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TMP21 regulates Aβ production but does not affect caspase-3, p53, and neprilysin

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ABSTRACT

The presenilin (PS)-dependent γ-secretase activity refers to a high molecular mass-complex including, besides PS1 or PS2, three other proteins recently identified, namely nicastrin, Aph-1, and Pen-2. This proteolytic complex has been shown to contribute to both γ- and ε-cleavages of the β-amyloid precursor protein (βAPP), thereby generating β-amyloid peptides (Aβ) and the APP intracellular domain (AICD), respectively. TMP21, a member of the p24 cargo protein family, was recently shown to interact with PS complexes. Interestingly, TMP21 modulates γ-secretase-mediated Aβ production but does not regulate ε-secretase-derived AICD formation [F. Chen, H. Hasegawa, G. Schmitt-ulms, T. Kawarai, C. Bohm, T. Katayama, Y. Gu, N. Sanjo, M. Glista, E. Rogaeva, Y. Wakutami, R. Pardossi-Piquard, X. Ruan, A. Tandon, F. Checler, P. Marambaud, K. Hansen, D. Westaway, P. St. George-Hyslop, P. Fraser, TMP21 is a presenilin complex component that modulates γ- but not ε-secretase activities, Nature 440 (2006) 1208–1212]. Here we investigate the functional incidence of the over-expression or depletion of TMP21 on both intracellular and secreted Aβ recoveries and AICD-associated phenotypes. First we confirm that TMP21 depletion yields increased levels of secreted Aβ40. However, we demonstrate that both staurosporine-stimulated caspase-3 activation, p53 and neprilysin expression and activity were not affected by TMP21 over-expression or depletion. Overall, our functional data further reinforce the view that TMP21 behaves as a regulator of γ- but not ε-cleavages generated by PS-dependent γ-secretase complex.

Alzheimer's disease is a neurodegenerative pathology characterized by the cerebral deposition of senile plaques that are basically composed of Aβ peptides [2]. Aβ results from the sequential cleavage of βAPP by β- and γ-secretases that liberate the N- and C-terminal moieties of Aβ peptides, respectively [3]. γ-Secretase activity plays a likely pivotal role in Alzheimer's disease pathology since this proteolytic activity conditions the nature of the C-terminal end of Aβ peptides that can be 40- or 42-amino-acid long. A downstream catalytic event occurring on βAPP, referred to as ε-secretase cleavage, leads to the generation of AICD (APP Intracellular Domain) [4].

There likely exist both presenilin (PS)-dependent and PS-independent γ-secretase activities [5–8]. The former proteolytic activity consists in a multi-protein complex that includes, at least, PS1 or PS2 as well as three other partners, nicastrin, Aph-1, and Pen-2 [9]. One of the questions that remained to be addressed concerns the possibility that γ-and ε-secretase could be either due to distinct proteolytic activities or, alternatively, be differently regulated within the γ-secretase complex. In this context, it is noteworthy that PS mutations, several γ-secretase inhibitors that physically interact with PS as well as various drugs trigger distinct effects on γ- and ε-cleavages [10–13]. More recently, Chen and colleagues demonstrated that TMP21, a protein of the p24 cargo family [14] could modulate the γ-site cleavage of βAPP without affecting the ε-cleavage [1]. This was evidenced by the ability of TMP21 to modulate Aβ production without altering ε-secretase-mediated AICD production [1].

Others and we recently showed that AICD could act as a transcription factor able to modulate a series of proteins including APP, GSK3β, EGFR, neprilysin, and p53 [15–19]. More recently, we documented the fact that AICD could modulate p53-dependent caspase-3 activation [20]. The aim of the present work was to analyze the functional consequence of TMP21 over-expression or depletion on both Aβ production and AICD-mediated phenotypes. We confirm that TMP21 depletion enhances the production of Aβ. However, both TMP21 over-expression and reduction did not modulate staurosporine-associated caspase-3 activation, p53 immunoreactivity, and neprilysin expression and activity. These data confirm that

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TMP21 selectively regulates PS-dependent γ-secretase activity without influencing ε-secretase-derived phenotypes.

Materials and methods

Cells culture and transfection experiments. Stably transfected HEK293 cells expressing wild-type PS2 (wt-PS2) or mutated PS2 (N141I-PS2) and telencephalon murine (TSM1) cell lines were cultured as previously described [21,22]. Transient transfection of empty pcDNA4 vector or pcDNA4 encoding wild-type or human Flag TMP21 (1–2 μg) were carried out into 6-well plates using Lipofectamine-2000 reagent (Invitrogen, Cergy-Pontoise, France) as described previously [23].

Detection of secreted and intracellular Aβ. Stably transfected HEK293 cells expressing Swedish-mutated βAPP [24] were cultured in 6-well plates were transfected with control and TMP21 cDNAs or with TMP21 siRNA as previously described [1]. Twenty-four hours after second transfection, media were changed and replaced with DMEM containing fetal calf serum (1%), in presence of phosphoramidon (10 μM) in order to prevent Aβ degradation. After 8 h, media were collected (secreted Aβ) and cells were rinsed with phosphate-buffered saline and lysed (intracellular Aβ) in 1 ml lysis buffer (Tris–HCl 10 mM, NaCl 150 mM, EDTA 5 mM SDS 0.1%, natrium deoxycholate 0.5%, and NP40 1%). Secreted and intracellular Aβ were immunoprecipitated with FCA3340 [25], electrophoresed on a 16.5% Tris–tricine acrylamide gel and analyzed by Western blotting with 6E10 antibody.

Caspase activity measurements. HEK293 cells expressing either empty vector, wt-PS2 or N141I-PS2 were cultured in 6-well plates, as previously described then transiently transfected with empty pcDNA4 vector or wild-type or Flag-tagged human TMP21 cDNA as described above then incubated for 16–24 h at 37 °C in presence or absence of staurosporine (1–2 μM, Sigma). Cells were then rinsed, gently scraped, pelleted by centrifugation and assayed for their caspase-3-like activity, as extensively detailed [20]. Fluorescence was recorded at 360 and 460 nm for excitation and emission wavelengths, respectively.

Fluorimetric assay of neprilysin activity. Neprilysin (NEP) activity was measured on intact cells with Suc-Ala-Ala-Phe-7AMC (0.2 mM; Sigma, St. Louis, MO, USA) in absence or presence of phosphoramidon, (10 μM, Sigma) as described previously [18]. In transfection experiments with siRNA directed towards TMP21, neprilysin was measured 48 h after the second transfection.

Lactate deshydrogenase assay. HEK293 cells over-expressing Swedish-mutated APP were grown in a 5% CO_2 atmosphere in 12-well plates. At 80% confluency, cells were transfected with empty vector or TMP21 cDNA as described previously above. Twenty-four hours after transfection, media were changed and replaced with DMEM containing fetal calf serum (1%). After 8 h, 40 μl of secretion media were analyzed by means of CytoTox-OneTM homogenous membrane integrity assay (Promega) according to manufacturer's conditions.

Western blot analyses. Equal amounts of protein (50 μg) were separated on 12 and 8% SDS–PAGE gels for the detection of Flag TMP21, endogenous TMP21, p53 or neprilysin, respectively, and wet transferred to Hybond-C (Amersham Pharmacia Life Science) membranes. Membranes were blocked with non-fat milk and incubated overnight with the following primary antibodies: rabbit polyclonal anti-C-terminal of TMP21 (peptide antigen corresponding to residues 211–219 of human TMP21), mouse monoclonal anti-Flag (Flag TMP21, Sigma, St. Quentin-Fallavier, France), mouse monoclonal anti-p53 (Santa Cruz Biotechnology, CA), 18B5 (anti-neprilysin, kind gift from Dr. G. Boileau) and anti-actin (monoclonal antibody, Sigma). Immunoreactive bands were identified with anti-rabbit IgG (Immunotech) or anti-mouse IgG antibodies coupled to peroxidase (Amersham Life Science). Chemi-

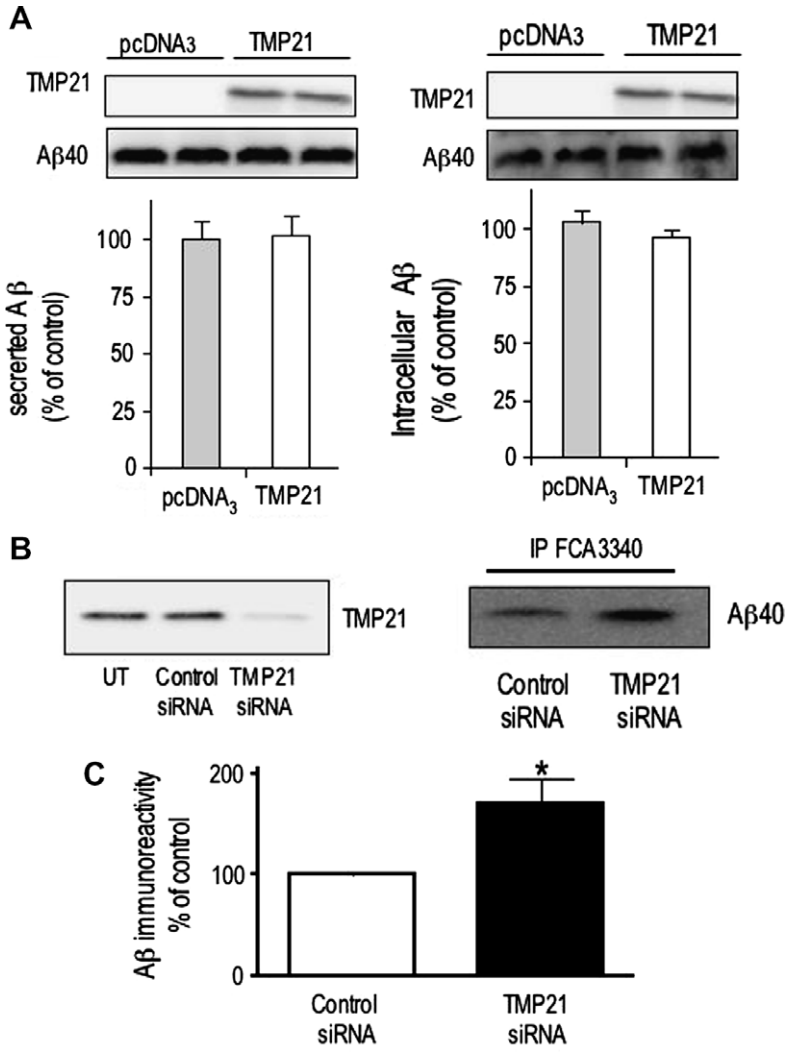

Fig. 1. Influence of TMP21 expression and depletion on Aβ production. Stably transfected HEK293 cells over-expressing βAPP harboring the Swedish mutation were transiently transfected with empty pcDNA3 or TMP21 cDNA (A) or with control (scrambled nonsense) or TMP21 siRNA (B) and analyzed for TMP21 immunoreactivity as described in Methods. Intracellular (A) or secreted (B) Aβ40 was immunoprecipitated with FCA3340 antibody, western-blotted, and analyzed as described in Methods. Note that intracellular Aβ label corresponds to long exposure of the gels. Bars represent the means ± of Aβ40 densitometric analyses of 3–5 independent immunoprecipitation experiments and are expressed as percent of corresponding Aβ40 recovered in control conditions. $^{*}p < 0.05$.

luminescence was recorded using a Luminescence Image Analyzer Las-3000 (Raytest Courbevoie, France) and quantification of captured images was performed using the AIDA Image Analyser software (Raytest).

Statistical analysis. Statistical analyses were performed with PRISM software (Graph-Pad Software, San Diego) by using the Newmans–Keuls multiple comparison test for one-way analysis of variance and *t*-Student test.

Protein concentrations. Protein concentrations were established by the Bio-Rad method [26].

Results and discussion

There is a "biochemical paradox" concerning the theoretical molecular weight of the PS-dependent γ-secretase complex. Thus, functional γ-secretase activity is recovered under immunopurified complexes of at least 650 kDa [27,28] while the arithmetical sum of the four protein members of the complex, based on a 1:1:1:1 reported stoichiometry [29] predicts a 220 kDa molecular weight. Although recent studies indicate that the apparent molecular weights of the active complex could vary according to solubilizing detergents [29], the poor activity recovered when co-expressing the four members of the complex only [9,30,31] lend support to the possibility that additional endogenous regulators could participate in the PS-dependent activity. Very few proteins harboring this potential have been yet described. Among them, TMP21, a member of the p24 cargo family [14], was recently reported as a component of the PS-dependent γ-secretase [1]. Remarkably, TMP21 apparently regulated γ- but not ε-secretase-associated cleavages of βAPP and other substrates. Thus, the depletion of TMP21 drastically enhances Aβ recovery but did not modify the production of ε-secretase-derived ICDs produced from βAPP, Notch, E- and N-cadherins [1].

We have further examined the influence of TMP21 on Aβ40 production and used staurosporine-induced caspase-3 activation and neprilysin activity and expression as functional read outs of ε-secretase-associated phenotypes. First we show that the over-expression of TMP21 in HEK293 cells over-expressing βAPP harboring the Swedish mutation does not influence intracellular and secreted Aβ production (Fig. 1A). However, the reduction of endogenous TMP21 expression enhances the recovery of secreted Aβ. Thus, TMP21 expression was drastically reduced by a siRNA approach and that was accompanied by a statistically significant increase of Aβ40 (Fig. 1B).

ε-Secretase cleavage of βAPP liberates an intracellular fragment called AICD that was shown to trigger a proapoptotic phenotype [32]. We also showed that AICD regulates the transcription of the tumor suppressor p53 and that was associated to increased cell death and caspase-3 activation [20]. As a functional read out of a putative influence of TMP21 on the control of cell death, we examined whether over-expression or down-regulation of TMP21 could modulate staurosporine-induced caspase-3 in HEK293 cells. Transient transfection of HEK293 cells with a cDNA construct encoding tagged-TMP21 leads to the over-expression of a tagged protein (Fig. 2A, upper) which is also labeled with anti-TMP21 antibodies that identifies both tagged and endogenous TMP21 (Fig. 2A, middle) without any sign of cellular toxicity as evidenced by the lack of significant lactate deshydrogenase (LDH) leaking (less than 5% of total LDH) (Fig. 2D). As expected, anti-TMP21 antibodies only label the endogenous protein in mock-transfected cells (Fig. 2A, middle). Clearly, the over-expression of TMP21 does not modify HEK293 cells responsiveness to staurosporine as demonstrated by similar caspase-3 activation regardless of the cell system examined (Fig. 2B). In order to confirm that endogenous TMP21 did not modulate staurosporine-stimulated caspase-3 activation in HEK293 cells, we examined the responsiveness of HEK293 cells in which TMP21 expression was reduced by siRNA targeting. We demonstrate that the reduction of endogenous TMP21 did not influence caspase-3 activation (Fig. 2C).

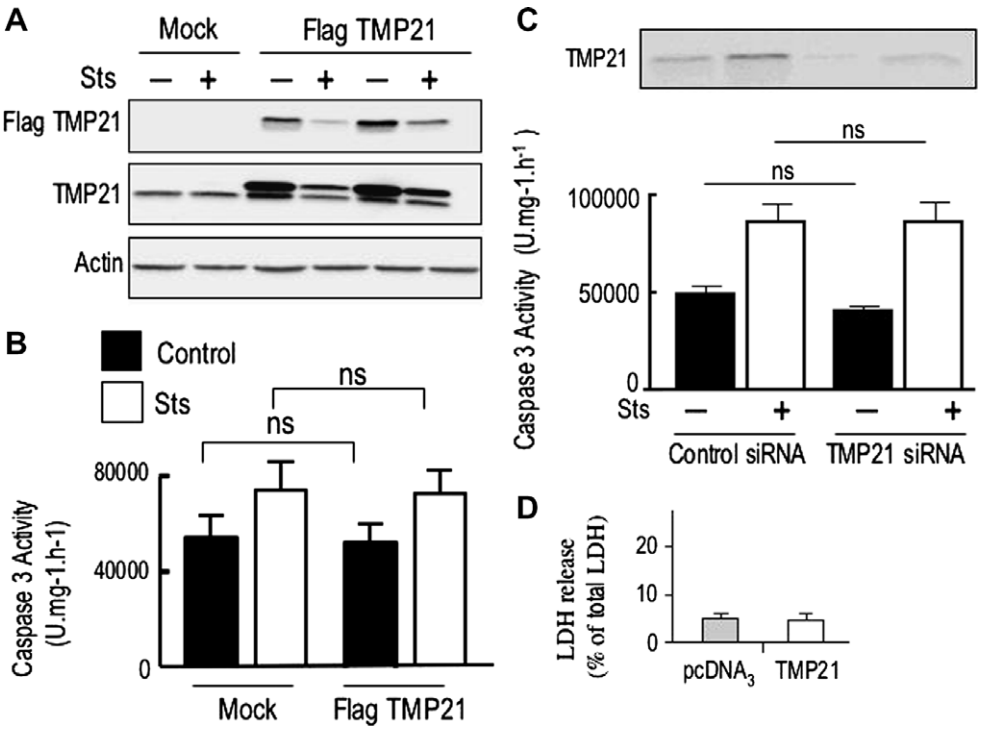

Fig. 2. Over-expression or reduction of TMP21 does not modulate staurosporine-induced caspase-3 activation in HEK293 cells. HEK293 cells were transiently transfected with either empty pcDNA4 vector (mock, A and B), tagged-TMP21 (Flag TMP21, A and B), TMP21 siRNA or control (scrambled nonsense, C) then treated for 24 h (A,B) or overnight (C) without (control) or with staurosporine (STS, 1 μM). Tagged (A) and endogenous (C) TMP21 expression and caspase-3 activity (B,C) were analyzed as described in Methods. Bars are means ± SEM of four independent determinations. NS, not statistically different. (D) Cells were treated as in A then LDH activity was measured as described in Methods. Bars correspond to release LDH expressed as percent of total cellular LDH and are means ± SEM of three independent determinations.

We previously established that HEK293 cells over-expressing wild-type PS2 display exacerbated staurosporine-stimulated caspase-3 activation and that this was accompanied by an enhancement of p53 expression [22]. We demonstrated that this

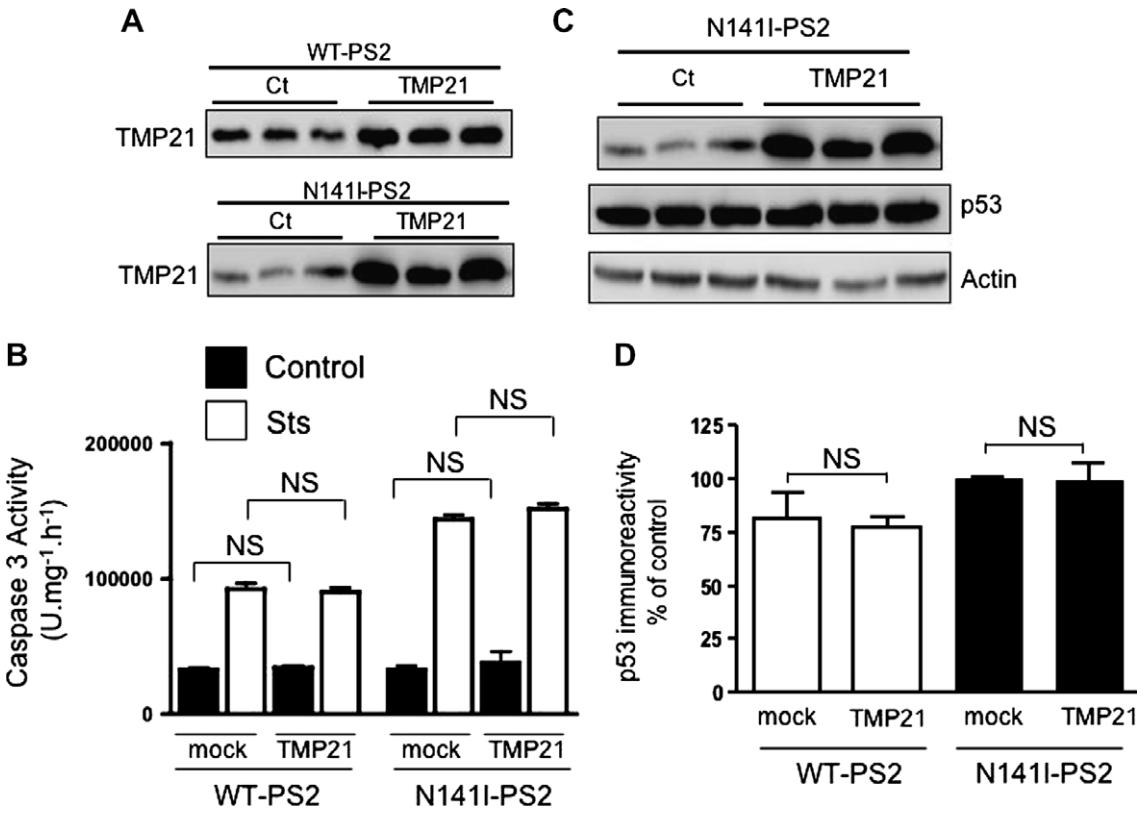

Fig. 3. Over-expression of TMP21 does not modulate staurosporine-induced caspase-3 activation and p53 expression in HEK293 cells over-expressing wild-type or mutated PS2. HEK293 cells expressing either wild-type PS2 or N141I-PS2 were transiently transfected with empty pcDNA4 vector (mock) or pcDNA4 encoding TMP21 (TMP21). Twenty-four hours after transfection, cells were either treated for 16 h without (control) or with staurosporine (STS, 2 μM) and analyzed for their caspase-3 activity (A,B) or examined for their p53 immunoreactivity (C,D) as described in Methods. Bars are means ± SEM of three independent determinations. NS, not statistically different.

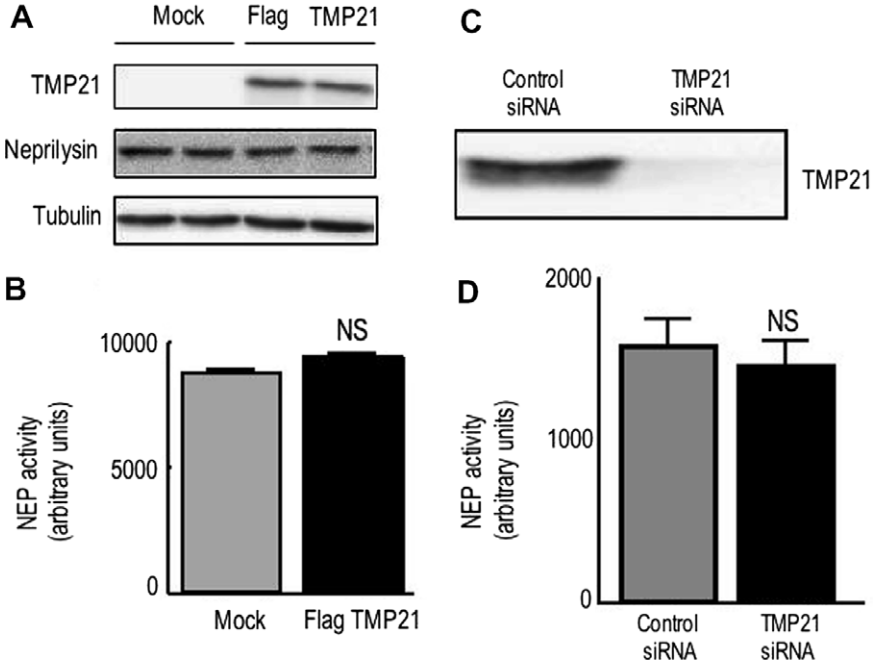

Fig. 4. Over-expression or reduction of TMP21 does not modulate neprilysin expression and activity. HEK293 cells were transiently transfected with empty pcDNA4 vector (mock, A, B), pcDNA4 encoding either tagged-TMP21 (Flag TMP21, A, B), TMP21 siRNA (C,D) or control (scrambled nonsense, C and D) then neprilysin expression (A) activity (B,D) and TMP21 expression (C) were analyzed as described in Methods. Bars are means ± SEM of four independent determinations. NS, not statistically different.

phenotype was potentiated by the pathogenic mutation N141I [22] and mediated by AICD [20]. We show that the over-expression of TMP21 (Fig. 3A and C) does not modify staurosporine-induced caspase-3 activation (Fig. 3B) and p53 expression (Fig. 3C and D) in both wt-PS2 and N141I-PS2-expressing HEK293 cells.

We recently showed that presenilins control the expression of the Aβ-degrading enzyme neprilysin via AICD production [18,33]. We therefore examined whether TMP21 could modulate neprilysin activity and expression. Our study shows that neither the over-expression of TMP21 (Fig. 4A) nor the reduction (Fig. 4D) of endogenous TMP21 affects neprilysin expression (Fig. 4B) and activity (Fig. 4C and E).

The demonstration that βAPP could undergo an epsilon proteolytic cleavage downstream to the canonical γ-site yielding the C-terminus of Aβ40 and Aβ42 has brought another level of complexity in the understanding of βAPP physiopathological maturation but has also raised a set of poorly resolved questions. First, the fact that this cleavage is strikingly reminiscent of the S3 cleavage occurring on Notch [34,35] led to the hypothesis that the intracellular fragment derived from ε-cleavage could harbor transcription factors properties [15]. Although still discussed, a consistent network of data based on AICD nuclear localization and identification of transcriptional targets support the idea that AICD could indeed behaves as a genuine direct or indirect modulator of gene transcription [15–20,33]. As a corollary, several questions concerning the nature of the enzyme involved in γ- and ε-cleavages, the kinetic sequence of these catalytic events and the regulation of these cleavages remained important. Thus, although both γ- and ε-secretase cleavages appear to be dependent upon PS, this by no mean demonstrates that the PS-dependent complex generates both cleavages since any rate-limiting hydrolysis (see [36,37]) controlled by PS would ultimately lead to the absence of subsequent cleavage even if the latter were due to a PS-independent process. In this context, it is interesting to note that γ- and ε-secretase cleavages can be clearly discriminated by PS-directed inhibitors [38,39], as well as by mutations on βAPP and PS [10,11,13,38,40,41]. Furthermore, several compounds were described as able to block Aβ-production without affecting ε-cleavages such as JLK inhibitors [12], non-steroidal anti-inflammatory drugs [42], the Abl kinase inhibitor Gleevec [43] as well as the anti-depressive and GSK3β inhibitor agent lithium [44]. The present study reinforces the previous demonstration that endogenous modulators of secretases could discriminate between γ- and ε-sites. This support the view that therapeutic strategies aimed at selectively blocking γ-secretase-derived Aβ production remains of actuality.

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