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Niemann–Pick type C cells show cholesterol dependent decrease of APP expression at the cell surface and its increased processing through the β-secretase pathway

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ABSTRACT

The link between cholesterol and Alzheimer's disease has recently been revealed in Niemann–Pick type C disease. We found that *NPC1*$^{-/-}$ cells show decreased expression of APP at the cell surface and increased processing of APP through the β-secretase pathway resulting in increased C99, sAPPβ and intracellular Aβ40 levels. This effect is dependent on increased cholesterol levels, since cholesterol depletion reversed cell surface APP expression and lowered Aβ/C99 levels in *NPC1*$^{-/-}$ cells to the levels observed in wt cells. Finding that overexpression of C99, a direct γ-secretase substrate, does not lead to increased intracellular Aβ levels in *NPC1*$^{-/-}$ cells *vs.* CHOwt suggests that the effect on intracellular Aβ upon cholesterol accumulation in *NPC1*$^{-/-}$ cells is not due to increased APP cleavage by γ-secretase. Our results indicate that cholesterol may modulate APP processing indirectly by modulating APP expression at the cell surface and thus its cleavage by β-secretase.

1. Introduction

Altered processing of the β-amyloid precursor protein (APP) leading to increased formation of amyloid-β (Aβ) peptide is considered to be a central event in the pathogenesis of Alzheimer's disease (AD) [7]. Aβ is generated from the proteolytic processing of APP by the sequential action of β- and γ-secretases. APP cleavage by α- and β-secretase generates soluble sAPPα and sAPPβ fragments and membrane-bound C-terminal fragments C83 (CTFα) and C99 (CTFβ), respectively. C83 and C99 are both substrates of γ-secretase. While γ-secretase cleavage of C99 generates Aβ peptides, γ-secretase cleavage of C83 liberates a non-amyloidogenic species, p3. In addition, γ-secretase cleavage of C83 and C99 liberates a C-terminal product APP-intracellular domain (AICD) [25]. Although it has been previously shown that within the transmembrane domain (TM) of APP the site of γ-secretase cleavage generating Aβ/p3 is distinct from the site of γ-cleavage generating AICD ($\gamma_{40/42}$ and ε-site, respectively) [25], the mechanism of dual γ-secretase action is still controversial [31]. While β-secretase cleavage of APP is thought to happen primarily within endosomal or secretory compartments [6,24,32], cleavage of APP by α-secretase has been suggested to occur mainly at the cell surface [29].

There is growing evidence that cholesterol contributes to the pathogenesis of Alzheimer's disease. Elevated dietary cholesterol uptake increased amyloid plaque formation in rabbits and transgenic mice [23,30], and cholesterol loading and depletion affects Aβ generation in cultured cells [5,28]. In addition to the indirect effects of cholesterol on APP processing, recent findings suggest that cholesterol may directly modulate the activity of β-secretase (BACE1) [11], while contradictory results have been reported for the effect of cholesterol on γ-secretase cleavage of APP [34,35].

The link between cholesterol and Aβ has recently been revealed in Niemann–Pick type C (NPC) disease as well as AD. NPC is an inherited lysosomal storage disorder caused by recessive loss of function mutations within the *NPC1/2* gene. The mutant NPC1/2 proteins fail to transport cholesterol to the plasma membrane and endoplasmic reticulum (ER) resulting in cholesterol accumulation in late endosomal/lysosomal compartments [18]. Recent *in vitro* and *in vivo* findings indicate that loss of NPC1 leads to a significant increase in C99 and intracellular Aβ production [2,10,38]. With additional reports now linking changes in lipid homeostasis to altered APP processing and Aβ generation, NPC pathobiology has become an important model to study the link between cholesterol and APP processing.

Abbreviations: AD, Alzheimer's disease; AICD, APP-intracellular domain; APP, β-amyloid precursor protein; ApoE, apoliprotein E; Aβ, amyloid-β; CTFs, C-terminal fragments; DAPT, *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester; FA, formic acid; $flAPP_m$, mature full-lenght APP; $flAPP_{im}$, immature full-lenght APP; LPDS, lipid-deficient serum; NPC, Niemann–Pick type C; SEAP, secreted alkaline phosphatase; sAPP, soluble APP

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The goal of this work was to investigate the mechanism of the cholesterol-effect on Aβ in *NPC1*$^{-/-}$ model cells. Our data show that cholesterol accumulation upon loss of NPC1 function affects APP processing by altering expression of APP at the cell surface and by favouring APP cleavage through the β-secretase pathway. These findings add to the role of cholesterol on Aβ production and support a link between cholesterol, AD and NPC disease.

2. Materials and methods

2.1. Antibodies

The following primary antibodies were used: 5313 (polyclonal N-terminal APP), 6687 (polyclonal C-terminal APP, kindly provided by Dr. H. Steiner and Dr. C. Haass), W02 (kindly provided by Dr. Konrad Beyreuther), 9E10 (monoclonal anti-*c-myc*, Sigma-Aldrich) and 22C11 (monoclonal N-terminal APP, Chemicon International). Antibodies 192wt and 192sw specific for the C-terminus of sAPPβ were a generous gift from Dr. Dale Schenk.

2.2. Cell lines, cDNA constructs and transfection

Chinese hamster ovary wild-type cells (CHOwt), CHO *NPC1-null* cells (*NPC1*$^{-/-}$) and *CFP-NPC1wt*-stably transfected *NPC1-null* CHO cells (all kindly provided by Dr. Daniel Ory) were maintained in DMEM:F12 medium (1:1) containing 0.5 mM Na-pyruvate supplemented with 10% FBS, 2 mM L-glutamine and antibiotic/antimycotic (all from Sigma-Aldrich).

For transient expression, cells were transfected using GeneJuice (Novagen, Merck) or Lipofectamine 2000 (Invitrogen) according to the supplier's instructions. *C99* and *APPsw 6myc-tagged* constructs were generated using pCS2+6MT vector [8]. Twenty-four hours after transfection, medium was removed, fresh medium was added and further incubated for 24 h. To monitor transfection efficiency between the cell lines, the cells were transiently transfected with a secreted alkaline phosphatase (*SEAP*) construct (kindly provided by Drs. Stefan Lichtenthaler and Raphael Kopan) in parallel wells/dishes. Forty-eight hours after transfection, alkaline phosphatase activity was measured in the medium as described previously [16] and corrected for the protein concentration in the cell lysate.

2.3. Cholesterol depletion

The cells were plated in a 10-cm dish in 10% FBS DMEM/F12 medium. The next day, medium was removed and cells were washed in PBS to remove the remaining FBS. Fresh medium containing 10% lipid-deficient serum (LPDS, Cocalico Biologicals Inc.) instead of 10% FBS was added [21] and then the cells were transiently transfected. Twenty-four hours after transient transfection, the media were replaced with fresh 10% LPDS medium (5 ml/plate) for cholesterol depletion and further incubated for 24 h. The media and cell lysates were then collected (as described in the Sample preparation and immunoblotting section) and used for biochemical assays.

2.4. Sample preparation and immunoblotting

The medium was collected, 1 mM AEBSF (Roche Applied Science) was added and centrifuged at 4 °C for 10 min at 16000×*g*. The supernatant was used for analysis of secreted Aβ40 (see *ELISA Aβ assay*). For detection of secreted APP (sAPP) by western blot, aliquots of conditioned medium normalized according to protein concentration in the cell lysate and SEAP activity were directly loaded onto an electrophoresis gel.

Cells were washed with PBS and lysed in RIPA buffer (0.5% Na-deoxycholate, 0.1% SDS, 1% NP40, 5 mM EDTA, 150 mM NaCl, 50 mM Tris–HCl pH 8.0) containing a protease inhibitor cocktail (Roche Applied Science) on ice for 10 min and centrifuged at 4 °C for 10 min at 16000×*g*. Supernatants were mixed with 6× sample buffer (60% glycerol, 12% SDS, 3% DTT, 1/8 v/v 0.5 M Tris pH 6.8, bromphenol blue) and heated at 70 °C for 10 min.

Equivalent amounts of protein, stratified according to SEAP activity, were loaded onto SDS–PAGE gels. Immunoblot detection was carried out using the indicated antibodies. Proteins were visualized using chemiluminescence.

For endogenous APP–CTF analysis, cells were plated in 10-cm dish in 10% FBS DMEM/F12 medium. Cells were treated with 1 μM DAPT (Sigma-Aldrich) for 24 h for γ-secretase inhibition. For cholesterol depletion, cells were grown for 48 h in 10% LPDS DMEM/F12 medium. Cells were lysed in CoIP lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP40) containing protease inhibitor cocktail (Roche Applied Science). Cell lysates were normalized according to protein concentration and incubated with 6687 antibody and Protein A-Sepharose (Sigma-Aldrich) at 4 °C overnight with constant rocking. Samples were heated at 70 °C for 10 min in 2× sample buffer and subjected to SDS–PAGE on Tris–Tricine gels [26].

2.5. ELISA Aβ assay

The levels of secreted and intracellular Aβ40 were determined by ELISA Aβ40 kit (Invitrogen) as previously described [8]. The colorimetric detection system was used. Aβ concentration is expressed per mg of protein and is stratified according to SEAP activity.

2.6. In vitro γ-secretase assay

Cells were transiently transfected with the *C99-6myc* construct and 48 h after transfection total cellular membranes were isolated [36]. *In vitro* γ-secretase assays were performed as previously described [8]. To inhibit γ-secretase activity, we used γ-secretase inhibitor WPE-III-31C (10 μM, Calbiochem). The reactions were stopped by chilling on ice. After centrifugation (10 min at 16000×*g* and 4 °C) supernatants were analyzed for Aβ40 levels using the ELISA assay and for AICD levels by immunoblotting.

2.7. α-, β- and γ-secretase activity assay

A FRET-based assay was used to measure α-, β- and γ-secretase activities (R&D Systems Inc.). All procedures were performed as described in the manufacturer's protocol, except for γ-secretase.

The end-point α- and β-secretase activity was determined after 2 h of reaction at 37 °C. The fluorescence was read in a fluorescent microplate reader (Fluoroskan Ascent FL, Thermo Electron Corporation) at 355 nm (excitation) and 538 nm (emission).

To measure γ-secretase activity, the postnuclear supernatant (PNS) and total cellular membranes were collected as previously described [8]. Membranes (pellet), stratified according the protein levels, were resuspended in Tris–CHAPSO assay buffer (50 mM Tris, pH 6.5, 2 mM EDTA and 0.25% CHAPSO) [40]. Aliquots were incubated with fluorogenic γ-secretase substrate (R&D Systems Inc.) containing either none or 10 μM γ-secretase inhibitor WPE-III-31C (Calbiochem). After 2 h of reaction at 37 °C, samples were cleared by centrifugation at 16000×*g* for 15 min at 4 °C. Supernatants were transferred into 96-well plate and the fluorescence was read in a fluorescent microplate reader (Fluoroskan Ascent FL, Thermo Electron Corporation, excitation/emission at 355/538 nm). Specific γ-secretase activity is determined after subtracting the fluorescence obtained in the presence of WPE-III-31C (10 μM).

2.8. Cell surface biotinylation

Cell surface biotinylation was performed using EZ-Link™ Sulfo-NHS-SS-Biotin (1 mg/ml) and Neutravidin™ Protein beads (all from

Pierce) as described [22]. During the biotinylation procedure, all reagents and cell cultures were kept on ice. The cells were washed three times in PBS pH 8.5 (measured at 4 °C), then incubated in 1 mg/ml EZ-Link™ Sulfo-NHS-SS-Biotin solution (Pierce) in PBS pH 8.5 for 30 min and washed twice with 100 mM glicine in PBS on plates. The cells were then lysed in lysis buffer (1% NP40, 0.1% SDS in PBS pH 8.5) in the presence of a protease inhibitor cocktail (Roche Applied Science, Germany). Lysates were incubated for 20 min on ice, centrifuged at 4 °C for 10 min at 16000×*g*. Supernatants (normalized per mg of protein) were incubated overnight with 50 µl of Neutravidin™ Protein beads (Pierce) with constant rocking. Neutravidin beads were washed three times with PBS then dried by aspiration, resuspended in 2× sample buffer, heated for 10 min at 70 °C and used for Western blot analysis of the cell surface APP. For detection of the total APP levels, the cell lysates were blotted and analyzed as indicated. The signals were quantified and expressed as the ratio of the cell surface and total (mature + immature) APP levels.

2.9. Cholesterol levels

The levels of total cholesterol were determined in cell lysates using the Amplex Red Cholesterol Assay kit (Molecular Probes, Invitrogen). The fluorescence was read in a fluorescent microplate reader (Fluoroskan Ascent FL, Thermo Electron Corporation) at 530 nm (excitation) and 590 nm (emission).

2.10. Filipin staining and immunocytochemistry

The cells were grown on coverslips. Filipin staining was done as described [17]. The cells were mounted (Polyvinyl alcohol mounting medium with DABCO antifading, Fluka) and were viewed by fluorescence microscopy (Olympus BX51).

Non-permeabilized cells were stained with 5313 antibody (N-terminal APP) for 10 min on ice to detect cell surface APP. Cells were then fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min and were further incubated for 1 h with secondary anti-rabbit-Alexa488 antibody (Molecular Probes, Invitrogen). For detection of intracellular APP, cells were fixed, permeabilized with 0.2% saponin (Sigma-Aldrich) and immunostained with antibody 6687 (C-terminal APP) overnight following 1 h of incubation with secondary anti-rabbit-Alexa488 antibody (Molecular Probes, Invitrogen). The cells were mounted (Polyvinyl alcohol mounting medium with DABCO antifading, Fluka) and were viewed by Leica inverted fluorescent confocal microscope.

3. Results

3.1. sAPPβ and CTFβ/C99 are increased in NPC1$^{-/-}$ cells

It has been previously reported that accumulation of cholesterol in late endosomal/lysosomal compartments as a result of NPC1 dysfunction causes increased C99/CTFβ and Aβ formation [2,10,38]. Since C99 and Aβ generation require β-secretase cleavage, we investigated whether this pathway may be favoured by cholesterol accumulation in *NPC1*$^{-/-}$ cells. To test this, we compared APP processing in CHO *NPC1-null* cells (NPC1$^{-/-}$ cells) and parental CHOwt cells. By analyzing endogenous levels of cholesterol and endogenous C-terminal APP fragments (CTFs), we show that *NPC1*$^{-/-}$ cells behave as previously observed U18666A-treated cells or *NPC1-mutant* cell lines/mouse model [2,10,38]: they exhibit ~2-fold higher total cholesterol levels than CHOwt cells (Fig. 1A, $p<0.01$), show accumulation of cholesterol in punctuate endocytic structures (Fig. 1B) and show substantially increased levels of endogenous APP C-terminal fragments (CTFs) (Fig. 1C). The origin of these APP–CTF fragments was confirmed by their accumulation after DAPT treatment (Fig. 1D). Using *NPC1* stably transfected *NPC1*$^{-/-}$ cells, we confirmed that this phenotype was dependent upon NPC1 function and was not a secondary effect of *NPC1* deletion because in NPC1 expressing *NPC1*$^{-/-}$ cells, cholesterol levels/accumulation as well as the levels of APP–CTFs were reversed to the levels seen in wt cells (Fig. 1A, B and C).

Surprisingly, analysis of endogenous levels of total sAPP in conditioned media showed no difference between wt and *NPC1*$^{-/-}$ cells. However, further analysis of sAPPβ species revealed its marked increase in *NPC1*$^{-/-}$ cells *vs.* CHOwt (Fig. 1E), suggesting that the loss of *NPC1* increases β-secretase cleavage of APP. We could not specifically analyze the sAPPα secreted from the CHO cell lines because antibodies to human Aβ1-16 do not detect hamster APP sequence (GenBank accession number AF030413), which is the same as the sequence in other rodents. For this reason, we only analyzed endogenous levels of total sAPP and sAPPβ by immunoblotting with antibodies 22C11 and 192wt, respectively, which recognize both human and rodent sAPP.

To investigate APP processing between wt and *NPC1*$^{-/-}$ cells in more detail and in order to be able to specifically detect the α-secretase cleavage products of APP, the cells were transiently transfected with the *APPsw-6myc* construct [8]. We observed that C99 levels were significantly increased in *NPC1*$^{-/-}$ *vs.* CHOwt cells (Fig. 2A, $p<0.05$), while levels of C83 were not significantly altered (Fig. 2A). To further analyze α- and β-secretase cleavage of APP in *NPC1*$^{-/-}$ cells, we measured secreted APP levels (sAPPβ, sAPPα and sAPPtotal) in the media of APP-transfected cells. In accord with our previous results on endogenous levels (Fig. 1E), we detected a significant increase in sAPPβ (Fig. 2B, $p<0.05$) levels and, additionally, a decrease in sAPPα levels (Fig. 2B, $p<0.01$) in *NPC1*$^{-/-}$ *vs.* CHOwt cells. These results suggest that cholesterol accumulation upon loss of NPC1 may favour APP processing through the β-secretase pathway and lower cleavage by α-secretase.

3.2. NPC1 loss causes increase of intracellular form of Aβ but does not cause an increase of AICD

The levels of intracellular and secreted Aβ were analyzed using an ELISA assay (Biosource Int. Inc., Invitrogen) in *APPsw-6myc* transiently transfected cells. We detected increased levels of soluble (RIPA-extractable) and insoluble (formic acid (FA)-extractable) intracellular Aβ40 in *NPC1*$^{-/-}$ cells compared to CHOwt cells (Fig. 2C, left and right panel, respectively), while secreted Aβ40 levels were not changed (Fig. 2D). In agreement with Yamazaki et al. [38] and Jin et al.[10], the levels of aggregated FA-extractable form of intracellular Aβ40 were significantly increased (by ~10-fold, $p<0.01$) in *NPC1*$^{-/-}$ cells compared to a slight increase in the soluble RIPA-extractable form (~2-fold, $p<0.05$) with no increase in secreted Aβ40, indicating that accumulation of cholesterol upon loss of NPC1 mainly affects the intracellular pool of Aβ and not secreted Aβ. In addition, we confirmed that this increase in intracellular Aβ in *NPC1*$^{-/-}$ cells was directly dependent on NPC1 function, as we observed for cholesterol and APP–CTF levels (Fig. 1), since expression of NPC1 in *NPC1*$^{-/-}$ cells reverted Aβ levels to those seen in wt cells (Fig. 2C). Intracellular Aβ42 levels were below the detection limit of the ELISA assay used (Biosource Int. Inc., Invitrogen) and thus are not shown. Using *APPwt-6myc* construct, we observed the same effect of intracellular Aβ40 increase upon NPC1 loss (not shown); however, the extent of Aβ40 accumulation using *APPsw* construct was far more substantial resulting in more reliable read-outs by ELISA assay.

Since γ-secretase cleavage of APP generates two products: Aβ, generated by γ-cleavage at the γ-site and AICD, generated by γ-cleavage at the ε-site, we investigated whether accumulation of cholesterol in NPC1-deficient cells would cause an increase in AICD levels in parallel with an increase in intracellular Aβ. In contrast to intracellular Aβ levels (Fig. 2C), we did not detect increased AICD levels in *APPsw-6myc* transfected *NPC1*$^{-/-}$ cells *vs.* CHOwt (Fig. 2A). This result indicates that the loss of NPC1 affects Aβ generation by

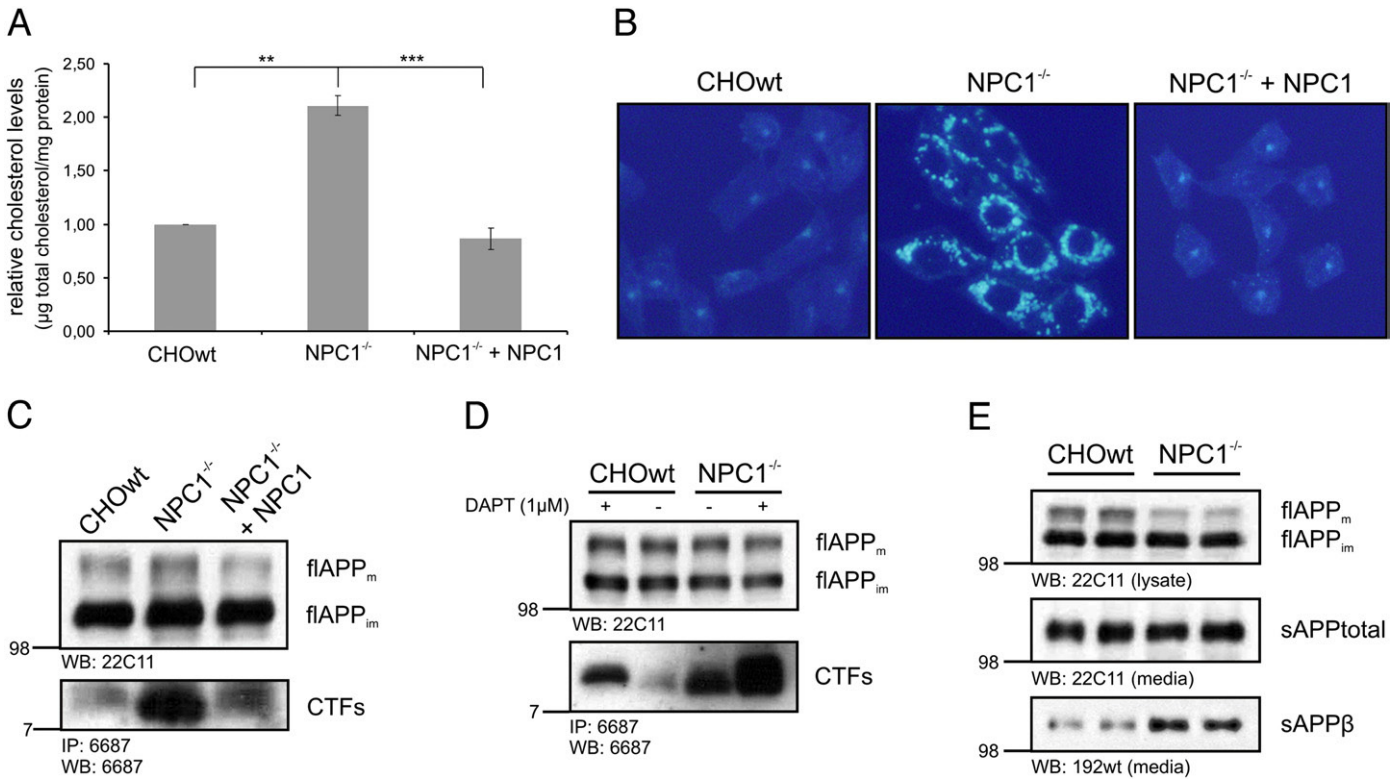

Fig. 1. *NPC1*$^{-/-}$ cells show increased levels of total cholesterol, accumulation of free cholesterol and increased levels of endogenous C-terminal APP-stubs (CTFs) and sAPPβ. Total cholesterol levels, free cholesterol accumulation and endogenous levels of APP–CTFs and sAPPβ were analyzed between CHOwt, *NPC1*$^{-/-}$ cells and *NPC1*-transfected *NPC1*$^{-/-}$ cells. (A) Total cholesterol levels were determined in cell lysates by AmplexRed cholesterol assay (Molecular probes, Invitrogen). Shown are mean and SEM of three independent experiments. Statistical analysis was performed using Student's *t*-test: $^{**}p<0.01$, $^{***}p<0{,}001$. (B) Filipin staining (Sigma-Aldrich) shows punctuate accumulation of free cholesterol in *NPC1*$^{-/-}$ cells compared to CHOwt cells and *NPC1*-transfected *NPC1*$^{-/-}$ cells. (C) The levels of APP–CTF stubs (CTFs) were determined in cell lysates by immunoprecipitation and western blotting as indicated. (D) Treatment with a γ-secretase inhibitor DAPT (1 µM) confirmed that the CTF stubs analyzed were indeed γ-secretase substrates since they accumulated upon DAPT treatment. (E) The levels of sAPPtotal and sAPPβ were determined in the media by western blotting as indicated.

increasing β-secretase cleavage but not by increasing γ-secretase cleavage.

3.3. Increased Aβ levels in NPC1$^{-/-}$ cells are not due to increased C99-cleavage by γ-secretase

To directly assess γ-secretase cleavage of APP in *NPC1*$^{-/-}$ cells, without the confounding effects of NPC1 loss on α-/β-secretase processing of APP, we overexpressed a *C99* construct in *NPC1*$^{-/-}$ cells and compared its processing to that in CHOwt cells. Interestingly, we found that the levels of soluble (RIPA-extractable) and insoluble (FA-extractable) Aβ40 were similar in *C99*-transfected CHOwt and *NPC1*$^{-/-}$ cells (Fig. 3A, left and right panel, respectively). This result suggests that the effect on intracellular Aβ upon cholesterol accumulation in *NPC1*$^{-/-}$ cells is not due to increased APP cleavage by γ-secretase. Since an increase in intracellular Aβ was observed in *APP*- (Fig. 2C) but not *C99*-transfected (Fig. 3A) *NPC1*$^{-/-}$ *vs.* CHOwt cells, this result provides further support for the hypothesis that increased Aβ in *NPC1*$^{-/-}$ cells is due to favoured APP processing by β-secretase, which is in agreement with the increased sAPPβ levels. In addition, we observed similar levels of AICD between *C99*-transfected CHOwt and *NPC1*$^{-/-}$ cells (Fig. 3B), further confirming that γ-secretase dependent formation of AICD is not altered in *NPC1*$^{-/-}$ cells (Fig. 2A).

3.4. Activities of α-, β- and γ-secretase are not altered in NPC1$^{-/-}$ cells

To investigate whether altered C99 and sAPPα/β levels in *NPC1*$^{-/-}$ cells are due to altered activities of α- and/or β-secretase, we performed a FRET-based assay (R&D Systems Inc.). Since these fragments are direct products of APP cleavage by α- and β-secretase, we hypothesized that cholesterol accumulation in *NPC1*$^{-/-}$ cells may alter APP processing by modulating the activities of α- and/or β-secretase. However, we observed that the kinetics (not shown) and the end-point activities of both α- and β-secretases were similar in CHOwt and *NPC1*$^{-/-}$ cells (Fig. 4A), indicating that the altered processing of APP through α-/β-secretase pathways in *NPC1*$^{-/-}$ cells is not due to altered activities of either α- or β-secretase.

To analyze γ-secretase activity in CHOwt and *NPC1*$^{-/-}$ cells, we performed a FRET-based assay (R&D Systems Inc.) (Fig. 4A) and an *in vitro* γ-assay (Fig. 4B). Total cellular membranes isolated from *C99*-transfected cells showed similar rates of *in vitro* generation of Aβ40 (Fig. 4B, upper panel) and AICD (Fig. 4B, lower panel) in CHOwt and *NPC1*$^{-/-}$ cells upon 1 and 4 h incubation at 37 °C. Treatment with the γ-secretase inhibitor WPE-III-31C (10 µM) completely blocked *in vitro* formation of both Aβ40 and AICD in both cell lines (Fig. 4B), confirming that the activity measured *in vitro* was indeed γ-secretase. In addition, a FRET-based assay showed similar levels of γ-activity between wt and *NPC1*$^{-/-}$ cells (Fig. 4A). Together, these findings support that activity of γ-secretase is similar between CHOwt and CHO *NPC1-null* cells and that increased intracellular Aβ levels in *NPC1*$^{-/-}$ cells are not due to increased activity of γ-secretase.

3.5. Increased formation of intracellular Aβ and APP–CTFs in NPC1$^{-/-}$ cells is dependent on cholesterol levels

To test whether increased intracellular Aβ/CTFs levels in *NPC1*$^{-/-}$ cells are related to higher cholesterol levels, we reduced the levels of cholesterol in both CHOwt and *NPC1*$^{-/-}$ cells by growing the cells in a lipid-deficient serum (LPDS) and we compared APP processing in untreated and cholesterol-depleted cells (Fig. 5). In *NPC1*$^{-/-}$ cells

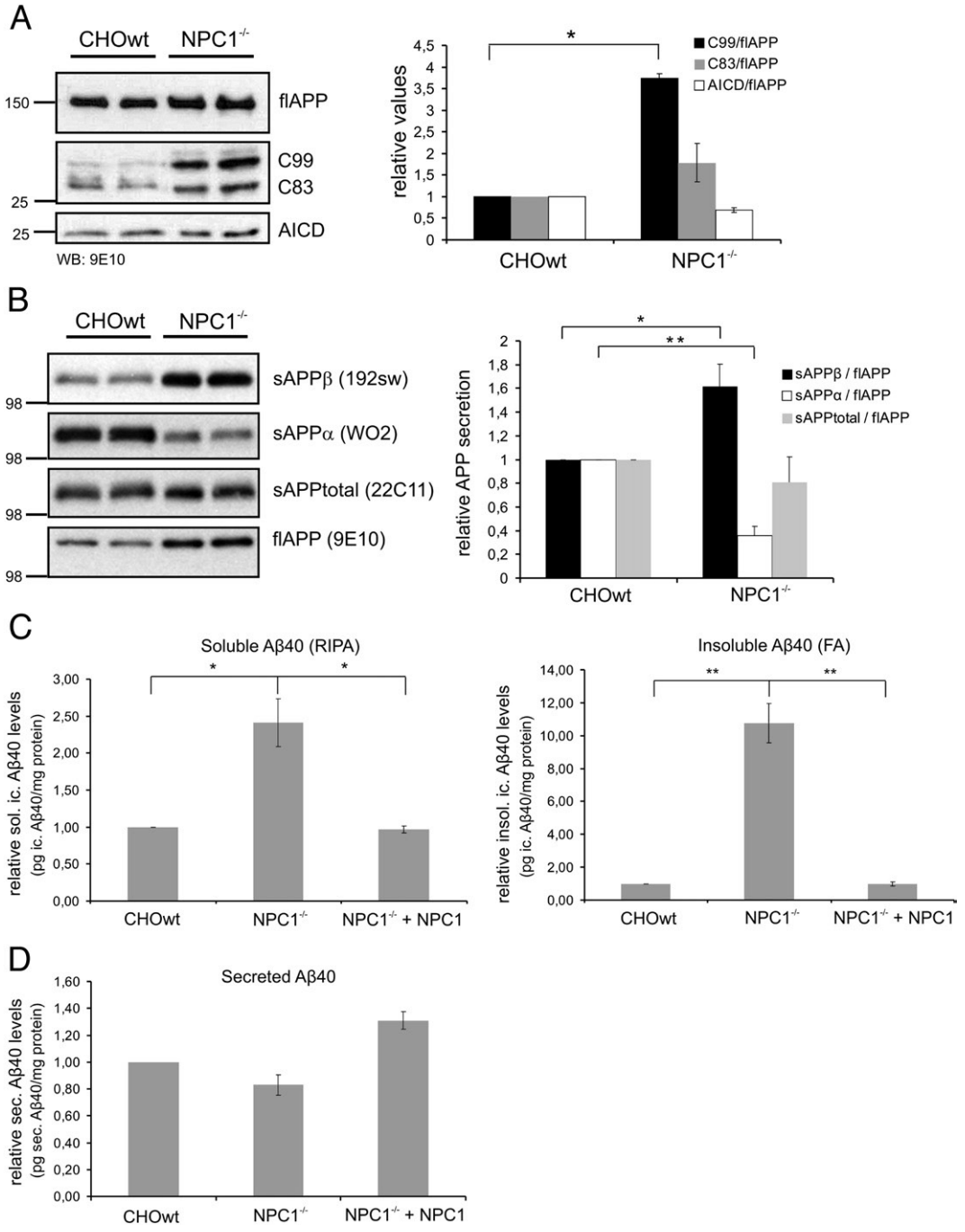

Fig. 2. *NPC1*$^{-/-}$ cells show increased APP processing through the β-secretase pathway. CHOwt, *NPC1*$^{-/-}$ cells and *NPC1*-transfected *NPC1*$^{-/-}$ cells were transiently transfected with *APPsw-6myc* and we monitored (A) C99, C83 and AICD levels in the cell lysate by western blotting (left panel) and quantification of the results with Image J (right panel); (B) sAPPβ, sAPPα and sAPP total levels by western blotting (left panel) and quantification of the results (right panel); (C) soluble (RIPA-extractable, left panel) and insoluble (FA-extractable, right panel) intracellular Aβ40 levels (ELISA assay, BioSource International Inc.); and (D) secreted Aβ40 levels (ELISA assay, BioSource International Inc.). Shown are mean and SEM of three independent experiments. Statistical analysis was performed using Student's *t*-test: $^{*}p<0.05$, $^{**}p<0.01$.

grown in LPDS media, cholesterol levels were decreased (Fig. 5A, $p<0.001$) to levels comparable to untreated wt cells (Fig. 5A). Filipin staining confirmed a strong reduction in the accumulation of free cholesterol in LPDS-grown *NPC1*$^{-/-}$ cells compared to cells grown in FBS media (Fig. 5B). Cholesterol depletion lowered the levels of both soluble ($p<0.05$) and insoluble ($p<0.01$) Aβ40 in *NPC1*$^{-/-}$ cells (Fig. 5C, left and right panel, respectively) to levels comparable with wt cells. The same effect was observed when we depleted cholesterol levels using statin treatment in LPDS media (not shown). Since we observed that cells under statin treatment were less viable compared to the cells grown in LPDS media alone, causing the same effect on cholesterol reduction, we used LPDS-grown cells as a model of cholesterol reduction. Lowering cholesterol levels in CHOwt cells (Fig. 5A, $p<0.001$) caused a significant decrease of insoluble (FA-extractable) intracellular Aβ40 ($p<0.01$) as well (Fig. 5C, left and right panel, respectively).

Cholesterol depletion in *NPC1*$^{-/-}$ cells also caused a strong reduction of both endogenous and exogenous APP–CTFs levels (Fig. 5D and E, respectively), supporting the hypothesis that increased C-terminal APP fragment formation upon NPC1 loss is related to high cholesterol levels/accumulation. The observation that cholesterol depletion in *NPC1*$^{-/-}$ cells exerts the same effect on APP–CTFs and

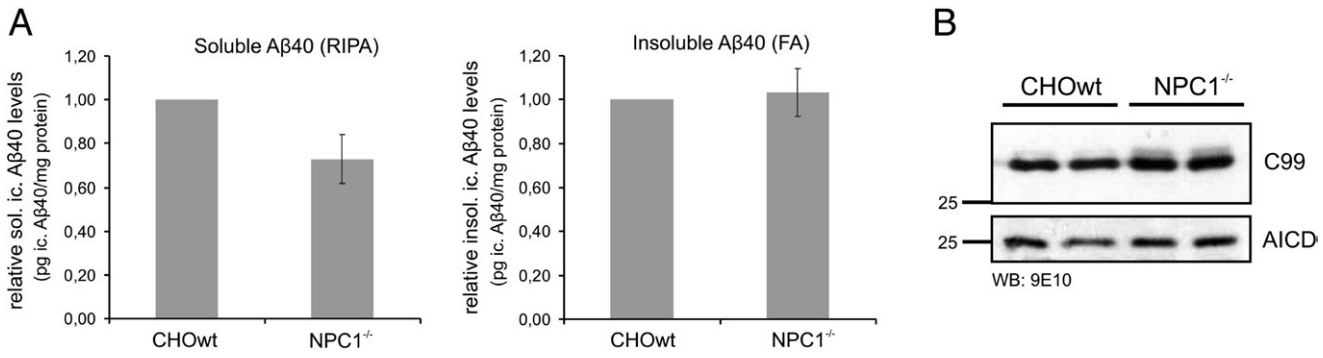

Fig. 3. Overexpression of C99, a direct γ-secretase substrate, in *NPC1*$^{-/-}$ cells does not lead to increased Aβ levels. The CHOwt and *NPC1*$^{-/-}$ cells were transiently transfected with *C99-6myc* construct. (A) In *C99*-transfected *NPC1*$^{-/-}$ cells, the levels of both soluble (left panel) and insoluble (right panel) intracellular Aβ40 were similar to the levels seen in wt cells (ELISA assay, BioSource International Inc.). (B) Processing of *C99-6myc* revealed similar levels of AICD between CHOwt and *NPC1*$^{-/-}$ cells.

intracellular Aβ as NPC1 expression (Figs. 1C and 2C, respectively) further confirms that the effect on intracellular Aβ and APP–CTFs upon loss of NPC1 is due to the cholesterol levels/accumulation caused by NPC1 loss rather than NPC1 function directly.

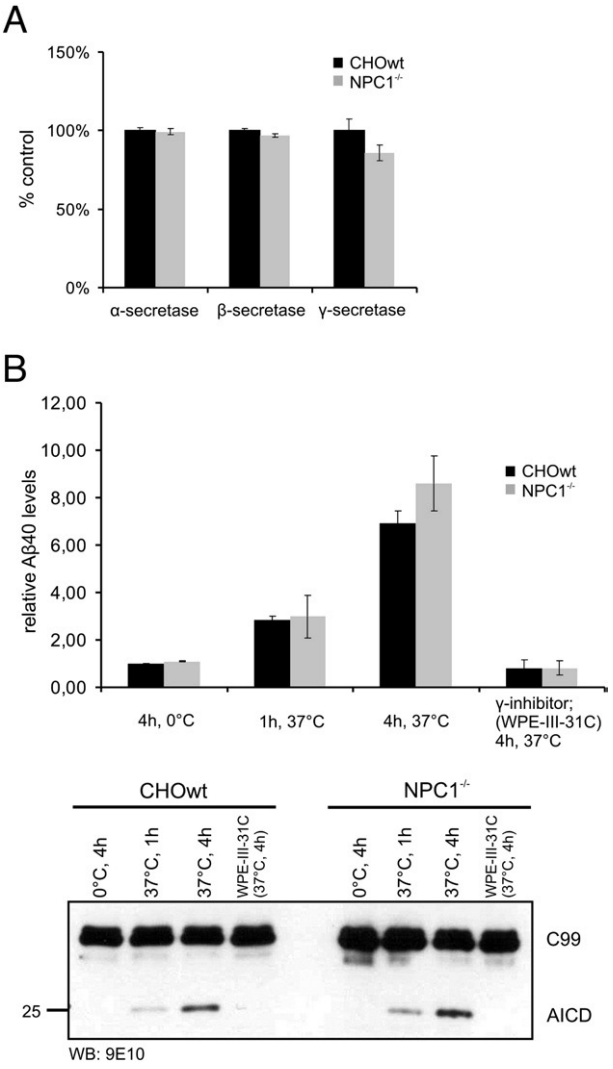

Fig. 4. Activities of α-, β- and γ-secretase are not altered in *NPC1*$^{-/-}$ *cells*. (A) Activities of α-, β- and γ-secretase were measured by a FRET-based assay (R&D Systems Inc.). (B) *In vitro* γ-secretase assay using total cellular membranes showed similar rate of Aβ40 (upper panel) and AICD synthesis (lower panel) in CHOwt and *NPC1*$^{-/-}$ cells. Shown are mean and SEM of three independent experiments. Statistical analysis was performed using Student's *t*-test.

3.6. APP expression at the cell surface is markedly decreased in NPC1$^{-/-}$ *cells*

To further investigate the effect of NPC1 loss on APP processing through α- and β-secretase pathways, we analyzed the levels of endogenous APP at the cell surface in CHOwt and *NPC1*$^{-/-}$ cells. We hypothesized that cell surface expression of APP would be altered upon cholesterol accumulation in *NPC1*$^{-/-}$ cells leading to altered processing through either α- or β-secretase pathways. Indeed, the biotinylation assay showed a significant decrease in cell surface APP in *NPC1*$^{-/-}$ cells compared to CHOwt ($p<0.001$, Fig. 6A and B). These findings, together with our results of APP processing in CHOwt and *NPC1*$^{-/-}$ cells (Figs. 1 and 2), further suggest that cholesterol accumulation upon loss of NPC1 causes a shift in APP localization which leads to increased cleavage of APP by β-secretase. We also observed a marked difference in endogenous APP localization between CHOwt and CHO-*NPC1*$^{-/-}$ cells using confocal microscopy (Fig. 6C). To detect cell surface APP, we stained non-permeabilized cells with APP ectodomain 5313 antibody. We observed a pronaunced dotted cell membrane staining in CHOwt cells which was reduced in *NPC1*$^{-/-}$ cells. Furthermore, the analysis of APP localization using 6687 antibody on permeabilized cells showed strong perinuclear signal in CHOwt cells, while in *NPC1*$^{-/-}$ cells we observed a strong vesicular staining of APP at a periphery (Fig. 6C).

Either NPC1 expression or cholesterol depletion in *NPC1*$^{-/-}$ cells reversed the levels of endogenous APP at the cell surface (Fig. 6A and B), supporting the hypothesis that decreased APP localization at the cell surface due to cholesterol accumulation upon NPC1 loss may be the primary cause of altered APP processing in *NPC1*$^{-/-}$ cells.

4. Discussion

Cholesterol levels have been implicated in the pathogenesis of Alzheimer's disease (AD) [9,37] and a non-AD disorder with disturbed cholesterol homeostasis, Niemann–Pick type C (NPC), parallels AD by showing neurodegeneration, tau pathology and increased formation of Aβ [1,2,4,10,38]. The results of this work show that increased levels of intracellular Aβ upon loss of NPC1 function are tightly linked to cholesterol-mediated defects in *NPC1*$^{-/-}$ cells, supporting a link between altered cholesterol metabolism/levels and Aβ production. In addition, we show that increased formation of intracellular Aβ upon cholesterol accumulation in *NPC1*$^{-/-}$ cells is due to increased APP cleavage by β-secretase most likely the result of decreased APP expression at the cell surface.

In contrast to previous reports that used U18666A compound to mimic NPC disease or *NPC1* mutant cells/mouse model, we used CHO cells that are null for the *NPC1* gene to study how the loss of NPC1 function modulates APP processing. Our results on Aβ and APP–CTF levels confirm that the NPC model we have used shows an AD-like

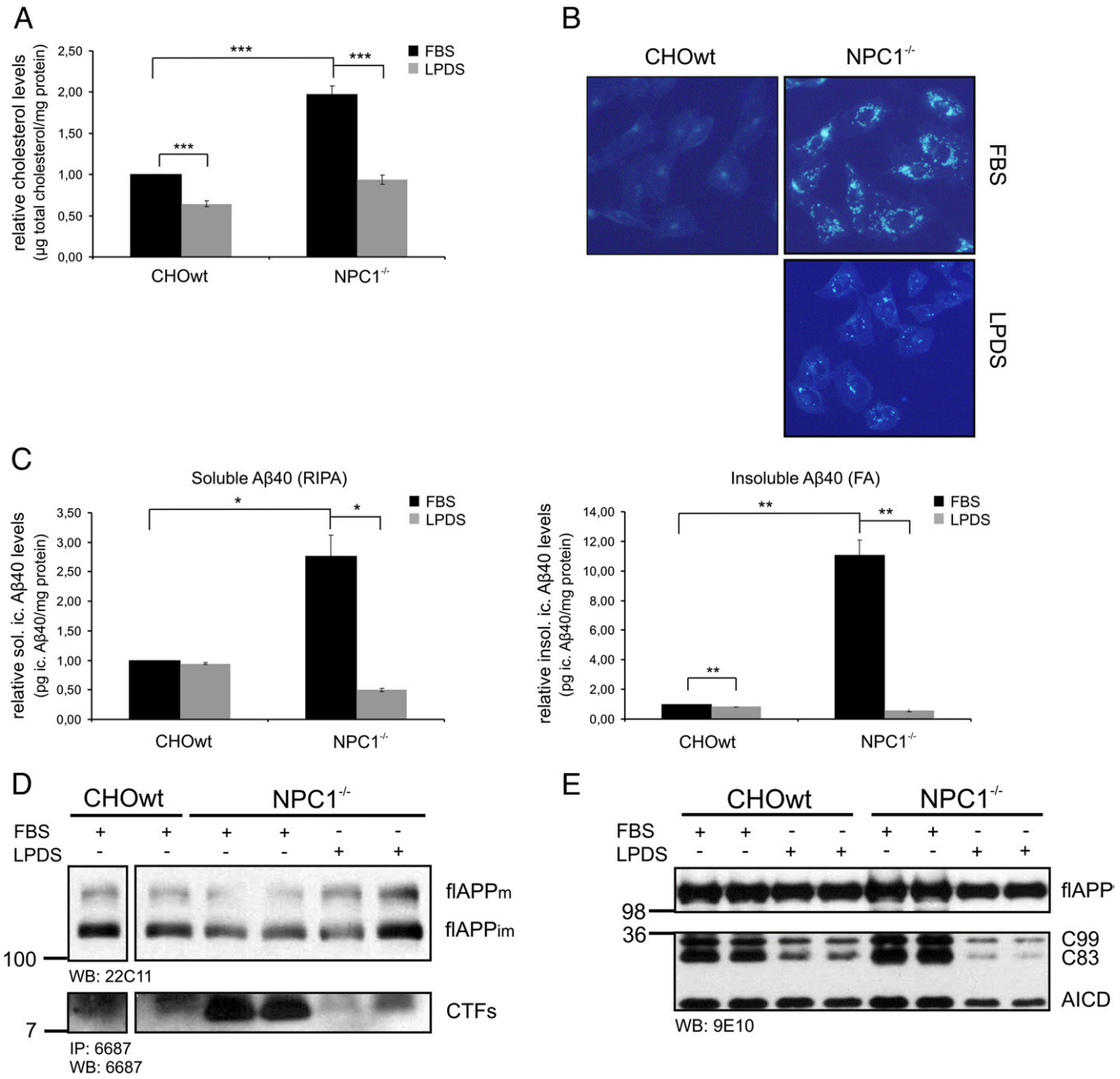

Fig. 5. Cholesterol depletion in *NPC1*$^{-/-}$ cells reverts intracellular Aβ/CTF levels to that seen in CHOwt cells. To deplete cholesterol, *APPsw-6myc* transiently transfected cells were grown in the medium containing 10% LPDS for 48 h. We measured (A) the levels of total cholesterol by AmplexRed cholesterol assay (Molecular Probes, Invitrogen), (B) accumulation of free cholesterol by filipin staining, (C) the levels of soluble (left panel) and insoluble Aβ40 (right panel) by ELISA assay (BioSource International Inc.), (D) the levels of endogenous APP–CTF stubs and (E) the levels of exogenous APP–CTF stubs by western blotting. Shown are mean and SEM of three independent experiments. Statistical analysis was performed using Student's *t*-test: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

phenotype comparable to that observed in previous reports [2,10,38]. CHO *NPC1*$^{-/-}$ cells showed increased intracellular Aβ levels as well as increased levels of APP–CTFs compared to CHOwt cells. Interestingly, *NPC1*$^{-/-}$ in CHO cells caused a significant increase in the insoluble (aggregated) pool of intracellular Aβ compared to the soluble intracellular pool with no effect on secreted Aβ levels, similar to what was previously observed in U18666A-treated CHO cells [38] and in U18666A-treated primary mouse cortical neurons [10]. While we observed a strong increase in intracellular Aβ40 species (Aβ42 levels were below the detection limit of the ELISA assay used), previous studies showed an increase of Aβ42 only [10,38] or both Aβ40 and Aβ42 [2]. Although these discrepancies may be due to the different cell lines used and/or the way NPC disease was modeled, they all show that cholesterol accumulation upon loss of NPC1 leads to increased C99 levels and increased levels of the intracellular pool of aggregated Aβ. Using *NPC1*-stably transfected CHO *NPC1*$^{-/-}$ cells, we confirmed that NPC1 expression rescued the Aβ and C99 phenotype observed in the *NPC1*$^{-/-}$ cells.

The three main findings in this work support the hypothesis that increased formation of intracellular Aβ upon cholesterol accumulation in NPC disease is due to altered APP localization leading to increased APP cleavage by β-secretase. First, steady-state levels of APP–CTF/C99 and sAPPβ, the two direct products of APP cleavage by β-secretase, were increased in *NPC1*$^{-/-}$ cells compared to CHOwt.

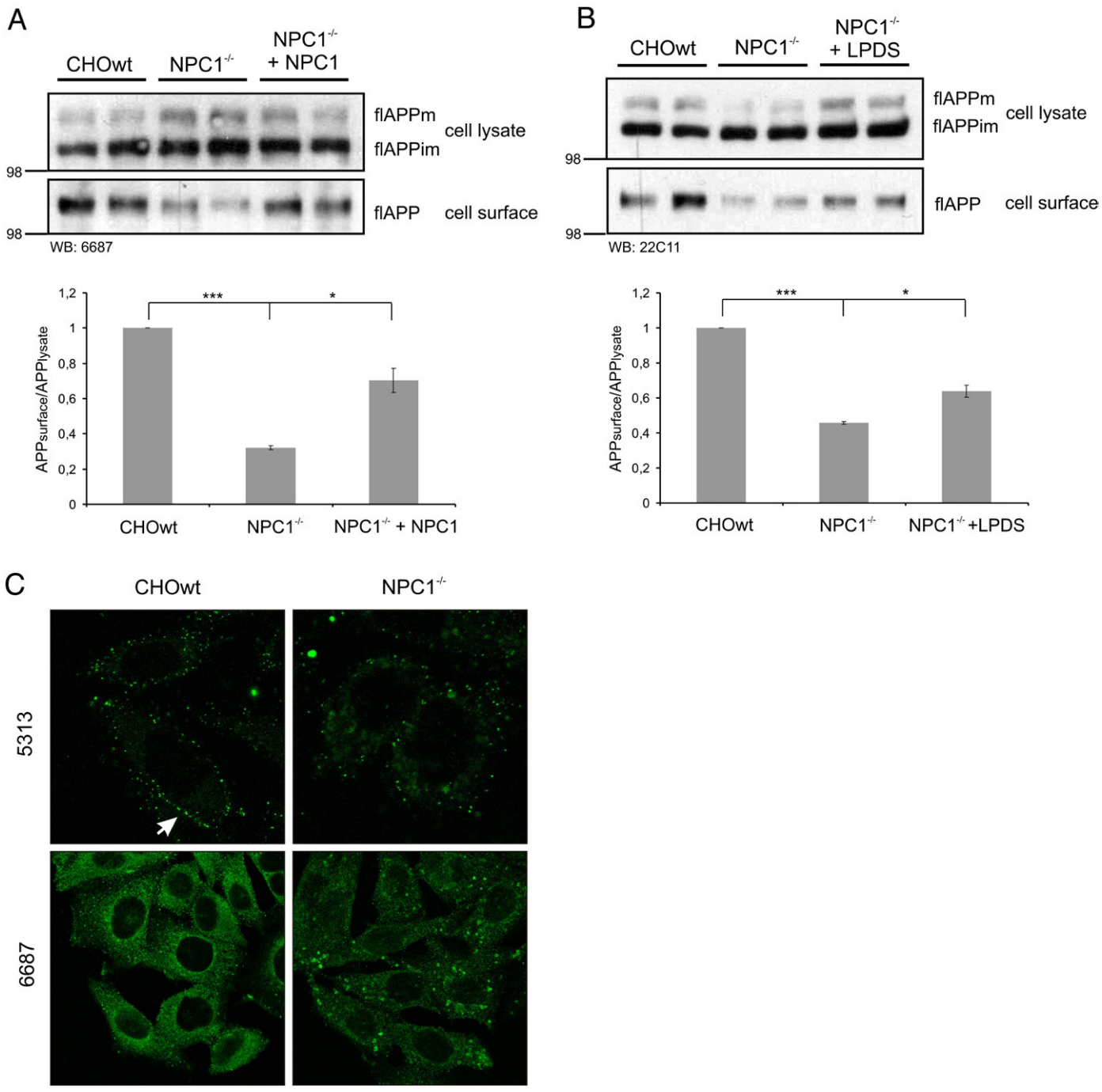

Fig. 6. *NPC1*$^{-/-}$ cells show decreased expression of APP at the cell surface and accumulation of APP in a punctuate vesicular structures. Endogenous levels of APP at the cell surface of CHOwt, *NPC1*$^{-/-}$ cells and *NPC1*-transfected *NPC1*$^{-/-}$ cells were monitored using a biotinylation assay. A significant decrease of cell surface APP was observed in *NPC1*$^{-/-}$ cells. Note that both NPC1-expression (A) and cholesterol depletion (B) reversed the levels of cell surface APP to levels similar to those seen in wt cells. Blots were quantified with Image J. Statistical analysis was performed using Student's *t*-test: $^{*}p<0.05$, $^{***}p<0.001$. (C) Immunostaining with 5313 and 6687 antibody revealed a marked difference in APP staining between CHOwt and *NPC1*$^{-/-}$ cells. Cell surface staining of non-permeabilizied cells with ectodomain 5313 antibody revealed stronger dotted signal at the cell surface of wt cells. The 6687 antibody staining of permeabilizied cells showed that APP was localized more perinuclearly in wt cells, while upon NPC1 loss it accumulated in vesicular punctuate structures at a periphery. *Arrow* indicates the strong cell surface staining in CHOwt cells.

Second, experiments using a *C99* construct, a direct γ-secretase substrate and the product of β-secretase APP cleavage, showed that increased β-secretase generation of C99 is a prerequisite for the Aβ increase in *NPC1*$^{-/-}$ cells. The observation that intracellular Aβ levels are not increased in C99-expressing *NPC1*$^{-/-}$ cells further indicates that increased Aβ levels upon loss of NPC1 are not due to decreased clearance nor are due to cholesterol-mediated accumulation of intracellular Aβ/C99 *per se*. Our findings show that the effect on Aβ in *NPC1*$^{-/-}$ cells is due to altered APP processing upstream of C99 cleavage by γ-secretase.

Third, biotinylation experiments showed a marked decrease in cell surface APP in *NPC1*$^{-/-}$ cells compared to wt cells, suggesting that altered APP processing by β-secretase in *NPC1*$^{-/-}$ cells may primarily be caused by altered APP localization, *i.e.* decreased APP expression at the cell surface. Since APP cleavage by α-secretase is thought to mainly occur at the cell surface [29], while β-secretase cleavage is

suggested to happen within endosomal and secretory compartments [6,24,32], finding less APP at the cell surface upon loss of NPC1 would be expected to lead to decreased processing by α-secretase with a parallel increase in β-secretase cleavage. Indeed, an increase of sAPPβ was observed either at endogenous (*APPwt*) or exogenous (using *APPsw* construct) levels as well as a decrease of sAPPα. To further address β-secretase cleavage of APP in *NPC1*$^{-/-}$ cells, the goal of future studies would be to examine whether cholesterol accumulation in these cells causes increased APP endocytosis and/or increased APP/BACE1 colocalization within endosome/secretory compartments. Indeed, in *NPC1*$^{-/-}$ cells APP staining revealed punctuate structures, suggesting APP accumulation in a certain intracellular compartments. Previous reports have shown that cholesterol may modulate endocytosis [14]. Von Arnim et al. [33] demonstrated that cholesterol modulates APP cleavage by β-secretase and APP/β-secretase proximity within endosome compartments. These authors found that cholesterol enrichment increases sAPPβ levels and decreases sAPPα secretion, similar to our observation upon cholesterol accumulation in *NPC1*$^{-/-}$ cells. In addition, we have recently shown that cholesterol accumulation in NPC1$^{-/-}$ cells leads to increased APP–CTF partitioning within cholesterol-rich lipid rafts [13].

Our cholesterol depletion experiments in CHOwt and CHO *NPC1*$^{-/-}$ cells showed that increased intracellular Aβ/C99 upon loss of NPC1 is linked to cholesterol levels/accumulation. Cholesterol depletion in *NPC1*$^{-/-}$ cells significantly lowered C99 as well as aggregated intracellular Aβ levels to levels similar to those observed in wt cells and in *NPC1* transfected *NPC1*$^{-/-}$ cells. Importantly, either cholesterol depletion or NPC1-expression in *NPC1*$^{-/-}$ cells markedly increased the levels of APP at the cell surface, further supporting the hypothesis that cholesterol may modulate APP trafficking and thus its cleavage by α-secretase (at the cell surface) and β-secretase (within endosome/secretory compartments). Finding that lowering cholesterol levels corrects the AD-like phenotype in *NPC1*$^{-/-}$ cells by increasing APP expression at the cell surface indicates that altered APP trafficking may be the primary cause of the cholesterol-effect on APP metabolism and on the pathogenesis of Alzheimer's disease. However, since we used LPDS-grown cells as a model for cholesterol depletion, we cannot exclude the possibility that the observed effect on APP processing could be due to ApoE depletion as well [39].

Although it has been suggested that cholesterol may modulate activities of α-, β- and/or γ-secretase, we show that cholesterol accumulation upon NPC1 deficiency does not alter the activities of α-, β- and/or γ-secretase. While similar activities of α- and β-secretase were previously detected in NPC and wt mouse, γ-secretase activity was reported to be slightly increased in NPC mouse brain homogenates [2]. However, using an *in vitro* γ-secretase assay, we further confirmed no change in γ-activity between wt and *NPC1*$^{-/-}$ cells.

It is intriguing that increased processing of APP through the β-secretase pathway in *NPC1*$^{-/-}$ cells generated an increase of only the intracellular and not the secreted pool of Aβ, a finding that was observed in this study as well as by Yamazaki et al. [38] and Jin et al. [10]. The effect of cholesterol accumulation on intracellular Aβ could be explained by either decreased recycling of APP to the plasma membrane, and thus less APP secretion, and/or an impairment of the autophagic pathway in *NPC1*$^{-/-}$ cells. Indeed, decreased recycling of the transferrin receptor (TfR) as well as Bodipy-labeled lactosylceramide (Bodipy-LacCer) in *NPC1*$^{-/-}$ cells has been previously reported [12,20]. In addition, altered trafficking within the endocytic pathway has been observed for several other proteins including cation-independent mannose-6-phosphate receptor (CI-MPR) [21], Rab4 [3] and TfR (Pipalia et al., 2007). However, since sAPPβ levels are increased in *NPC1*$^{-/-}$ cells, we speculate that recycling of APP upon NPC1 dysfunction may not be affected but rather that an accumulation of autophagosomes and defective autophagosome-lysosome fusion, previously observed in lysosomal storage disorders [27], could explain the specific effect of NPC1-loss on intracellular Aβ and not on its secreted pool. Although cholesterol-mediated activation of autophagy was reported in NPC disease by increased beclin 1 expression [15,19], Settembre et al. [27] showed inefficient degradation of exogenous aggregate-prone proteins (expanded huntingtin and mutated α-synuclein) in lipid storage disorders multiple sulfatase deficiency (MSD) and mucopolysaccahridosis type IIIA (MPSIIIA). Since macroautophagy, *i.e.* accumulation of autophagosomes and late autophagic vacuoles (AV), has been observed early in Alzheimer's disease and is a major reservoir of intracellular Aβ [40], we speculate that cholesterol accumulation upon loss of NPC1 sequesters APP and β-secretase (BACE1) within accumulated autophagosomes [41] resulting in increased β-secretase cleavage of APP and thus increased CTFβ/C99 and intracellular Aβ generation. However, further studies are necessary to confirm this.

5. Conclusions

In summary, our results demonstrate that cholesterol accumulation upon NPC1 loss of function affects APP partitioning between α- and β-secretase pathways favouring APP cleavage by β-secretase. Furthermore, our findings suggest that altered APP cell surface expression/localization may be a primary cause of altered APP processing upon cholesterol accumulation in *NPC1*$^{-/-}$ cells. We postulate that accumulation of cholesterol upon loss of NPC1 alters APP compartmentalization causing increased cleavage by β-secretase and increased C99 and Aβ levels. These findings add to the mechanism of the cholesterol-effect on APP metabolism and demonstrate that there is a link between cholesterol homeostasis, APP metabolism and the pathogenesis of Alzheimer's disease.

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