteasome inhibition. FOXRED2(1–250) protein containing one D box was barely detected in cells and then only in the presence of MG132, and FOXRED2(251–684) protein comprising one degradation box and one KEN box was not detected even in the presence of MG132 (data not shown). These boxes were characterized in more detail using site-directed mutagenesis. FOXRED2 D box mutants FOXRED2(D_1)–R246G/L249V and FOXRED2(D_2)–R582G/L585V and FOXRED2 KEN box mutant FOXRED2(K)–K460A were more stable than FOXRED2 (Fig. 4c). In addition, all of the FOXRED2 mutants increased GFP^U accumulation more efficiently than FOXRED2 (Fig. 4c). Measurement of protein stability after treatment with cycloheximide revealed a rapid turnover of FOXRED2 protein with less than 3 h of half-life in cells (Fig. 4d). These results suggest that the FOXRED2 protein is unstable, mainly due to the presence of the degradation and KEN boxes at the C-terminus.

From an in vitro ubiquitination assay using purified 6× His-tagged FOXRED2ΔC protein, we found that incubation of SH-SY5Y cell extracts with FOXRED2ΔC protein apparently increased the accumulation of Ub-conjugates; however, it did not affect the protein levels of other UPS components, such as S5a and 20S core (Fig. 4e, upper and middle). Enzymatic assays of the same reaction products showed that FOXRED2ΔC protein also inhibited proteasome activity in cell extracts in a time- and dose-dependent manner (Fig. 4e, lower; ESM Fig. 2A). Proteasome activity was about 20% lower at 24 h of incubation with FOXRED2ΔC protein than with the control. It is interesting to note that purified FOXRED2ΔC protein did not show any inhibitory effect on proteasome activity when incubated together with purified 26S proteasome (ESM Fig. 2B). On the contrary, FOXRED2ΔN protein failed to inhibit proteasome activity and to induce the accumulation of Ub-conjugates (ESM Fig. 2C). These results indicate that purified FOXRED2ΔC protein containing an FAD-dependent oxidoreductase domain can inhibit proteasome activity indirectly and also increase the accumulation of Ub-conjugates in vitro.

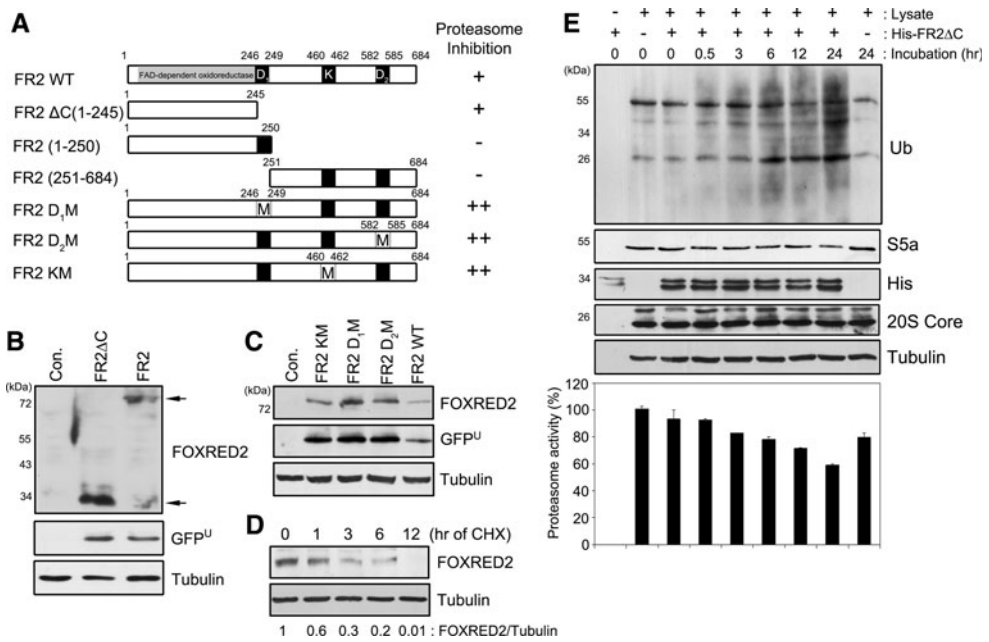
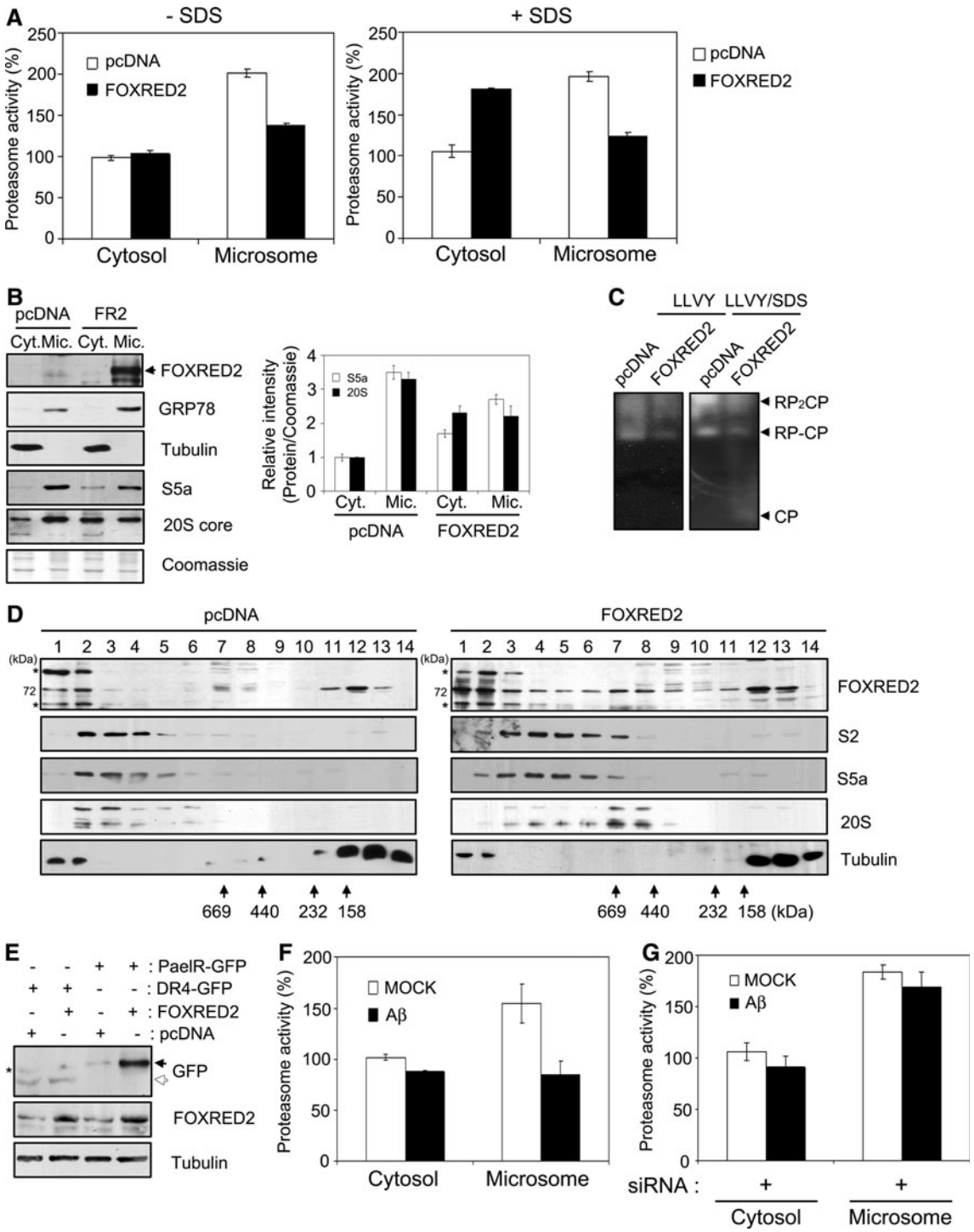


Fig. 4 The FAD-dependent oxidoreductase domain of FOXRED2 is required for proteasome inhibition. **a** Schematic diagram showing FOXRED2 (*FR2*) and its mutants. *D* Degradation box, *K* KEN box of FOXRED2, *numbers in parenthesis* number of amino acid residues. FOXRED2(D_1)–R246G/L249V and FOXRED2(D_2)–R582G/L585V mutants (*M*) in the D box and the FOXRED2(K)–K460A mutant (*M*) in the KEN box are described. **b**, **c** Effects of FOXRED2 mutants on accumulation of GFP-degron. SH-SY5Y cells were transfected with $pGFP^u$ and pcDNA, pFOXRED2ΔC, or pFOXRED2 (**b**), or with FOXRED2(D_1M), FOXRED2(D_2M), or FOXRED2(KM) (**c**) for 36 h. Cell lysates were analyzed by Western blotting using anti-FOXRED2, anti-GFP, and anti-tubulin antibodies. *Arrows* in **b** indicate FOXRED2 and FOXRED2ΔC. **d** Instability of FOXRED2 protein. SH-SY5Y cells were treated with 30 μg/ml cycloheximide (*CHX*) for the indicated times, and cell lysates were analyzed by Western blotting using anti-FOXRED2 antibody. Signals on the blot were quantitated using densitometry analysis, and the relative ratios of each signal to the control (time 0) (FOXRED2/tubulin) are indicated. **e** In vitro ubiquitination assay using purified His-FOXRED2ΔC protein. Cell extracts (250 μg) prepared from SH-SY5Y cells were incubated with bacterially purified His-FOXRED2ΔC protein (3 μg) for the indicated times. Reaction products were then analyzed by Western blotting using anti-Ub (*upper*), anti-S5a, anti-His, and anti-20S core antibodies (*middle*) or were measured for proteasome activity using a fluorogenic substrate (Suc-LLVY-AMC) (*bottom*)

FOXRED2 decreases microsomal proteasome activity and interferes with proteasome assembly

To elucidate a mechanism by which ER-resident FOXRED2 regulates proteasome activity, we prepared SH-SY5Y cell extracts and separated the ER-enriched microsomal fraction from the cytosolic fraction by fractionation analysis using centrifugation. By Western blotting, we observed that GRP78, an ER protein, and tubulin, a cytosolic protein, were detected only in the microsomal fraction and the cytosolic fraction, respectively (Fig. 5b). Both FOXRED2 and exogenous FOXRED2 were detected exclusively in the microsomal fraction (Fig. 5b). From enzyme activity assays, we observed that proteasome activity in the microsomal fraction was twofold higher than that in the cytosolic fraction (Fig. 5a). Of particular interest, when FOXRED2 was over-expressed in SH-SY5Y cells, proteasome activity was reduced by about 30% in the microsomal fraction but not in the cytosolic fraction (Fig. 5a, left). Then, when 0.01% sodium dodecyl sulfate (SDS) was added to the fractions in order to open the inactive 20S proteasome [24], we found that proteasome activity was increased by about 70–80% only in the cytosolic fraction (Fig. 5a, right). From Western blotting, we noted that the amounts of S5a and 20S subunits were significantly decreased in the microsomal fraction and increased in the cytosol fraction of cells over-expressing FOXRED2 (Fig. 5b). These results suggest that FOXRED2 may relocalize S5a and 20S core proteasome subunits from the microsomal fraction to the cytosolic fraction.

The enzymatic activities of 20S and 26S proteasome complexes were also analyzed and compared using native gel assay. Ectopic expression of FOXRED2 decreased the enzyme activities of the 26S proteasome complexes (RP_2CP and RP–CP), but increased 20S proteasome (CP) activity (Fig. 5c). Moreover, the interaction of S2 or S5a 19S subunit with 20S core subunits was decreased by FOXRED2 over-expression, as assessed with immunoprecipitation assays using anti-20S core antibody (Fig. ESM 3A). The disassembly of proteasome complexes was examined using a gel filtration assay. Western blot analysis of the fractions separated by this gel filtration assay showed that, compared to the control cell



extracts (pcDNA), the S2 and S5a subunits of the 19S proteasome were detected in late fractions (fraction numbers 6 and 7) of cell extracts over-expressing FOXRED2 (Fig. 5d), suggesting that increased expression of FOXRED2 results in the detection of S2 and S5a proteins in proteasome complexes with a lower molecular weight. In a similar manner, the majority of 20S core proteins which were detected in fraction numbers 2–6 of the control cell extracts were found in fraction numbers 7 and 8 of cell extracts over-expressing FOXRED2 (Fig. 5d). FOXRED2 did not affect the expression levels of other proteasome subunits (data not shown). These results indicate that an increase of FOXRED2 protein may interfere with the assembly between 19S and 20S proteasome complexes, thereby allowing the detection of abnormal proteasome complexes.

◀ **Fig. 5** FOXRED2 regulates proteasome activity in the microsomal fractions containing the endoplasmic reticulum (ER) and modulates proteasome assembly. **a**, **b** Fractionation assays showing the suppression of proteasome activity in the microsomal fractions by ectopic expression of FOXRED2. SH-SY5Y cells were transfected with pcDNA or pFOXRED2 for 36 h, and cytosolic and microsomal fractions were then separated by centrifugation, as described in the Materials and methods. Proteasome activities in the fractions were measured using a fluorogenic substrate (Suc-LLVY-AMC) in the absence or presence of 0.01% sodium dodecyl sulfate (*SDS*) (**a**). Each fraction was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting using anti-FOXRED2, anti-GRP78, anti-tubulin, anti-S5a, and anti-20S antibodies. Equal amounts of proteins on the blot were visualized with Coomassie staining (**b**, *left*). Signal intensities of S5a and 20S core on the Western blots were quantitated with densitometric analysis and normalized by those of Coomassie staining (protein/Coomassie). Signals of the S5a and 20S core in the cytosol fraction (*Cyt*) of control cells (*pcDNA*) were adjusted to unit 1, and relative ratios of other signals are shown (**b**, *right*). **c** Reduced activity of 26S proteasome complexes by FOXRED2 in a native gel assay. Following transfection of HEK293T cells with pcDNA or pFOXRED2 for 36 h, cell lysates were prepared, separated by native-PAGE, and subjected to overlay assays using Suc-LLVY-AMC in the absence or presence of 0.01% SDS, as described in the Materials and methods. The regulatory particle (*RP*) and core particle (*CP*) are indicated. **d** Interference in proteasome assembly by FOXRED2 over-expression. Following transfection of HEK293T cells with pcDNA (*left*) or pFOXRED2 (*right*) for 36 h, cell extracts were subjected to gel filtration analysis using a Superose 6 column, as described in the Materials and methods. Fractions were then analyzed by Western blotting using the indicated antibodies. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) were used as molecular weight markers. *Asterisks* Non-specific signals. Tubulin served as an internal control. **e** Ectopic expression of FOXRED2 induced the accumulation of PaelR-GFP. SH-SY5Y cells were cotransfected with pcDNA or pFOXRED2 and either DR4-GFP or PaelR-GFP for 24 h, and Western blot analysis was then performed using anti-GFP, anti-FOXRED2, and anti-tubulin antibodies. *Black arrow* PaelR-GFP, *white arrow* DR4-GFP, *asterisk* non-specific signal. **f** Suppression of microsomal proteasome activity by Aβ42 treatment. Following treatment of SH-SY5Y cells with 5 μM Aβ42 for 48 h, cytosolic and microsomal fractions were separated and assayed for proteasome activity, as in **a**. *Mock* Untreated SH-SY5Y cells. **g** Rescue of Aβ42-induced inhibition of microsomal proteasomal activity by down-regulation of FOXRED2 expression. Following transfection with FOXRED2 siRNA for 24 h, SH-SY5Y cells were left untreated (Mock) or treated with 5 μM Aβ42 for 24 h. Cytosolic and microsomal fractions were separated and assayed for proteasome activity, as in **a**. *Mock* Untreated SH-SY5Y cells. *Bars*: Mean ± SD ($n > 3$)

Since proteasome activity was changed by proteasome disassembly in the microsomal fraction, we further assessed the degradation of the ERAD substrate Parkin-associated endothelin-receptor-like receptor (PealR), which is a G-protein-coupled orphan receptor residing on ER and degraded by Parkin via ERAD [25]. We used DR4 (death receptor 4) as a control because it is a non-ERAD substrate localized in the plasma membrane [26]. Compared to control, ectopic expression of FOXRED2 increased the accumulation of PaelR-GFP, but not DR4-GFP (Fig. 5e). Together, these results imply that FOXRED2 alters proteasome activity in the microsomal fraction, probably by affecting ERAD.

Because Aβ treatment inhibits proteasome activity, we examined the effect of Aβ on microsomal proteasome activity. Treatment of SH-SY5Y cells with Aβ inhibited 40% of proteasome activity in the microsomal fraction, but very little cytosolic proteasome activity (Fig. 5f). Notably, down-regulation of FOXRED2 expression using siRNA abolished the Aβ-induced inhibition of microsomal proteasome activity and did not affect proteasome activity in the cytosolic fraction (Fig. 5g). Equal amounts of proteins were used in the analysis (ESM Fig. 3B). These results suggest that FOXRED2 mediates Aβ-induced inhibition of proteasome activity in the microsomal fraction.

FOXRED2 mediates Aβ toxicity via ER stress response

We then examined the contribution of FOXRED2 to Aβ neurotoxicity. First, we observed that ectopic expression of FOXRED2 in SH-SY5Y cells induced significant amounts of cell death after 24 h of transfection (Fig. 6a, left). Also, FOXRED2ΔC, which was as potent as FOXRED2 in inhibiting proteasome activity, induced cell death on the same scale as FOXRED2. The FOXRED2(D_1) mutant was even more efficient than FOXRED2 in the induction of cell death, showing a close association between the proteasome-inhibitory activity of FOXRED2 and its pro-apoptotic activity (Fig. 6a). FOXRED2-induced cell death was inhibited by IDN6556 pan-caspase inhibitor (Fig. 6b, left). However, IDN6556 did not affect the accumulation of GFP-degron triggered by FOXRED2 over-expression (Fig. 6b, right), suggesting that FOXRED2-induced inhibition of proteasome activity is an earlier event than cell death. In addition, FOXRED2-mediated cell death in mouse HT22 cells accompanied proteolytic activation of caspase-12, a pro-apoptotic molecule in the ER stress response (Fig. 6c). FOXRED2 also increased the expression level of GRP78, an ER stress marker (Fig. 6c). Salubrinal, an inhibitor of ER stress-mediated cell death, was able to suppress FOXRED2-induced cell death (Fig. 6d, left), but did not affect the accumulation of GFP-degron (Fig. 6d, right). These results suggest that FOXRED2-induced cell death is mediated through an ER stress response.

Next, we attempted to answer the question of whether expressional regulation of FOXRED2 affects Aβ-induced cell death. While over-expression of FOXRED2 enhanced Aβ-induced cell death and induced caspase-3 activation in human SH-SY5Y cells, down-regulation of FOXRED2 expression by siRNA suppressed both Aβ-induced cell death and GRP78 expression (Fig. 7a, b). These observations suggest that FOXRED2 plays a role in Aβ-induced cell death by modulation of the ER stress response.

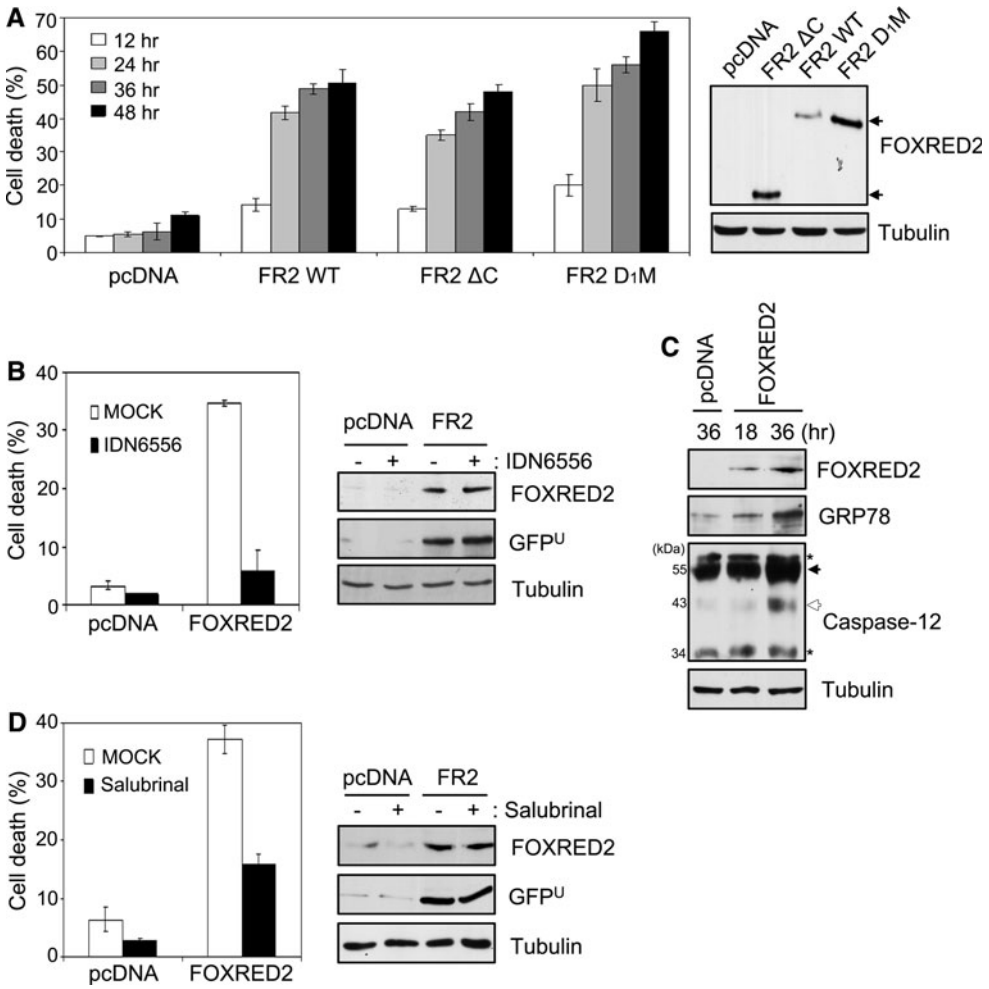


Fig. 6 FOXRED2 mediates ER stress-mediated neuronal cell death. **a** FOXRED2 over-expression induces neuronal cell death. Following transfection of SH-SY5Y cells with pcDNA, pFOXRED2 (*FR2 WT*), pFOXRED2ΔC(1–245) (*FR2 ΔC*), or pFOXRED2(D_1M) (*FR2D_1M*) for the indicated times, cell death was examined using ethidium homodimer staining (*left*). Cell lysates were analyzed by Western blotting using anti-FOXRED2 antibody (*right*). *Arrows* indicate the FOXRED2ΔC, FOXRED2, and FOXRED2(D_1) mutant. **b** Inhibition of FOXRED2-induced cell death by caspase inhibitor. Following cotransfection of SH-SY5Y cells with $pGFP^u$ and either pcDNA or pFOXRED2 in the absence or presence of 25 μM IDN6556 for 24 h, cell death was examined using ethidium homodimer staining (*left*). Western blot analysis was performed using anti-FOXRED2, anti-GFP, and anti-tubulin antibodies (*right*). *Mock* Untreated SH-SY5Y cells. **c** Increase in GRP78 expression and proteolytic cleavage of caspase-12 by FOXRED2 over-expression. HT22 cells were transfected with pcDNA or pFOXRED2 for the indicated times, and cell extracts were examined by Western blot analysis using anti-FOXRED2, anti-GRP78, anti-caspase-12, and anti-tubulin antibodies. *Asterisks* Non-specific signals, *black arrow* pro-form of caspase-12, *white arrow* processed form of caspase-12. **d** Inhibition of FOXRED2-induced cell death by Salubrinal. Following cotransfection of SH-SY5Y cells with $pGFP^u$ and either pcDNA or pFOXRED2 for 24 h in the absence or presence of 75 μM Salubrinal, cell death was examined as in **b** (*left*), and Western blot analysis was performed using anti-FOXRED2, anti-GFP, and anti-tubulin antibodies (*right*). *Mock* Untreated SH-SY5Y cells

Discussion

FOXRED2 is an unstable protein with two degradation (D) boxes and one KEN box, and it is rapidly degraded under culture conditions. Our observations that the introduction of mutations into the D or KEN box of FOXRED2 increases both protein stability and its ability to modulate proteasome activity indicate that the cellular level of the FOXRED2 protein is critical to the modulation of the proteasome. In particular, the FOXRED2 deletion mutant containing the N-terminus but lacking the D and KEN boxes, was more stable than FOXRED2 and efficient in the inhibition of proteasome activity. The N-terminus of FOXRED2 possesses a FAD-dependent oxidoreductase domain, which may catalyze the transfer of electrons from reductant (electron donor) to oxidant (electron acceptor) and induce oxidative stress using cofactors FAD or NADH [27]. However, we did not observe any significant alteration in protein carbonylation or in the intracellular level of reactive oxygen species (ROS) in cells over-expressing

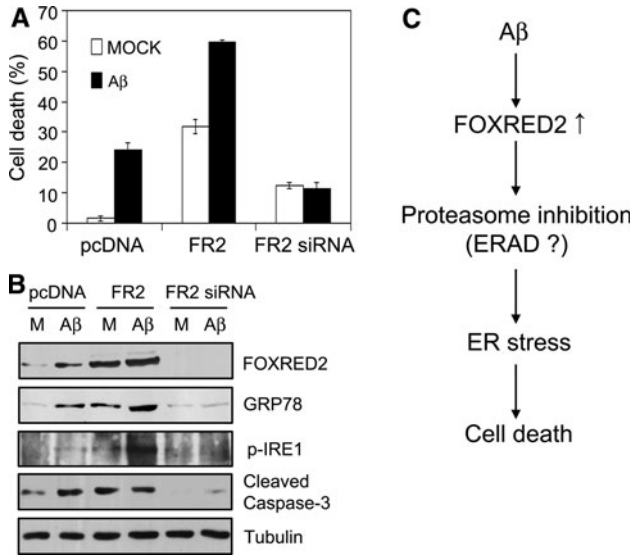


Fig. 7 Knockdown of FOXRED2 expression suppresses Aβ42-induced cell death and the endoplasmic reticulum (*ER*) stress response. **a**, **b** Suppression of Aβ42-induced cell death by FOXRED2 knockdown. **a** Following transfection with pcDNA, pFOXRED2, or FOXRED2 siRNA for 24 h, SH-SY5Y cells were left untreated (*Mock*) or treated with 10 μM Aβ42 for 24 h. Cell death was examined using ethidium homodimer staining. **b** Cell extracts were analyzed by Western blotting using anti-FOXRED2, anti-GRP78, anti-p-IRE1, anti-caspase-3, and anti-tubulin antibodies. *M* Mock. **c** Proposed model for the role of FOXRED2 in Aβ neurotoxicity. Treatment with Aβ increases the FOXRED2 level, inhibiting proteasome activity by modulating proteasome assembly and leading to neuronal cell death via ER stress. *ERAD* ER-associated degradation system

FOXRED2 (data not shown) [17]. Moreover, anti-oxidant treatment could not rescue the FOXRED2-induced inhibition of proteasome activity (data not shown); therefore, it is not likely that FOXRED2 inhibits proteasome activity through oxidative stress.

APC/C (anaphase-promoting complex/cyclosome) is an E3 ligase and recognizes substrates by a specific motif, such as the D box, KEN box, or A box with two cofactors, Cdc20 and Cdh1 [28, 29]. Control of the cell cycle and regulation of axonal growth and neuronal cell death are major functions of APC/C [30]. FOXRED2 has the D- and KEN-box motif; therefore, it is possible that FOXRED2 is a substrate of APC/C. The results of the fluorescence activated cell sorter (FACS) analysis, however, revealed that over-expression of FOXRED2 had little effect on cell cycle progression (data not shown). Nonetheless, the question of whether FOXRED2 is a substrate of APC/C during neuronal cell death remains to be elucidated.

Proteasomes are localized in the cytoplasm, the nucleus, and the cytoplasmic surface of the ER in mammalian cells [32]. Since FOXRED2 resides in the ER lumen, the occurrence of proteasome inhibition via the direct interaction between the proteasome and FOXRED2 is difficult. Findings from a recent report show that Aβ oligomers trap nascent and transiently misfolded proteins, leading to their ubiquitination by E3 ligases and the inhibition of proteasome activity [31]. However, we observed that FOXRED2 did not bind directly to Aβ in vitro (ESM Fig. 4) [37]; therefore, it is unlikely that Aβ oligomers directly link FOXRED2 to the proteasome. Regulation of proteasome activity by FOXRED2 may possibly occur through relocalization of the proteasome from the ER to the cytosol. Previous reports have shown that subcellular localization of the proteasome can be modulated. In yeast, the majority of the 26S proteasome is localized in the nucleus throughout the cell cycle, while there is a massive cytoplasmic relocalization of proteasome subunits in the quiescence stage [33–35]. In addition, the proteasome can be relocalized into intracytoplasmic inclusions or aggresomes under proteasome inhibitory conditions, such as neurodegenerative diseases [36].

Another possibility is that FOXRED2, which has been proposed to form a protein complex with ERAD components, such as SEL1L, OS-9, and ERdj5 [17], interferes with proteasome activity in the ER via the ERAD. Our results indicate that FOXRED2 may be a regulator of the ERAD and enhance ER stress-mediated cell death. This proposal is in line with our observation that PealR, an ERAD substrate, is accumulated and proteasome activity in microsome fraction is inhibited by FOXRED2 and that FOXRED2 plays a role in Aβ neurotoxicity as a pro-apoptotic molecule. Aβ neurotoxicity is known to be mediated consistently through an ER stress response [10]. Examination of the question of whether the N-terminus of FOXRED2 interacts with ERAD components is of particular interest.

We believe that increased levels of FOXRED2 affect proteasome assembly, leading to the inhibition of proteasome activity, which is in line with an earlier report of the dysregulation of proteasome-assembly chaperones inhibiting proteasome activity [2]. Our results are a starting point towards understanding the regulation of proteasome activity/assembly by FOXRED2 at the molecular level. It is very possible that FOXRED2 is a key molecule linking the modulation of UPS to neurodegeneration, such as amyloid neurotoxicity in AD.

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