**Matters Arising**

**Familial Alzheimer Disease mutations in Presenilin generate amyloidogenic Aβ peptide seeds**

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**Summary**

Recently it was proposed that the familial Alzheimer disease (FAD) causing presenilin (PSEN) mutations PSEN1-L435F and PSEN1-C410Y do not support the generation of Aβ-peptides from the amyloid precursor protein (APP). This challenges the amyloid hypothesis and disagrees with previous work showing that PSEN1 FAD causing mutations generate invariably long Aβ and seed amyloid. We contrast here the proteolytic activities of these mutant PSEN alleles with the complete loss-of-function PSEN1-D257A allele. We find residual carboxy- and endo-peptidase γ-secretase activities, similar to the formerly characterized PSEN1-R278I. We conclude that the PSEN1-L435F and -C410Y mutations are extreme examples of the previously proposed 'dysfunction' of γ-secretase that characterizes FAD associated PSEN.

**Introduction**

More than 150 different mutations in the presenilin genes *PSEN1&2* cause familial Alzheimer Disease (FAD (Sherrington et al., 1995)) (http://www.alzforum.org/mutations). Presenilins provide the catalytic core to the γ-secretases and process APP-CTF to generate Aβ peptides (Matsumura et al., 2014; De Strooper et al., 1998; Wolfe et al., 1999).

The fact that mutations in the substrate (APP) and the protease (Presenilin/γ-secretase) generating Aβ cause Alzheimer disease is in strong support for the amyloid cascade hypothesis, which proposes that disturbances in Aβ metabolism are central to AD pathogenesis (Hardy and Selkoe, 2002; Karran et al., 2011, De Strooper and Karran, 2016). However, the intriguing observation of Shen, Kelleher and

colleagues that total loss of PSEN function ($Psen1^{-/-}$, $Psen2^{-/-}$) in the adult mouse brain causes progressive cognitive decline and neurodegeneration in the absence of Aβ generation led to hypothesize that PSEN full loss-of-function could be the underlying mechanism of (familial) AD neurodegeneration (Saura et al., 2004). In order to challenge this hypothesis, Shen and Kelleher have recently studied the effects of three FAD presenilin mutant alleles,PSEN1-R278I, -L435F and -C410Y, which according to their data fully inactivate PSEN/γ-secretase function (Heilig et al., 2013; Xia et al., 2015). In several assays none of these mutants supported $Aβ_{40}$ or $Aβ_{42}$ generation. Furthermore, they also generated homozygous knock-in mice containing the Psen1-L435F or the Psen1-C410Y mutation in a *Psen2* null background. These *knock-in* (KI/KI) mice display, according to Shen and colleagues, a loss-of-function phenotype that cannot be distinguished from the previously investigated ($Psen1^{-/-}$, $Psen2^{-/-}$) knockout mice (Saura et al., 2004). The results provided the basis to propose that Aβ is an epiphenomenon in FAD (Xia et al., 2015).

It should however be noticed that patients carrying FAD-linked *PSEN1* mutations express in almost all cases also one normal copy of *PSEN1* and two normal copies of *PSEN2* alleles which could compensate for the loss of activity of the diseased allele. Therefore, the question arises whether the conclusions based on complete *Psen* deficiency models can be extrapolated to FAD.

Our data suggest an alternative hypothesis: FAD-linked *PSEN* mutations affect in a variable way the endoproteolytic activities of γ-secretase (Fig.1A), while no examples of full inactivation (complete loss-of-function) were found (Chávez-Gutiérrez et al., 2012; Szaruga et al., 2015), with the potential exception of the ones discussed above (Xia et al., 2015). Significantly, all *PSEN*-FAD mutations affect invariably the carboxypeptidase-like efficiency or processivity of γ-secretase which results in the release of long ($\geq Aβ_{42}$) amyloidogenic Aβ peptides at the expense of the short ones ($Aβ_{40}$ and $Aβ_{38}$). This model was directly tested in brain extracts from 22 *PSEN1*-FAD patients covering 9 different mutations (Szaruga et al., 2015). The study confirmed that pathogenic mutations affect γ-secretase carboxypeptidase-like activities in a disease relevant context, i.e. heterozygosity of the *PSEN* alleles in the human brain, while the endopeptidase activity (epsilon-cleavage) was not altered (Szaruga et al., 2015). Thus, our data in a large cohort of FAD patients clearly shows no inactivating effect on total brain γ-secretase activity, as proposed recently by Xia et al., but in contrast, demonstrate changes in its processivity as a common characteristic. Moreover, experiments in human neuronal cultures derived from FAD patient fibroblast support the same concept (Moore et al., 2015). Thus experiments *in vitro*, in cell cultures and in brain material of patients suggest strongly that *PSEN*-FAD mutations consistently reduce γ-secretase processivity, resulting in "qualitative" changes in Aβ profiles, i.e. changes in relative concentrations of different Aβ peptides, while the γ-secretase signaling function is largely or completely preserved in patients (Chávez-Gutiérrez et al., 2012; Szaruga et al., 2015).

An important piece of data (Saito et al., 2011) was noticeably absent in the report by Xia et al (Xia et al., 2015). This previous work used very similar approaches i.e. 'knock-in' of the clinical *Psen1-R278I* mutation allele into the endogenous mouse gene and the mice developed the same severe phenotype as the one described in the Shen and Kelleher study. In contrast to the report by Shen and colleagues which stated(that this mutation fully inactivates PSEN/γ-secretase based on cellular experiments (Heilig et al., 2013)), we found that the mutant allele releases highly aggregation-prone and likely pathogenic Aβ$_{43}$-peptides (Saito et al., 2011). Despite the relevance of this observation, Aβ$_{43}$ peptide was not measured in the Shen and Kelleher study (Xia et al., 2015).

The conflicting data led us to re-investigate whether the two *PSEN*-FAD alleles L435F and C410Y-PSEN1 are indeed different from all previously investigated *pathogenic* alleles and cause a full loss-of-function. We present here strong evidence that these two mutants display residual activity and release longer Aβ and conclude therefore that they are only extreme examples of 'dysfunction' of γ-secretase that characterizes FAD associated PSEN mutations.

**Results**

We expressed the FAD-linked PSEN1-R278I, PSEN1-L435F, and PSEN1-C410Y mutations and the catalytically inactive full loss-of-function PSEN1-D257A mutation in *Psen1/2* deficient mouse embryonic fibroblasts (*Psen$^{1/2-/-}$* MEF) and measured Aβ$_{40}$, Aβ$_{42}$, and Aβ$_{43}$ directly from the conditioned medium. This approach has been proven to provide reliable data that could be extrapolated to human brain samples (Szaruga et al., 2015). Western blot data show that PSEN1-R278I, -C410Y and -L435F mutants reconstitute γ-secretase complex, as indicated by similar levels of mature Nicastrin expression in the different cell lines (Figure 1B). Accumulation of full length PSEN1 is seen in the 4 cell lines expressing the mutant PSEN alleles, compared to the control line reconstituted with wild type PSEN1. Thus, all four mutations impair PSEN1 autoproteolysis (presenilinase activity), which was also observed in the mouse brain extracts (Xia et al., 2015). Clearly, the steady state levels of PSEN1-NTF and -CTF in the cell lines expressing the FAD-linked mutations are higher than the fragment levels seen in the cell line expressing the fully inactive PSEN1-D257A allele, which are generated by an alternative caspase cleavage, and do not reflect remaining presenilinase activity in this catalytically inactive mutant (Nyabi et al., 2003). Overall the data show that the cell lines express comparable levels of PSENs, resulting in similar γ-secretase levels, allowing to calibrate any residual activity in the mutant expressing lines towards full activity in the wild type (WT) and full loss-of-function in the PSEN-D257A reconstituted cell lines.

We addressed next to what extent γ-secretase activity was reconstituted in the FAD mutant cell lines. Endogeneous APP levels are very low and mouse APP is not processed efficiently by BACE-1 (De Strooper et al., 1995). We therefore transduced the cell lines with *APP$^{NL/KM}$* (APP containing the Swedish mutation). APP containing the Swedish mutation is a substrate efficiently

processed by β-secretase, resulting in the generation of wild type (WT-)APP-CTF, the direct substrate for γ-secretase. We quantified $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$ peptides in the extracellular medium of the cells (Figure 1C). Significantly, all investigated mutants drastically reduced the production of $A\beta_{40}$, but sustain secretion of longer $A\beta_{42}$ (Figure 1C). Most strikingly, PSEN1-R278I, -C410Y and -L435F mutants cause significant increments in the secretion of $A\beta_{43}$, relative to the control cell line (at the expense of $A\beta_{40}$). Similar results were obtained when using APP-WT or APP-C99 as substrate (not shown). No $A\beta$ was generated from the real loss-of-function PSEN-D257A expressing cell line. Importantly, the changes in $A\beta$ secretion observed for the pathogenic mutants are not explained by differences in γ-secretase expression levels, as all mutants reconstitute Nicastrin and Pen2 expression, and thus γ-secretase complexes to similar levels (Figure 1B). Thus, these data unequivocally demonstrate that the three PSEN1-FAD mutants are partially active and display low carboxypeptidase-like efficiency (processivity), which is in line with previous findings regarding other FAD associated alleles of PSEN1.

We next sought to confirm these results in cell lines with endogenous expression levels of all γ-secretase components; however, cell lines with the PSEN-C410Y and -L435F mutations were not made available to us. So we restricted this experiment to fibroblasts derived from the previously described *Psen1*[R278I] knock-in mice (Saito et al., 2011) which, as discussed, display the same phenotype as the mice with *Psen* knock-in mutations described by Xia et al. We tested *Psen1*-WT, heterozygous (*Psen1*-WT; *Psen1*[R278I]) and homozygous *Psen1*[R278I] and transduced them with WT-APP. In these cell lines Psen1 is expressed under its endogenous promoter and WT-APP substrate is processed by endogenous β-secretase levels. Similar to our observations in *Psen*[1/2-/-] MEF rescued with *Psen1*[R278I], secreted $A\beta_{40}$ and $A\beta_{42}$ peptides were drastically reduced but very high $A\beta_{43}$ levels were observed in the homozygous mutant cell line (Figure 1E). Thus the increased $A\beta_{43}$ production levels in the homozygous mutant cell lines cannot be attributed to potential experimental artifacts related to overexpression and/or integration of the transgene during the reconstitution of γ-secretase complexes and/or to expression of APP containing the Swedish mutation. These results also confirm entirely the previous observations (Saito et al., 2011) and validate our results using rescued *Psen*[1/2-/-] MEF .

During the revision process, Shen and Kelleher communicated in their response to our manuscript that wild type mouse brain detergent extracts generate measurable amounts of $A\beta_{43}$, while no $A\beta_{43}$ is detected in brain detergent extracts of the KI/KI mutant PSEN (C410Y or L435F) mice. To determine $A\beta_{43}$ production, Shen and Kelleher used a detergent-based γ-secretase assay, which has been extensively used in the past and, when its limitations are taken into account, can yield important and reliable information (Chávez-Gutiérrez et al., 2012; Kakuda et al., 2006; Quintero-Monzon et al., 2011; Shimojo et al., 2008; Takami et al., 2009). However, we would like to highlight that detergent extraction decreases γ-secretase processivity (for instance, detergent solubilization results in the generation of long $A\beta_{43}$ at the expense of $A\beta_{40}$ from WT γ-secretase) and may also decrease its

endopeptidase activity. Several reports, including ours have shown lower processivity by WT γ-secretase in detergent-solubilized conditions (production of longer Aβ peptides) in comparison to activity assays in a membrane-like environment (Chávez-Gutiérrez et al., 2012; Matsumura et al., 2014; Quintero-Monzon et al., 2011; Shimojo et al., 2008; Takami et al., 2009; Winkler et al., 2012). Thus, when evaluating γ-secretase activity it is crucial to check any results obtained in 'cell-free' detergent assays, also in intact cells or in assays where the protease is maintained in a membrane-like environment before taking any conclusions with regard to γ-secretase activity (Acx et al., 2014; Chávez-Gutiérrez et al., 2012; Matsumura et al., 2014; Szaruga et al., 2015; Takami et al., 2009; Yagishita et al., 2008).

Thus, we wondered whether the discrepancies between ours and Shen/Kelleher's observations with regard to production of Aβ43 from L435F and C410Y mutants (high production vs. no production, respectively) could be explained by the experimental protocol used in the respective studies. We measured γ-secretase activity in CHAPSO solubilized membranes from the cell lines described above using purified C99-3XFLAG as substrate, which is a protocol similar to the one used by Shen and Kelleher to analyze the activity of γ-secretase in brain detergent extracts of their mice. Under these conditions we measured high $A\beta_{43}$ production in wild type cell extracts (Figure 1D), something which is not observed when analyzing intact cells. PSEN1-C410Y and -L435F mutant γ-secretase complexes, extracted from the fibroblast cell lines, produced extremely low $A\beta_{43}$, similar to what Shen and Kelleher observe in brain detergent extracted samples. We conclude that the experimental conditions (detergent solubilization) used by Shen and Kelleher compromised the carboxypeptidase efficiencies (processivity) of the wild type and mutant enzymes. This explains the decrease in $A\beta_{40}$ and the concomitant elevation of $A\beta_{43}$ production in the WT situation in their brain detergent extract assay.With the clinical mutations this reduction of processivity is further enhanced as shown in our experiments with detergent extracted cell lines compared to intact cell assays.

**Discussion**

In contrast to the recent reports by Shen, Kelleher and colleagues (Heilig et al., 2013; Xia et al., 2015) our data demonstrate that the PSEN1-R278I, -C410Y and -L435F mutants do reconstitute active γ-secretase complexes, although at low levels. In fact, Xia et al. (2015) also found increased PSEN1-NTF and -CTF levels in L435F and C410Y homozygous mice, compared to null *Psen1* alleles (see Fig 1 panel D in the publication), but the mutant PSEN fragments were not taken as potential indications of residual activity. More significantly, we demonstrate here that the three PSEN1-FAD mutants (PSEN1-R278I, C410Y and -L435F) are able to promote secretion of highly amyloidogenic $A\beta_{42}$- and $A\beta_{43}$-peptides which is not seen when expressing the real loss-of-function allele PSEN1-D257A.We want to point out that our data is in complete agreement with our previous study showing high $A\beta_{43}$ levels in *Psen*[R278I] knock-in mice (Saito et al., 2011) and with data from the group of Steiner

and colleagues which show large accumulation of $A\beta_{43}$ in patient's brain material of a PSEN1-L435F carrier (Kretner et al., 2016). Clearly, the three PSEN1-FAD-linked mutant alleles studied here are not fundamentally different from other pathogenic PSEN1 alleles. The observation that pathogenic PSEN-FAD alleles *variably* affect the endopeptidase γ-secretase activity (ε-cleavage) but *consistently* impair the carboxypeptidase-like efficiency of γ-secretase, also applies to the PSEN1-R278I, -C410Y and -L435F mutants. We have called this 'dysfunction' of γ-secretase, which *invariably* leads to the release of longer, more aggregation-prone Aβ peptides (Chávez-Gutiérrez et al., 2012; Szaruga et al., 2015). In conclusion, the pathogenic mutations analyzed here are no exception on this rule.

The proposed interpretation provides a coherent explanation for all available data in cell culture, *in vitro* assays, in mouse brain, and in patient brain on the relationship between pathological PSEN-FAD alleles and APP processing. One example for instance is the amyloid plaque accumulation in the heterozygous $Psen^{L435F\ KI/+}$ mice crossed with human WT overexpressing APP mice (Xia et al., 2015). According to our data, the $Psen^{L435}$ allele provides very small amounts of longer peptides (e.g. $A\beta_{42}$ and $A\beta_{43}$) which induce amyloid plaque formation by recruiting Aβ produced by the normal allele. This is only an example of the seeding or nucleation effect of long Aβ described several decades ago (Jarrett and Lansbury, 1993).

The study by Xia et al., 2015 raises a series of additional interesting questions with regard to the use of mice to model Alzheimer Disease. On one hand, the heterozygous animals ($Psen^{L435F\ KI/+}$ mice) have very good constructive value for the study of FAD as they mimic closely the genetic situation in the patients. Similar to other mouse experiments, these animals do develop amyloid plaques but no neuronal tangles or Aβ induced neurodegeneration. It is known that mouse brain is not very sensitive to Aβ toxicity for reasons that remain unclear, but might partially reflect differences in Tau biochemistry and/or other unknown factors (Ashe and Zahs, 2010; Zahs and Ashe, 2010). Even in the human carriers of PSEN-FAD alleles it takes several decades before the initial Aβ alterations translate into overt neurodegeneration.

On the other hand, the expression of the FAD mutant alleles in combination with $Psen2^{-/-}$ knockout alleles in brain caused a progressive neurodegeneration in mice (Xia et al., 2015). The experiment creates however a dramatic reduction of overall γ-secretase activity in brain which is not seen in patients (Szaruga et al., 2015). Under these conditions, membrane bound γ-secretase substrates will no longer be cleared (Kopan and Ilagan, 2004) and γ-secretase signaling events will be seriously hampered. Again, such extreme situation has never been encountered in familial patients and the relevance of this type of neurodegeneration in the process occurring in familial and certainly in sporadic Alzheimer disease is questionable. One should notice that neurodegeneration, an umbrella term, can be caused by many different mechanisms not necessarily related to the processes associated to AD. Furthermore, this neurodegeneration occurs in the absence of amyloid plaques and neuronal

tangles, which are hallmarks of Alzheimer disease. All data together strongly indicate that although combined inactivation (*Psen 1$^{-/-}$* ; *Psen2$^{-/-}$*) of γ-secretase triggers neurodegeneration in the adult mouse brain, this mechanism is not related to the neurodegenerative process observed in human Alzheimer disease, where normal *PSEN1/PSEN2* alleles compensate for potential mutant inactivating effects.

Our recent work analyzing the ε-cleavage in brain material of 22 PSEN1-FAD patients covering 9 different mutations (Szaruga et al., 2015) confirm that the signaling and clearing function of γ-secretase remains well preserved in patients. In fact, this study included the analysis of one FAD patient's brain sample carrying the R278I-*PSEN1*-FAD mutation, for which we observed a non-significant reduction in the endopeptidase γ-secretase activity levels. We would like to point out that γ-secretase endoprotease activity was unaffected in most of the FAD brain samples and therefore general loss-of-function cannot be an essential part of the PSEN associated pathogenic mechanism. Most likely, the three additional healthy PSEN alleles present in FAD patients' brains compensate for signaling and clearance deficits associated with the disease allele, which is completely in line with the fact that heterozygous *Psen1$^{+/-}$* mice are normal. Moreover, human heterozygote carriers of full (non-sense) loss-of-function alleles of γ-secretase components were reported and these patients suffer from inherited Acne Inversa without further indications of increased risk for dementia, as the work from Shen and Kelleher would predict (Chen, 2015; Pink et al., 2012a, 2012b; Wang et al., 2010). Finally, six FAD-PSEN1 homozygous individuals (*PSEN1$^{E280A}$*/ *PSEN1$^{E280A}$*) have been identified recently in Colombia (Kosik et al., 2015). If the mutant E280A allele was a severe loss-of-function we would expect in the homozygous situation developmental defects or at least Notch signaling problems in the skin, gastrointestinal or immunological system (Tournoy et al., 2004). This is however not observed.

In conclusion, our proposal integrates the available data in a coherent model for FAD: amyloidogenic Aβ generated by the defective allele will drive the neurodegenerative process by seeding the aggregation of normal Aβ generated by WT *PSEN1* and *PSEN2* alleles. Such interpretation also provides an explanation for the more than twenty APP mutations, which are all affecting Aβ generation and aggregation and cause very similar forms of FAD as the Presenilin mutations (Chávez-Gutiérrez et al., 2012; Citron et al., 1992; Games et al., 1995; Scheuner et al., 1996; Simons et al., 1996)(reviewed in Wilquet and De Strooper, 2004). The hypothesis that β amyloid is triggering the neurodegeneration in FAD patients (which is different from driving the whole process see Karran et al, 2011 and De Strooper &Karran, 2016) remains the most parsimonious and consistent explanation for all experimental data.

Finally, we want to note that some PSEN mutation carriers display other disease symptoms than AD, such as spastic paraparesis, myoclonus, epileptic seizures, parkinsonism, language deficits, frontotemporal dementia and cerebellar ataxia (reviewed in (Bergmans and De Strooper, 2010; Ryan and Rossor, 2010)). Misprocessing of other substrates than APP, at the endopeptidase level, may

contribute to this phenotypic variation. This does however not take away from the concept that the Alzheimer dementia in these patients is ultimately initiated by abnormal Aβ generation in their brains.

## Materials and Methods

### Antibodies

Rabbit Polyclonal antibody against mouse PEN-2 (B126.2) and monoclonal 9C3 against the C-terminus of Nicastrin have been described before (Annaert et al., 2001; Esselens et al., 2004). PSEN1-NTF and -CTF were detected with monoclonal antibodies MAB1563 (Chemicon) and MAB5232 (Millipore/Chemicon), respectively. Antibodies used in the quantification of $A\beta_{40}$ and $A\beta_{42}$ peptides by ELISA were obtained through collaboration with Janssen Pharmaceutica: JRF/cAb40/28 for Aβ1-40, JRF/cAb42/26 for Aβ1-42 and detection antibody JRF/AbN/25 labeled with SULFO-TAG.

### Immortalization of primary mouse embryonic fibroblasts

Mouse embryonic fibroblasts derived from *Psen1*-R278I knock-in mice (*Psen1*-wt, *Psen1*-wt;*Psen1*-R278I$^{+/−}$ and *Psen1*-R278I$^{+/+}$), were immortalized by transfection with the plasmid pMSSVLT (driving the expression of the large T-antigen). Immortalized mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) containing 10% fetal bovine serum (Sigma) and 200 μg/ml G418 Sulfate (ThermoFisher Scientific).

### Generation of Stable Cell Lines

*Psen1/Psen2*-deficient mouse embryonic fibroblasts (MEFs *Psen1/2$^{-/-}$*)(Nyabi et al., 2002) were cultured in DMEM/F-12 containing 10% fetal bovine serum. MEFs *Psen1/2$^{-/-}$* were transduced using a replication-defective recombinant retroviral expression system (Clontech) to stably express either WT or mutant human PSEN1. WT and mutant PSEN1 cell lines were selected with puromycin (5μg/ml) and maintained in DMEM/F-12 containing 10% serum and 3μg/ml puromycin. PSEN1 expression levels and reconstitution of the γ-secretase complex in the different cell lines was analyzed by SDS-PAGE/western blot.

### Transduction with APP Adenovirus

WT and mutant PSEN1 MEF cell lines were transduced with the recombinant adenovirus Ad5/CMV-APP bearing human APP-695 (wt or Swedish) as previously described (Chávez-Gutiérrez et al., 2008). Medium was refreshed at 7 h post-transduction and extracellular media collected after 24h.

**Enzyme-linked Immunosorbent Assay**

$A\beta_{40}$ and $A\beta_{42}$ peptide levels in the extracellular media of WT or mutant PSEN1 MEF cell lines were quantified on Meso Scale Discovery (MSD) 96 well plates. MSD plates were coated with 50µl/well of anti-$A\beta_{40}$ or anti-$A\beta_{42}$ antibodies (1.5 µg/ml in PBS) overnight at 4°C. Plates were blocked with 150 µl/well 0.1 % casein buffer for 1.5 h at room temperature (600 rpm) and rinsed 5 times with 200 µl/well washing buffer (PBS + 0.05 % Tween-20). 25 µl of SULFO-TAG detection antibody diluted in blocking buffer (concentration) was mixed with 25 µl of standards (synthetic human $A\beta$1-40 or $A\beta$1-42 peptide) or 25 µl extracellular media and 50 µl of the mix was loaded per well.

After overnight incubation at 4°C, plates were rinsed with washing buffer and 150 µl/well of the 2x MSD Read Buffer T (tris-based buffer containing tripropylamine, purchased from Meso Scale Discovery) was added. Plates were immediately read on MSD Sector Imager 6000. $A\beta$1-43 levels were measured using an $A\beta$43 ELISA kit (IBL, Japan).

***In vitro* activity assays using detergent extracted γ-secretase complexes**

*In vitro* activity assays were done as previously described (Chavez- Gutierrez et al, 2008) with minor modifications. MEF's microsomal fractions were prepared in 20mM Pipes, pH 7.0, 0.25M sucrose, 1mM EGTA, complete PI and 1% CHAPSO. *In vitro* reactions were carried out in 20mM Pipes, pH 7.0, 0.25M sucrose, 1mM EGTA, 1x EDTA-free complete proteinase inhibitors (Roche), 2.5% DMSO, 1% phosphatidylcholine and 0,25% CHAPSO. Reactions were incubated for 11h at 37°C with 1,5 µM C99-3xFLAG substrate.

**Expression and purification of C99-3xFLAG**

Substrate purification was performed as previously described (Chavez-Gutierrez et al, 2008). Purity was assessed by SDS–PAGE and Coomassie staining (GelCode reagent, Pierce).

**Figure Legend**

A) γ-Secretase processing of APP-CTF occurs in different consecutive steps. The intracellular domain of APP is released first by an endopeptidase-like activity called ε-cleavage (Kakuda et al., 2006; Matsumura et al., 2014) . This process also releases the Notch intracellular domain and is linked to the signaling function of γ-secretase (De Strooper et al., 1999). The remaining transmembrane part of APP is consequently processed by consecutive carboxypeptidase-like activities which ultimately leads to the release of Aβ peptides, somewhat variable in length but mostly $A\beta_{40}$ (Matsumura et al., 2014).

B) Expression of D257A, R278I, L435F and C410Y mutants in $Psen1/2^{-/-}$ deficient MEFs (dKO) rescue Nicastrin maturation (upper arrow in the NCT row), indicating that all mutants reconstitute γ-secretase complex. Elevated full length PSEN1 steady-state levels and drastic reductions in the levels of N- and C- terminal PSEN1 fragments in the D257A, R278I, C410Y and L435F mutant cell lines, relative to control human PSEN1, indicate that the activation of the protease complex is substantially affected by these substitutions. 12 µg solubilized membrane proteins were loaded per lane. Solubilized membrane proteins prepared from the WT and dKO cell lines were loaded in a 1:10 mix ratio in lane " WT/dKO $^{1/10}$ ".

C) Quantification of $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$ peptide levels in the extracellular media of control or mutant PSEN1 cell lines after 24h post-transduction with mutant APP Swedish ($APP^{NL/KM}$ )(n=3).

D) *De novo* $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$ peptides produced by detergent-extracted membranes from $Psen1/2^{-/-}$ deficient MEFs rescued with wild type or mutant PSEN1 (D257A, R278I, C410Y and L435F) after 11 h incubation with recombinant C99-3XFLAG (n≥3).

E) Quantification of $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$ peptide levels in the extracellular media of MEFs derived from PS1-R278I knock-in mice (WT Psen1, Psen1-R278I$^{+/-}$ and Psen1-R278I$^{+/+}$) after 24h post-transduction with WT APP (n=3). All graphs shown average ± SE.

**Author contributions:** LCG and BDS conceived the study and planned experiments. TS and TS provided critical material and intellectual input. SV performed the experiments. All authors interpreted the data. BDS and LCG wrote the manuscript. All authors read, corrected, and approved the final version.

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