Alzheimer's disease-causing proline substitutions lead to presenilin 1 aggregation and malfunction

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Abstract

Do different neurodegenerative maladies emanate from the failure of a mutual protein folding mechanism? We have addressed this question by comparing mutational patterns that are linked to the manifestation of distinct neurodegenerative disorders and identified similar neurodegeneration-linked proline substitutions in the prion protein and in presenilin 1 that underlie the development of a prion disorder and of familial Alzheimer's disease (fAD), respectively. These substitutions were found to prevent the endoplasmic reticulum (ER)-resident chaperone, cyclophilin B, from assisting presenilin 1 to fold properly, leading to its aggregation, deposition in the ER, reduction of γ -secretase activity, and impaired mitochondrial distribution and function. Similarly, reduced quantities of the processed, active presenilin 1 were observed in brains of cyclophilin B knockout mice. These discoveries imply that reduced cyclophilin activity contributes to the development of distinct neurodegenerative disorders, propose a novel mechanism for the development of certain fAD cases, and support the emerging theme that this disorder can stem from aberrant presenilin 1 function. This study also points at ER chaperones as targets for the development of counter-neurodegeneration therapies.

Keywords Alzheimer's disease; cyclophilin B; presenilin 1; proteostasis

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

To mature properly, newly synthesized polypeptides undergo complex folding and modification events that are assisted and supervised by specialized chaperones (Kim *et al*, 2013). Despite these chaperones' activities, not all nascent proteins attain their desired spatial conformations. Cellular quality control surveillance mechanisms identify terminally misfolded molecules and designate

them for degradation by autophagy (Arias & Cuervo, 2011) or by the ubiquitin–proteasome system (UPS) (Schrader *et al*, 2009). However, in some cases, misfolded polypeptides escape degradation and form insoluble aggregates that lead to the development of diseases that were collectively termed “proteinopathies” (Walker *et al*, 2006). Prion disorders (Aguzzi & Calella, 2009), frontotemporal dementia (FTD) (Roberson, 2012), Huntington's disease (HD), and Alzheimer's (AD) disease (Selkoe, 2003) are late-onset neurodegenerative disorders that emanate from toxic protein aggregation (proteotoxicity) and consist of a group of proteinopathies.

Most neurodegenerative disorders exhibit more than one pattern of emergence. While the majority of AD and prion disease cases onset sporadically during the patient's seventh decade of life or later, fewer cases manifest during the fifth or sixth decade as familial, mutation-linked maladies [certain prion diseases can also be infectious (Prusiner, 1998)]. This common temporal emergence pattern defines aging as the major risk factor for the development of neurodegeneration (Amaducci & Tesco, 1994) and suggests that aging-associated decline in the efficiency of protein quality control mechanisms underlies the etiology of these illnesses. This theme is strongly supported by the finding that the alteration of aging protects model worms (Morley *et al*, 2002; Cohen *et al*, 2006) and mice (Cohen *et al*, 2009; Freude *et al*, 2009) from proteotoxicity.

The detailed molecular mechanisms that lead to the development of AD, the most prevalent human neurodegenerative disorder, are largely obscure. However, according to the amyloid hypothesis, the highly aggregative family of A β peptides plays a crucial role in the development of the disease (Hardy & Higgins, 1992). A β peptides are released as a result of a dual proteolytic digestion of the type 1 transmembrane amyloid precursor protein (APP) by the β -secretase (BACE) and γ -secretase proteolytic complex which is composed of presenilin 1, presenilin 2 (PS1 and PS2, respectively), presenilin enhancer 2 (Pen-2), nicastrin, and the anterior pharynx defective 1 (APH-1) (Selkoe & Wolfe, 2007). Shortly after translation, PS1 undergoes a rapid auto-cleavage to generate the PS1 N-terminal domain (NTF) and C-terminal domain (CTF) (Thinakaran *et al*, 1996). Mutations in PS1, a multi-spanning transmembrane aspartic

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protease which possesses the proteolytic activity of the γ -secretase complex (De Strooper *et al*, 1998), are accountable for the majority of familial AD cases (Bertram & Tanzi, 2008). PS1 was shown to play crucial roles in key biological functions including autophagy (Lee *et al*, 2010), the mediation of correct interactions between the ER and mitochondria (Area-Gomez *et al*, 2009), as well as in the maintenance of calcium homeostasis (Bezprozvanny & Mattson, 2008).

Although rare, mutation-linked neurodegeneration cases provide invaluable hints that can help decipher the mechanisms that underlie the development of these maladies. Interestingly, many AD-causing mutations in the sequence of PS1 do not elevate total A β levels (Chavez-Gutierrez *et al*, 2012), suggesting that non-canonical mechanisms are accountable for the development of certain AD cases by attenuating PS1 function. Moreover, while most PS1 mutations cause familial AD (fAD), certain amino acid substitutions in the sequence of PS1 were reported to initiate FTD (Mendez & McMurtry, 2006).

Similarly, mutations in the sequence of the highly aggregative prion protein (PrP) are associated with the emergence of at least three familial neurodegenerative disorders: Creutzfeldt–Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann–Sträussler–Scheinker syndrome (GSS) (reviewed in Aguzzi & Polymenidou, 2004). The indications that one protein can be involved in the development of more than one neurodegenerative malady and the common temporal emergence pattern of different disorders have led us to hypothesize that a common mechanism may underlie the emergence of distinct neurodegenerative diseases.

To test this hypothesis, we adopted a comparative approach, searching for similar mutational patterns that are linked to the onset of different neurodegenerative diseases. This comparison was based on the assumption that the substitution of amino acids in a sequence that serves as a recognition site for a folding chaperone impedes the functional interaction between the chaperone and its client, preventing the mutated protein from folding properly. The misfolding and aggregation of the protein may initiate the etiological process that eventually causes neurodegeneration. Our search unveiled that similar proline substitutions in the sequences of PrP and of PS1 underlie the development of GSS and of fAD, respectively.

The amino acid proline is known to serve as an axis for the isomerization of polypeptides from *cis* to *trans* and, thus, to play key roles in protein folding. At least three groups of peptidyl prolyl *cis/trans* isomerases, chaperones that catalyze this conformational conversion, have been identified: cyclophilins, FK506-binding

proteins (FKBPs), and parvulins (Schiene-Fischer, 2014). Among these, the cyclophilins are most abundant within different cellular organelles. The drug cyclosporin-A (CsA) specifically and efficiently inhibits the activity of cyclophilins (Handschumacher *et al*, 1984). Here, we found that inhibiting the activity of the ER-resident chaperone cyclophilin B results in PS1 aggregation, deposition in an ER-derived quality control compartment (ERQC) and loss or reduction of its proteolytic activity as well as impairment of mitochondrial distribution and function. Reduced levels of the processed, active form of PS1 were also observed in the brains of mice that lack cyclophilin B, confirming that these findings are conserved in the mammalian brain. Since cyclophilin activity is also needed for the correct folding of PrP (Cohen & Taraboulos, 2003), our discoveries indicate that the failure of one folding mechanism can underlie the development of more than one neurodegenerative malady and support the emerging notion that in some cases AD emanates from the attenuation of PS1 activity.

Results

Similar mutational patterns underlie the development of familial Alzheimer's disease and of GSS

Speculating that analogous mutational patterns in distinct neurodegeneration-linked proteins can serve as hints for the identification of failed chaperone–client interactions (Fig 1A), we searched for common mutated motifs and found that both PrP and PS1 carry disease-linked proline substitutions in the motif PXXP, residing in basic regions (Fig 1B). While the substitution of either P102 (Hsiao *et al*, 1989) or P105 (Yamazaki *et al*, 1999) in the sequence of PrP underlies the development of GSS, the replacement of either proline P264 (Campion *et al*, 1995) or P267 (Hutton *et al*, 1996) in the motif PXXP of PS1 with other amino acids causes early-onset fAD. Previously, we found that the GSS-associated proline substitutions prevent folding chaperones, members of the cyclophilin family of peptidyl prolyl *cis/trans* isomerases (PPIase) from assisting PrP to fold properly (Cohen & Taraboulos, 2003). Analogously, the inhibition of cyclophilin activity by CsA leads to PrP misfolding, aggregation, and deposition in cellular sites that were termed “aggresomes” (Johnston *et al*, 1998) that serve as quality control compartments (Ben-Gedalya *et al*, 2011). The nearly identical disease-linked proline substitutions in the sequences of PrP and PS1 suggested that cyclophilins are also required for the correct folding of PS1.

Figure 1. The inhibition of cyclophilin activity leads to the aggregation of presenilin 1.

- A A conceptual model suggests that the abolishment of similar folding chaperone recognition sites in discrete neurodegeneration-linked proteins can lead to the development of distinct maladies by analogous mechanisms.
- B The prion protein (PrP) and presenilin 1 (PS1) share similar mutational patterns. The substitution of proline in residue 102 or 105 in the sequence of PrP causes GSS, while the replacement of proline in residue 264 or 267 leads to the development of familial AD.
- C The inhibition of chaperones of the cyclophilin family by the drug cyclosporin-A (CsA) results in the aggregation of PS1 in CHO cells that stably over-express wild-type PS1. This phenomenon is concentration-dependent.
- D The aggregation of the endogenous mouse PS1 was observed by Western blot (WB) analysis in CsA-treated MEFs, indicating that the over-expression of PS1 is not a prerequisite for its aggregation when cyclophilin activity is inhibited by CsA.
- E WB analysis indicates that CsA-induced PS1 aggregates cross-react with antibodies toward the N- and C-terminal fragments of the protein (lanes 5 and 11), implying that the full-length protein forms aggregates prior to its self-cleavage. In contrast, proteasome inhibition by MG132 does not induce the aggregation of PS1 (lanes 6 and 12).

Figure 1.

Aggregated wild-type PS1 accumulates in CsA-treated cells

To test whether cyclophilins assist the folding of PS1, we created Chinese hamster ovary cells (CHO) that stably over-express moderate amounts of the human wild-type PS1 (Appendix Fig S1A) (CHO-PS1 cells). CHO-PS1 cells were treated for 16 h with increasing CsA concentrations, and aggregated proteins were isolated from soluble forms of the protein by high-speed centrifugation. Using Western blot analysis (WB) and a PS1 antibody that reacts with the protein's CTF, we discovered that PS1 forms aggregates in cells that were treated with 60 $\mu\text{g}/\text{ml}$ or more CsA (Fig 1C). Thus, this concentration was used for cyclophilin inhibition in the experiments described below.

To examine whether this phenotype emanates from PS1 over-expression, we used wild-type mouse embryonic fibroblasts (MEFs) and tested whether endogenous PS1 forms aggregates in response to CsA treatment. The cells were treated either with ethanol [the vehicle of CsA (Ve)] or with 60 $\mu\text{g}/\text{ml}$ CsA, lysed, and subjected to high-speed centrifugation. Supernatants and pellets were analyzed by WB. Our results (Fig 1D) show that endogenous PS1 aggregates accumulate in pellets of CsA-treated cells, indicating that PS1 aggregation in cells in which cyclophilin activities were inhibited is not a result of over-expression.

To ascertain whether CsA induces the aggregation of PS1 in an early processing stage, presumably prior to its self-cleavage, or in a later maturation step, we used antibodies that react with the protein's NTF or CTF. We also assessed the possibility that aggregated PS1 accumulates due to proteasome overtaxing emanating from the misfolding of other cyclophilin substrates and not from the requirement of cyclophilins for PS1 maturation. CHO-PS1 cells were treated for 16 h with CsA or for 4 h with the proteasome inhibitor MG132 and subjected to high-speed sedimentation, and PS1 was probed in the supernatants and pellets by NTF and CTF antibodies. The absence of aggregated PS1 in the supernatants and pellets of MG132-treated cells indicated that proteasome malfunction (Appendix Fig S1B) is not sufficient for the accumulation of PS1 aggregates (Fig 1E and Appendix Fig S1C).

The observation that aggregated PS1 reacts with both PS1 NTF and CTF antibodies demonstrates that CsA induces the aggregation of the full-length wild-type PS1 prior to its self-cleavage, implying that PS1 forms aggregates in an early maturation stage within the ER.

Aggregated PS1 accumulates in the ER quality control compartment (ERQC) of CsA-treated cells

In order to determine where aggregated PS1 accumulates within CsA-treated cells, we used immunofluorescence. CHO-PS1 cells

were either treated for 16 h with CsA or for 5 h with MG132 or left untreated, and PS1 was labeled with NTF (green) and CTF (red) antibodies. While both PS1 antibodies indicated that the protein is distributed throughout untreated and MG132-treated cells (Fig 2A), PS1 accumulated in a juxta-nuclear ringlike shape in cells that were exposed to CsA (Figs 2A and EV1A, CsA arrows). Similar ringlike shapes were seen in untransfected, CsA-treated NIH 3T3 cells (Fig EV1B), indicating that this phenomenon is not cell line-specific, and further show that it does not emanate from PS1 over-expression. The observation that both PS1 antibodies label the ringlike deposition site suggests that this structure contains the full-length PS1 molecules, corroborating the theme that PS1 forms aggregates within the ER, prior to the self-cleavage event.

Using a battery of antibodies, we characterized the PS1-containing ringlike structure. First, we examined whether it is caged by collapsed vimentin filaments, a hallmark of aggresomes (Johnston *et al*, 1998), and could not identify a distinct caging (Fig EV1C). We also found that the ringlike structure does not contain ubiquitinated proteins (Fig EV1D) but overlaps with the ER membrane-integral chaperone calnexin (Brodsky & Skach, 2011) in CsA-treated cells (Fig 2B). This observation proposes that aggregated PS1 accumulates in the ER-derived quality control compartment (ERQC), a sub-organelle that was previously shown to contain misfolded ER-resident proteins (Kamhi-Nesher *et al*, 2001). To test this hypothesis, we transiently expressed in CHO-PS1 cells a fluorescently tagged chimera of the secreted form of the asialoglycoprotein receptor H2a (H2a-RFP), a well-established ERQC marker (Kamhi-Nesher *et al*, 2001). The cells were treated for 16 h with CsA, and PS1 was labeled using the PS1-NTF antibody. Our results revealed co-localization of PS1 and H2a-RFP [Fig 2C, no such H2a-RFP-containing structure was seen in untreated cells (Fig EV1E)].

To examine whether the PS1-containing ringlike shape is present at a pericentriolar localization, a known feature of the ERQC (Kondratyev *et al*, 2007), CHO-PS1 cells were treated with CsA as described above and PS1 and γ -tubulin (a marker of the centriole) were labeled by specific antibodies. Our results revealed that the PS1-containing ringlike structures (Fig 2D, green) are located around the centriole (red). Finally, we found that the PS1-containing structure is segregated from the non-ERQC-resident chaperone BiP (Kondratyev *et al*, 2007) (Fig 2E).

While the inhibition of autophagy by the drugs pepstatin A and E64 (Yu *et al*, 2010) resulted in elevated quantities of the endogenous PS1 in naive 3T3 cells, the protein did not accumulate in the ERQC in response to this treatment (Appendix Fig S2), suggesting that PS1 molecules that fail to fold properly within the ER are directed for proteasomal degradation.

Figure 2. Aggregated PS1 accumulates in the ER quality control compartment (ERQC) of CsA-treated cells.

- A Fluorescent immunocytochemistry using PS1 NTF and CTF antibodies performed on CHO cells over-expressing wild-type PS1 and treated with CsA (60 μM) uncovers PS1 accumulation in a juxta-nuclear, ringlike structure (arrows). This phenomenon was not observed in cells that were treated with 10 μM MG132 (for 5 h) or with the vehicle of CsA (EtOH). Scale bar, 5 μm .
- B–D Fluorescent immunocytochemistry using PS1 NTF and calnexin or γ -tubulin antibodies or the expression of fluorescent asialoglycoprotein receptor H2a (H2a-RFP) performed on CHO cells expressing wild-type PS1 and treated with CsA. The CsA-induced, PS1-containing ringlike structure overlaps with the ER-residing chaperone calnexin (B), co-localizes with the ERQC marker H2a-RFP (C), and organizes around the microtubule-organizing center ("Ce," arrows) (D). Scale bar, 5 μm .
- E PS1 signal does not overlap with BiP, a chaperone that is known not to reside within the ERQC.
- F Fluorescence microscopy unveiled that GFP-PS1 accumulates in ERQC of CHO cells over-expressing wild-type PS1 that were treated with siRNA toward the ER-resident cyclophilin B (arrows). No such phenomenon was seen in cells that were treated with siRNA toward cyclophilin A.

Figure 2.

The observation that PS1 accumulates within the ER predicts that cyclophilin B, an ER-resident member of this family, is the chaperone that is critically required for the correct folding of PS1. To scrutinize this hypothesis, we fused the green fluorescent protein (GFP) to PS1 and expressed this construct in CHO cells (CHO-GFP-PS1 cells). The cells were treated for 48 h with small interfering RNA (siRNA) toward either cyclophilin B or the cytosolic family member, cyclophilin A (48 h of treatment is sufficient to notably reduce the levels of cyclophilin A and B, Fig EV1F). Visualization by fluorescence microscopy indicated that GFP-PS1 accumulates in ERQC of cells that were treated with cyclophilin B-targeting siRNA (red) but not in cells that were transfected with siRNA toward cyclophilin A (Fig 2F). These results indicate that cyclophilin B is the chaperone that is functionally required for the correct folding of nascent PS1 molecules.

Collectively, our results clearly show that the inhibition of cyclophilin B by CsA results in the misfolding and aggregation of full-length PS1 and in its deposition in the ERQC.

P264L and P267S PS1 accumulate in ERQC upon proteasome inhibition

If cyclophilin B assists the maturation of PS1 by promoting *cis/trans* isomerization that is based on proline 264, 267 or both, it is expected that the fAD-linked substitution of these prolines will result in the accumulation of aggregated, mutated PS1 in the ERQC. To test this hypothesis, we created mutated human PS1 constructs that carry either one of these mutations: P264L, P267S, or both [double mutant (DM)]; and expressed them in CHO cells (CHO-PS1-P264L, CHO-PS1-P267S, and CHO-PS1-DM, respectively). First, we examined the effects of CsA on DM PS1 molecules expressed in these cells and found that the inhibition of cyclophilins induces their aggregation as tested by a high-speed sedimentation assay (Fig EV2A). Next, we examined the effect of CsA treatment on the cellular distribution of P264L, P267S, and the DM PS1 and found that the inhibition of cyclophilins leads to their accumulation in the ERQC (Fig EV2B). We also asked whether proteasome inhibition leads to the deposition of the mutated PS1 molecules in the ERQC. CHO cells expressing either the wild-type PS1 or one of the aforementioned mutants were treated for 5 h with either vehicle or 10 μ M MG132, to inhibit proteasomes (as demonstrated in Appendix Fig S1B), and the cellular distribution of PS1 was visualized. While proteasome inhibition led to the accumulation of wild-type PS1 in a reticular pattern throughout the cell but not in its deposition in the ERQC (Fig 3A), MG132 treatment directed P264L and P267S PS1 mutants to the ERQC (Fig 3B and C, arrows) in ~10% of the cells. Similarly, proteasome inhibition induced the aggregation (Fig EV2A) and accumulation of DM PS1 in the ERQC (Fig EV2C), but neither the inactive D257A PS1 (Wolfe *et al*, 1999) (Fig EV2D) nor the A246E fAD-associated PS1 mutant (Sherrington *et al*, 1995) accumulated in this structure (Fig EV2E). In addition, CsA does not induce the accumulation of the YFP-fused transmembrane dopamine transporter in the ERQC of CsA-treated cells (Fig EV2F), indicating that not all multi-transmembrane proteins are directed to the ERQC upon cyclophilin inhibition. Finally, ERQC formation could not be seen in CHO cells expressing the wild-type or mutated PS1 in resting conditions (vehicle treatment) as displayed by the H2a RFP reticular appearance (Appendix Fig S3). This result indicates that these mutants are not deposited in the ERQC when proteasomes are active.

To further test the conclusion that proline-substituted PS1 accumulates within the ER as a result of endoplasmic reticulum associated protein degradation (ERAD) impairment, we blocked the retro-translocation of proteins from the ER to the cytosol using the potent ERAD inhibitor Eeyarestatin I (Wang *et al*, 2008). Our observations showed that similar to MG132, this treatment induced the accumulation of P264L PS1 but not of wild-type PS1 in the ring-like structure, supporting the theme that misfolded, P264L PS1 molecules are retained within the ER and accumulate within the ERQC (Fig 3D).

These findings imply that PS1 molecules that harbor the fAD-linked proline substitutions are degraded by proteasomes. However, when proteasome activity is impaired, these molecules accumulate within the ERQC. The similar cell biological features seen in CsA-treated CHO-PS1 cells and in cells which express the mutated PS1 molecules suggest that P264 and P267 are critical for the correct folding of PS1 and that the abolishment of the cyclophilin recognition site plays a key role in the development of fAD in individuals who carry these mutations. The possible role of the ERQC in the etiology of AD has prompted us to further characterize the physical properties of PS1 species that reside in this structure.

ERQC-trapped PS1 molecules are immobile

At least two types of cellular deposition sites have been described: dynamic quality control compartments in which resident molecules exhibit high mobility, and structures that accumulate terminally aggregated, immobile proteins (Kaganovich *et al*, 2008). To investigate the rate of mobility of ERQC-resident PS1 molecules, we used CHO-GFP-PS1 cells which were treated with CsA and subjected to a fluorescence recovery after photobleaching (FRAP) assay. This technique is based on a high-power laser pulse which bleaches the fluorescent signal of tagged molecules in a limited area within the examined compartment, followed by a kinetic analysis of the signal recovery (Lippincott-Schwartz *et al*, 2003). High mobility rate enables rapid recovery of the fluorescent signal in the affected area while immobility results in slow recovery. While the fluorescent signal in vehicle-treated cells recovered quickly (from ~55% after bleach to ~93% at 34 s) (Appendix Fig S4A and B), almost no recovery was seen in the bleached area of PS1 ERQC 15 min after the bleach (from ~55% after bleach to ~65% at 34 s) (Fig 4A and C), indicating that ERQC-resident PS1 is terminally aggregated.

Utilizing the fluorescence loss in photobleaching (FLIP) technique (Lippincott-Schwartz *et al*, 2003), we measured the rate of molecular exchange between the ERQC and its surrounding. This method is based on continuous bleaching of a small area outside of the examined cellular structure by a laser beam. High rate of exchange results in a rapid decline in the deposit's fluorescence over time, while the outcome of low rate of exchange is a stable fluorescence level. The fluorescent signal seen in the PS1 ERQC exhibited only a marginal decline over time (Fig 4B and D), demonstrating a very low level of molecular exchange between this deposition site and its vicinity.

Together, the analyses of GFP-PS1 dynamics in live cells and the sedimentation experiments (Fig 1E) propose that highly aggregated PS1 is trapped within the ERQC upon cyclophilin B inhibition. These results raise the question of whether the aggregation and deposition of PS1 results in the attenuation of γ -secretase activity.

Figure 3. Proteasome inhibition directs mutated PS1 carrying either P264L or P267S substitution to the ERQC.

A–C Fluorescent immunocytochemistry using PS1 NTF antibody (green) and PS1 CTF (red in merge) shows that the inhibition of proteasome activity by 10 μ M MG132 (5 h) did not induce the accumulation of wild-type PS1 in the ERQC of CHO cells (A), but directed PS1 carrying the fAD-linked P264L (B) or P267S (C) mutation to this structure (arrows). These results imply that in unstressed cells, proteasomes degrade PS1 molecules that misfold due to failed interaction with cyclophilin B.

D Similar to MG132 treatment, the inhibition of ER-associated degradation (ERAD) by Eeyarestatin I (1 h, 10 μ M) directs P264L PS1 but not wild-type protein to the ERQC (arrows).

Figure 4. ERQC-resident PS1-GFP molecules are immobile.

- A** Fluorescence recovery after photobleaching (FRAP) experiment performed on CHO cells over-expressing GFP-PS1 shows slow recovery of the fluorescent signal in a bleached area within the ERQC. This result indicates a low rate of mobility of PS1-GFP molecules within the ERQC, implying that they are aggregated.
- B** Fluorescence loss in photobleaching (FLIP) experiment indicates low rate of molecular exchange between the cytosol and the ERQC in GFP-PS1-over-expressing CHO cells.
- C, D** Quantitative analysis of (C) 6 FRAP experiments as in (A), and (D) 6 FLIP experiments as in (B). Signal was normalized to the initial signal prior to bleaching. Error bars represent SEM of 6 independent experiments.

Reduced γ -secretase activity in cells expressing mutated P264L or P267S PS1

To address this question, we tested whether the fAD-associated proline substitutions affect γ -secretase maturation and activity.

Auto-cleavage of PS1 to generate the NTF and CTF fragments is an early maturation step of the γ -secretase complex (Xia, 2008). We stably expressed the human wild-type PS1 or either one of the fAD-linked proline substituted PS1, P264L or P267S or the double mutant DM P264L-P267S in mouse embryonic fibroblasts (MEFs) that were

derived from *psen 1* knockout mice (PS1-KO MEF, Fig EV3A), and thus lacking endogenous PS1 activity (Herreman *et al*, 1999, 2003). As a negative control, we expressed in PS1-KO MEF cells the human PS1 gene that harbors the artificial D257A mutation which abolishes the proteolytic activity of the γ -secretase complex (Wolfe *et al*, 1999). To examine the levels of the full-length uncleaved PS1 in relation to the NTF fragment, we used WB analysis (Fig 5A) and found that while the P264L mutation reduces the efficiency of endo-cleavage compared to the wild-type PS1 (lanes 2 and 3, respectively), the introduction of the P267S mutation results in the disappearance of PS1 (lane 4). Similarly to the D257A PS1 (lane 6), no auto-cleavage could be detected in cells that expressed the DM PS1 (lane 5) implying loss of γ -secretase activity.

To examine whether cellular degradation mechanisms are accountable for the disappearance of the P267L PS1 mutant, we used MEF cells, which lack both endogenous PS1 and PS2 (PS1/2-KO MEF) (Herreman *et al*, 1999, 2003), and transiently expressed either the wild-type PS1 gene or the P267L PS1 mutant. The cells were exposed to the proteasome inhibitor MG132 (20 μ M, 6 h) or to the combination of autophagy inhibitors E64 and pepstatin A (20 μ g/ml each, 2 h), and PS1 quantities in the cells were compared by WB using the NTF PS1 antibody. Our results (Fig 5B) clearly show that MG132 stabilized the full-length wild-type PS1. No significant stabilization of transiently expressed PS1 was observed when the cells were treated with the autophagy inhibitors. Interestingly, MG132 stabilized the NTF fragment of the P267S PS1 mutant but not the full-length protein, suggesting that this mutant undergoes self-cleavage prior to proteasomal degradation.

We further tested the effect of the fAD-linked proline substitutions on γ -secretase activity by stably expressing the human wild-type and mutated PS1 constructs described above, in PS1/2-KO MEF cells. The cells were transiently transfected with myc-tagged APP C99, and the level of γ -secretase activity was assessed by measuring

the amounts of its cleaved product, the APP intracellular domain (AICD) (Hecimovic *et al*, 2004). Using WB and a myc antibody, we detected a prominent band, corresponding to the AICD, in cells expressing the wild-type PS1 but a band of ~50% lower intensity in cells expressing P264L PS1. Similar to PS1/2-KO MEF cells (Fig EV3B), no γ -secretase activity could be detected in cells expressing P267S PS1, DM PS1, or the D257A inactive PS1 (Fig 5C and D). Similar results were obtained by transient transfection of both the APP C99-myc and PS1 constructs into the PS1/2-KO cells (Fig EV3C and D). A reciprocal experiment indicated that the inhibition of cyclophilin activity by CsA reduces γ -secretase activity in cells over-expressing wild-type PS1 (Fig EV3E).

As a complementary technique, we used an *in vitro* γ -secretase activity assay based on a C-terminal β -APP-fluorescent peptide. In this assay, the proteolysis of the internally quenched peptide at the A β 40-, A β 42-, and A β 43-generating cleavage sites results in enhanced fluorescence. A calibration experiment using purified membranes containing γ -secretase complex (Sato *et al*, 2007) showed a dose-dependent increase in fluorescence (Fig EV3F). A direct comparison of fluorescence levels generated by membrane fractions of wild-type cells versus either PS1- or PS2-deficient cells indicated a reduction in PS1 activity of 49 and 23%, respectively (Fig EV3G). Comparison of γ -secretase activity of the PS1 KO cells stably expressing the different human constructs showed that membrane extracts of cells that expressed the P264L PS1 displayed ~25% reduction in activity compared to extracts of cell expressing the wild-type PS1, while extracts of cells expressing the P267S mutant showed 33% reduced activity level (Fig 5E).

To further test γ -secretase activity in cells that express the proline-substituted PS1 mutants, we performed ELISAs and measured the amounts of A β in media collected from PS1/2-KO MEF cells in which mutated PS1 constructs together with myc-APP-C99 were expressed. While the amounts of A β _{1–40} were ~70%

Figure 5. P264L and P267S PS1 mutations reduce γ -secretase activity.

- A PS1 self-cleavage was examined by WB analysis of the myc-conjugated APP intracellular domain (AICD), using MEFs that were derived from a PS1/2 knockout mouse and stably infected with the indicated PS1 constructs, carried by the pBABE retroviral vector. APP C99-myc was transiently expressed in these cells. While wild-type PS1 exhibited complete self-cleavage (lane 2), P264L PS1 showed partial digestion (lane 3), and P267S (lane 4) disappeared. The DM PS1 and D257A mutant exhibited no activity (lanes 5 and 6).
- B PS1/2-KO MEFs were transiently transfected with plasmids carrying either the wild-type or P267S PS1 constructs. The cells were treated with either MG132 (20 μ M for 6 h) to inhibit proteasomes or with E64 + pepstatin A (20 μ g/ml each for 2 h) to inhibit autophagy. PS1 levels were analyzed by WB using the NTF PS1 antibody. Inhibition of proteasomes results in the accumulation of wild-type and mutated PS1 (lanes 2 and 5 respectively). No PS1 accumulation was observed when autophagy was inhibited (lanes 3 and 6) in MEF cells lacking endogenous PS1 and transiently over-expressing PS1. These results imply no role for this mechanism in the digestion of PS1.
- C, D Western blot analysis of myc-tagged APP (C) unveils that while the expression of wild-type PS1 in PS1/2 knockout MEF cells efficiently restores APP digestion (AICD-myc, lane 3, arrow), the expression of P264L PS1 in these cells (lane 4) exhibits reduced level of APP digestion. P267S (lane 5), DM (lane 6), or D257A PS1 expression in the cells results in no detectable γ -secretase activity (D). Error bars represent SEM of 4 independent experiments, $*P < 0.01$ (Student's *t*-test).
- E Membranes purified from MEFs of PS1 KO mice that stably express either P264L or P267S human PS1 exhibit reduced γ -secretase activity relative to wild-type PS1 as detected using the fluorogenic γ -secretase substrate NMA-Gly-Gly-Val-Ile-Ala-Thr-Val-Lys(DNP)-D-Arg-D-Arg-D-Arg-NH₂. Bars indicate relative levels of fluorescence $\pm$ SEM, $*P < 0.01$ (Student's *t*-test).
- F PS1/2-KO MEF cells were transiently transfected with the indicated PS1 constructs as well as with APP. The levels of A β _{1–40} in the cell media were measured using a peptide-specific ELISA kit. While ~70% reduction in the levels of both peptides was detected in media of cells that express the P264L PS1 mutant compared to the amounts detected in media of cells that express the wild-type PS1 construct, a 90% reduction was seen in the media of cells that express the P267S or the DM PS1 mutants. These A β amounts were similar to the background level (broken line) that was observed in media of cells that were transfected with the empty vector or with the inactive D257A PS1. Error bars represent SEM of three independent experiments, $*P < 0.01$ (Student's *t*-test).
- G The relative amounts of A β _{1–42} in the cell media were measured by a specific ELISA kit as in (F). While the A β _{1–42} signals that were observed in the media of cells that express the P267S or the DM PS1 mutants are indistinguishable from the background level (broken line), merely 33% reduction was detected in the media of P264L PS1-expressing cells compared to the level observed in media of cells that expressed wild-type PS1 (this difference was not significant). Error bars represent SEM of three independent experiments, $*P < 0.01$ (Student's *t*-test).
- H The expression of P264L PS1 elevates the relative ratio of A β _{1–42}/A β _{1–40} as measured by ELISA. This observation shows that the P264L mutation affects the AD-related A β form ratio differently than the P267S mutation.

Figure 5.

lower in media of cells that expressed the P264L PS1 mutant compared to WT, the replacement of P267 or of both prolines (DM mutant) reduced the amounts of this peptide by nearly 90% (Fig 5F). These levels were similar to the background level (broken line) seen in media of cells that expressed the empty plasmid or the inactive D257A PS1 mutant. These results were consistent with the observations that P264L-mutated PS1 exhibits only residual γ -secretase activity and P267S mutation leads to nearly complete loss of function (Fig 5A–E).

We also used an ELISA kit to measure the relative amounts of $A\beta_{1-42}$ in media of PS1/2-KO MEF cells as described above and found that the reduction in the amounts of this aggregative peptide in the media of cells that express the P264L mutant was merely 33% and not significantly different from the levels detected in the media of cells that were transfected with wild-type PS1 (Fig 5G). The levels of $A\beta_{1-42}$ in media of cells that expressed the P267S or the double-mutated PS1 (DM) were similar to background level and significantly lower than those detected in media of wild-type PS1-expressing cells.

Besides modulating the proteolytic activity of the γ -secretase complex, AD-causing mutations in the sequence of PS1 have been shown to change the ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$, a feature which is believed to be associated with the development of the disease (Chavez-Gutierrez *et al*, 2012). A comparison of the $A\beta_{1-40}/A\beta_{1-42}$ ratio (as measured by the ELISAs) revealed that this AD-associated parameter is increased in PS1/2-KO MEFs that express the P264L PS1 mutant but not in those which express either the P267S, DM, or D257A PS1 mutants (Fig 5H).

Collectively, our experiments revealed that PS1 carrying the fAD-linked proline substitution P264L exhibits notable reduction in γ -secretase activity and increases the ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$, while the P267S mutation appears to cause a near-complete loss of PS1 function.

PXXP mutations in PS1 impair mitochondrial distribution and function

The involvement of PS1 in the formation of mitochondria-associated ER membranes (MAMs) (Area-Gomez *et al*, 2009) and the observations that PS1 accumulates in the ER (Fig 2) and exhibits attenuated function (Fig 5) have led us to ask whether mitochondrial distribution and function are impaired in cells that possess PS1-containing ERQC. We simultaneously followed the localization of GFP-PS1 and mitochondria in living CsA-treated CHO-GFP-PS1 cells and observed concurrent accumulation of GFP-PS1 in the ERQC (Fig 6A, green channel) and clustering of the mitochondria around this structure (red channel and Movies EV1 and EV2). Since presenilin 2 (PS2) is also present and functions in MAMs (Area-Gomez *et al*, 2012) and contains a PXXP motif (at positions 270–273), we asked whether this protein also accumulates in the ERQC upon CsA treatment. CHO cells expressing GFP-tagged PS2 were exposed to CsA for 16 h, fixed, and visualized. Similar to PS1, PS2 accumulates in the ERQC of CsA-treated CHO and NIH3T3 cells (Fig EV4A and B, respectively). To test whether PS2-containing ERQC and mitochondria also co-localize, we recorded the localization of GFP-PS2 (Fig 6B, green) and mitochondria (Fig 6B, red channel, and Movies EV3 and EV4) as described above and found that they redistribute to create nearly identical patterns within the cell.

To further examine whether the accumulation of PS1 in the ERQC leads to impaired mitochondrial distribution and to directly test whether the fAD-linked proline substitutions in PS1 are associated with this phenomenon, we treated CHO-PS1 and CHO-PS1-DM cells for 5 h with 10 μ M MG132 and examined them by transmission electron microscopy (TEM). Our results unveiled that while the mitochondria of untreated (Fig 6C) and MG132-treated (Fig 6E) CHO-PS1 cells were evenly distributed, the mitochondria of CHO-PS1-DM cells accumulated in a juxta-nuclear localization upon proteasome inhibition (Fig 6D).

These observations imply that the accumulation of mutated PS1 in the ERQC leads to impaired mitochondrial distribution due to PS1-induced change of MAMs, which may be related to a phenomenon seen previously in cells derived from patients with AD (Area-Gomez *et al*, 2012). Thus, we next tested whether P264L-mutated PS1 also affects mitochondrial function. PS1-KO MEF cells were stably infected with retroviruses carrying wild-type human PS1, or the P264L PS1 mutant or the double mutant (DM). These cells were stained by MitoTracker Green to label total mitochondria (Fig 7A, green) and tetramethylrhodamine methyl ester perchlorate (TMRM) (red) to stain active mitochondria (Petronilli *et al*, 2001). The cells were visualized by confocal microscopy, and the signals were quantified (Fig 7B). Our results show that the lack of PS1 resulted in lower rate of mitochondrial activity as judged by low TMRM signal. While the expression of wild-type human PS1 restored mitochondrial function and fragmentation to levels nearly double than seen in MEFs of wild-type mouse, no change in TMRM signal could be detected in cells expressing the P264L or the DM PS1.

As an additional approach to assess whether the expression of mutated PS1 impedes mitochondria activity, we measured ATP production in PS1-KO MEFs stably expressing the wild-type PS1 or either one of the mutated forms of the protein, P264L, P267S or DM, using the ATPlite luminescence assay. We found (Fig 7C) that while the restoration of wild-type PS1 expression elevated the rates of ATP compared to cells infected with the empty viral vector, the expression of the mutated PS1 proteins had remarkably lower effects on the levels of ATP. Together, the reduced TMRM signal and lower ATP levels indicate that mitochondria are not only aberrantly distributed but also malfunction in cells expressing PS1 that carries the AD-associated proline substitutions.

Reduced quantities and elevated PS1 aggregation in brains of cyclophilin B knockout mice

To evaluate the relevance of our findings to the mechanism that underlies the development of fAD in individuals who carry the 264 or 267 proline substitutions (and perhaps in some sporadic cases), we sought to test whether cyclophilin B is required for the correct folding of PS1 in the mammalian brain. Thus, we examined the levels and distribution of the endogenous PS1 in brain of mice that lack cyclophilin B (CyPB KO mice) (Cabral *et al*, 2014). Brains of four CyPB KO mice and of four matched genetic background wild-type mice were homogenized and cleared by low-speed centrifugation, and soluble proteins were separated from aggregated proteins by high-speed centrifugation. The levels of PS1 in both the soluble and insoluble fractions were compared using WB analysis and the CTF PS1 antibody. Our results clearly show lower levels of processed PS1 in the soluble fractions of brains that were obtained

Figure 6. The accumulation of aggregated PS1 in the ERQC impairs mitochondrial distribution.

A, B Live CHO cells expressing either GFP-PS1 (A) or GFP-PS2 (B) were treated for 16 h with CsA and visualized overnight by a confocal microscope. Our results indicate that both GFP-tagged presenilins 1 and 2 were deposited in the ERQC (arrows) following the inhibition of cyclophilins by CsA. This phenomenon was accompanied by the clustering of mitochondria (tagged by mito-DsRed expression or stained by MitoTracker, arrows) around this structure (scale bars, 5 μ m).

C–E Transmission electron microscopy (TEM) shows an even distribution of mitochondria in an untreated (vehicle) CHO cell over-expressing the wild-type PS1 (C). In contrast, MG132 treatment (10 μ M, 5 h) causes mitochondria clustering in cells over-expressing the DM PS1 (D), but not in wild-type PS1-expressing cells (E).

Figure 7. The inhibition of cyclophilins impairs mitochondrial function.

A, B PS1-KO MEFs were stably infected with an empty virus or with a virus carrying either human wild-type, P264L, or DM PS1. Total mitochondria were stained with MitoTracker (green), and active mitochondria were labeled using tetramethylrhodamine methyl ester perchlorate (TMRM). The cells were visualized by confocal microscopy (A), and the signals were quantified and normalized to the wild-type mouse cells expressing only the endogenous mouse PS1. Error bars represent SEM of three independent experiments, * $P < 0.01$ (Student's *t*-test) (B). While the expression of wild-type PS1 restored mitochondria activity to higher levels than those of MEFs of wild-type mice (left panels), the expression of P264L or DM PS1 did not restore mitochondria activity levels.

C A luminescence-based ATP assay performed on PS1-KO MEFs expressing the indicated PS1 constructs displays lower ATP levels in cells expressing the proline-substituted PS1 compared to their wild-type-expressing counterparts. This result shows that the substitution of prolines of the PXXP motif in PS1 impairs mitochondrial activity. Error bars represent SEM of four independent experiments.

from CyPB KO mice compared to the amounts detected in brains of control WT animals (Fig 8A and C). In contrast, no reduction in PS1 levels was observed in the insoluble fractions (pellets) (Fig 8B and D).

Using immunohistochemistry (IHC) and the CTF PS1 antibody, we further compared the PS1 levels in brains of CyPB KO animals and of their wild-type counterparts. Since PS1 is highly expressed in the hippocampus (Quarteronnet *et al*, 1996), we focused on this brain structure and visualized the dentate gyrus (DG). The levels of PS1 in the DG of CyPB KO animals were remarkably lower than those seen in DG of wild-type animals (Figs 8E and EV5A). A similar phenomenon of reduced PS1 levels was detected when PS1 levels were compared in the cortices of CyPB KO and wild-type mice (Fig EV5B).

To examine whether the absence of cyclophilin B affects γ -secretase activity, we purified membranes of wild-type and CyPB KO mouse brains and utilized the C-terminal β -APP-fluorescent peptide-based assay as described above. Our results (Fig 8F) indicated that the activity of the endogenous γ -secretase is significantly reduced ($P < 0.02$) in brains of CyPB KO animals compared to the activity levels seen in their WT counterparts. The observed reduction of ~20% in fluorescence level was analogous to that seen in cells that express the P264L or P267S PS1 (Fig 5E).

Together, these results indicate that cyclophilin B activity is crucially required for the proper maturation of PS1 and activity of the γ -secretase complex in the mouse brain. They also suggest that a misfolded PS1 subpopulation escapes degradation and forms aggregates in the brain.

P264L PS1 forms aggregates in the hippocampus of mice

We also asked whether the fAD-linked proline 264 substitution affects PS1 distribution in the mouse brain. To address this question, we created GFP-labeled lentiviral vectors that drive the expression of either the human wild-type or P264L PS1 and injected them into the hippocampi of young naïve mice (strain BALB/c). The animals' brains were harvested 5 weeks after injection and human PS1 was labeled using the NTF antibody (Figs 8G and EV5C, red), nuclei were stained with Hoechst (blue), and GFP was enhanced by an antibody (green). While human wild-type PS1 was diffused throughout the cell, the P264L-mutated protein accumulated in foci (Figs 8G and EV5C, insets (arrows)), suggesting that this mutation induces the aggregation of PS1 within the brain. To compare the number and average area of PS1 deposits in brains of mice injected

with the mutated or wild-type PS1-expressing virus, we used image processing software (ImageJ). Our results indicated that mice injected with the virus that drives the expression of P264L PS1 had much more PS1 foci than their counterparts that expressed the wild-type PS1 (average of 34.8 and 8.8 foci/slice, respectively). Furthermore, the PS1 foci observed in P264L PS1-expressing mice were much smaller in size (Figs 8H and EV5D). These results are consistent with the observation that PS1 accumulates in foci in the brain of humans that carry the P264L, fAD-linked mutation (Martikainen *et al*, 2010).

Discussion

The common temporal emergence patterns of different neurodegenerative maladies (Amaducci & Tesco, 1994) and the involvement of certain aggregative proteins in the development of more than one disorder have led us to speculate that in some cases, one mechanism underlies the manifestation of distinct neurodegenerative diseases. Here, we show that similar to its key role in the correct folding of PrP (Cohen & Taraboulos, 2003; Ben-Gedalya *et al*, 2011), cyclophilin B activity is critically required for the correct folding and processing (Fig 9) of PS1. The inhibition of cyclophilin activity results in PS1 misfolding (III), aggregation (IV), and deposition in the ERQC (V). Similarly, the substitution of proline 264 or 267 abolishes a cyclophilin recognition site which is vital for the proper maturation of PS1 (VI) leading to its digestion by the proteasome or aggregation (VII). Interestingly, our results indicate that while P264L PS1 mutant is preferably deposited in the ERQC, molecules that carry the P267S substitution are highly prone to proteasomal degradation. The attenuation of PS1 proteolytic activity impairs mitochondrial distribution and function, plausibly impedes additional PS1 functions, and initiates the pathological process that underlies the development of fAD (VIII).

Our discoveries point at the comparison of mutational patterns as a valid approach for investigating mechanisms that trigger the manifestation of neurodegeneration, support the concept that distinct disorders can emanate from common mechanisms, and strengthen the emerging idea that in some cases, the attenuation of PS1 activity is accountable for the emergence of AD (Shen & Kelleher, 2007; Xia *et al*, 2015).

How misfold polypeptides are sorted to be deposited in different cellular sites is largely unknown. While PrP accumulates in cytosolic aggresomes following CsA treatment (Cohen & Taraboulos,

Figure 8. Cyclophilin B is required for the maturation and integrity of PS1 in the mouse brain.

- A–D Brains of cyclophilin B knockout (KO) mice and of their wild-type siblings were homogenized and subjected to high-speed sedimentation assay. WB analysis using a PS1 CTF antibody revealed reduced levels of soluble PS1 in the supernatants of cyclophilin B KO animals compared to supernatants of control animals (A, C). In contrast, PS1 quantities in the pellets of wild-type and cyclophilin B KO mouse brains were similar (B, D). Error bars represent SEM of 4 mice for each genotype, * $P < 0.013$ (Student's *t*-test).
- E Similar results were obtained by immunohistochemistry (IHC) and visualization of PS1 in the paraffin-embedded hippocampi of wild-type and cyclophilin B KO animals using the CTF PS1 antibody.
- F A C-terminal β -APP-fluorescence-based activity assay shows reduced γ -secretase activity in membranes of brains of cyclophilin B KO mice compared to membranes isolated from brains of wild-type animals. Error bars represent SEM of three mice for each genotype, * $P < 0.02$ (Student's *t*-test).
- G Wild-type or P264L human PS1 was expressed in the hippocampi of BALB/c mice by GFP-labeled lentiviral vectors (green channel). The mice were sacrificed 5 weeks after the injection of the viruses, and brain slices were visualized by confocal microscopy. While wild-type human PS1 exhibits diffuse cellular pattern (upper panels, red), the human P264L mutant accumulates in foci throughout the dentate gyrus (DG) (lower panels, arrows). Scale bar, 20 μ m.
- H Image analysis indicated that PS1 foci in brains of mice that were injected with P264L PS1 virus occupy smaller areas than those seen in brains of their counterparts that express wild-type PS1 (a total of 5 brain slices per genotype were analyzed; error bars represent SEM).

Figure 8.

Figure 9. A model for the development of fAD in individuals that carry the P264L or P267L/S mutations.

To attain functionality, nascent PS1 molecules require the assistance of cyclophilin B (I) in order to fold properly and undergo additional modifications (II). Due to aging-associated decline in the efficiency of cyclophilin activity or as a result of inhibition by CsA, PS1 molecules fail to fold properly (III), aggregate (IV), and are deposited at the ERQC (V). Similarly, cyclophilins cannot support the folding of mutated PS1 molecules which carry the P264L or P267L/S substitutions (VI). These molecules are designated to proteasomal degradation (VII) or deposited at the ERQC when proteasomes are overtaxed (V). The aggregation and deposition in the ERQC lead to attenuated PS1 function and to the development of Alzheimer's disease (VIII).

2003), PS1 is deposited in the ERQC as a result of the same treatment. Moreover, while proteasome inhibition directs PS1 molecules that carry the P264L and/or P267S substitutions to the ERQC, the same treatment sends PS1 molecules that carry the fAD-linked A264E mutation to aggresomes (Johnston *et al*, 1998). These findings raise the question of what determines the fate of a specific misfolded PS1 conformer and directs it to the adequate deposition site. It is plausible that the accumulation of an aggregative protein in an aggresome, where it can be recycled (Ben-Gedalya *et al*, 2011), is preferred compared to its deposition in the ERQC where it is trapped and may impair ER function. Thus, it is probable that a rapid and robust aggregation averts the retro-translocation of P264L and P267S PS1 to the cytosol rendering its deposition in the ERQC. This theme is supported by the finding that ERQC-resident PS1 molecules are immobile (Fig 4), while PrP in aggresomes is dynamic (Ben-Gedalya *et al*, 2011).

The nature of the aggregating protein may also be a determining factor in the triage to different quality control compartments. While the PrP is a GPI anchored protein, PS1 is a multi-pass transmembrane protein, a feature that may present a challenge to the cellular degradation machinery. Recently, an alternative route involving intermembrane proteolysis was suggested (Fleig *et al*, 2012), thus allowing dislocation of clipped products.

Another interesting question is why P264L PS1 exhibits partial activity (Fig 5A and C–E) while P267S PS1 appears to undergo

degradation (Fig 5A and B), thereby showing no γ -secretase activity (Fig 5C and D). It is possible that the substitutions of prolines 264 and 267 in the sequence of PS1 differentially affect the spatial structure of the protein. This explanation proposes that P264L PS1 molecules attain a three-dimensional structure which is more similar to the wild-type conformation than that of the P267S-mutated PS1. Thus, P264L PS1 molecules escape degradation and exhibit partial γ -secretase activity, while PS1 molecules that carry the P267S mutation are directed for digestion by the proteasome. Structural analyses are required to examine this idea.

The widely accepted amyloid hypothesis suggests that AD stems from the over-production and aggregation of A β peptides which lead to synaptic malfunction (Selkoe, 2011). Our study strongly suggests that the substitution of proline 264 or 267 in the sequence of PS1 leads to AD by an alternative, non-canonical mechanism. The reduced γ -secretase activity and mitochondrial aberrant distribution and function support the notion that the failure of vital cellular functions that emanate from PS1 aberrant folding can cause AD late in life (Shen & Kelleher, 2007; Chavez-Gutierrez *et al*, 2012). A recent study strongly supports this idea by showing that certain fAD-causing mutations in the sequence of PS1 lead to the abolishment of γ -secretase activity (Xia *et al*, 2015). Moreover, the aggregation of PS1 itself may cause ER stress and an additional burden on the proteasome, leading to cell death and disease. This concept is strengthened by the reduced levels of PS1 observed in brains of

cyclophilin B knockout mice (Fig 8A–E), the findings that PS1-containing cellular inclusions are present in brains of mice that express the P264L PS1 (Fig 8G) and of individuals carrying this mutated protein (Martikainen *et al*, 2010) as well as in brain sections of patients who had sporadic AD (Busciglio *et al*, 1997; Chui *et al*, 1998).

Why fAD which stems from the proline substitutions in PS1 onsets late in life is another key enigma. One possibility suggests that early in life, sufficient efficiencies of two cellular mechanisms prevent AD from emerging. First, a small fraction of the nascent mutated PS1 molecules that are formed in *cis* conformation exhibit sufficient PS1 activity, and second, an increased degradation capacity clears the mutated PS1 molecules that are synthesized in *trans* conformation. According to this model, aging-associated decline in the competence of protein degradation mechanisms exposes the aged organism to proteotoxicity and disease. This hypothesis is reinforced by the findings that the alteration of aging by the inhibition of IGF1 signaling protects mice (Cohen *et al*, 2009; Freude *et al*, 2009) and worms (Cohen *et al*, 2006; Teixeira-Castro *et al*, 2011; El-Ami *et al*, 2014) from proteotoxicity. Furthermore, cyclophilins were found to be modifiers of proteostasis (Silva *et al*, 2011) that aggregate and sediment in aged worms (David *et al*, 2010; Kirstein-Miles *et al*, 2013).

The requirement of cyclophilins for the correct maturation of PrP and PS1 highlights the key roles of proline *cis/trans* isomerization for the maintenance of proteostasis and the prevention of proteopathies. For instance, the activity of the prolyl isomerase Pin1 restores functionality of microtubule-associated protein TAU (Lu *et al*, 1999). Our study points at ER-resident cyclophilin activity as critical for the correct folding of PS1. In contrast, FKBP51 was reported to enhance proteotoxicity and to be more abundant in brains of old individuals and patients with AD than in those of healthy controls (Blair *et al*, 2013). Similarly, cyclophilin D deficiency was found to attenuate mitochondrial perturbation and alleviate learning deficiencies of AD-model mice (Du *et al*, 2008), implying that elevated activity of PPIase chaperones is not always beneficial.

Finally, it is also possible that an aging-associated decline in the integrity of deposition sites leads to the release of toxic species, transforming these structures from protective entities to sources of toxicity in late life stages (Ben-Gedalya & Cohen, 2012).

In conclusion, our study highlights the complexity of mechanisms that lead to the development of neurodegenerative disorders, indicating that while one mechanism underlies the development of two distinct maladies, fAD may emanate from different types of proteostasis failures. It also emphasizes the key role of the aging process in enabling the manifestation of neurodegeneration late in life.

Materials and Methods

Materials

Cell culture reagents were purchased from Biological Industries (Beit Haemek, Israel). Protein concentration was determined using BCA kit (Pierce 23223). Cyclosporin-A (CsA) (C1832 and MG132 (C2211) and all other reagents were from Sigma.

Cell cultures

Cells were grown at 37°C in DMEM supplemented with 10% fetal calf serum. Transfections were achieved with the reagent TransIT-LT1 (Mirus MC-MIR-2300) according to the manufacturer's instructions. CHO cells stably expressing moderate levels of wild-type human PS1 were selected by G418 (1 mg/ml). To generate stable cell lines of PS1 constructs on PS1 or PS1 and PS2 null background, presenilin KO cells (gift from Dr. Bart De Strooper; Herreman *et al*, 1999, 2003) were infected with the pBABE-puro retroviral vectors expressing either empty, wild-type PS1 P264L, P267S, P264L/P267S, or D257A (all based on the human wild-type PS1). Media was replaced 24 h after infection, and 24 h later, infected cells were selected with 2 µg/ml puromycin for 72 h.

Antibodies and dyes

PS1 NTF and PS1 CTF antibodies were purchased from Chemicon (MAB1563 and MAB5232, respectively) and used for both immune fluorescence assays and WB. PS2 antibody (Abcam ab51249) was used to detect PS2 CTF by WB. BML-Pw8810-0500 for mono- and poly-ubiquitinated conjugates (FK2) was from Enzo. Calnexin C-20, sc-6465, was from Santa Cruz. γ -tubulin (T6557) and vimentin (V6630) antibodies were from Sigma. MitoTracker Red CMXRos M7512 was purchased from Invitrogen (San Diego, CA). DAPI staining was achieved by Vectashield-DAPI mounting media (VE-H-1200). BiP antibody was purchased from Abcam (ab21685).

γ -secretase activity assays

Myc-tagged substrate method

PS1 and PS2 knockout MEFs (a generous gift of Bart De Strooper, Leuven, Belgium) were maintained in DMEM F12 (Invitrogen, Carlsbad, CA) supplemented with 10% HIFCS and penicillin–streptomycin. PS1 constructs were expressed either transiently or by retroviral infection producing stable clones. The γ -secretase substrate APP C99-myc (4°C) (kindly provided by A. Goate, Washington University) was expressed by transient transfection using TransIT X2™ (Mirus Bio LLC, Madison, WI). Cells were harvested 20–22 h post-transfection and lysed in modified buffer containing 1% CHAPSO, 50 mM Hepes pH 7.2, 150 mM NaCl, 1% TX-100, and protease inhibitor cocktail (Calbiochem #539134). Samples were spun at 16,000 × g for 10 min, and supernatant was tested for protein concentration by BCA assay. Cell lysates were subjected to SDS–PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with TBST/5% milk and probed with mouse anti-myc antibody (Sigma, clone 9E10) for the AICD and C99.

Fluorescent substrate method

Membrane fraction was isolated by homogenizing the cells in 50 mM Hepes (pH 7), 250 mM sucrose, 5 mM EDTA, and complete protease inhibitor (Roche) (which does not contain pepstatin A—an aspartyl protease inhibitor). Homogenates were centrifuged at 3,000 g for 10 min to remove debris and nuclei. The supernatants were then centrifuged at 100,000 g for 1 h at 4°C. To extract the dissolved proteins from the crude membranes, the pellets were dissolved by shaking for 90 min in 100 µl of the same buffer supplemented with 1% CHAPSO at 4°C. The supernatants were collected

after an additional 100,000 g centrifugation for 1 h at 4°C. Aliquots were removed for protein concentration, and CHAPSO was diluted to 0.25%. To measure the γ -secretase activity *in vitro*, samples containing 10 μ g protein were incubated with 8 μ M of a fluorescence-conjugated peptide γ -secretase substrate (NMA-GGVVIA TVK (DNP)-DRDRDR-NH₂). The proteolysis at the A β 40-, A β 42-, and A β 43-generating cleavage sites results in enhanced fluorescence (excitation 320 nm, emission 460 nm).

Quantification of soluble A β 42/40 using sandwich ELISA

Millipore (Temecula, CA USA) high-sensitivity human amyloid β 42 (EZHS42) and β 40 (EZHS40) ELISA kits were used according to the manufacturer's instructions. Samples were prepared from media collected from PS1/2-KO MEF cells expressing both the specified PS1 construct and APP C99-myc.

Immunofluorescence microscopy and live imaging

To detect PS1, cells were grown on poly-D-lysine-coated chamber slides (Nunc, #155411), fixed (10% formalin in PBS, 30 min, RT), quenched with cold 1% NH₄Cl in PBS, permeabilized (0.1% TX-100 in PBS, 2 min, RT), and blocked with 2% BSA (30 min, RT). The cells were then incubated overnight with the primary antibody (in 1% BSA, 4°C) and rinsed, and the secondary antibody conjugated to fluorescent probe as mentioned (diluted 1:200 in 1% BSA) was added for 1 h (RT). The labeled cells were mounted using Vecta-shield-DAPI mounting media VE-H-1200 and viewed with a Zeiss LSM 710 Axio Observer.Z1 laser scanning confocal microscope.

For time-lapse microscopy and FRAP and FLIP experiments, cells were plated on poly-L-lysine (Sigma)-coated glass-bottom plates (MatTek Corp., #P35GC-1.0-14-C) or on a chambered cover glass system (Nunc, #177445). Confocal microscopy was conducted using Zeiss LSM 710 scanning microscope with a 63 $\times$ oil for FRAP, FLIP, and immunofluorescence, and an LD Plan-Neofluar 40 $\times$ /0.6 Corr M27 objective for time-lapse microscopy. For time-lapse experiments, Z-stack series of 1- μ m scans were collected in 10-min intervals for 16 h. For FRAP, a region of interest was bleached using a 488-nm laser for 2 s at full laser power, and single-scan images were collected every 1 s for 1 min following the bleach. Fluorescence of the bleached region of interest (F) was calculated as $F = (I_i - I_b)/(I_r - I_b)$, where " I_i " is fluorescence intensity in the region of interest, " I_r " is intensity in a reference area, and " I_b " is background intensity (outside all cells). Intensity data were recorded using Zeiss ZEN software. Reported values are the average of at least three data points. Zeiss ZEN and ImageJ software were used for processing and quantification. For FLIP experiments, a 2 $\times$ 2 μ m area of the cytosol was bleached continuously (with each scan), while the fluorescence of the inclusion and a control cytosolic region was measured.

Transmission electron microscopy (TEM)

Cells were seeded on poly-D-lysine-coated chamber slides (Nunc, #155411) and treated with either vehicle or MG132 (10 μ M for 5 h). Cells were rinsed 3 times with PBS and fixed by incubation in 2.5% paraformaldehyde and 0.1 M PB buffer (22 mM NaH₂PO₄, 78 mM Na₂HPO₄) for 1 h at RT. After three rinses with cold PB buffer containing 1% NH₄Cl, cells were permeabilized with 0.05% TX-100

for 3 min at RT followed by three rinses with PB buffer and blocking with 4% BSA in PBS for 30 min at RT. The cell monolayer was fixed, dehydrated, embedded, and cut into thin sections as described previously (Cohen & Taraboulos, 2003).

The analysis of PS1 in the brain of cyclophilin B knockout mice

CyPB KO mice (Cabral *et al*, 2014) and their wild-type siblings (strain C57BL/6, which were bred from Het X Hat mating) were sacrificed at the age of 2 months, and brains were removed. One hemisphere was snap-frozen and used for biochemical assays, while the other half was fixed in 3% PFA for 24 h, then transferred to 1% PFA, dehydrated, cleared with xylene, and embedded in paraffin. PS1 immunohistochemistry was performed on 8-micron-thick paraffin sections, precleared for endogenous peroxidase activity by incubation in 3% H₂O₂. Antigen retrieval was performed by boiling in pressure cooker 110°C for 5 min in 10 mM citrate buffer. Brain sections were blocked by CAS block (00-8120; Invitrogen San Diego, CA). Immunostaining was performed with the PS1 CTF antibody (MAB5232; Millipore, MA USA). Anti-mouse Ig peroxidase ImmPRESS reagent (MP-7402) was used as a secondary antibody, and immunoreactivity was visualized using DAB substrate kit (SK-4100), both from Vector laboratories (Burlingame, CA USA). Slides were developed in parallel and then stained with Mayer's hematoxylin, rinsed, and mounted prior to visualization.

Image analysis brain sections

Images of brain slices were taken using a Zeiss LSM 710 confocal microscope. PS1 foci were counted and measured using ImageJ software (using the "analyze particles" option, threshold was set to 0–60).

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Author contributions

EC and TBG initiated and designed the study and wrote the manuscript. TBG performed cloning procedures, WB, IF, EM, FRAP and FLIP experiments as well as *in vitro* assays and mouse brain analyses. LM performed WB experiments, and MBS created mutated PS1 plasmids. JCM and WAC created cyclophilin B knockout mice. DFM created PS1-expressing lentiviruses, and IS and SF injected mouse brains with the lentiviruses. TBC performed mouse brain sectioning and IHC experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Aguzzi A, Polymenidou M (2004) Mammalian prion biology: one century of evolving concepts. *Cell* 116: 313–327
- Aguzzi A, Calella AM (2009) Prions: protein aggregation and infectious diseases. *Physiol Rev* 89: 1105–1152
- Amaducci L, Tesco G (1994) Aging as a major risk for degenerative diseases of the central nervous system. *Curr Opin Neurol* 7: 283–286
- Area-Gomez E, de Groof AJ, Boldogh I, Bird TD, Gibson GE, Koehler CM, Yu WH, Duff KE, Yaffe MP, Pon LA, Schon EA (2009) Presenilins are enriched in endoplasmic reticulum membranes associated with mitochondria. *Am J Pathol* 175: 1810–1816
- Area-Gomez E, Del Carmen Lara Castillo M, Tambini MD, Guardia-Laguarta C, de Groof AJ, Madra M, Ikenouchi J, Umeda M, Bird TD, Sturley SL, Schon EA (2012) Upregulated function of mitochondria-associated ER membranes in Alzheimer disease. *EMBO J* 31: 4106–4123
- Arias E, Cuervo AM (2011) Chaperone-mediated autophagy in protein quality control. *Curr Opin Cell Biol* 23: 184–189
- Ben-Gedalya T, Lyakhovetsky R, Yedidia Y, Bejerano-Sagie M, Kogan NM, Karpuj MV, Kaganovich D, Cohen E (2011) Cyclosporin-A-induced prion protein aggregates are dynamic quality-control cellular compartments. *J Cell Sci* 124: 1891–1902
- Ben-Gedalya T, Cohen E (2012) Quality control compartments coming of age. *Traffic* 13: 635–642
- Bertram L, Tanzi RE (2008) Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses. *Nat Rev Neurosci* 9: 768–778
- Bezprozvanny I, Mattson MP (2008) Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci* 31: 454–463
- Blair LJ, Nordhues BA, Hill SE, Scaglione KM, O'Leary JC III, Fontaine SN, Breydo L, Zhang B, Li P, Wang L, Cotman C, Paulson HL, Muschol M, Uversky VN, Klengel T, Binder EB, Kaye R, Golde TE, Berchtold N, Dickey CA (2013) Accelerated neurodegeneration through chaperone-mediated oligomerization of tau. *J Clin Invest* 123: 4158–4169
- Brodsky JL, Skach WR (2011) Protein folding and quality control in the endoplasmic reticulum: recent lessons from yeast and mammalian cell systems. *Curr Opin Cell Biol* 23: 464–475
- Busciglio J, Hartmann H, Lorenzo A, Wong C, Baumann K, Sommer B, Staufenbiel M, Yankner BA (1997) Neuronal localization of presenilin-1 and association with amyloid plaques and neurofibrillary tangles in Alzheimer's disease. *J Neurosci* 17: 5101–5107
- Cabral WA, Perdivara I, Weis M, Terajima M, Blissett AR, Chang W, Perosky JE, Makareeva EN, Mertz EL, Leikin S, Tomer KB, Kozloff KM, Eyre DR, Yamauchi M, Marini JC (2014) Abnormal type I collagen post-translational modification and crosslinking in a cyclophilin B KO mouse model of recessive osteogenesis imperfecta. *PLoS Genet* 10: e1004465
- Campion D, Flaman J-M, Brice A, Hannequin D, Dubois B, Martin C, Moreau V, Charbonnier F, Didierjean O, Tardieu S, Penet C, Puel M, Pasquier F, Le Doze F, Bellis G, Calenda A, Heilig R, Martinez M, Mallet J, Bellis M et al (1995) Mutations of the presenilin 1 gene in families with early-onset Alzheimer's disease. *Hum Mol Genet* 4: 2373–2377
- Chavez-Gutierrez L, Bammens L, Benilova I, Vandersteen A, Benurwar M, Borgers M, Lismont S, Zhou L, Van Cleynebreugel S, Esselmann H, Wiltfang J, Serneels L, Karran E, Gijzen H, Schymkowitz J, Rousseau F, Broersen K, De Strooper B (2012) The mechanism of gamma-Secretase dysfunction in familial Alzheimer disease. *EMBO J* 31: 2261–2274
- Chui DH, Shirotani K, Tanahashi H, Akiyama H, Ozawa K, Kunishita T, Takahashi K, Makifuchi T, Tabira T (1998) Both N-terminal and C-terminal fragments of presenilin 1 colocalize with neurofibrillary tangles in neurons and dystrophic neurites of senile plaques in Alzheimer's disease. *J Neurosci Res* 53: 99–106
- Cohen E, Taraboulos A (2003) Scrapie-like prion protein accumulates in aggregates of cyclosporin A-treated cells. *EMBO J* 22: 404–417
- Cohen E, Bieschke J, Perciavalle RM, Kelly JW, Dillin A (2006) Opposing activities protect against age-onset proteotoxicity. *Science* 313: 1604–1610
- Cohen E, Paulsson JF, Blinder P, Burstyn-Cohen T, Du D, Estepa G, Adame A, Pham HM, Holzenberger M, Kelly JW, Masliah E, Dillin A (2009) Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. *Cell* 139: 1157–1169
- David DC, Ollikainen N, Trinidad JC, Cary MP, Burlingame AL, Kenyon C (2010) Widespread protein aggregation as an inherent part of aging in *C. elegans*. *PLoS Biol* 8: e1000450
- De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 391: 387–390
- Du H, Guo L, Fang F, Chen D, Sosunov AA, McKhann GM, Yan Y, Wang C, Zhang H, Molkentin JD, Gunn-Moore FJ, Vonsattel JP, Arancio O, Chen JX, Yan SD (2008) Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease. *Nat Med* 14: 1097–1105
- El-Ami T, Moll L, Carvalhal Marques F, Volovik Y, Reuveni H, Cohen E (2014) A novel inhibitor of the insulin/IGF signaling pathway protects from age-onset, neurodegeneration-linked proteotoxicity. *Aging Cell* 13: 165–174
- Fleig L, Bergbold N, Sahasrabudhe P, Geiger B, Kaltak L, Lemberg MK (2012) Ubiquitin-dependent intramembrane rhomboid protease promotes ERAD of membrane proteins. *Mol Cell* 47: 558–569
- Freude S, Hettich MM, Schumann C, Stohr O, Koch L, Kohler C, Udelhoven M, Leiser U, Muller M, Kubota N, Kadowaki T, Krone W, Schroder H, Bruning JC, Schubert M (2009) Neuronal IGF-1 resistance reduces Abeta accumulation and protects against premature death in a model of Alzheimer's disease. *FASEB J* 23: 3315–3324
- Handsbumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* 226: 544–547
- Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256: 184–185
- Hecimovic S, Wang J, Dolios G, Martinez M, Wang R, Goate AM (2004) Mutations in APP have independent effects on Abeta and CTFgamma generation. *Neurobiol Dis* 17: 205–218
- Herreman A, Hartmann D, Annaert W, Saftig P, Craessaerts K, Serneels L, Umans L, Schrijvers V, Checler F, Vanderstichele H, Baekelandt V, Dressel R, Cupers P, Huylebroeck D, Zwijsen A, Van Leuven F, De Strooper B (1999) Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. *Proc Natl Acad Sci USA* 96: 11872–11877
- Herreman A, Van Gassen G, Bentahir M, Nyabi O, Craessaerts K, Mueller U, Annaert W, De Strooper B (2003) Gamma-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. *J Cell Sci* 116: 1127–1136

- Hsiao K, Baker HF, Crow TJ, Poulter M, Owen F, Terwilliger JD, Westaway D, Ott J, Prusiner SB (1989) Linkage of a prion protein missense variant to Gerstmann-Sträussler syndrome. *Nature* 338: 342–345
- Hutton M, Busfield F, Wragg M, Crook R, Perez-Tur J, Clark RF, Prihar G, Talbot C, Phillips H, Wright K, Baker M, Lendon C, Duff K, Martinez A, Houlden H, Nichols A, Karran E, Roberts G, Roques P, Rossor M et al (1996) Complete analysis of the presenilin 1 gene in early onset Alzheimer's disease. *NeuroReport* 7: 801–805
- Johnston JA, Ward CL, Kopito RR (1998) Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* 143: 1883–1898
- Kaganovich D, Kopito R, Frydman J (2008) Misfolded proteins partition between two distinct quality control compartments. *Nature* 454: 1088–1095
- Kamhi-Nesher S, Shenkman M, Tolchinsky S, Fromm SV, Ehrlich R, Lederkremer GZ (2001) A novel quality control compartment derived from the endoplasmic reticulum. *Mol Biol Cell* 12: 1711–1723
- Kim YE, Hipp MS, Bracher A, Hayer-Hartl M, Hartl FU (2013) Molecular chaperone functions in protein folding and proteostasis. *Annu Rev Biochem* 82: 323–355
- Kirstein-Miles J, Scior A, Deuerling E, Morimoto RI (2013) The nascent polypeptide-associated complex is a key regulator of proteostasis. *EMBO J* 32: 1451–1468
- Kondratyev M, Avezov E, Shenkman M, Groisman B, Lederkremer GZ (2007) PERK-dependent compartmentalization of ERAD and unfolded protein response machineries during ER stress. *Exp Cell Res* 313: 3395–3407
- Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, Wolfe DM, Martinez-Vicente M, Massey AC, Sovak G, Uchiyama Y, Westaway D, Cuervo AM, Nixon RA (2010) Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. *Cell* 141: 1146–1158
- Lippincott-Schwartz J, Altan-Bonnet N, Patterson GH (2003) Photobleaching and photoactivation: following protein dynamics in living cells. *Nat Cell Biol* 5: S7–S14
- Lu PJ, Wulf G, Zhou XZ, Davies P, Lu KP (1999) The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. *Nature* 399: 784–788
- Martikainen P, Pikkariainen M, Pontynen K, Hiltunen M, Lehtovirta M, Tuisku S, Soininen H, Alafuzoff I (2010) Brain pathology in three subjects from the same pedigree with presenilin-1 (PSEN1) P264L mutation. *Neuropathol Appl Neurobiol* 36: 41–54
- Mendez MF, McMurtry A (2006) Frontotemporal dementia-like phenotypes associated with presenilin-1 mutations. *Am J Alzheimers Dis Other Dement* 21: 281–286
- Morley JF, Brignull HR, Weyers JJ, Morimoto RI (2002) The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 99: 10417–10422
- Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F (2001) The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings *in situ*. *J Biol Chem* 276: 12030–12034
- Prusiner SB (1998) Prions. *Proc Natl Acad Sci USA* 95: 13363–13383
- Quarteronnet D, Pradier L, Czech C, Delalande L, Burgevin MC, Doble A, Petitot F (1996) Localization of presenilin-1 mRNA in rat brain. *NeuroReport* 7: 2587–2591
- Roberson ED (2012) Mouse models of frontotemporal dementia. *Ann Neurol* 72: 837–849
- Sato T, Diehl TS, Narayanan S, Funamoto S, Ihara Y, De Strooper B, Steiner H, Haass C, Wolfe MS (2007) Active gamma-secretase complexes contain only one of each component. *J Biol Chem* 282: 33985–33993
- Schiene-Fischer C (2014) Multidomain peptidyl prolyl cis/trans isomerases. *Biochim Biophys Acta* 1850: 2005–2016
- Schrader EK, Harstad KG, Matouschek A (2009) Targeting proteins for degradation. *Nat Chem Biol* 5: 815–822
- Selkoe DJ (2003) Folding proteins in fatal ways. *Nature* 426: 900–904
- Selkoe DJ, Wolfe MS (2007) Presenilin: running with scissors in the membrane. *Cell* 131: 215–221
- Selkoe DJ (2011) Alzheimer's disease. *Cold Spring Harb Perspect Biol* 3: pii: a004457
- Shen J, Kelleher RJ III (2007) The presenilin hypothesis of Alzheimer's disease: evidence for a loss-of-function pathogenic mechanism. *Proc Natl Acad Sci USA* 104: 403–409
- Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I et al (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375: 754–760
- Silva MC, Fox S, Beam M, Thakkar H, Amaral MD, Morimoto RI (2011) A genetic screening strategy identifies novel regulators of the proteostasis network. *PLoS Genet* 7: e1002438
- Teixeira-Castro A, Ailion M, Jalles A, Brignull HR, Vilaca JL, Dias N, Rodrigues P, Oliveira JF, Neves-Carvalho A, Morimoto RI, Maciel P (2011) Neuron-specific proteotoxicity of mutant ataxin-3 in *C. elegans*: rescue by the DAF-16 and HSF-1 pathways. *Hum Mol Genet* 20: 2996–3009
- Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, Kim G, Ratovitsky T, Davenport F, Nordstedt C, Seeger M, Hardy J, Levey AI, Gandy SE, Jenkins NA, Copeland NG, Price DL, Sisodia SS (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. *Neuron* 17: 181–190
- Walker LC, Levine H III, Mattson MP, Jucker M (2006) Inducible proteopathies. *Trends Neurosci* 29: 438–443
- Wang Q, Li L, Ye Y (2008) Inhibition of p97-dependent protein degradation by Eeyarestatin I. *J Biol Chem* 283: 7445–7454
- Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* 398: 513–517
- Xia W (2008) From presenilinase to gamma-secretase, cleave to capacitate. *Curr Alzheimer Res* 5: 172–178
- Xia D, Watanabe H, Wu B, Lee SH, Li Y, Tsvetkov E, Bolshakov VY, Shen J, Kelleher RJ III (2015) Presenilin-1 knockin mice reveal loss-of-function mechanism for familial Alzheimer's disease. *Neuron* 85: 967–981
- Yamazaki M, Oyanagi K, Mori O, Kitamura S, Ohshima M, Terashi A, Kitamoto T, Katayama Y (1999) Variant Gerstmann-Sträussler syndrome with the P105L prion gene mutation: an unusual case with nigral degeneration and widespread neurofibrillary tangles. *Acta Neuropathol* 98: 506–511
- Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, Mi N, Zhao Y, Liu Z, Wan F, Hailey DW, Oorschot V, Klumperman J, Baehrecke EH, Lenardo MJ (2010) Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature* 465: 942–946