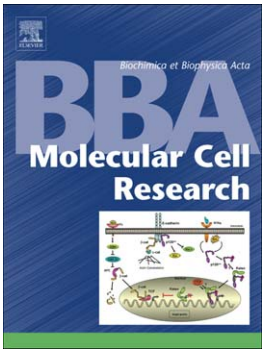

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Tetraspanin 3:

A central endocytic membrane component regulating the expression of ADAM10, presenilin and the amyloid precursor protein

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Abstract

Despite existing knowledge about the role of the A Disintegrin and Metalloproteinase 10 (ADAM10) as the α-secretase involved in the non-amyloidogenic processing of the amyloid precursor protein (APP) and Notch signalling we have only limited information about its regulation. In this study, we have identified ADAM10 interactors using a split ubiquitin yeast two hybrid approach. Tetraspanin 3 (Tspan3), which is highly expressed in the murine brain and elevated in brains of Alzheimer´s disease (AD) patients, was identified and confirmed to bind ADAM10 by co-immunoprecipitation experiments in mammalian cells in complex with APP and the γ-secretase protease presenilin. Tspan3 expression increased the cell surface levels of its interacting partners and was mainly localized in early and late endosomes. In contrast to the previously described ADAM10-binding tetraspanins, Tspan3 did not affect the endoplasmic reticulum to plasma membrane transport of ADAM10. Heterologous Tspan3 expression significantly increased the appearance of carboxy-terminal cleavage products of ADAM10 and APP, whereas N-cadherin ectodomain shedding appeared unaffected. Inhibiting the endocytosis of Tspan3 by mutating a critical cytoplasmic tyrosine-based internalization motif led to increased surface expression of APP and ADAM10. After its downregulation in neuroblastoma cells and in brains of Tspan3-deficient mice, ADAM10 and APP levels appeared unaltered possibly due to a compensatory increase in the expression of Tspans 5 and 7, respectively. In conclusion, our data suggest that Tspan3 acts in concert with other tetraspanins as a stabilizing factor of active ADAM10, APP and the γ-secretase complex at the plasma membrane and within the endocytic pathway.

Introduction

Tetraspanins were discovered in the early 90s as proteins which span the membrane four times. Today, the 33 known mammalian members of the tetraspanin family are involved in diverse biological processes including the regulation of immune responses, infection with pathogens and cancer progression [1]. As glycosylated and palmitoylated proteins, tetraspanins interact with each other and with many transmembrane proteins including integrins, cell adhesion proteins, growth factor and cytokine receptors and membrane embedded enzymes [2-5]. Such an interaction network of tetraspanins and transmembrane proteins was previously referred to as the "tetraspanin web" [2]. The transmembrane domains and the large extracellular loop of tetraspanins as well as the surrounding lipid composition contribute to these protein interactions. Most likely, different tetraspanins act in concert in a tissue-specific context in the regulation of the stability and intracellular trafficking of membrane proteins. This affects the steady state level and localization of tetraspanin partner proteins in a given cellular or membrane compartment [1].

Members of the ADAM (A Disintegrin And Metalloproteinase) family which can mediate the proteolytic processing of membrane proteins referred to as ectodomain shedding [6] were also found to interact with certain tetraspanins. In particular, the interaction of tetraspanins with ADAM10 has raised attention due to its proteolytic function as an amyloid precursor protein (APP) α-secretase, as the sheddase of the cellular prion protein (PrP^c) and as a Notch1 receptor site 2 proteinase [7-10] was found to interact with members of the tetraspanin superfamily. A subfamily of tetraspanins, TspanC8, with eight cysteines in the large extracellular domain, appeared to have high affinity to ADAM10. Tspan5, 10, 14, 15, 17 and 33 interact with ADAM10 and regulate its exit from the ER and transport to the plasma membrane where ectodomain shedding occurs [11-14]. Overexpression or RNAi-mediated downregulation of the TspanC8s caused changes in the maturation process and stabilized the surface expression of ADAM10 [12]. The interaction between different TspanC8 proteins and ADAM10 starts in the ER [12] and mainly involves the large

extracellular loop at the side of the TspanC8s and the membrane-proximal domain as well as the disintegrin domain in the ADAM10 protein [15]. The interaction of ADAM10 with different TspanC8s may differentially affect the shedding of ADAM10 substrates including Notch1 [16]. It is of note that the TspanC8 family was also recently implicated in a possible functional clustering of the γ-secretase complex and the APP α-secretase, ADAM10 [17].

Tetraspanin-3 (Tspan3) is another member of the tetraspanin superfamily but only contains six cysteines in the large luminal domain. It was shown to associate with claudin-11 and beta1 integrin thereby regulating the proliferation and migration of oligodendrocytes [18,19]. Tspan3 is expressed in all stages in oligodendrocyte development but is most prominently expressed in neurons and astrocytes [19]. In brain, Tspan3 associates with the Nogo-A receptor sphingosine-1-phosphate receptor 2 (S1PR2). Tspan3 knockdown reduced the Nogo-A-induced S1PR2 clustering, cell spreading and the inhibition of neurite outgrowth [20]. In *Xenopus leavis* embryos Tspan3 is expressed in migratory neural crest cells [21]. Tspan3 was also found to be a causative factor in the development of acute myelogenous leukemia (AML) and deficiency of Tspan3 led to an impaired stem cell-renewal and disease propagation [22].

Using a yeast split-ubiquitin based screening system we identified Tspan3 as a binding partner of ADAM10. This interaction did not affect the trafficking or maturation of the protease or its ability to cleave the neuronal cadherin (N-cadherin). However, cell-based experiments revealed that Tspan3 affected the stabilization of ADAM10 and APP at the cell surface. Our experiments suggest that Tspan3 is part of an interaction scaffold that associates with APP and α- and γ-secretases at the plasma membrane and within the endocytic pathway.

Material and Methods

Antibodies

The following antibodies were used: anti-ADAM10 antibody EPR5622 (IB, Abcam, Cambridge, UK previously GenTex, Irvine, USA), anti-ADAM10 antibody raised against a the C-terminus of ADAM10 (IP, [12]), MAB946 anti-ADAM10 ectodomain (FACS, R&D Systems, Minneapolis, USA), anti-ADAM17 antibody A300D [23], anti-actin (IB, Sigma-Aldrich, Munich, Gemany), B63.2 anti-APP C-terminus (IB, a kind gift of Wim Annaert, Leuven, Belgium), anti-EEA1 (IF, Cell Signaling Technology, Danvers, USA), FL-335 anti-GAPDH (Santa Cruz Biotechnology, Dallas, USA), 9B11 anti-myc (IB and IF, Cell Signaling Technology, Danvers, USA), anti-myc (IP, GTX30518, GenetTex, Irvine, USA), anti-N-Cadherin (BD Transduction Laboratories, Heidelberg, Germany), anti-PDI (A6) (Abcam, Cambridge, UK), MAB 5232 anti-Presenilin-1 C-terminal loop (IB, Merck Millipore, Darmstadt, Germany), MAB1563 anti-Presenilin-1 N-terminal (IB, Merck Millipore, Darmstadt, Germany), L2T2 anti-LIMP-2 (IF, IB [24]), anti-PDI (IF, Santa Cruz Biotechnology, Heidelberg, Germany), POM1 anti-PrP^c (IB, Prof. Dr. A. Aguzzi, Zürich, Switzerland), anti-Rab5 (IF, Synaptic Systems, Göttingen, Germany), H4B4 anti-LAMP2 (IF, DSHB, Iowa Ciy, USA), anti-E7 beta-Tubulin (DSHB Iowa Ciy, USA), anti-Tspan3 (IB, IP, IF, IHC, antibody produced by Pineda, Berlin, Germany against a synthetic peptide corresponding to the last 19 amino acids of the C-terminus of mouse Tspan3).

Split-Ubiquitin Membrane Yeast Two Hybrid Screening

The split-ubiquitin yeast two hybrid screen was performed as already described [Prox et al, 2012]. Briefly, cDNA of murine ADAM10 was cloned into a yeast expression vector (pTMBV4) and C-terminally fused to a Cub-LexA-VP16 cassette. Expression of the ADAM10 bait construct was monitored using positive (NubI) and negative (NubG) controls as supplied by the manufacturer. ADAM10 bait construct was co-transfected with N-terminally NubG-tagged murine cDNA brain library (MoBiTec) into NMY51 yeast and seeded on selective media plates (-Leu, -Trp, -His). After

incubation for 3-5 days at 30°C clones were selected and expanded. Prey plasmids were extracted and analyzed by sequencing (GATC).

Cell culture, transfection and plasmids

All cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, USA) with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C, 5% CO_2 atmosphere and 95% relative humidity. HEK293 and N2A cells stably overexpressing APP695 or APP695swe (Swedish mutant provided by Michael Willem, DZNE, Munich) were additionally supplemented with 200 µg/ml of the antibiotic G418. HEK293 cells deficient for ADAM10 (A10-/-) or ADAM17 (A17-/-)were generated by using the CRISPR/Cas9 system. The cells were transiently transfected with Polyethylenimin Max (PEI, Polysciences, Hirschberg, Germany). For inhibition of ADAM10 cell culture medium was supplemented with 10 µM of the hydroxamate-based inhibitor GI254023X (Iris Biotech, Marktredwitz, Germany) for 12 h. Mammalian expression vectors were pCI-neo (human APP695), pcDNA3.1Hygro (ADAM10) and pFrog3 (murine Tspan3-myc and Tspan15-myc) derived from pcDNA3 (Thermo Fisher Scientific, Waltham, USA) [25]. The myc-epitope was inserted after the last amino acid of Tspan3 or Tspan15, respectively. The myc-tagged murine Tspan7 expression plasmid was purchased from OriGene Technologies Rockville, USA (MR221859).

Generation of the Tspan3$_{Y243L}$ mutant

Tspan3$_{Y243L}$ was generated by mutating the cDNA sequence of myc-tagged wild type Tspan3. cDNA sequence coding for the amino acid Tyrosine (Y) at position 243 was exchanged by the sequence coding for Leucine (L). Myc-tagged Tspan3$_{Y243L}$ was again subcloned into the pFrog3 vector. The following primers were used:

5‘-CAGTGATGAGGAGCTCAGCGGCAGGATCTCTACTCC-3’; 5’-GGAGTAGAGATCCTGC CGCTGAGCTCCTCATCACTG-3’

Protein extraction from cells and organs

Cells were washed three times with cold PBS, scraped off in 1 mL PBS plus 0.04% proteinase inhibitor cocktail (Complete™, Roche, Diagnostics, Mannheim, Germany) and centrifuged for 20 min at 4000 g. The cell pellet was resuspended in lysis buffer (1 mM EGTA, 5 mM Tris, 250 mM Saccharose, pH 7,4) containing 1% Triton X-100 and 0.04% proteinase inhibitor cocktail. Following lysis, cells were sonicated two times for 15 sec at 60 Hz, incubated for 1 h on ice and sonicated again. Cell lysates were centrifuged for 10 min at 14,000 g at 4°C to remove cellular debris and nuclei.

For protein extraction from organs, tissue was homogenized in RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 2.5 mM sodium deoxocholate, 0.04% proteinase inhibitor cocktail) using the Precellys homogenization system (Bertin Corp., Rockville, USA). Thereafter, homogenates were sonicated two times for 15 sec at 60 Hz, incubated on ice for 1 h and centrifuged for 10 min at 14,000 g at 4°C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). Finally, lysates were prepared for SDS-PAGE by incubation with reducing SDS-PAGE loading buffer (625 mM Tris base, 10% SDS, 50% glycerol, 250 mM DTT) and boiling for 5 min at 95°C.

Peptide N-Glycosidase F (PNGase F) digestion

Mouse brain homogenates were prepared as described above. After homogenization, equal amounts of total protein were incubated for 5 min at 95°C with 5x denaturing buffer (250 mM Na_2PO_4, pH 8.0, 1% SDS, 4% β-mercaptoethanol). After denaturation 5x Triton solution (7.5% Triton X-100, 50 mM EDTA) and proteinase inhibitor cocktail were added. The samples were incubated with PNGase F (Roche, Diagnostics, Mannheim, Germany) or ddH_2O for 2 h at 37°C and prepared for SDS-PAGE.

SDS-PAGE and immunoblotting

Equal amounts of extracted proteins were loaded on 10% SDS-PAGE or 4-12% gradient BIS/Tris NuPAGE™ gels (Thermo Fisher Scientific, Waltham, USA) and separated for 1.5 . Thereafter, proteins were transferred to nitrocellulose membranes (Whatman, GE Healthcare, Little Chalfont, UK) by tank blotting for two hours at 0.8 A and 4°C. For the detection of APP C-terminal fragments blotting was performed at 0.25 mA for two hours at 4° C. To prevent unspecific antibody binding the membranes were incubated with 5% milk solution in TBS plus 0.1% Tween (TBS-T). The membranes were incubated over night with primary antibodies diluted in 5% milk solution at 4°C. After three washes in TBS-T, immunoblots were incubated with peroxidase-coupled secondary antibodies for 1 h at room temperature. Membranes were washed again and developed using the Lumigen ECL Ultra detection solution (Lumigen Inc., Southfield, USA) and the chemiluminescence detection system LAS4000 (GE Healthcare, Little Chalfont, UK). Signal intensities were quantified with the quantification software ImageJ and statistically analyzed.

Co-Immunoprecipitation experiments

Cells or brain tissues were lysed with immunoprecipitation buffer (120 mM NaCl, 50 mM Tris-HCl, 0.04% complete proteinase inhibitor cocktail, pH 7.4) containing 0.5% NP-40 (Sigma-Aldrich, Munich, Gemany) as described above. 60 µL of lysates were removed as input controls. Residual lysate amounts were incubated with primary antibodies over night at 4°C under continuous rotation. 50 µL of Protein G coupled Dynabeads (Thermo Fisher Scientific, Waltham, USA) were washed with lysis buffer and incubated with SEA-blocking buffer (Thermo Fisher Scientific, Waltham, 1:1 diluted with lysis buffer) over night at 4°C to reduce unspecific binding of proteins. Next day, Dynabeads were added to the lysate-antibody complexes and incubated for 30 min at room temperature. Beads were washed three times with lysis buffer and incubated with 50 µL of reducing SDS-PAGE loading buffer for 20 min at 65°C to elute precipitated proteins. Input controls and immunopreciptates (IP) were then loaded on 10% SDS-PAGE or 4-12% gradient BIS/Tris

NuPAGE™ gels (Thermo Fisher Scientific, Waltham, USA) and respective amounts were determined by immunoblotting.

Surface Biotinylation

Biotinylation of surface proteins was performed with the biotinylation reagent EZ-Link™ Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific, Waltham, USA). All steps were done on ice (4 °C) to minimize endocytosis of cell surface proteins. Cells were washed three times with ice-cold PBS-CM (0.1 mM $CaCl_2$, 1 mM $MgCl_2$ in PBS) and incubated with 3 ml biotin solution (1 mg/ml in PBS-CM) (+biotin) or PBS-CM (-biotin) for 30 min. Unreacted Sulfo-NHS-SS-Biotin was removed by incubation with quenching buffer (50 mM Tris-HCl/PBS-CM, pH 8.0) for 10 min. Cells were washed again three times with PBS-CM, harvested and lysed with biotinylation lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X100, 0.04% proteinase inhibitor cocktail) as described above. 50 µL of the total cell lysates were removed as expression control (total) and prepared for SDS-PAGE. Equal amounts of residual lysates were incubated with 70 µL of washed Pierce™ Streptavidin Agarose beads (Thermo Fisher Scientific, Waltham, USA) for 1 hour at 4°C and continuous rotation. Afterwards, beads were washed five times with lysis buffer and incubated with reducing SDS-PAGE loading buffer for 20 min at 65°C to elute bound proteins. Finally, samples were prepared for SDS-PAGE and immunoblotting.

Flow Cytometry

Cells were washed with cold PBS and incubated with 2 ml Accutase (PAA Laboratories, Dartmouth, USA) for 5 min at 37°C to detach cells from cell culture dishes. Cells were counted and $5x10^5$ cells per sample incubated with anti-ADAM10 antibody (MAB946, 1:100) for 1 h at 4°C. Cells were washed with MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA) and incubated with an IgG2b goat anti-rat Alexa-488 labeled secondary antibody for 1 h at 4 C. Cells only incubated with secondary antibody were used as negative control. After another washing step cells

were analyzed by flow cytometry using the BD FACS Canto™ II system (BD Biosciences, Heidelberg, Germany).

Immunofluorescence analysis

HeLa cells were grown on coverslips in six-well cell culture dishes. 48 h past transfection cells were washed three times with ice-cold PBS and fixed for 20 min with 4% (w/v) paraformaldehyde in PBS. Cells were permeabilized with 0.2% saponin in PBS, followed by 10 min incubation with 0.2% saponin, 0.12% glycine in PBS. Afterwards cells were incubated for 1 h with blocking solution (10% (v/v) FCS, 0.2% saponin, PBS). Blocking solution was also used as diluent for primary and secondary antibodies. Coverslips were incubated with 50 µL antibody solution over night at 4 °C. After five times of washing with 0.2% saponin in PBS cells were incubated for 1 h with Alexa®-488 and -594 labeled secondary antibodies. Again, cells were washed five times with washing buffer plus two additional washing steps in ddH_2O. Coverslips were mounted with DAPCO/mowiol containing 0.1 µg/mL DAPI (4-,6-diamidino-2-phenylindole; Sigma-Aldrich, Munich, Gemany) for nucleus staining. Confocal pictures were acquired with an Olympus FV1000 confocal laser scanning microscope.

siRNA-based knockdown experiments

Cells were seeded in 6 cm cell culture dish plates. According to the manufacturer's instructions cells were transfected with 5 nM of human Tspan3 (ON-TARGETplus® smart pool #10099 siRNA, GE Healthcare Dharmacon, Chicago, USA), murine ADAM10 siRNA (Stealth RNAi™ siRNA #1320001, Thermo Fisher Scientific, Waltham, USA) or respective non-targeting control siRNA using INTERFERin® (PolyPlus, Illkirch-Graffenstade, France). The Procedure was repeated after 48 h and cells were harvested additional 24 h later. For the analysis of the effect of Tspan3 expression on APP processing, cells were additionally transfected with the Tspan3-myc expression construct as described above.

Quantitative RT-PCR

RNA was extracted from cells or brain tissue using the NucleoSpin® RNA Kit (Macherey Nagel, Düren, Germany). 1 µg of extracted RNA was reverse transcribed with the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) using a Random Hexamer primer according to the manufacturers protocol. Gene transcription was determined by real-time PCR analysis of 0.5 µL cDNA on a LightCycler® 480 Real-time PCR System (Roche, Diagnostics, Mannheim, Germany) in 10 µL reaction volume in triplicates. Relative gene transcription was calculated by using ΔCt values normalized to the transcription level of a housekeeping gene. The actual PCR efficiency of each assay was determined by serial dilution of standards and these values were used for calculation. T-tests were performed on the mean ΔCt values of the technical triplicate measurements.

Generation of Tspan3 mutant mice

Tspan3 knockout mice were created by transcription activator-like effector nucleases (TALEN) mediated genome editing. The TALEN were designed to target exon2 of the Tspan3 gene. The following TALEN-repeat domain sequences were used. Left TALEN: NN NG NN NG NI HD NI HD NN HD NG HD NG NG HD HD HD NG NN HD, right TALEN: NN NI NI NI NI NN HD NI NI NN NN HD NG HD HD NG NI HD NI. The Tspan3-TALEN mRNA was co-injected with a single stranded synthetic oligodeoxynucleotide, bearing three stop codons and one XbaI restriction site, into zygotes and transferred into mice, ssOligodeoxynucleotide sequence: 5'-ACGACTATGACCACTTCTTCGAGGATGTGTACACGCTCTAACCTGCCGTGGTGATAG CATAGTAGTATGAGTCTAGATTTTCATCATTGGCCTGATCGGCTGCTGTGC-3'. TALEN RNA precursors were generated and microinjected into male nucleoli of zygotes isolated from C57BL/6N mice as described elsewhere [26]. Founder mice were genotyped for TALEN-mediated frameshift mutations. Resulting from this, mice with a 26 bp insertion mutation inducing a

translational stop codon within exon2 of the Tspan3 gene were used for further breeding and analysis.

Animal experiments

All animal works were approved by the Animal welfare committee of the Christian Albrechts-University of Kiel (Germany) and were performed according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Bioinformatics and Statistical analysis

Sequence alignment of amino acid sequences of human TspanC8 and TspanC6 tetraspanins was performed with the multiple sequence alignment tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Statistical significances were tested with unpaired Student's t-tests or one-way ANOVA followed by Bonferroni's multiple compariosons test using statistical analysis software GraphPad Prism. Error bars indicate the mean +/- standard deviation (SD). Statistical significance was considered as p-values:* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Results

ADAM10-Tspan3 interaction in yeast and mammalian cells

We have previously identified Tspan15 as an ADAM10 interacting protein using a split-ubiquitin yeast screening assay [12] (Fig.1A(I)). Applying the same approach using mouse brain cDNAs encoding for N-terminal tagged proteins as prey and mADAM10 as bait, we found Tspan3 as an

additional ADAM10 interactor. Since yeast cells expressing both mouse ADAM10 and Tspan3 or Tspan15 as a positive control grew on tryptophan/leucine/histidine-deficient media plates we concluded a close proximity of bait and prey since only then ubiquitin was reconstituted and the histidine 3 gene was transcriptionally activated (Fig. 1A). In contrast to Tspan15 belonging to a subfamily of tetraspanins characterized by the presence of eight cysteines (TspanC8) in their large extracellular loop Tspan3 is evolutionary and structurally different with only six cysteines (TspanC6) in the extracellular domain (Fig. 1B). By analysing the expression of Tspan3 in different mouse tissues we found it most abundantly expressed in brain and intestine with hardly any expression in lung, liver and bone marrow (Fig. 1C). Deglycosylation experiments revealed that Tspan3 is glycosylated and the degree of glycosylation differs between different tissues (Fig. 1C,D). Interestingly, by analysing post mortem human cortex samples (overview in Suppl. Fig.S1) we found a twofold increased expression of Tspan3 in brains of AD patients compared to age-matched control samples (Fig. 1E). Tspan3 expression was predominantly found in murine neurons of the frontal cortex, the hippocampus and in Purkinje cells of the cerebellum (Suppl. Fig. S2).

To also validate the interaction of Tspan3 and ADAM10 in mammalian cells we expressed a C-terminally myc-tagged mouse Tspan3 and wild type mouse ADAM10 in HeLa cells and co-precipitated both proteins with anti-myc and anti-ADAM10 antibodies, respectively (Fig. 1F). Tspan3 could be found in the precipitate after the pulldown of overexpressed and endogenous ADAM10 (Fig. 1F(I)). Vice versa, ADAM10 was found in the precipitate after Tspan3 pulldown (Fig. 1F(II)). As a positive control we included the co-expression of Tspan15 and ADAM10 revealing the established association [12] between both proteins (Fig.1F). As negative controls immunoprecipitations of cells were included which were transfected with constructs expressing ADAM10 alone or the structurally related ADAM17 protease. We only found co-precipitation of Tspan3 with ADAM10 and not with ADAM17 (Suppl. Fig. S3). To also confirm the interaction between ADAM10 and Tspan3 at the endogenous level we used mouse brain homogenates from

wild type and Tspan3 knockout mice and precipitated Tspan3 (Fig.1G). We found Tpsan3 in the precipitates from wild type brain but not in the knockout precipitate nor in the antibody or bead control samples. Importantly, ADAM10 as well as full length APP (fl-APP) and the APP carboxy-terminal fragment (APP-CTF) was only found in the precipitate from wild type brain. These experiments demonstrate that Tspan3 is an endogenous relevant interaction partner of ADAM10 and APP.

Our data suggest that Tspan3 is a tetraspanin member preferentially expressed in the central nervous system with an apparently higher expression in AD brains. Studies in yeast and mammalian cells suggest a specific interaction of Tspan3 with ADAM10 (α-secretase) and APP.

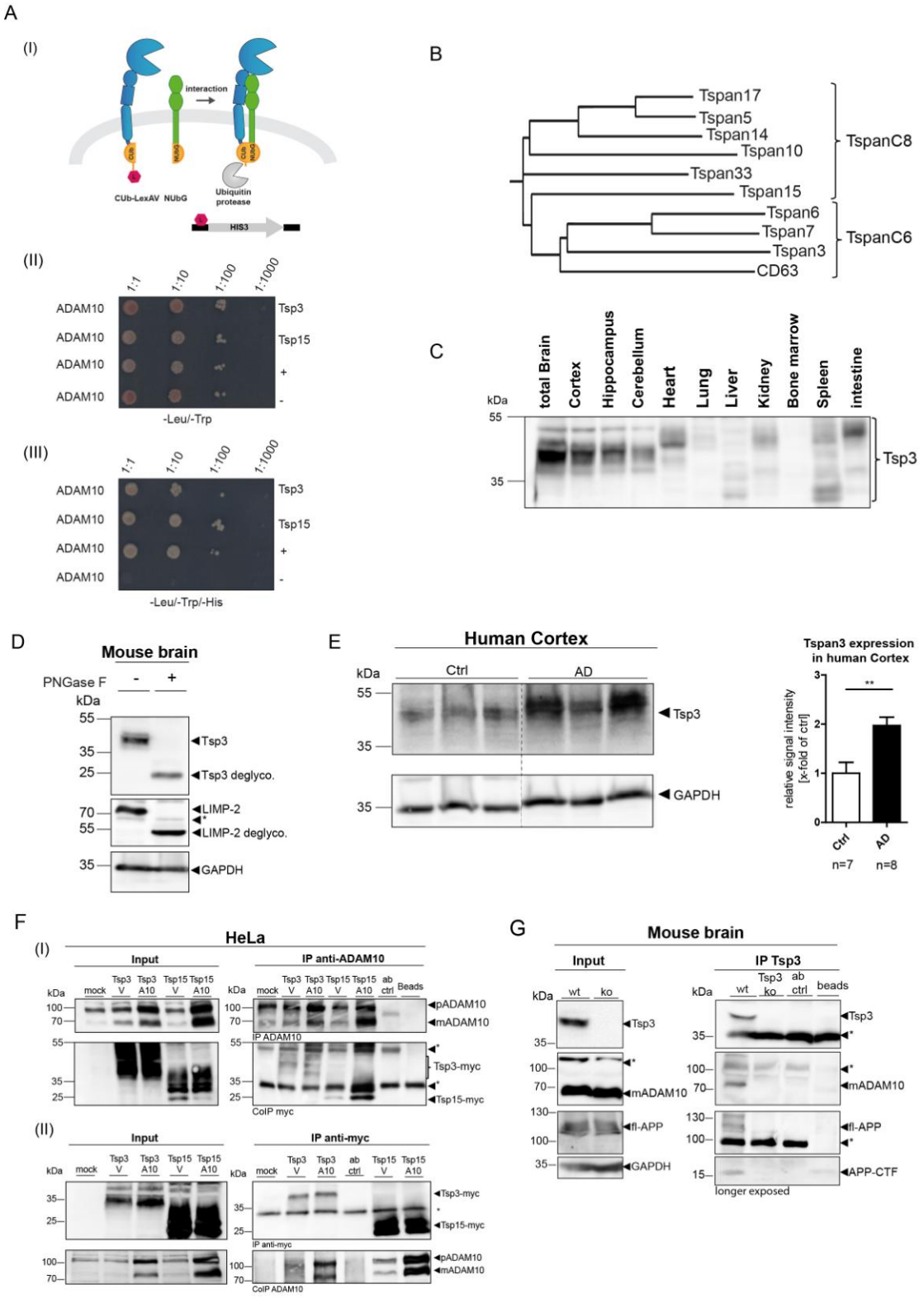


Figure 1: Tspan3 is a new ADAM10 interaction partner. (**A)** (I) The principle of the Split-Ubiquitin yeast two Hybrid System is depicted (modified from [27]). ADAM10 is fused to the C-terminal part of ubiquitin (Cub) and an artificial transcription factor LexA (LexA-VP16) expressed in yeast together with N-terminal NubG tagged proteins from a murine brain cDNA library. The close proximity between the ADAM10 bait protein and an interaction partner leads to the reconstituion of ubiquitin. Cellular ubiquitin proteases release the transcription factor LexA, which activates HIS3 gene transcription and allows yeast clones to grow on selective media plates lacking histidin (His). (II-III) ADAM10 bait protein was co-expressed with the identified Tspan3 (Tsp3)

and Tspan15 (Tsp15) prey proteins and controls in yeast. Expression of bait and prey proteins is verified by growth of yeast clones on Leu/Trp-deficient media and the interaction of ADAM10 and Tspan3 is monitored on Leu/Trp/His-lacking media plates in comparison to controls ("+" and "-"). (**B)** Tspan3 belongs to the tetraspanins characterized by six cysteines in the large extracellular loop and is evolutionary distinct from the group of tetraspanins with eight cysteines (TspanC8 which e.g. includes Tspan15). **(C)** Immunoblot analysis of murine tissues reveals prominent Tspan3 expression in the central nervous system. Tspan3 is differentially glycosylated in different tissues. **(D)** Tspan3 is a glycosylated protein. PNGase F digestion of murine brain homogenates decreases the molecular weight of Tspan3 from 40 kDa in untreated (-) to a molecular weight of 25 kDa in PNGase F treated (+) samples. Successful deglycosylation by PNGase F digestion is verified by the decrease of the molecular weight of the lysosomal integral membrane protein-2 (LIMP-2), which is a highly glycosylated protein. **(E)** Upregulation of Tspan3 expression in post-mortem prefrontal cortex samples (more information in Suppl. Fig. S1) of control and Alzheimer`s disease (AD) patients as revealed by immunoblot of the tissue samples and quantification. **(F)** Mammalian expression constructs of murine ADAM10, murine Tspan3-myc and Tspan15-myc were transiently co-expressed in HeLa cells. (I) Tspan3-myc (40 kDa) and Tspan15-myc (25 kDa) were precipitated (IP) using an anti-myc antibody. Co-precipitation (CoIP) of ADAM10 was detected with an anti-ADAM10 antibody, pro (p) ADAM10, 95 kDa, mature (m) ADAM10, 75 kDa. (II) ADAM10 was precipitated and co-precipitation of Tspan3- and Tspan15-myc was detected. **(G)** Tspan3 was precipitated from murine brain homogenates (wt: wild type; Tspan3 ko: Tspan3 knockout) using a C-terminal specific anti-Tspan3 antibody and co-precipitation of mADAM10, fl-APP and APP-CTF was observed. Abbreviations: murine ADAM10 (A10), murine Tspan3-myc (Tsp3), murine Tspan15-myc (Tsp15), empty vector (V), full length (fl), C-terminal fragment (CTF), antibody control (ab ctrl), asterisk marks immunoglobulin signals.

Tspan3 does not affect the maturation and surface expression of ADAM10 as compared to Tspan15

To address the question of whether the interaction between ADAM10 and Tspan3 has a similar impact on the function of ADAM10 as described for Tspan15 which regulated the ER exit and the maturation of ADAM10 [12] we expressed C-terminally myc-tagged Tspan3 or Tspan15 in HeLa cells (Fig. 2A(I)) and in the murine neuroblastoma cell line N2A (Fig. 2A,(II)), respectively. As described previously, overexpression of Tspan15 led to an increase in the mature form of endogenous ADAM10 [12]. In contrast, despite its considerable overexpression, Tspan3 did not alter the ratio between the pro- and mature forms of the protease in both cell types (Fig. 2A). An interesting observation is that the carboxy-terminal fragments of ADAM10 (ADAM-CTFs of about 15 kDa), which are proposed to be generated by ADAM9 and ADAM15-mediated shedding followed by γ-secretase cleavage [28] or by autocatalysis [29], were markedly increased upon Tspan3 expression in both cell types (Fig. 2A). Tspan3 overexpression in murine neuroblastoma

N2A cells mainly increased the possible intracellular domain 15 kDa CTF of ADAM10 which is most likely a product of γ-secretase processing (Fig. 2A,(II)).

To analyse a possible change in the surface expression of ADAM10, we analyzed N2A cells using surface biotinylation (Fig. 2B(I)) and fluorescence activated cell sorting (Fig. 2B(II)). Tspan15 led to a considerable increase in the surface levels of ADAM10 but Tspan3 did not affect the amount of ADAM10 at the surface (Fig. 2B). Taken together, our data suggests that Tspan3 modulates the ectodomain shedding and intramembrane proteolysis of ADAM10 without affecting the maturation of the protease and its delivery to the cell surface.

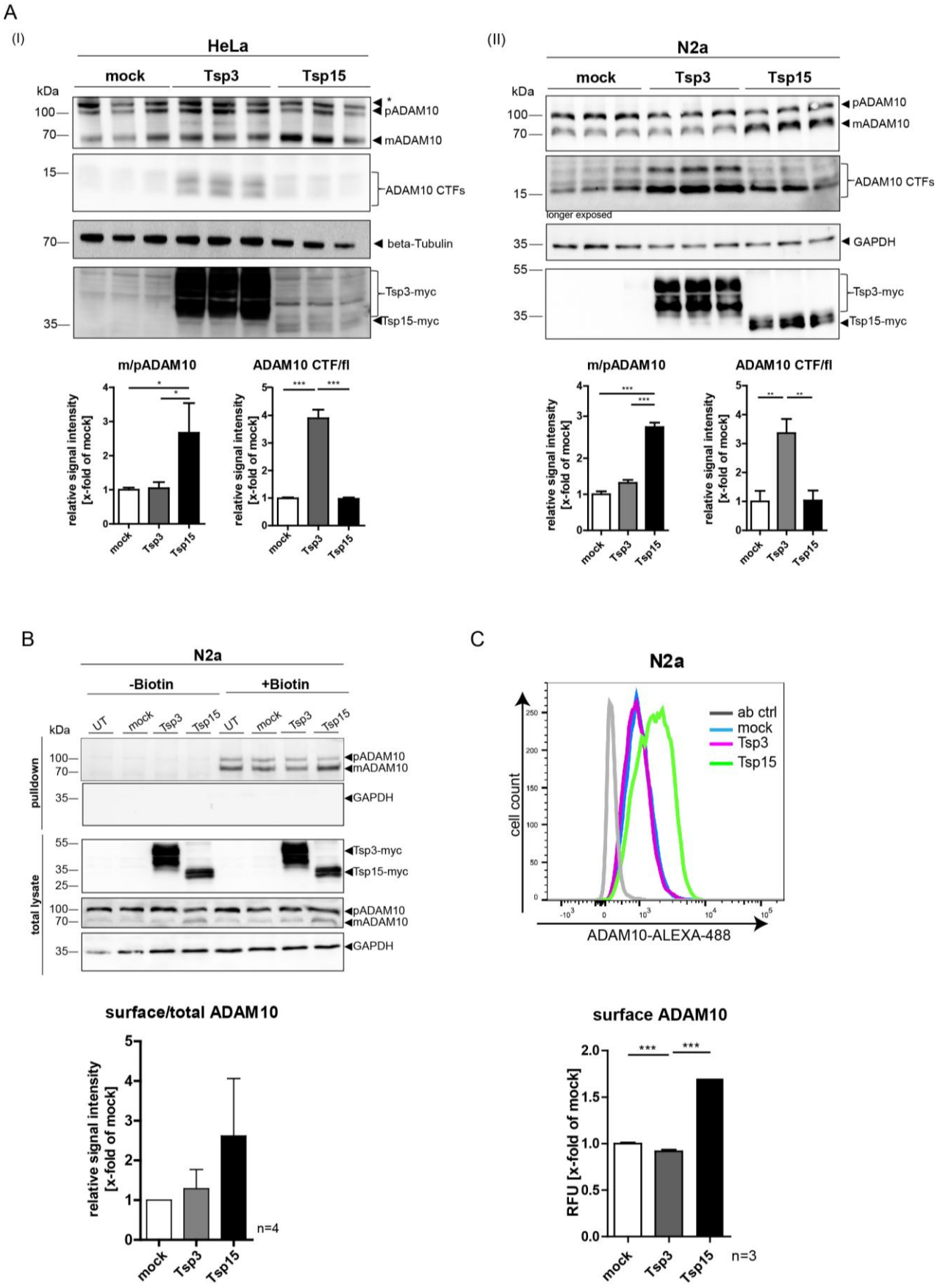


Figure 2: Tspan3 expression accelerates ADAM10 CTF generation without increasing ADAM10 surface levels. (A) Murine Tspan3-myc (Tsp3) and Tspan15-myc (Tsp15) expressed in HeLa cells (I). After immunoblotting ADAM10 was detected using an ADAM10-specific C-

terminal antibody and Tspan3-myc or Tspan15-myc were detected using an anti-myc antibody. Carboxy-terminal fragments (CTFs) of ADAM10 are increased 3.5 fold after Tspan3 expression. No change in the mature (m) form of ADAM10 was observed after Tspan3 but after Tspan15 expression. (II) Murine Tspan3-myc and Tspan15-myc expressed in murine N2A neuroblastoma cells. The 15 kDa ADAM10 CTF is predominantly increased after Tspan3 expression. Tspan15 shifted the ADAM10 proform (p) to the mature form (m); Tspan3 did not show such an effect. **(B)** Surface biotinylation and **(C)** fluorescence activated cell sorting (FACS) analysis confirmed a higher level of surface ADAM10 in N2A cells after expression of Tspan15 but not after Tspan3 expression. N2A cells were biotinylated after transfection with either an empty vector (mock), murine Tspan3-myc or Tspan15-myc. Following cell lysis total protein samples were taken and biotin-labeled proteins precipitated (pulldown). After immunoblotting Tspan3-myc, Tspan15-myc, ADAM10 and GAPDH as a negative control were detected. Abbreviations: Ab: antibody control; UT: untransfected. A one-way ANOVA statistical analysis followed by Bonferroni's multiple comparisons test was performed (*, p<0,05, **, p<0,01***, p<0,001).

Tspan3 expression affects ADAM10-mediated APP carboxy-terminal C83 fragment generation

Although Tspan3 overexpression did not overtly affect the cellular localization of ADAM10, we also determined what effect it would have on ADAM10 activity. To do this we analyzed its impact on ADAM10-dependent generation of membrane-bound APP carboxy-terminal fragment (APP-CTF) as well as the generation of the CTF of the ADAM10 substrate, N-cadherin. Whereas Tspan3 expression led to a 2.9 fold increase in APP-CTF, it had no apparent effect on the generation of the N-cadherin-CTF (Fig. 3A, Suppl. Fig. S4). In contrast, Tspan15 overexpression increased N-cadherin cleavage (CTF-generation) while only mildly affecting the generation of APP-CTFs (Fig. 3A, Suppl. Fig. S4). Inhibition of ADAM10 after Tspan3 overexpression using a well described hydroxamate-based drug (GI254023X) [30] also reduced the level of the APP-CTFs to almost mock transfected levels confirming that the Tspan3 effect was to a large part mediated by ADAM10 (Fig. 3B). Downregulation of the protease by ADAM10-specific siRNA in N2A cells led to a similar effect (i.e. a reduction of the Tspan-3-dependent appearance of APP-CTFs) (Fig. 3C). Also, in human embryonic kidney 293 (HEK) cells CRISPR/Cas9-mediated deletion of ADAM10 but not ADAM17 slightly reduced the appearance of the APP-CTF after Tspan3 expression (Fig. 3D).

In agreement with the identified role of ADAM10 as the major APP α-secretase [8,9,31] in HEK cells overexpressing the Swedish mutant of APP, overexpression of Tspan3 preferentially increased

levels of the C83 APP fragment (α-CTF, Fig. 3E). Likewise, in murine N2A cells a gel system allowing a separation of the APP-derived CTFs revealed that Tspan3 mainly affected the production of the endogenous murine C83 species and not the C99 fragments of APP-CTFs and inhibition of β- and γ-secretase did not alter the amount of this fragment (Suppl. Fig. S5).

Moreover, using HEK cells that stably expressed the Swedish mutant form of APP (K595N/M596L), we observed that Tspan3 significantly reduced the level of amyloid-β protein (Aβ) in the supernatant of these cells but only mildly altered the level of the soluble sAPPα ectodomain (Fig. 3F) suggesting that in the mutant APP-swe expressing cells the generation of intracellular α-CTF and extracellular sAPPα follow different kinetics, or that their stability is differentially regulated by Tspan3. Alternatively, the α-CTF may be generated by ADAM10-mediated trimming of the β-CTF.

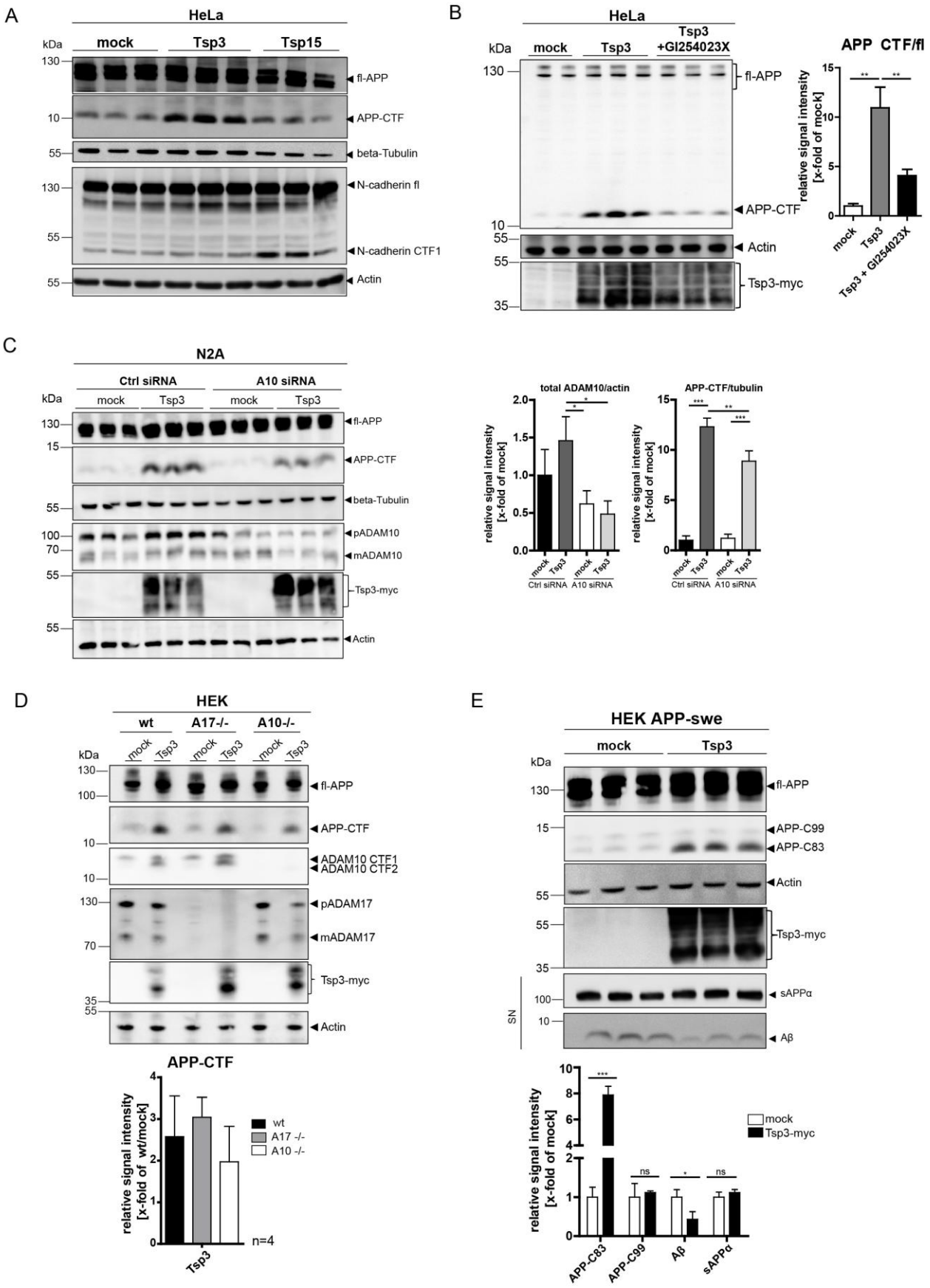


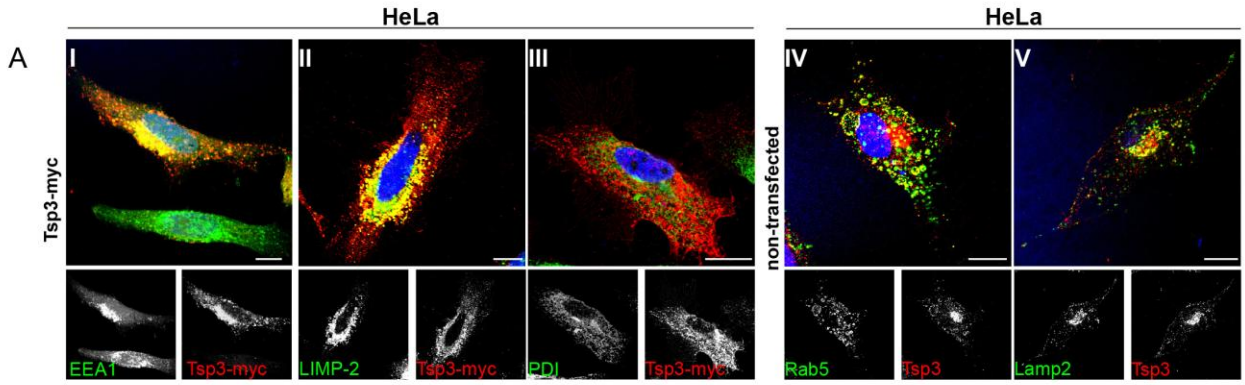
Figure 3: Tspan3 expression leads to an ADAM10-mediated increase of the APP α-CTF (C83). (A) Tspan3-myc (Tsp3) expression in HeLa cells increases the levels of the APP-CTFs but does not affect the N-Cadherin CTF production. Expressing Tspan15-myc (Tsp15) only moderately

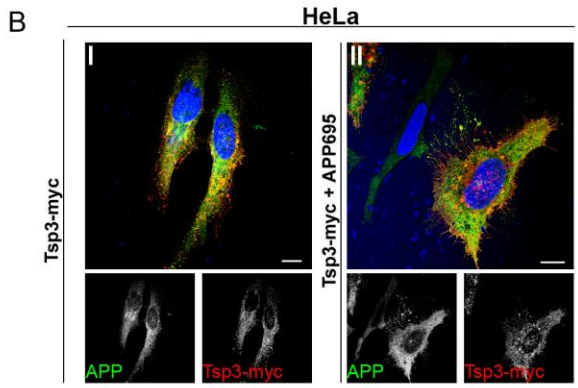
affects APP-CTF generation yet clearly increases the formation of the N-Cadherin CTF. **(B)** Addition of a hydroxamate-based inhibitor of ADAM10 (GI254023X) reduced the Tspan3-induced generation of the APP-CTF in HeLa cells. Actin loading and the transfected Tspan3 are shown in the lower panels. A quantification of the APP-CTF generation in the presence and absence of the inhibitor is shown. **(C)** siRNA-mediated downregulation of ADAM10 in N2A cells also reduced the generation of APP-CTFs after Tspan3 expression. Scrambled control siRNA was used as a control. A quantification of total ADAM10 and APP-CTF levels is shown next to the blot. **(D)** HEK cells with CRISPR/Cas9-mediated-knockout of ADAM10 or ADAM17 were used to transfect Tspan3-myc or a vector control (mock). APP-CTF expression was slightly reduced in ADAM10-deficient cells. The expression of ADAM10 and ADAM17 is controlled by immunoblots. A quantification of APP-CTF levels relative to the wild-type mock control is shown below the blot (n=4 experiments)**. (E)** Stably transfected HEK cells expressing the Swedish (swe) mutation of APP were transfected with Tspan3-myc and immunoblot analysis revealed an accumulation of the APP-C83 CTF. In the same cells immunoblot analysis showed that Tspan3 expression reduced the level of Aβ but did not affect the generation of sAPPα. Quantification is shown below the blot. Statistical significance was determined using Student's and ANOVA-test (*, p<0,05, **, p<0,01***, p<0,001).

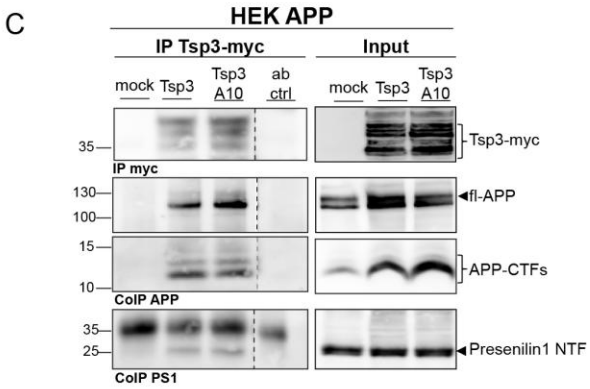
Tspan3 is mainly localized in endocytic vesicles and interacts with the amyloid precursor protein and presenilin-1

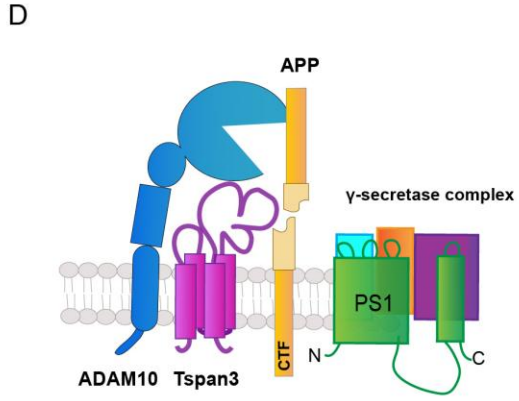
To better understand the effect of Tspan3 on APP processing and CTF generation we first analyzed the subcellular localization of Tspan3 in HeLa cells. Myc-tagged Tspan3 showed a prominent co-localization with the early endosome protein EEA1 as well as the lysosomal membrane protein LIMP-2 (Fig. 4A(I,II)) with minor amounts at the plasma membrane. No apparent co-localization was observed with the protein disulfide-isomerase (PDI) as an endoplasmic reticulum (ER) marker (Fig. 4A(III)). Also at the endogenous level, Tspan3 co-localized in part with Rab5 and LAMP-2 (Fig. 4A(IV,V)) suggesting that the steady state distribution of Tspan3 is mainly in endosomes and lysosomes, respectively. Both endogenous but also overexpressed APP showed a prominent co-localization with myc-tagged Tspan3 in HeLa cells (Fig. 4B) suggesting that APP is part of a Tspan3 containing protein complex. Co-immunoprecipitation experiments in human APP-expressing HEK cells confirmed that both full length APP (fl-APP) and also APP-CTFs could be pulled down by precipitation of Tspan3 (Fig. 4C). Interestingly, we also found the N-terminal fragment of presenilin-1 in the Tspan3 precipitate suggesting that Tspan3 provides an interaction platform for α- and γ-secretases as well as for their common substrate APP (Fig. 4D). Expression of Tspan3 but not Tspan15 in N2A cells caused a trend towards an increase in surface expression of

full length APP and a significant increase in surface APP-CTF (Fig. 4E, Suppl. Fig. S6) as well as the presenilin-1-CTF (Suppl. Fig. S6) as revealed in a surface biotinylation experiment arguing for an APP and presenilin stabilizing role of Tspan3 at both the plasma membrane and possibly also within the endocytic pathway.









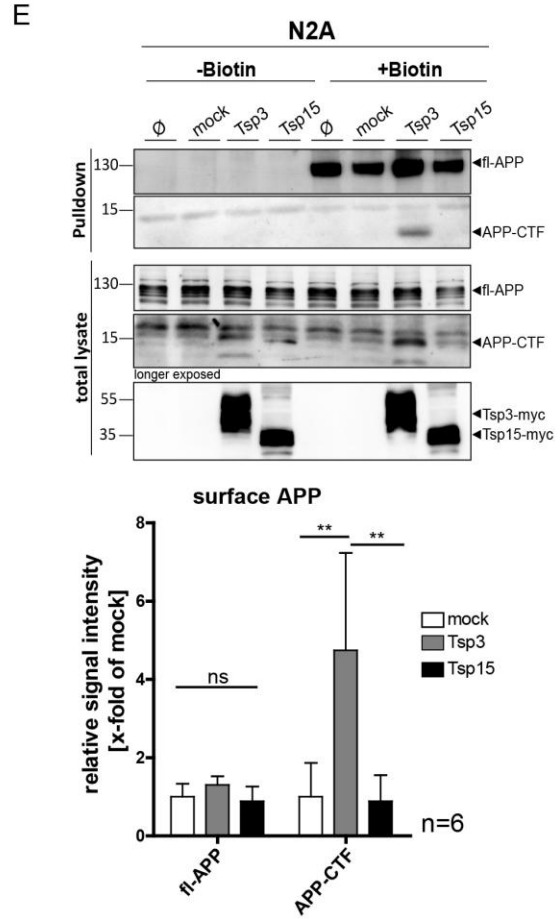


Figure 4: Tspan3 localizes to the endocytic pathway and interacts with APP and presenilin-1 **(A)** I, II, III: Murine Tspan3-myc expressed in HeLa cells. IV, V: Endogenous Tspan3 in HeLa cells. Confocal immunofluorescence pictures were taken using an anti-PDI antibody (III), an anti-LIMP-2 antibody (II), an anti-EEA1 antibody, an anti-Rab5 antibody (IV) and an anti -LAMP-2 antibody (V). **(B)** I, II Co-localization of endogenous APP (I) or transfected APP695 (II) with Tspan3-myc in HeLa cells. Scale bars for A and B represent 10 µm. **(C)** Expression constructs of murine Tspan3-myc and murine ADAM10 were transiently co-expressed in HeLa cells. Tspan3-myc (35-37 kDa) was immunoprecipitated (IP) using an anti-myc antibody and co-precipitation (coIP) of APP and presenilin-1 was detected with an anti-APP and anti-presenilin antibody. Abbreviations: murine ADAM10 (A10), murine Tspan3-myc (Tspan3), amyloid precursor protein (APP), fl: full length protein; CTF: carboxy-terminal fragments; NTF: amino terminal fragments. **(D)** Hypothetical model of the Tspan3 complex comprised of ADAM10, APP, APP-CTF and the γ-secretase complex. **(E)** N2A cells were biotinylated after transfection with either the mock vector or murine Tspan3-myc or Tspan15-myc. Following cell lysis total protein samples were taken and after precipitation of biotin-labeled proteins immunoblotting of total lysates and bound fractions was performed. Tspan3-myc, and APP full length (fl) and APP carboxy-terminal fragments (APP-CTF) were detected. Quantification of the experiment (n=6) is provided below the blot. Statistical significance was determined using ANOVA (**, p<0,01).

Mutation of a Tspan3 endocytosis motif causes decreased turnover of APP-CTFs

The increased levels of APP caused by the overexpression of Tspan3 prompted the question of whether modulating surface levels of Tspan3 would alter the expression of full length APP and APP-CTFs. We identified a "YELL" motif within the C-terminal cytoplasmic domain of Tspan3 (Fig. 5A) which fits well with the common YXXΦ endocytosis motif, where Φ is a bulky hydrophobic amino acid [32]. Mutating the Tspan3 tyrosine residue 243 to alanine (Tspan3Y243A) resulted in a more prominent surface expression of Tspan3 and reduced co-localization with the early endosomal marker EEA1 in transfected HeLa cells (Fig. 5B). Surface biotinylation in APP-expressing N2A cells confirmed the increased surface levels of the Tspan3Y243A mutant (Fig. 5C) which was accompanied by an increased surface expression of full length endogenous APP and APP-CTF (Fig. 5D).

Our experiments point to a role of Tspan3 as an interaction scaffold for ADAM10. siRNA-mediated downregulation of endogenous Tspan3 in human neuroblastoma SH-SY5Y cells did not obviously affect the expression levels of APP, APP-CTFs and ADAM10 (Fig. 5E(I)). To evaluate if the absence of Tspan3 is compensated by other tetraspanins, the transcription level of TspanC6

tetraspanins (Tspan6, Tspan7, Tspan12 and CD63) and Tspan5 was analyzed (Fig. 5E(II) but no significant changes were observed. Tspan7 transcription was not detected in this cell-line.

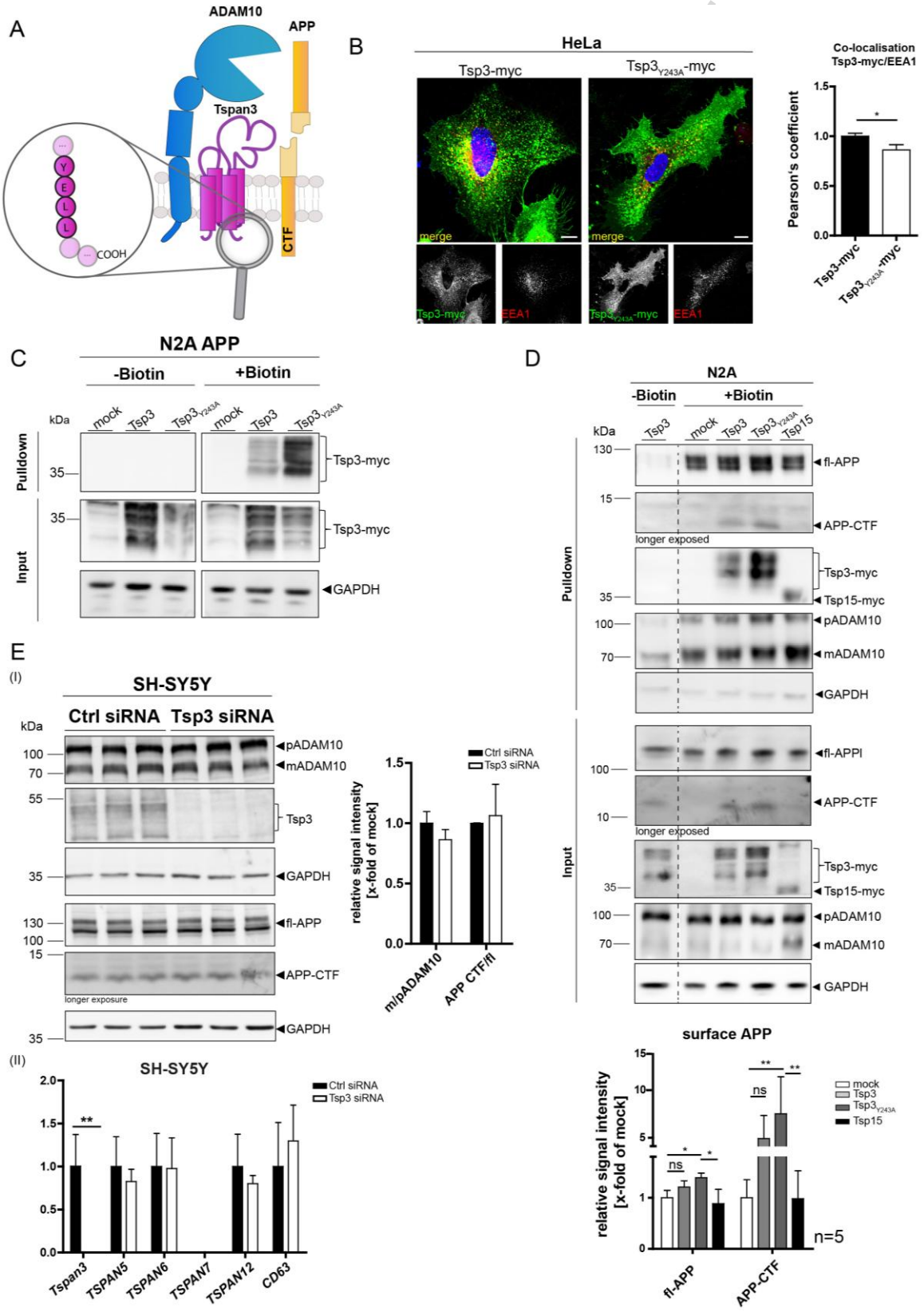


Figure 5: Mutation of a Tspan3 cytoplasmic endocytosis motif affects Tspan3 and APP surface expression. (A) Scheme of Tspan3 in complex with ADAM10 and APP. In the magnified view the "YELL" endocytosis motif within its carboxy-terminal cytoplasmic domain of Tspan3 is depicted. **(B)** Localization of the wild type (wt) Tspan3-myc and the $Tspan3_{Y243A}$-myc mutant in transfected HeLa cells as revealed by fluorescence confocal microscopy. Scale bars: 10 μm. Co-localization of Tspan3 with EEA1 was quantified by determining the Pearsons'correlation coefficient. **(C)** HeLa cells were biotinylated after transfection with either the wt Tspan3-myc or the mutant $Tspan3_{Y243A}$-myc. Following cell lysis total protein samples were taken and after biotin-labeled proteins were precipitated immunoblotting of total lysates and bound fractions was performed. Tspan3-myc was detected. **(D)** Surface biotinylation of N2A APP cells transfected with wt Tspan3-myc (Tsp3), mutant $Tspan3_{Y243A}$-myc ($Tsp3_{Y243A}$) or Tspan15-myc. Cells were lysed, total protein lysates taken and biotin-labeled proteins precipitated. Total lysates and precipitates were subjected to immunoblot. Tspan3, $Tspan3_{Y243A}$ and Tspan15, ADAM10, full length (fl)APP and carboxy-terminal fragments (CTFs) were detected and quantified (shown below the blots) **(E)** (I) siRNA-mediated knockdown of Tspan3 in human neuroblastoma SH-SY5Y cells. A quantification of the ratio of mature and proform of ADAM10 as well as APP-CTFs is provided next to the blot. (II) qPCR analysis of Tspan3, 5, 6, 7, 12 and CD63 gene transcription in Tspan3 (Tsp3) siRNA or control (ctrl) siRNA treated SH-SY5Y cells. The histogram shows the the calculated mean value ± SD. Statistical significance was tested using Student's t-test (** $p<0,01$).

Tspan3-deficiency is most likely compensated in vivo

To address the *in vivo* role of Tspan3 we have generated Transcription activator-like effector nuclease (TALEN)-mediated Tspan3 knockout mice. The resulting mice contain an insertion of 26 nucleotides with a new XbaI restriction site in exon 2 of the *Tspan3* locus leading to a premature stop codon (Fig. 6A). The deficiency of Tspan3 expression was validated by immunoblot analysis of different tissues (Suppl. Fig. S7) including brain (Fig. 6B). Tspan3-deficient mice did not reveal an overt phenotype and obvious morphological alterations in the brain by the age of six months. Interestingly, similar to the siRNA experiment in cultured cells (Fig. 5E), the level of ADAM10, PrP^c, fl-APP and APP-CTFs were not obviously affected by the lack of Tspan3 (Fig. 6B) suggesting that compensatory mechanisms (e.g. other tetraspanins) exist *in vivo* which can replace Tspan3 and contribute to the stabilization of APP and APP-cleavage products. Analyzing the expression level of selected tetraspanins by qRT-PCR in brains from wild type and Tspan3-deficient littermates indeed revealed about a five-fold increase in the expression of *Tspan7* (belonging to the TspanC6 family) but also an approximate 30-fold increase of *Tspan5* (as a member of the TspanC8 family) (Fig. 6C). Other tetraspanins were only slightly (*Cd63*, *Tspan12*) or clearly (*Tspan6*) downregulated. The

possible compensatory role of Tspan7 was validated by heterologous expression in N2A cells. Similar to Tspan3 also Tspan7 led to a significant increase in the generation of APP-CTFs (Fig. 6D).

Our *in vivo* data indicate that the lack of Tspan3 causes a complex adaptation of the expression of other tetraspanin family members which may contribute to an apparent normal function of ADAM10 and processing of its substrates despite a depletion of Tspan3.

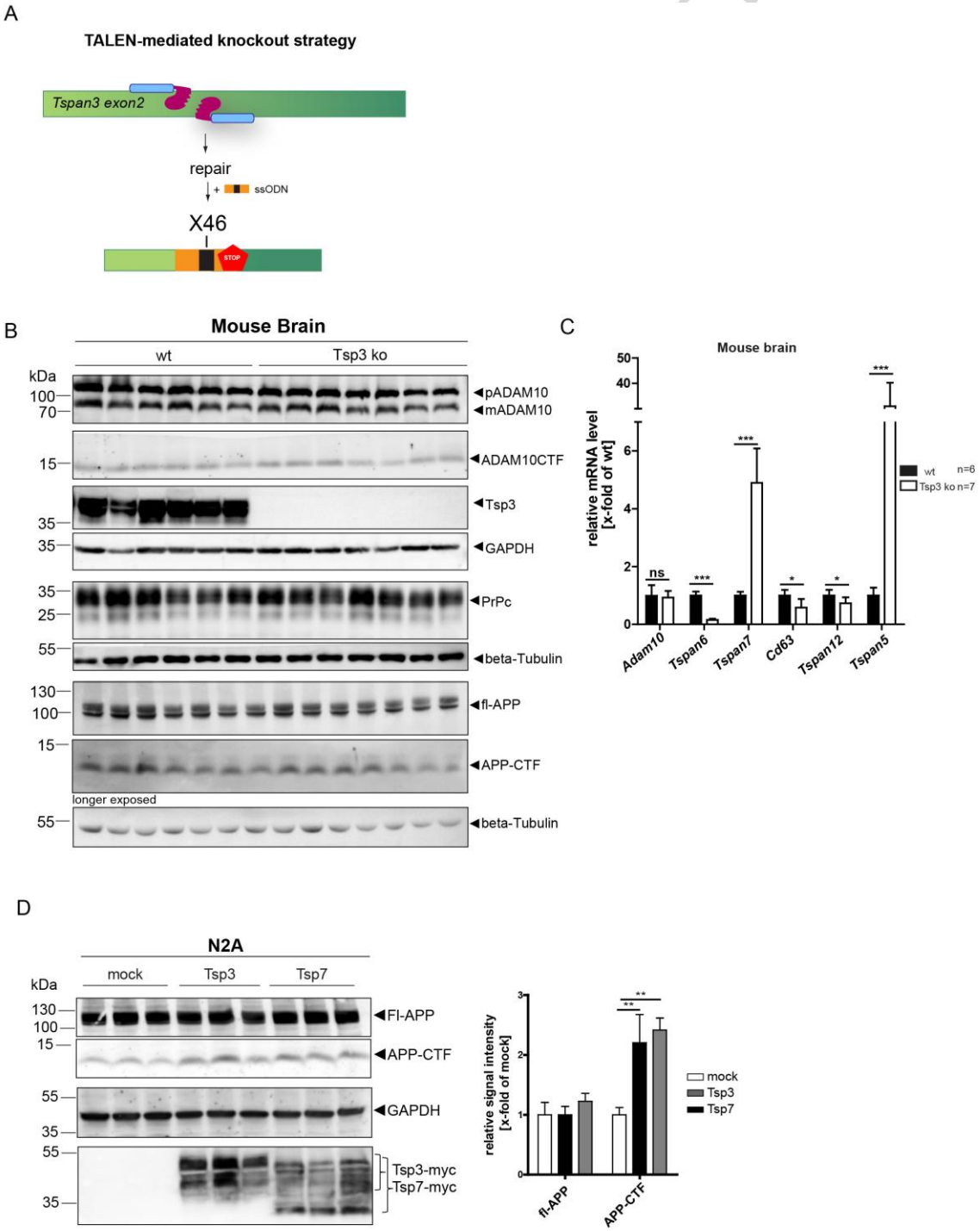


Figure 6: Knockout of Tspan3 in mice is likely compensated by other tetraspanins. **(A)** Scheme of the used targeting strategy for the generation of Tspan3 knockout mice. Tspan3 exon 2-specific TALEN oligonucleotides were used to induce a double-strand break. Following, an additional 46 bp single stranded Oligodeoxynulceotid (ssODN) was partially incorporated by homology-directed repair, resulting in a frameshift mutation and a premature stop codon. **(B)** Analysis of brain tissue from wild type (wt, n=6) and Tspan3 knockout (ko, n=7) mice. Brain lysates were immunoblotted and the expression of ADAM10, Tspan3, the cellular prion protein PrP^c, GAPDH, APP and beta-tubulin was analyzed. **(C)** qPCR Analysis of *Adam10*, *Tspan6*, *Tspan7*, *Cd63*, *Tspan12 and Tspan5* gene transcription in brain tissues isolated from wild type (wt) or Tspan3 knockout (Tsp3 ko) mice. The data is represented as histogram showing the mean value ± SD of the calculated, relative mRNA level. Statistical significance was determined using Student's t-test (** $p<0,01$, *** $p<0,001$). **(D)** Murine myc-tagged Tspan3 (Tsp3) or Tspan7 (Tsp7) were expressed in N2A cells. Transfection of an empty vector served as a mock control. Full length (fl) APP and APP-CTFs were analyzed by immunoblot using a C-terminal APP antibody. Quantification is shown beside the blots. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's multiple comparisons test (** $p<0,01$).

Discussion

The functional analysis of tetraspanins has been partially hampered by their redundant functions and their ability to form membrane-embedded protein domains which are able to interact with a number of membrane proteins. Such tetraspanin enriched microdomains [5] organize a spatial arrangement of membrane proteins thereby affecting biological processes like cell adhesion, signaling, endocytosis and proteolysis [5,1,33,34]. The pathogenic relevance of APP proteolysis by the α- and γ-secretases is also affected by tetraspanins [35,36,11,14,12,37]. A recent study revealed that tetraspanins may physically associate with the α- and γ-secretase complex in an active multiprotease complex at the plasma-membrane [17]. In agreement with this study we observed that Tspan3 interacts with both ADAM10 and presenilins. Importantly, it also assists to group APP as a substrate and the C-terminal cleavage product of APP in this complex. Our cell-based experiments reveal that Tspan3 is mainly found within the endocytic compartment and its endocytosis affects the cell surface expression and stability of its protein partners. We found Tspan3 to act in a different manner as the hitherto described tetraspanins belonging to the Tspan C8 family [35,36,11,14,12,16] by not having an obvious impact on the maturation and intracellular transport of ADAM10 from the endoplasmic reticulum to the plasma membrane. Tspan3, a tetraspanin member with a pronounced expression in murine and human brain and in migratory neural crest cells [21] seems to excert stabilizing functions on ADAM10 and certain associated substrates (including APP and PrP^{c}) once they have reached the plasma membrane and become endocytosed. In this regard, it is of note that presenilins are partially found in late endosomes/lysosomes and they are involved in intracellular Aβ production [38]. It is attractive to speculate that Tspan3 and other intracellular Tspans modulate the activity of intracellular amyloidogenesis. In how far Tspan3 also participates in the cell surface control and endocytosis of other cell surface molecules like claudin-11 and beta 1 integrin in oligodendrocytes or with the Nogo-A receptor S1PR2 in a similar manner [18-20] has not yet been addressed. Mechanisms of how exactly endocytosis of Tspan3 together with its bindings partners are controlled, remain to be determined. In case of the tetraspanin CD63, an association with

adaptor protein complexes 2 and 3 was revealed leading to internalization of the H^+,K^+-ATPase beta-subunit [39]. Our findings are in agreement with a role of certain tetraspanins in regulating the specific tetraspanin-interaction partner network by endocytosis.

Since Tspan3 was also found in lysosomes of cortical neurons and its overexpression increased levels of the APP-C83 fragment (α-CTF) it might be speculated that its association with the full length form of APP and APP-CTFs after internalization has a protective effect preventing premature degradation of the proteins by lysosomal proteases [40]. It is known for some time that α- and β-secretase-derived APP-CTFs are found in late endocytic compartments [41] where Aβ generation may also take place [42]. APP endocytosis and its potential control by Tspan3 may also affect Aβ production as revealed by experiments where the cytosolic tail of APP was removed [43,44]. The observation that Tspan3 expression is increased in AD brains may simply reflect an upregulation of the endocytic system in AD [45,46]. However, elevated levels of Tspan3 may also directly contribute to C-terminal APP fragments and intracellular Aβ production.

We could show that Tspan3 also physically interacts with ADAM10 and this binding directly affects its proteolysis of APP as revealed by pharmacological inhibition, siRNA-mediated knockdown or complete depletion of protease expression. Since overexpression of Tspan3 preferentially causes an increase in APP-C83 fragments and not of the APP-C99 fragments an inhibitory role on the γ-secretase function seems unlikely. By increasing and stabilizing the APP α-CTF, Tspan3 could also promote γ-secretase cleavage of APP. The biological effects we observed in our cell-based assays may reflect a mixture of effects related to a stabilization of the protein complexes and the embedded secretase substrates within the endocytic pathway and a possible selective effect of Tspan3 on ADAM10-mediated proteolysis of APP but not N-cadherin, another well-known ADAM10 substrate [47]. This substrate specificity may be explained by specific interactions within the tetraspanin-enriched domains that allow more direct contact between the Tspan3/ADAM10 complex and APP as compared to N-cadherin.

Surprisingly, deficiency of Tspan3 in human neuroblastoma cells and in Tspan3 knockout mice neither directly affect the stability of ADAM10 nor the stability and proteolytic processing of APP. A compensation by other members of the tetraspanin family seems likely and is supported by our observation that Tspan5 and Tspan7 are dramatically upregulated in the brains of the Tspan3 knockout mice. A lack of an upregulation in the neuroblastoma cell line SH-SY5Y suggest that the regulation of Tspan expression is cell-type specific. Tspan7 belongs to the TspanC6 subfamily and it is likely that similar to Tspan5,10,14,15,17 and 33 [11-14] its increased expression could accelerate the plasma membrane delivery of ADAM10. A functional effect may therefore be masked by the redundant role and an efficient replacement of certain tetraspanins by others in order to ensure an efficient protein network. The lack of an overt phenotype in the Tspan3 knockout mice but also many other Tspan-deficient mice [33] supports such a view. Future Tspan knockout studies are required to better understand the ADAM10/Tspan interaction networks and their impact on the proteolysis of APP and other substrates. Such studies are also required to define Tspans as novel drug targets, allowing a more selective modulation of APP secretase activity which usually show a broad spectrum of substrates.

In conclusion, Tspan3 is revealed as a novel interactor of ADAM10 involved in its stabilization and that of the γ-secretase and APP. Due to its predominant localization in endosomes, it may exert major roles within the endocytic pathway, modulating the stability of proteolytic products of APP and contributing to intracellular Aβ generation.

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Highlights

- Tspan3 was identified as a novel ADAM10 interaction partner
- Tspan3 modulates ectodomain shedding and intramembrane proteolysis of ADAM10.
- Tspan3 increases ADAM10-mediated generation of APP α-CTFs and reduces Aβ liberation.
- Tspan3 stabilizes ADAM10, APP and the γ-secretase complex at the plasma membrane
- Deficiency of Tspan3 is (most likely) compensated by other tetraspanins.