



Title	Prefoldin prevents aggregation of alpha-synuclein
Author(s)	Takano, Mariko; Tashiro, Erika; Kitamura, Akira; Maita, Hiroshi; Iguchi-Ariga, Sanae M. M.; Kinjo, Masataka; Ariga, Hiroyoshi
Citation	Brain research, 1542, 186-194 <a href="https://doi.org/10.1016/j.brainres.2013.10.034">https://doi.org/10.1016/j.brainres.2013.10.034</a>
Issue Date	2014-01-13
Doc URL	<a href="http://hdl.handle.net/2115/54875">http://hdl.handle.net/2115/54875</a>
Type	article (author version)
File Information	WoS_64420_Ariga.pdf



[Instructions for use](#)

## **Prefoldin prevents aggregation of $\alpha$ -synuclein**

*Mariko Takano<sup>a,b</sup>, Erika Tashiro<sup>a,b</sup>, Akira Kitamura<sup>c</sup>, Hiroshi Maita<sup>a</sup>, Sanae M.M.*

*Iguchi-Ariga<sup>d</sup>, Masataka Kinjo<sup>c</sup>, and Hiroyoshi Ariga<sup>a,\*</sup>*

*<sup>a</sup>Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060-0812, Japan*

*<sup>b</sup>Graduate School of Life Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan*

*<sup>c</sup>Laboratory of Molecular Cell Dynamics, Faculty of Advanced Life Science, Hokkaido University, Kita-ku, Sapporo 001-0021, Japan*

*<sup>d</sup>Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan*

\*Corresponding author: Hiroyoshi Ariga, Faculty of Pharmaceutical Sciences, Hokkaido

University, Kita 12, Nishi 6, Kita-ku, Sapporo 060-0812, Japan. Tel.: +81 11 706 3745; Fax: 81

11 706 4988; E-mail: hiro@pharm.hokudai.ac.jp

## **ABSTRACT**

Protein aggregation is observed in various neurodegeneration diseases, including Parkinson's disease (PD). Alpha-synuclein, a causative gene product of familial PD, is a major component of large aggregates (inclusion bodies) in PD. Prefoldin, a molecular chaperone comprised of six subunits, PFD1~6, prevents misfolding of newly synthesized nascent polypeptides and also prevents aggregation of protein such as a pathogenic form of Huntingtin, a causative gene product of Huntington disease. In this study, we first found that aggregation of TagRFP-tagged wild-type  $\alpha$ -synuclein and its pathogenic mutants, but not that of GFP-tagged  $\alpha$ -synuclein, occurred in transfected Neuro-2a cells. The fluorescence of GFP is weakened under the condition of pH 4.5-5.0, and TagRFP is a stable red fluorescence protein under an acidic condition. Aggregated TagRFP-wild-type  $\alpha$ -synuclein and its pathogenic mutants in Neuro-2a cells were ubiquitinated and were colocalized with the prefoldin complex in the lysosome under this condition. Furthermore, knockdown of PFD2 and PFD5 disrupted prefoldin formation in  $\alpha$ -synuclein-expressing cells, resulting in accumulation of aggregates of wild-type and pathogenic  $\alpha$ -synuclein and in induction of cell death. The levels of aggregation and cell death in pathogenic  $\alpha$ -synuclein-transfected cells tended to be higher than those in wild-type  $\alpha$ -synuclein-transfected cells. These results suggest that prefoldin works as a protective factor in aggregated  $\alpha$ -synuclein-induced cell death.

*Key words:* prefoldin; chaperone;  $\alpha$ -synuclein; protein aggregation; cell death

## **1. Introduction**

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and

Huntington disease (HD) are caused by neuronal cell death, which is induced by various factors including genetic and environmental factors. In these diseases, proteins with improper conformation are oligomerized and aggregated into  $\beta$ -sheet-rich fibrils, thereby forming large aggregates (inclusion bodies) in affected neurons, suggesting that dysfunction of repair systems converting improper conformation to normal conformation of proteins leads to the onset of these neurodegenerative diseases (see recent reviews, references therein: Cohen, 2012; Takalo et al., 2013; Chhangani and Mishra, 2013 ). It is well known that molecular chaperones play a role in formation of properly folded proteins through binding to newly synthesized unfolded proteins or through preventing protein aggregations (Morimoto, 2008; Broadley and Hartl, 2009; Clare and Saibil, 2013; Chaari et al., 2013; Kim et al., 2013). It has been reported that Hsp70 prevents aggregation of amyloid  $\beta$ ,  $\alpha$ -synuclein and pathogenic Huntington in AD, PD and HD, respectively (Warrick et al., 1999; Kazemi-Esfarjani and Benzer, 2000; Muchowski et al., 2000; Evans et al., 2006; Wacker et al., 2004; Klucken et al., 2004) and that chaperonin TriC/CCT prevents aggregation of pathogenic Huntingtin (Behrends et al., 2006; Kitamura et al., 2006; Tam et al., 2006). These findings suggest that elucidation of the molecular mechanisms underlying prevention of protein aggregation by chaperones will lead to an understanding of one of the mechanisms of the onset of neurodegenerative diseases.

Prefoldin is a molecular chaperone that assists folding of newly synthesized polypeptide chains such as actin and tubulin (Vainberg et al., 1998; Geissler et al., 1998). Prefoldin binds to proteins that have been synthesized in ribosomes and transports them to chaperonin TriC/CCT in cooperation with Hsp70 and Hsp40 (Vainberg et al., 1998; Geissler et al., 1998; Siegers et al., 1999; Hartl and Hayer-Hartl, 2002). Prefoldin is comprised of six subunits including two  $\alpha$ -subunits (PFD3 and PFD5) and four  $\beta$ -subunits (PFD1, PFD2, PFD4 and PFD6). The

coiled-coiled regions present in both N- and C-terminal regions in prefoldin form a "jellyfish-like" structure and bind to substrates with their tentacle-like structures (Siegert et al., 2000; Martin-Benito et al., 2007). Furthermore, it has been reported that archaeal prefoldin stimulates formation of soluble amyloid  $\beta$  oligomers to inhibit their fibril formation *in vitro* (Sakono et al., 2008) and that human prefoldin inhibits A $\beta$  fibrillation to form nontoxic A $\beta$  aggregates (Sörgjerd et al., 2013). We have reported that prefoldin inhibited aggregate and inclusion formation of exogenously added pathogenic Huntingtin in cells (Tashiro et al., 2013) and that knockdown of prefoldin expression and mutation of PFD5 caused accumulation of ubiquitinated protein aggregates in cells and mice, resulting in reduced cell viability (Abe et al., 2013). These findings suggest that prefoldin plays a modifier role against the toxicity of misfolded proteins, including proteins that cause neurodegenerative diseases.

PD is a movement disorder that occurs due to dopaminergic cell death in the substantia nigra, resulting in a reduced level of dopamine in the striatum. Although familial PD cases account for 10% of total cases of PD, investigations of the functions of familial PD gene products have provided great insights into the molecular mechanisms of the onset of PD, and familial PD gene products are thought to also play roles in the pathogenesis of sporadic PD (see recent reviews, Sai et al., 2012; Hauser and Hastings, 2013; Ariga et al., 2013). The  $\alpha$ -synuclein gene is the familial PD gene *park1*, and three missense mutations coding for A30P, A53T and E46K  $\alpha$ -synuclein have been found (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004). Furthermore, duplication and triplication of the  $\alpha$ -synuclein gene are also known as *park4* (Zarranz et al., 2004; Chartier-Harlin et al., 2004; Ibáñez et al., 2004). Alpha-synuclein is a major component of the inclusion body termed Lewy body and is ubiquitinated in Lewy bodies of PD patients (Singleton et al., 2003; Liani et al., 2004). The monomer of  $\alpha$ -synuclein is

assumed to be misfolded by various stresses and to form fibrils and large inclusions (see recent review: Chaari et al., 2013). Although inclusion bodies containing  $\alpha$ -synuclein have long been thought to be a causative factor for PD, accumulating evidence suggests that formation of the inclusion is not correlated with neuronal cell death and that the inclusion body acts as a deposit of aggregated proteins to decrease the risk of neuronal cell death (Zarranz et al., 2004; Chaari et al., 2013).

In this study, we investigated the effect of prefoldin on aggregation of TagRFP-tagged  $\alpha$ -synuclein and cell death induced by aggregated  $\alpha$ -synuclein and we found that knockdown of prefoldin caused accumulation of ubiquitinated  $\alpha$ -synuclein aggregates in co-localization with the prefoldin complex and decreased cell viability, suggesting that prefoldin works as a protective factor in aggregated  $\alpha$ -synuclein-induced cell death.

## **2. Results**

### *2.1. Establishment of a monitoring system for detection of $\alpha$ -synuclein aggregation in cells.*

Since wild-type and mutant  $\alpha$ -synuclein are known to be prone to aggregation (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004; Chaari et al., 2013), GFP-tagged wild-type  $\alpha$ -synuclein and three mutants of  $\alpha$ -synuclein, A30P, A53T and E46K, were transfected into human SH-SY5Y and mouse Neuro-2a cells and proteins were visualized 48 h after transfection. No or little aggregation of  $\alpha$ -synuclein, however, was observed (data not shown). Since it has been reported that ubiquitinated  $\alpha$ -synuclein and its aggregates are degraded by the ubiquitin-proteasome system or by lysosome-mediated autophagy in which various proteinases degrade protein aggregates under pH 4.5-5.0 (see a recent review, references therein: Nixon et al., 2013) and since the fluorescence of GFP is weakened under pH conditions of 4.5-5.0,

$\alpha$ -synuclein was tagged with TagRFP (pKa 3.8), which is stable red fluorescence protein under an acidic condition (Merzlyak et al., 2007). Forty-eight h after transfection of TagRFP-wild-type and mutant  $\alpha$ -synuclein into Neuro-2a cells, the expression levels of transfected  $\alpha$ -synuclein in cell extracts were examined by Western blotting with anti-TagRFP and anti- $\alpha$ -synuclein antibodies and their fluorescence images were examined by using a confocal laser microscope (Figs. 1A and 1B, respectively). The results showed that the expression levels of wild-type  $\alpha$ -synuclein and its three mutants were similar (Fig. 1A). Since a band with a size identical to that of TagRFP was always obtained in Western blotting without any transfection, the band that appeared in a TagRFP-transfected lane is a non-specific band. Stained images and their quantified data showed that dot/aggregation of wild-type  $\alpha$ -synuclein and its three mutants, but not that of TagRFP alone, occurred in cells, and that the highest level of aggregation occurred in TagRFP-A53T  $\alpha$ -synuclein-transfected cells with similar levels of aggregation in wild-type, A30P and E46K  $\alpha$ -synuclein-transfected cells, being consistent with results of previous studies (Conway et al., 1998; Narhi et al., 1999; Winner et al., 2011) (Figs. 1B and 1C). Wild-type and A53T  $\alpha$ -synuclein were therefore used for further study.

## *2.2. Co-localization of aggregated $\alpha$ -synuclein with the prefoldin complex.*

Since aggregation of TagRFP- $\alpha$ -synuclein, but not that of GFP- $\alpha$ -synuclein, was observed in Neuro-2a cells, it was thought that aggregation of TagRFP- $\alpha$ -synuclein occurred in acidic organelles such as lysosomes. To examine this, Neuro-2a cells were transfected with wild-type and A53T  $\alpha$ -synuclein and stained with LysoTracker at 48 h after transfection, and cell images were obtained under a confocal laser microscope. As shown in merged figures of Fig. 2A, all of the dots/aggregates of TagRFP-  $\alpha$ -synuclein were co-localized with those of LysoTracker signals.

TagRFP-wild-type and TagRFP-A53T  $\alpha$ -synuclein-transfected Neuro-2a cells were then stained with an anti-ubiquitin antibody followed by an FITC-conjugated secondary antibody, and the results showed that aggregates of both wild-type and A53T  $\alpha$ -synuclein were colocalized with ubiquitin, indicating that TagRFP-wild-type and TagRFP-A53T  $\alpha$ -synuclein were ubiquitinated (Fig. 2B). We have established an anti-prefoldin antibody that recognizes the prefoldin complex but not its 6 subunits (Abe et al., 2013). By using this antibody, aggregates of both TagRFP-wild-type and TagRFP-A53T  $\alpha$ -synuclein were found to be co-localized with the prefoldin complex (Fig. 2C). These results indicate that ubiquitinated TagRFP- $\alpha$ -synuclein aggregates were co-localized with the prefoldin complex in the lysosome.

### *2.3. Stimulation of $\alpha$ -synuclein aggregation and cell death in prefoldin-knockdowned Neuro-2a cells.*

To examine the role of prefoldin in  $\alpha$ -synuclein aggregation, knockdown of the expression of prefoldin subunits in cells was carried out using siRNAs targeting prefoldin subunits. Since the prefoldin complex contains two  $\alpha$ -type subunits (PFD3 and PFD5) and four  $\beta$ -type subunits (PFD1, PFD2, PFD4 and PFD6), siRNAs targeting PFD5 ( $\alpha$ -type subunit) and PFD2 ( $\beta$ -type subunit) were chosen for knockdown. As previously reported (Miyazawa et al., 2011; Tashiro et al., 2013; Abe et al., 2013), knockdown of either PFD2 or PFD5 reduced the expression levels of other prefoldin subunits (Fig. 3A) and knockdown of PFD2 and PFD5 disrupted the prefoldin complex detected by a glycerol density gradient centrifugation (data not shown). Neuro-2a cells were transfected with PFD2 siRNA, PFD5 siRNA or non-specific siRNA as a control.

Twenty-four h after transfection, cells were transfected with expression vectors for TagRFP, TagRFP-wild-type  $\alpha$ -synuclein and TagRFP-A53T  $\alpha$ -synuclein. Forty-eight hr after transfection

of TagRFP proteins, proteins in cells were first examined by Western blotting with anti-TagRFP, anti- $\alpha$ -synuclein, anti-PFD2 and anti-PFD5 antibodies. As shown in Fig. 3A, expression levels of PFD2 and PFD5 were reduced, but those of TagRFP proteins were not changed after transfection of siRNAs into Neuro-2a cells. TagRFP signals in cells were then analyzed using a fluorescence microscope at 48 h after transfection of TagRFP proteins. As shown in Figs. 3B-3D, both PFD2 and PFD5 knockdown increased the number of dots/aggregates in TagRFP-wild-type  $\alpha$ -synuclein and TagRFP-A53T  $\alpha$ -synuclein-expressing cells but not in cells expressing TagRFP. The number of cells containing more than 2 aggregates/cell was greater in TagRFP-A53T  $\alpha$ -synuclein-expressing cells than in TagRFP-wild-type  $\alpha$ -synuclein-expressing cells.

Neuro-2a cells were transfected with PFD2 siRNA and PFD5 siRNA and then transfected with expression vectors for TagRFP, TagRFP-wild-type  $\alpha$ -synuclein and TagRFP-A53T  $\alpha$ -synuclein as described above. Forty-eight h after transfection of TagRFP proteins, cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. As shown in Fig. 4A, knockdown of both PFD2 and PFD5 significantly decreased the viability of TagRFP-wild-type  $\alpha$ -synuclein and TagRFP-A53T  $\alpha$ -synuclein-transfected cells but not that of TagRFP-transfected cells. LDH assays were also carried out to examine the effect of prefoldin-knockdown on viability of TagRFP- $\alpha$ -synuclein-transfected cells. As shown in Fig. 4B, prefoldin-knockdown using PFD2 siRNA and PFD5 siRNA specifically increased cell toxicity. These results suggest prefoldin-dependent protective reactions against toxicity of  $\alpha$ -synuclein aggregation in Neuro-2a cells.

### **3. Discussion**

In this study, we first found that TagRFP-wild-type  $\alpha$ -synuclein and pathogenic mutants of

$\alpha$ -synuclein were ubiquitinated and aggregated in co-localization with the prefoldin complex in the lysosome (Figs. 1 and 2). We then found that knockdown of prefoldin expression increased the level of aggregated  $\alpha$ -synuclein and reduced viability of transfected Neuro-2a cells (Figs. 3 and 4). These results suggest that prefoldin plays a role in preventing  $\alpha$ -synuclein-induced cell toxicity.

It is known that  $\alpha$ -synuclein is oligomerized and aggregated into  $\beta$ -sheet-rich fibrils in the cytoplasm, thereby forming large aggregates/inclusion termed Lewy bodies (Zarranz et al., 2004; Chaari et al., 2013). Alpha-synuclein in Lewy bodies was shown to be ubiquitinated (Singleton et al., 2003; Liani et al., 2004). It has been suggested that after disruption of the lysosome-mediated degradation system due to accumulation of some  $\alpha$ -synuclein aggregates in the lysosome, formation of  $\alpha$ -synuclein aggregates in the cytoplasm is further accelerated and then a Lewy body is formed (Zarranz et al., 2004). Since TagRFP-tagged  $\alpha$ -synuclein used in this study was aggregated and ubiquitinated (Figs. 1 and 2B), it is thought that aggregates of TagRFP- $\alpha$ -synuclein are aggregates before or during Lewy body formation. Aggregates of TagRFP-wild-type  $\alpha$ -synuclein and TagRFP-mutants of  $\alpha$ -synuclein were colocalized with the prefoldin complex in the lysosome, though most of the prefoldin was localized in the cytoplasm (Figs. 2A and 2C). This conclusion is consistent of the results in our previous report, in which the prefoldin complex was co-localized with ubiquitinated protein (Abe et al., 2013). Furthermore, the number of  $\alpha$ -synuclein aggregate-containing cells and ratio of cell death were increased in prefoldin-knockdown cells (Figs. 3 and 4). Small aggregates of  $\alpha$ -synuclein are thought to be more toxic than large aggregates (Chaari et al., 2013). Since prefoldin assists folding of newly synthesized unfolded proteins (Vainberg et al., 1998; Geissler et al., 1998) and since prefoldin reduced aggregation of  $\alpha$ -synuclein in this study, it is thought that prefoldin

inhibits early stages of the aggregation of  $\alpha$ -synuclein such as formation of small oligomers. Indeed, we have shown that prefoldin inhibits formation of pathogenic Huntingtin oligomers that are larger than a dimer and trimer (Tashiro et al., 2013). Alternatively, it is also possible that the effects of prefoldin knockdown on synuclein aggregation and toxicity are indirect, perhaps through the accumulation of unrelated misfolded nascent proteins that then compromise the overall folding capacity of the cell. Many of the  $\alpha$ -synuclein aggregates are known to be degraded by autolysosomes through macroautophagy (Yu et al., 2009; Spencer et al., 2009; Ebrahimi-Fakhari et al., 2011). Although prefoldin prevents aggregation of ubiquitinated proteins even in the absence of autophagy (Abe et al., 2013), the results in the present study also suggest that prefoldin assists autophagy-dependent degradation of  $\alpha$ -synuclein aggregates by delivering them into the lysosome.

## 4. Experimental procedures

### 4.1. Plasmids

Nucleotide sequences used for PCR primers are as follows:  $\alpha$ -synuclein5'-EcoRI:

5'-GGGAATTCATGGATGTATTCATGAAAGGA-3';  $\alpha$ -synuclein3'-SalI:

5'-GTCGACGGGGCTTCAGGTTTCGTA-3';  $\alpha$ -synuclein-A30Psense;

5'-GTGTGGCAGAAGCACAGGAAAGACAAAAG-3';  $\alpha$ -synuclein-A30Pantisense:

5'-CTTTTGTCTTTCCTGGTGCTTCTGCCACAC-3';  $\alpha$ -synuclein-A53Tsense:

5'-TGGTGCATGGTGTGACAACAGTGGCTGAGA-3';  $\alpha$ -synuclein-A53Tantisense;

5'-TCTCAGCCACTGTTGTCACACCATGCACCA-3';  $\alpha$ -synuclein-E46Ksense:

5'-GCTCCAAAACCAAGAAGGGAGTGGTGCATG-3' and  $\alpha$ -synuclein-E46Kantisense:

5'-CATGCACCACTCCCTTCTTGGTTTTGGAGC-3'. pTagRFP-N1 was obtained from

Evrogen (Moscow, Russia). For pTagRFP- $\alpha$ -synuclein, PCR was carried out using two primers ( $\alpha$ -synuclein5'-EcoRI and  $\alpha$ -synuclein3'-SalI) and pHM5- $\alpha$ -synuclein as a template. The resultant PCR product was then digested with *EcoRI* and *SalI* and inserted into *EcoRI/SalI* sites of pTagRFP-N1. For pTagRFP-A30P  $\alpha$ -synuclein, pTagRFP-A53T  $\alpha$ -synuclein and pTagRFP-E46K  $\alpha$ -synuclein, PCR was carried out using sense and antisense primers corresponding to their mutations described above and using pTagRFP- $\alpha$ -synuclein as a template. The resultant PCR product was then digested with *DpnI* and used for transformation into *E. coli*.

#### 4.2. Analysis of aggregate-containing cells

Neuroblastoma Neuro-2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cells were cultured on a 3.5-cm glass-bottom dish (Iwaki, Tokyo, Japan) coated with type IV collagen (Cellmatrix, Nitta Gelatin, Osaka, Japan) and transfected with 0.05  $\mu$ g of pTagRFP-N1, pTagRFP- $\alpha$ -synuclein and its mutant versions using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Forty-eight h after transfection, cells were fixed and stained with 100 nM LysoTracker Green DND-26 (Molecular Probes, Eugene, OR, USA) or with anti-multi-ubiquitin (1/50 dilution, DAKO, Glostrup, Denmark) and anti-prefoldin (1/50 dilution) antibodies followed by respective secondary antibodies. Cell images were visualized by using a confocal laser microscope (LSM510, Zeiss, Jena, Germany). An anti-prefoldin antibody was established by us as described previously (Abe et al., 2013). Aggregated dots in this study were defined as visible large spots of TagRFP signals in cells. About 300 cells were counted in each figure.

To examine the role of prefoldin in aggregation of  $\alpha$ -synuclein, knockdown of  $\alpha$ -synuclein expression was carried out using specific siRNAs. Nucleotide sequences of the upper and lower

strands for siRNA were as follows: 5'-GGAGCAUGUGCUUAUUGAUGU-3' and 5'-AUCAAUAAGCACAUGCUCAC-3' for PFD2, and 5'-GGAGCGGACUGUCAAGAATT-3' and 5'-UUCUUUGACAGUCCGCUCCTT-3' for PFD5. Neuro-2a cells were first transfected with PFD2 siRNA and PFD5 siRNA using Lipofectamine 2000 reagent. Allstars Negative control siRNA (Qiagen, Hilden, Germany) was used as a non-specific negative control (Qiagen). Twenty-four h after transfection, cells were transfected with pTagRFP-N1, pTagRFP- $\alpha$ -synuclein and its mutant versions as described above. Forty-eight h after transfection, cell images were obtained as described above.

#### *4.3. Analysis of cell death*

Neuro-2a cells in 96-well plates ( $3.3 \times 10^3$  cells/dish) were first transfected with 1.33  $\mu$ m PFD2 siRNA, PFD5 siRNA and control siRNA using Lipofectamine 2000 reagent. Twenty-four h after transfection, cells were transfected with  $1.67 \times 10^{-3}$   $\mu$ g of pTagRFP-N1, pTagRFP- $\alpha$ -synuclein and its mutant versions. Forty-eight h after transfection, cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

To assess cell viability, LDH assays were carried out using cell extracts from cultured Neuro-2a cells as described above. LDH assays were carried out using an LDL assay kit (CytoTox-ONE<sup>TM</sup> Homogeneous Membrane Integrity Assay kit, Promega) according to the supplier's protocol. Briefly, 300  $\mu$ l of cell extract was mixed with 300  $\mu$ l of CytoTox-ONE reagent and incubated at 2°C for 10 min, and its fluorescence intensity (Ex = 560 nm, Em = 590 nm) was measured using a fluorescent spectrophotometer (F-2500, Hitachi).

#### *4.4. Western blotting*

Neuro-2a cells in 6-well dishes ( $1.25 \times 10^5$  cells/well) were transfected with 0.05  $\mu$ g of pTagRFP-N1, pTagRFP- $\alpha$ -synuclein and its mutant versions using Lipofectamine 2000 reagent. Forty-eight h after transfection, proteins were extracted from cells after incubation of cells with a Hepes buffer containing 40 mM Hepes-NaOH (pH 7.4), 120 mM NaCl, 1 mM EDTA, 0.5% NP-40 and protease inhibitors and subjected to Western blot analysis with anti- $\alpha$ -synuclein (1/5000 dilution, BD, Franklin Lakes, NJ, USA), anti-TagRFP (1/5000 dilution, Evrogen), anti-PFD 2 (1/1000 dilution) and anti-PFD5 (1/1000 dilution, S-20, Santa Cruz, Santa Cruz, CA, USA) antibodies. Proteins were then reacted with an IRDye800- or Alexa Fluor 680-conjugated secondary antibody and visualized by using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE, USA). An anti-PFD2 antibody was established by us as described previously (Tashiro et al., 2013).

#### *4.5. Statistical analyses*

Data are expressed as means  $\pm$  S.D. or  $\pm$  S.E. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by the Tukey-Kramer test.

#### **Acknowledgment**

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and from New Energy and Industrial Technology Development Organization (NEDO) and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) in Japan.

## References

- Abe, A., Takahashi-Niki, K., Takekoshi, Y., Shimizu, T., Kitaura, H., Maita, H., Iguchi-Ariga, S.M.M, Ariga, H., 2013. Prefoldin plays a role as a clearance factor in preventing proteasome inhibitor-induced protein aggregation. *J. Biol. Chem.* 288, 27764-27776.
- Ariga, H., Takahashi-Niki, K., Kato, I., Maita, H., Niki, T., Iguchi-Ariga, S.M.M, 2013. Neuroprotective function of DJ-1 in Parkinson's disease. *Oxid. Med. Cell Longev.* 2013, 683920.
- Behrends, C., Langer, C.A., Boteva, R., Böttcher, U.M., Stemp, M.J., Schaffar, G., Rao, B.V., Giese, A., Kretschmar, H., Siegers, K., Hartl, F.U., 2006. Chaperonin TRiC promotes the assembly of polyQ expansion proteins into nontoxic oligomers. *Mol. Cell.* 23, 887-897.
- Broadley, S.A., Hartl, F.U., 2009. The role of molecular chaperones in human misfolding diseases. *FEBS Lett.* 583, 2647-2653.
- Chaari, A., Hoarau-Véchet, J., Ladjimi, M., 2013. Applying chaperones to protein-misfolding disorders: Molecular chaperones against  $\alpha$ -synuclein in Parkinson's disease. *Int. J. Biol. Macromol.* 60, 196-205.
- Chartier-Harlin, M.C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., Levecque, C., Larvor, L., Andrieux, J., Hulihan, M., Waucquier, N., Defebvre, L., Amouyel, P., Farrer, M., Destée A., 2004. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet.* 364, 1167-1169.
- Chhangani, D., Mishra, A., 2013. Protein quality control system in neurodegeneration: A healing company hard to beat but failure is fatal. *Mol. Neurobiol.* 48, 141-156.

- Clare, D.K., Saibil, H.R., 2013. ATP-driven molecular chaperone machines. *Biopolymers*. 99, 846-859.
- Cohen, E., 2012. Aging, protein aggregation, chaperones, and neurodegenerative disorders: mechanisms of coupling and therapeutic opportunities. *Rambam. Maimonides Med. J.* 3:e0021.
- Conway, K.A., Harper, J.D., Lansbury, P.T., 1998. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat. Med.* 4, 1318-1320.
- Ebrahimi-Fakhari, D., Cantuti-Castelvetri, I., Fan, Z., Rockenstein, E., Masliah, E., Hyman, B.T., McLean, P.J., Unni, V.K., 2011. Distinct roles in vivo for the ubiquitin-proteasome system and the autophagy-lysosomal pathway in the degradation of  $\alpha$ -synuclein. *J. Neurosci.* 31, 14508-14520.
- Evans, C.G., Wisén, S., Gestwicki, J.E., 2006. Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1-42) aggregation in vitro. *J. Biol. Chem.* 281, 33182-33191.
- Geissler, S., Siegers, K., Schiebel, E., 1998. A novel protein complex promoting formation of functional alpha- and gamma-tubulin. *EMBO J.* 17, 952-966.
- Hartl, F.U., Hayer-Hartl, M., 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*. 295, 1852-1858.
- Hauser, D.N., Hastings, T.G., 2013. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. *Neurobiol. Dis.* 51, 35-42.

- Ibáñez, P., Bonnet, A.M., Débarges, B., Lohmann, E., Tison, F., Pollak, P., Agid, Y., Dürr, A., Brice, A., 2004. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet*. 364, 1169-1171.
- Kazemi-Esfarjani, P., Benzer, S., 2000. Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science*. 287, 1837-1840.
- Kim, Y.E., Hipp, M.S., Bracher, A., Hayer-Hartl, M., Hartl, F.U., 2013. Molecular chaperone functions in protein folding and proteostasis. *Annu. Rev. Biochem.* 82, 323-355.
- Kitamura, A., Kubota, H., Pack, C.G., Matsumoto, G., Hirayama, S., Takahashi, Y., Kimura, H., Kinjo, M., Morimoto, R.I., Nagata, K., 2006. Cytosolic chaperonin prevents polyglutamine toxicity with altering the aggregation state. *Nat. Cell Biol.* 8, 1163-1170.
- Klucken, J., Shin, Y., Masliah, E., Hyman, B.T., McLean, P.J., 2004. Hsp70 reduces alpha-synuclein aggregation and toxicity. *J. Biol. Chem.* 279, 25497-25502.
- Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Eppelen, J.T., Schöls, L., Riess, O., 1998. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* 18, 106-108.
- Liani, E., Eyal, A., Avraham, E., Shemer, R., Szargel, R., Berg, D., Bornemann, A., Riess, O., Ross, C.A., Rott, R., Engelender, S., 2004. Ubiquitylation of synphilin-1 and alpha-synuclein by SIAH and its presence in cellular inclusions and Lewy bodies imply a role in Parkinson's disease. *Proc. Natl. Acad. Sci. USA.* 101, 5500-5505.

- Martín-Benito, J., Gómez-Reino, J., Stirling, P.C., Lundin, V.F., Gómez-Puertas, P., Boskovic, J., Chacón, P., Fernández, J.J., Berenguer, J., Leroux, M.R., Valpuesta, J.M., 2007. Divergent substrate-binding mechanisms reveal an evolutionary specialization of eukaryotic prefoldin compared to its archaeal counterpart. *Structure*. 15, 101-110.
- Merzlyak, E.M., Goedhart, J., Shcherbo, D., Bulina, M.E., Shcheglov, A.S., Fradkov, A.F., Gaintzeva, A., Lukyanov, K.A., Lukyanov, S., Gadella, T.W., Chudakov, D.M., 2007. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nat. Methods*. 4, 555-557.
- Miyazawa, M., Tashiro, E., Kitaura, H., Maita, H., Suto, H., Iguchi-Arigo, S.M.M, Arigo, H., 2011. Prefoldin subunits are protected from ubiquitin-proteasome system-mediated degradation by forming complex with other constituent subunits. *J. Biol. Chem.* 286, 19191-19203.
- Morimoto, R.I., 2008. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes Dev.* 22, 1427-3148.
- Muchowski, P.J., Schaffar, G., Sittler, A., Wanker, E.E., Hayer-Hartl, M.K., Hartl, F.U., 2000. Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proc. Natl. Acad. Sci. USA.* 97, 7841-7846.
- Narhi, L., Wood, S.J., Steavenson, S., Jiang, Y., Wu, G.M., Anafi, D., Kaufman, S.A., Martin, F., Sitney, K., Denis, P., Louis, J.C., Wypych, J., Biere, A.L., Citron, M., 1999. Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. *J. Biol. Chem.* 274, 9843-9846.

- Nixon, R.A., 2013. The role of autophagy in neurodegenerative disease. *Nat. Med.* 19, 983-997.
- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., Nussbaum, R.L., 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. 276, 2045-2047.
- Sai, Y., Zou, Z., Peng, K., Dong, Z., 2012. The Parkinson's disease-related genes act in mitochondrial homeostasis. *Neurosci. Biobehav. Rev.* 36, 2034-2043.
- Sakono, M., Zako, T., Ueda, H., Yohda, M., Maeda, M., 2008. Formation of highly toxic soluble amyloid beta oligomers by the molecular chaperone prefoldin. *FEBS J.* 275, 5982-5993.
- Siegers, K., Waldmann, T., Leroux, M.R., Grein, K., Shevchenko, A., Schiebel, E., Hartl, F.U., 1999. Compartmentation of protein folding in vivo: sequestration of non-native polypeptide by the chaperonin-GimC system. *EMBO J.* 18, 75-84.
- Siegert, R., Leroux, M.R., Scheufler, C., Hartl, F.U., Moarefi, I., 2000. Structure of the molecular chaperone prefoldin: unique interaction of multiple coiled coil tentacles with unfolded proteins. *Cell*. 103, 621-632.
- Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muentner, M., Baptista, M., Miller, D., Blancato, J., Hardy, J.,

- Gwinn-Hardy, K., 2003. alpha-Synuclein locus triplication causes Parkinson's disease. *Science*. 302, 841.
- Spencer, B., Potkar, R., Trejo, M., Rockenstein, E., Patrick, C., Gindi, R., Adame, A., Wyss-Coray, T., Masliah, E., 2009. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *J. Neurosci.* 29, 13578-13588.
- Sörgjerd, K.M., Zako, T., Sakono, M., Stirling, P.C., Leroux, M.R., Saito, T., Nilsson, P., Sekimoto, M., Saido, T.C., Maeda, M., 2013. Human prefoldin inhibits amyloid- $\beta$  (A $\beta$ ) fibrillation and contributes to formation of nontoxic A $\beta$  aggregates. *Biochemistry*. 52, 3532–3542.
- Takalo, M., Salminen, A., Soininen, H., Hiltunen, M., Haapasalo, A., 2013. Protein aggregation and degradation mechanisms in neurodegenerative diseases. *Am. J. Neurodegener. Dis.* 2, 1-14.
- Tam, S., Geller, R., Spiess, C., Frydman, J., 2006. The chaperonin TRiC controls polyglutamine aggregation and toxicity through subunit-specific interactions. *Nat. Cell Biol.* 8, 1155-1162.
- Tashiro, E., Zako, T., Muto, H., Itoo, Y., Sörgjerd, K., Terada, N., Abe, A., Miyazawa, M., Kitamura, A., Kitaura, H., Kubota, H., Maeda, M., Momoi, T., Iguchi-Ariga, S.M.M, Kinjo, M., Ariga, H., 2013. Prefoldin protects neuronal cells from polyglutamine toxicity by preventing aggregation formation. *J. Biol. Chem.* 288, 19958-19972.

- Vainberg, I.E., Lewis, S.A., Rommelaere, H., Ampe, C., Vandekerckhove, J., Klein, H.L., Cowan, N.J., 1998. Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. *Cell*. 93, 863-873.
- Wacker, J.L., Zareie, M.H., Fong, H., Sarikaya, M., Muchowski, P.J., 2004. Hsp70 and Hsp40 attenuate formation of spherical and annular polyglutamine oligomers by partitioning monomer. *Nat. Struct. Mol. Biol.* 11, 1215-1222.
- Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L., Bonini, N.M., 1999. Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat. Genet.* 23, 425-428.
- Winner, B., Jappelli, R., Maji, S.K., Desplats, P.A., Boyer, L., Aigner, S., Hetzer, C., Loher, T., Vilar, M., Campioni, S., Tzitzilonis, C., Soragni, A., Jessberger, S., Mira, H., Consiglio, A., Pham, E., Masliah, E., Gage, F.H., Riek, R., 2011. In vivo demonstration that alpha-synuclein oligomers are toxic. *Proc. Natl. Acad. Sci. USA.* 108, 4194-4199.
- Yu, W.H., Dorado, B., Figueroa, H.Y., Wang, L., Planel, E., Cookson, M.R., Clark, L.N., Duff, K.E., 2009. Metabolic activity determines efficacy of macroautophagic clearance of pathological oligomeric alpha-synuclein. *Am. J. Pathol.* 175, 736-747.
- Zarranz, J.J., Alegre, J., Gómez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Muñoz, D.G., de Yebenes, J.G., 2004. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann. Neurol.* 55, 164-173.

## Figure legends

**Fig. 1.** Aggregation of wild-type and mutant  $\alpha$ -synuclein in Neuro-2a cells.

A. Neuro-2a cells were transfected with pTagRFP-N1, pTagRFP- $\alpha$ -synuclein and its mutant versions. Forty-eight h after transfection, proteins were extracted from cells and analyzed by Western blotting with anti-TagRFP and anti- $\alpha$ -synuclein antibodies as described in Experimental procedures.

B. Neuro-2a cells were transfected with pTagRFP-N1, pTagRFP- $\alpha$ -synuclein and its mutant versions. Forty-eight h after transfection, cell images were obtained using a confocal microscope. Arrows indicate dots/aggregates of  $\alpha$ -synuclein. Aggregated dots in this study were defined as visible large spots of TagRFP signals in cells.

C. Aggregate-containing cells in Figure 1B were quantified. About 300 cells were counted in each figure. Ratios of aggregate-containing cells/total cells counted are shown as mean  $\pm$  S.D. of three experiments. Significance: \*\* $p < 0.01$ .

**Fig. 2.** Co-localization of  $\alpha$ -synuclein with lysosome and the prefoldin complex.

A-C. Neuro-2a cells were transfected with pTagRFP-wild-type  $\alpha$ -synuclein and pTagRFP-A53T  $\alpha$ -synuclein. Forty-eight h after transfection, cells were stained with LysoTracker Green DND-26 (A), an anti-ubiquitin antibody (B) and anti-prefoldin antibody (C), and cell images were visualized by using a confocal laser microscope.

**Fig. 3.** Stimulation of aggregation of  $\alpha$ -synuclein in prefoldin-knockdown cells.

A. Neuro-2a cells were transfected with PFD2 siRNA, PFD5 siRNA or non-specific siRNA (si-control). Twenty-four h after transfection, cells were transfected with pTagRFP,

pTagRFP-wild-type  $\alpha$ -synuclein and pTagRFP-A53T  $\alpha$ -synuclein. Proteins were extracted 48 h after transfection and analyzed by Western blotting with antibodies against PFD2, PFD5, TagRFP and  $\alpha$ -synuclein.

B. Neuro-2a cells were transfected with PFD2 siRNA, PFD5 siRNA or non-specific siRNA (si-control) and then with pTagRFP, pTagRFP-wild-type  $\alpha$ -synuclein and pTagRFP-A53T  $\alpha$ -synuclein as described in the legend for Fig. 3A. Cell images of TagRFP fluorescence were visualized by using a confocal laser microscope. Cells containing a dot or dots were marked.

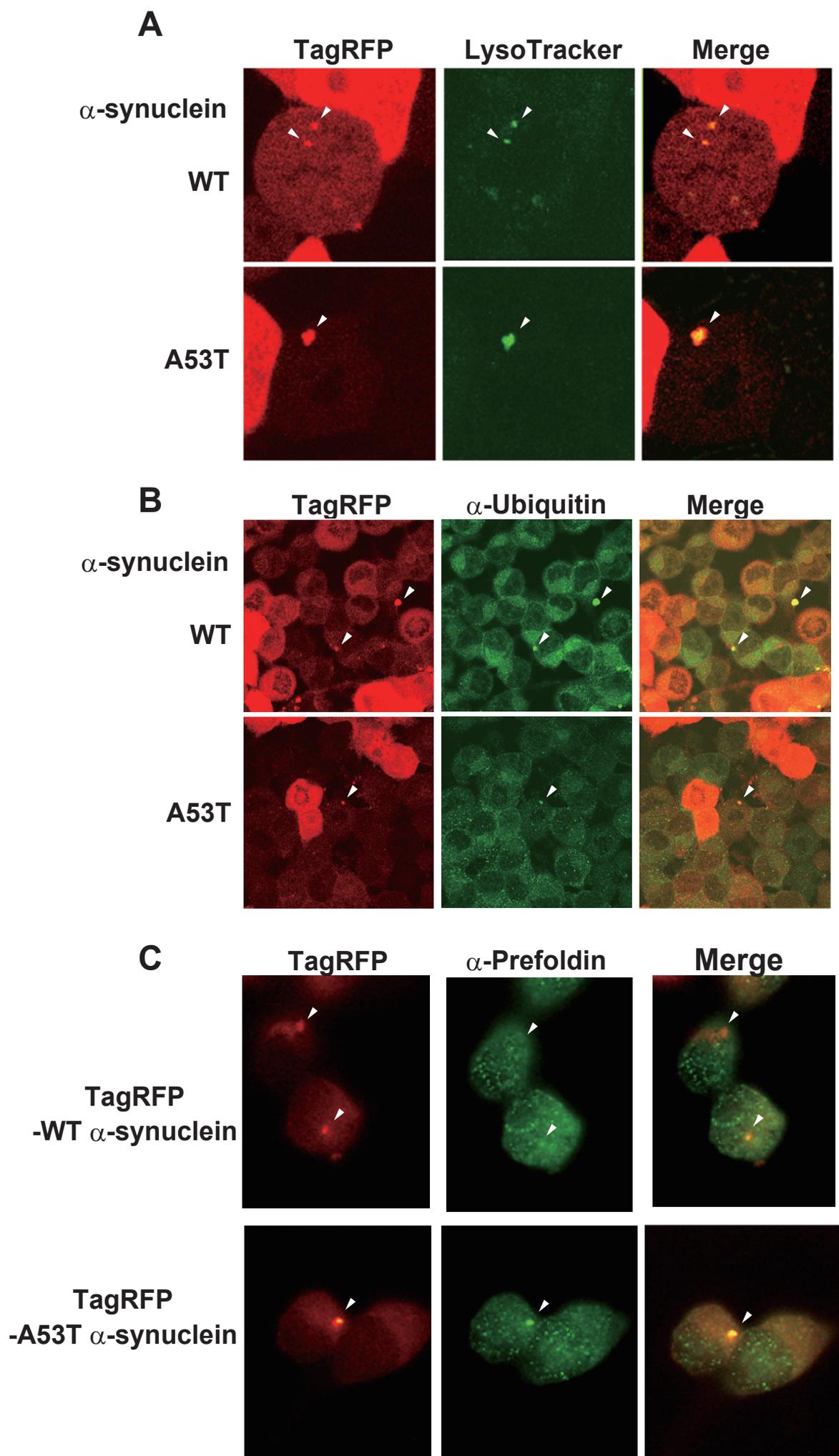
C. Aggregate-containing cells were counted in cell images of Fig. 3B, and data are shown as mean  $\pm$  S.D. of three experiments. Significance:  $**p < 0.01$ .

D. Numbers of cells containing one aggregate and more than two aggregates were counted in cell images of Fig. 3B, and data are shown as mean  $\pm$  S.D. of three experiments. Significance:  $*p < 0.05$ ,  $**p < 0.01$ .

**Fig. 4.** Stimulation of aggregated  $\alpha$ -synuclein-induced cell death in prefoldin-knockdown cells.

Neuro-2a cells were transfected with PFD2 siRNA, PFD5 siRNA or non-specific siRNA (si-control). Twenty-four h after transfection, cells were transfected with pTagRFP, pTagRFP-wild-type  $\alpha$ -synuclein and pTagRFP-A53T  $\alpha$ -synuclein. Forty-eight h after transfection, cell viability was assessed by MTT assays (A) and LDH assays (B). Data are shown as mean  $\pm$  S.E. of 15 experiments (A) and of 4 experiments (B). Significance:  $*p < 0.05$  and  $**p < 0.01$ .





**Fig. 2**

