The Amyloid Precursor Protein Copper Binding Domain Histidine Residues 149 and 151 mediate APP Stability and Metabolism

Loredana Spoerri¹², Laura J. Vella¹², Chi L. L. Pham¹², Kevin J. Barnham²³⁴, Roberto Cappai¹²

From the ¹Department of Pathology, ²The Bio21 Molecular Science and Biotechnology Institute, ³The Mental Health Research Institute of Victoria, ⁴Department of Pharmacology, University of Melbourne, Melbourne, University of Melbourne, Melbourne, Victoria 3010, Australia.

Running title: CuBD-mediated regulation of APP metabolism

To whom correspondence should be addressed: Roberto Cappai, Department of Pathology, The University of Melbourne, Melbourne, Victoria 3010, Australia, Tel.: +61-3-83442556; Fax: +61-3-83444004; email: r.cappai@unimelb.edu.au

Keywords: Amyloid Precursor Protein, Copper, Alzheimer's disease, Secretases, Amyloid beta.

CAPSULE

Backgrounds: Copper modulates the metabolism of the Alzheimer’s disease Amyloid Precursor Protein (APP).

Results: Histidines residues 149 and 151 of APP copper binding domain modulate APP ER-to-Golgi trafficking.

Conclusion: Histidines residues 149 and 151 of APP are pivotal for APP proteolytic processing.

Significance: Elucidating the copper binding domain elements involved in APP stabilization and metabolism is crucial for understanding the physiological role of APP.

SUMMARY

One of the key pathological hallmarks of Alzheimer’s disease (AD) is the accumulation of the APP-derived amyloid β (Aβ) peptide in the brain. Altered copper homeostasis has also been reported in AD patients and is thought to increase oxidative stress and to contribute to toxic Aβ accumulation and regulate APP metabolism. The potential involvement of the N-terminal APP copper binding domain (CuBD) in these events has not been investigated. Based on the tertiary structure of the APP CuBD we examined the histidine residues of the Cu binding site (His147, His149 and His151). We report that histidines 149 and 151 are crucial for CuBD stability and APP metabolism. Co-mutation of the APP CuBD His149 and His151 to asparagine decreased APP proteolytic processing, impaired APP ER-to-Golgi trafficking and promoted aberrant APP oligomerization in HEK293 cells. Expression of the triple H147,149,151N-APP mutant led to upregulation of the unfolded protein response. Using recombinant protein encompassing the APP CuBD, we found that insertion of asparagines at position 149 and 151 altered the secondary structure of the domain. This study identifies two APP CuBD residues that are crucial for APP metabolism and suggests an additional role of this domain in APP folding and stability beside its previously identified copper binding activity. These findings are of major significance for the design of novel AD therapeutic drugs targeting this APP domain.

INTRODUCTION

The Amyloid Precursor Protein (APP) is a type I transmembrane glycoprotein that has a central role in the pathogenesis of AD as the source of the neurotoxic amyloid β peptide (Aβ). During trafficking through the secretory pathway APP undergoes various post-translational modifications such as N- and O-glycosylation, phosphorylation and tyrosine sulfation (1-3). The N-glycosylated only, immature APP is mainly found in the ER/Cis-Golgi region whereas the N-O-glycosylated, mature, APP mostly localizes in the trans-Golgi/plasma membrane zone (3). Upon reaching the plasma membrane APP is rapidly
cuBD-mediated regulation of APP metabolism

cleaved by α-secretase to release the ectodomain or endocytosed as a full length protein in early endosomes (4,5). Following internalization, the majority of endocytosed APP is rapidly sorted to the lysosomal compartment for degradation while a smaller portion is recycled back to the plasma membrane (4).

APP is proteolytically processed by two different pathways. During amyloidogenic processing, APP is first cleaved at its β-site generating the sAPPβ ectodomain (6) and the membrane bound C-terminal domain (C99) species (7). C99 can subsequently be processed by γ-secretase giving rise to Aβ and the APP intracellular domain (AICD) (8). Amyloidogenic processing of APP occurs physiologically (9-11), therefore imbalances of this event are linked to AD pathology. As suggested by its name, the non-amyloidogenic pathway prevents Aβ formation by cleaving APP at the α-site located within the Aβ sequence (12,13). This event results in the shedding of the sAPPα ectodomain and the generation of the 83 amino acid C-terminal fragment (C83) (3) which can be further processed by γ-secretase in a similar way as for C99, giving rise to the analogous AICD and a small p3 (14) fragment instead of Aβ.

Several studies have demonstrated altered APP metabolism in AD patients with increased (15-17) or decreased (16,18) APP expression as well as up- or down-regulated β- and α-secretase activity respectively, supporting a mis-metabolism of APP in AD. Altered copper levels in hippocampus, amygdala, neuropil, cerebro-spinal fluid (CSF) and serum of AD patients have also been reported (19-23). Moreover, there is a negative correlation between copper levels and elevated amyloidogenic processing of APP in AD animal models (24,25). The APP CuBD is located in the N-terminal part of the protein, next to the growth factor domain (GFD). Its tertiary structure is composed by an α-helix (147-159) packed against three β-sheets (133-139, 162-167, 181-188) and stabilized by three disulfide bridges (C133-C187, C144-C174 and C158-C186) and a small hydrophobic core (Leu-136, Trp-150, Val-153, Ala-154, Leu-165, Met-170, Val-182, and Val-185) (26,27). Strand β3 is connected to strand β1 and to the helix via C133-C187 and C144-C174 bonds, respectively, C158-C186 bond links together two loop located at

copper levels and elevated amyloidogenic processing of APP in AD animal models (24,25).

The APP CuBD is located in the N-terminal part of the protein, next to the growth factor domain (GFD). Its tertiary structure is composed by an α-helix (147-159) packed against three β-sheets (133-139, 162-167, 181-188) and stabilized by three disulfide bridges (C133-C187, C144-C174 and C158-C186) and a small hydrophobic core (Leu-136, Trp-150, Val-153, Ala-154, Leu-165, Met-170, Val-182, and Val-185) (26,27). Strand β3 is connected to strand β1 and to the helix via C133-C187 and C144-C174 bonds, respectively, C158-C186 bond links together two loop located at

the end of the molecule. APP His147, His151 and Tyr168 appear to be the putative binding ligands for copper since all of them have been confirmed by NMR and crystallography studies to be bound to or to be in close proximity to copper. The role of Met170 remains unclear since the crystallography data excludes its involvement in copper binding. By employing a synthetic peptide encompassing APP residues 135 to156, earlier studies demonstrated that the CuBD can reduce copper(II) to copper(I) (28). The mutation of His147 to asparagine in this peptide decreased copper reduction (28) while leaving copper binding unaffected (29).

 Copper has been shown to regulate APP metabolism. In cellular models, increased intracellular copper levels up-regulated APP expression (30) and promoted the non-amyloidogenic processing of APP (31). Moreover, APP trafficked from the Golgi to the plasma membrane exhibited a decreased endocytosis in response to copper (32). Conversely, a decrease in intracellular copper levels led to decreased APP expression (33) and increased amyloidogenic processing of APP (34). In addition, APP is thought to modulate copper homeostasis by participating in copper efflux (32). Studies in both animal and cellular models demonstrated increased levels of copper when APP gene was knocked out (35,36). Since APP is a copper binding protein, these effects are likely to be related to APP’s ability to bind copper, however this link has not been elucidated yet.

In this study, we investigated the effect of mutating the putative copper coordinating residues of APP, His147 and His151, and a control histidine (His149) residue, to asparagine on APP cellular metabolism. We report that the presence of asparagines residues at positions 149 and 151 impairs APP maturation and APP ER- to-Golgi trafficking. We provide evidence that this effect is due to APP retention in the ER most likely caused by APP misfolding.

EXPERIMENTAL PROCEDURES

Antibodies and reagents - The following antibodies and dilutions were used for western blotting: WO2 at 1/1000 (37), Syntaxin6, EEA1, BIP, IRE1α, Ero1Lα, PERK, CHOP, PDI, Calnexin, GAPDH and β-tubulin all at 1/1000

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
CuBD-mediated regulation of APP metabolism

except EEA1 and GAPDH at 1/2500 and 1/5000 respectively (all Cell Signaling), secondary IgG anti-mouse conjugated to horse radish peroxidase at 1/10000 and secondary IgG anti-rabbit conjugated to horse radish peroxidase at 1/10000 (both GE Healthcare). The following antibodies were used for immunocytochemistry: W02 at 1/500 (37), anti-GRASP65 at 1/1000 (Abcam, kind gift from Ms. Fiona Houghton and Prof. Paul Gleson, The University of Melbourne), secondary IgG anti-mouse conjugated to AlexaFluor® 488 at 1/10000 (Invitrogen) and secondary IgG anti-rabbit conjugated to horse radish peroxidise at 1/10000 (both GE Healthcare). The following antibodies were used for immunocytochemistry: WO2 at 1/500 (37), anti-GRASP65 at 1/1000 (Abcam, kind gift from Ms. Fiona Houghton and Prof. Paul Gleson, The University of Melbourne), secondary IgG anti-mouse conjugated to AlexaFluor® 488 at 1/1000 (Invitrogen) and secondary IgG ant-rabbit conjugated to AlexaFluor® 594 at 1/1000 (Invitrogen). DAPI nucleic acid stain (Invitrogen) was used at 1/1000 to detect the cell nucleus. The FK2 antibody (Enzo Life Sciences, kind gift from Ms. Belinda Guo and A/Prof. Andrew Hill, The University of Melbourne) was used for the immunoprecipitation of ubiquitinated proteins (0.7μl/700μl of lysate).

Brefeldin A (Sigma) was employed at 1 μg/ml.

Capture and detection antibodies used for Aβ detection ELISA assay were a kind gift from Dr. Lisa Muenter and Prof. Gerd Multhaup (Freie Universität Berlin, licensed by The Genetics Company).

Cell culture - WT Human Embryonic Kidney 293 (HEK293) (ATCC) cells were maintained at 37°C in 5% CO₂ in DMEM medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). The same conditions and the same culture medium supplemented with 6 µg/ml Blasticidin (Invitrogen) or 500 µg/ml Geneticin (Invitrogen) were employed to maintain stably transduced HEK293 cells or WT 293FT producer cells.

Generation of APP-HEK293 stable cell lines - The wild type APP695 cDNA was subcloned into the pLenti6/V5-D-TOPO vector (Invitrogen) according to manufacturer’s instructions. Briefly, blunt-end APP695 was generated by amplification of APP695 cDNA and inserted into pLenti6/V5-D-TOPO vector by employing the TOPO cloning reaction. A STOP codon was included in the C-terminal primer to prevent fusion of APP with the V5 tag contained in the pLenti6/V5-D-TOPO vector. The wt-APP-pLenti construct was used as a template to introduce H147N, H149N, H151N, H147,149N, H147,151N and H147,149,151N mutations by site-directed mutagenesis (Quik-change System, Stratagene). Lentivirus particles were generated by co-transfection of the 293FT producer cell line with the construct of interest, the packaging mix (Invitrogen) and the Lipofectamine 2000™ reagent according to manufacturer’s instructions. HEK293 cells were transduced by overnight incubation with the lentivirus particles and cells stably expressing APP were selected with Blasticidin (6 µg/ml).

Aβ detection by sandwich enzyme-linked immunosorbent assay (ELISA) - Cells were plated in Poly-D-Lysine pre-coated 6 well plates at 0.5·10⁶ cells/well and cultured for 4 days. 96 well plates were coated overnight at 4°C with mouse W02 (0.55 µg/well) antibody diluted in carbonate buffer (100 mM, pH 9.6) and then blocked with Stabilcoat buffer (Surmodics) for 2 hours at room temperature. Plates were washed 3 x 3 minutes with low salt PBS-T buffer (3.8 mM NaH₂PO₄, 16.2 mM Na₂HPO₄, 150 mM NaCl, 0.1% (v/v), pH 7.4) then sample-binding buffer (80 mM PBS, pH7.4, 1.5% BSA, 0.5% Tween20, 0.1% Thiomerosal) was added together with cell culture media to the 96 well plate and incubated overnight at 4°C. The plates were again washed 3 x 3 minutes with low salt PBS-T buffer, then 4G8-HRP conjugated antibody (1/20'000 in sample-binding buffer) was added to the wells and the plate rocked at room temperature for 30 min. After 5 x 3 minutes washes, 100 µl of TMB substrate (One-Step Ultra TMB, Thermo Scientific) was added to each well, the plate incubated at room temperature in the dark for 30 min and the reaction stopped by adding 3% HCl (50 µl/well). Absorbance was read at 450 nm wavelength (FLUOstar Omega microplate reader, BMG Labtech).

Iodixanol fractionation - Cells were cultured in 175 cm² flasks until they reached ~90% confluence. The day of the fractionation cells were washed with PBS and homogenized in 1 ml ice cold homogenization buffer (20 mM Hepes/NaOH pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose) containing complete protease inhibitor cocktail (Roche) by 15 passages through a 26-gauge needle and 30 strokes with a Dounce homogenizer in ice. The cell homogenate was centrifuged at 1000 x g for 10 min at 4°C, the resulting post-nuclear-supernatant (PNS) collected.
and its iodixanol concentration adjusted to 25% with ice cold 50% iodixanol (5 volumes of 60% iodixanol (Optiprep, Sigma-Aldrich) diluted with one volume of dilution buffer (120 mM Hepes/NaOH pH 7.4, 6 mM EDTA, 6 mM EGTA, 0.25 M sucrose)) containing protease inhibitors. The sample was placed on the bottom of an ultracentrifugation tube and fractions of 20, 18.5, 17, 15.5, 14, 12.5, 11, 9.5, 8 and 6% ice cold iodixanol (50% iodixanol diluted with homogenization buffer) containing protease inhibitor were successively layered above it. After 20 h of centrifugation at 90,000 x g at 4°C (SW41Ti rotor, Beckman), fractions of 0.5 ml were collected from the top of the tube, precipitated using acetone, resuspended in sample buffer, boiled and equal volume of each fractions used for electrophoresis followed by immunoblot with WO2 (1:1000), BiP (1:1000), Syntaxin6 (1:1000) or EEA1 (1:1000) antibodies.

**Immunocytochemistry** - Cells were grown on poly-D-lysine pre-coated 13-mm glass coverslips for two days. Only for ER localization experiment, cells were transfected with a red fluorescent protein fused to an ER retention signal (CellLight ER-RFP BacMam 2.0, Invitrogen) before immunostaining according to manufacturer’s instructions. Following cell standard culture (Golgi localization experiment) or cell transfection (ER localization experiment), cells were fixed using 4% (w/v) paraformaldehyde (Acros Organics) in PBS for 20 min, permeabilized by incubation with 0.1% Triton X-100 (Ajax Finchem) in PBS for 3 min and non-specific sites were blocked in 2% (w/v) BSA (Sigma) in PBS (block buffer) for 1 h. WO2 (1:500 in block buffer) and anti-GRASP65 (1:1000 in block buffer) primary antibodies were used for immunocytochemistry. Primary antibodies were detected using secondary IgG antibodies conjugated to AlexaFluor® 488 or AlexaFluor® 594 fluorophores. Images were taken using a Leica TCS SP2 confocal microscope.

**Immunoprecipitation** - Cell lysate (1 ml) was pre-cleared with Protein-G sepharose beads (50 µl) for 1 hour at 4°C. Pre-cleared lysate was split in three aliquots and incubated first with FK2 or WO2 (both 0.7 µl for 300 µl lysate) or no antibody for 1 hour at 4°C and then with 20 µl of Protein-G sepharose beads overnight (14-18 hours) at 4°C. All incubation steps were performed on a rotating wheel. Isolated beads where then washed 3 times with STEN buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP-40, protease inhibitors), mixed with sample buffer, boiled and centrifuged. Supernatant was collected and analyzed by electrophoresis and WO2 antibody immunoblot. All centrifugation steps for pelleting beads were performed at 3’000 x g for 5 minutes at 4°C.

**Expression and purification of recombinant CuBD** - The cloning and the expression of the APP copper binding domain (CuBD) encompassing residues 133-189 of human wt APP has been described in detail elsewhere (38). Briefly, the protein was cloned into the pPIC-9 vector and expressed in Pichia pastoris. The protein was then concentrated using a centrifugal filter device (Amicon) with a molecular weight cut-off of 3kDa, purified by size exclusion chromatography (Hiload 16/60 Superdex 75 prep grade column, GE Healthcare) by eluting the proteins with 10mM sodium phosphate buffer pH 7.4 followed by HPLC chromatography (VYDAC 208TP C8 reversed phase column, Grace) with Buffer A = 0.1% trifluoroacetic acid in water and Buffer B = 0.1% TFA in acetonitrile (15 to 50% Buffer B over 30 minutes gradient). The peptides were then collected and lyophilized.

**Circular dichroism** - The purified and lyophilized proteins were resuspended in 10mM phosphate buffer pH 7.4 prior to CD analysis. Circular dichroism (CD) measurements were performed on a JASCO 815 CD spectrometer (Tokyo, Japan), using the Jasco software. Far UV-CD spectra were collected between 180-260 nm at 20°C. Measurements were recorded at 50 nm/min with a bandwidth of 1 nm and a response time of 8 s by averaging three accumulation scans per measurement. All spectra were background subtracted, and smoothed with the default algorithm in the Jasco software. Deconvolution of the spectra was performed using Dichroweb algorithm CDSSTR (39-42).

**Graphic simulation of protein three-dimensional structure**

Protein three-dimensional structures were displayed using the molecular graphic software MOLMOL (Reto Koradi, Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule-Hönggerberg, CH-8093,
Zurich, Switzerland) (43) and the 1OWT data from the Protein Data Bank (PDB) repository.

RESULTS

His147, His149 and His151 mutations decrease secreted Aβ levels
To assess whether the APP N-terminal CuBD is involved in regulating APP metabolism, we investigated the effect on Aβ secretion of mutating the putative copper coordinating histidine residues, His147 and His151, and the non-coordinating histidine 149 (Fig. 1A), to asparagine. The histidine-to-asparagine mutation is considered a moderately conservative mutation since it maintains steric characteristics despite it decreases acid-base and hydrogen bonding properties (the histidine imidazole ring increases hydrogen-transfer capabilities). We generated APP mutants bearing single mutations, double mutations or the triple mutation and stably overexpressed them in Human Embryonic Kidney 293 (HEK293) cells. Aβ levels were measured in conditioned cell culture media by sandwich ELISA assay using WO2 antibody and values normalized to total cellular protein concentration and total intracellular APP levels of the corresponding lysates (Fig. 1B). This allowed us to adjust for any variation in total expression levels between the mutants. Statistical analysis revealed that all mutations caused a significant decrease in Aβ levels. Compared to wt-APP, the H147N, H149N and H151N single mutations decreased Aβ levels to ~55, ~35 and ~45% respectively, all the double mutations (H147,149N, H147,151N and H149,151N) to ~20% and the triple mutation (H147,149,151N) to ~30%.

His149 is not part of the copper binding ligands (26,27) and was initially employed as a control mutation. Its unexpected involvement in controlling APP metabolism suggested that other mechanisms than copper binding (or at least than direct copper binding) might underlie the reduction in secreted Aβ levels caused by His147, His149 and His151 mutations.

His147, His149 and His151 mutations decrease secreted sAPPα levels
To assess whether the non-amyloidogenic processing of APP was affected by the mutation of His147, His149 and His151, conditioned cell culture media were examined for sAPPα by quantitative western blotting analysis using the WO2 antibody (Fig. 2A). As for Aβ, sAPPα levels were standardized to total intracellular APP levels of the corresponding lysates to adjust for any variation in total expression levels between the mutants. Compared to wt-APP-HEK, all the APP mutant HEK cells exhibited significantly decreased levels of sAPPα (note that the graph represents sAPPα levels standardized to the respective levels of total cellular APP after densitometry and not the absolute sAPPα values) (Fig. 2B). More precisely H147N, H149N and H151N mutations reduced sAPPα levels to about 65, 30 and 80% respectively. For the three double mutations sAPPα levels were decreased to about 45% and the triple mutant exhibited the strongest reduction dropping sAPPα levels to only 10%.

Together with the reduction in secreted Aβ levels, these findings suggested a general decrease in APP processing (both amyloidogenic and non-amyloidogenic processing) for the APP histidine mutants. This general reduction in processing might indicate a reduced intracellular trafficking through the secretory pathway.

Specific combinations of His147, His149 and His151 mutations decrease secreted sAPPβ levels
To further our understanding of the effect of the His147, His149 and His151 mutations on APP cellular metabolism, the secreted levels of sAPPβ were analysed. Conditioned cell culture media were examined by western blotting using an antibody specific to sAPPβ (not reacting with sAPPα or full length APP) and the resulting densitometry values were standardized to total intracellular APP levels of the corresponding lysates (Fig. 3). Secreted sAPPβ levels were reduced to approximately 65% by H147N, H147,149N and H149,151N mutations. A more pronounced decrease (approximately 40%) was observed for H149N and H147,149,151N mutations. Interestingly, H151N and H147,151N mutations didn’t affect secreted sAPPβ levels, despite exhibiting reduced levels of secreted Aβ.

When combined with the secreted Aβ and sAPPα data, these findings suggested that His147, His149 and His151 mutations can affect APP metabolism.
by various means, potentially altering different mechanisms related to APP metabolism such as APP cellular trafficking, membrane APP endocytosis and/or APP intrinsic ability to interact with the α-, β- and γ-secretase.

**Mutating His149 and His151 to asparagine impairs APP maturation in HEK293 cells**

We next investigated APP maturation, which constitutes the first step of APP cellular metabolism and includes O-glycosylation of N-glycosylated APP upon APP trafficking from the ER to the Golgi. Cells lysates were examined by quantitative western blotting analysis using the WO2 antibody (Fig. 4A) and the ratio of mature APP to immature APP (m/imAPP) calculated for each cell line (Fig. 4B). The single mutations H149N and H151N and the double mutations H147,149N and H147,151N significantly decreased the m/imAPP ratio by a similar manner (~30%). This phenotype was strongly exacerbated by the H149,151N double mutation and the triple mutation (H147,149,151N), which both reduced the m/imAPP ratio by ~80%. In contrast to all the other mutations, the single mutation H147N did not affect the m/imAPP ratio.

These data suggested two different degrees of impairment in APP maturation, a relatively mild impairment caused by mutating either His149 or His151 to asparagine, and a strong impairment when both His149 and His151 are mutated to asparagines.

With the premise that the observed decreased APP maturation is due to impaired APP ER-to-Golgi trafficking (3), this phenomenon would reduce extracellular APP metabolites levels since less APP would go through the secretory pathway and reach the plasma membrane. Thus the impairment in APP maturation observed for the His149 and His151 mutations was likely to underlie the decreased levels of secreted Aβ, sAPPβ and sAPPα. Nevertheless, the differences in Aβ and sAPPα levels between the mutants with a similar extent of impairment in APP maturation and the normal levels of secreted sAPPβ for H151N- and H147,151N-APP mutants, suggested the involvement of other events in addition to ER-to-Golgi trafficking.

**Mutating His149 and His151 to asparagine impairs APP ER-to-Golgi trafficking**

In WT cells immature APP (N-glycosylated) is mainly localized in the ER/cis-Golgi region, whilst mature APP (N- and O-glycosylated) is in trans-Golgi and post-Golgi subcellular compartments (3). With the premise that decreased APP maturation could be due to impaired APP ER-to-Golgi trafficking we studied the subcellular APP localization of the H147,149N-APP and the H147,149,151N-APP mutants. These mutants were chosen as they represented mild and strong impairment of APP maturation respectively. Analysis of APP subcellular distribution by iodixanol gradient fractionation revealed that wildtype mAPP and imAPP colocalized in the early endosomes/Golgi and in the ER containing fractions respectively (Fig. 5A). H147,149N-mAPP was correctly localized in the early endosomes and Golgi, however it constituted a smaller portion of total APP compared to wt-mAPP. H147,149,151N does not have any mature APP so no APP was detected in the early endosome or Golgi of this mutant. Conversely, the immature APP form of the H147,149N and H147,149,151N mutants correctly localized in the ER, however it constituted a higher percentage of total APP compared to wt-mAPP (Fig. 5B). These data suggested that the impairment of H147,149-APP and H147,149,151N-APP maturation was due to a decrease in APP trafficking from the ER to the Golgi. These data also suggested that mild and severe impairment of APP maturation were due to different extents of APP retention in the ER.

Impairment of H147,149,151N-APP trafficking from the ER to the Golgi was further confirmed using immunofluorescence confocal microscopy which revealed perinuclear punctuated staining for wt-APP (WO2) partially colocalizing with the ER (Fig. 6A). Compared to wt-APP, the H147,149,151N-APP mutant exhibited a different staining pattern, less punctuated and more evenly spread, with qualitatively higher ER colocalization (Fig. 6A). HEK cells expressing the vector alone demonstrated a very weak APP signal, indicating endogenous APP was contributing negligible fluorescence (Fig. 6A). Fluorescence confocal microscopy indicated partial colocalization of wt-APP to the Golgi, whereas there was no Golgi colocalization detectable for H147,149,151N-APP.
in accordance with the absence of this mutant in the Golgi (Fig. 6B).

The subcellular localization data suggested the impairment in APP maturation was due to hampered APP ER-to-Golgi trafficking, rather than APP’s inability to be O-glycosylated when properly trafficked. To test the ability of H147,149,151N-APP to undergo O-glycosylation, when localized in the same compartment as the O-glycosylation enzymes, the cells were treated with Brefeldin A (BFA). BFA causes the fusion of Golgi membranes with the ER leading to partial redistribution of Golgi enzymes into the ER (44), except for the TGN enzymes (45,46). BFA treatment is known to cause abnormal glycosylation of wt-APP which results in an intermediate molecular weight band between the immature and mature APP species (47). Following BFA treatment a new wt-APP species was detected with a molecular mass, bigger than immature APP but smaller than mature APP (Fig. 7), as previously reported for BFA treated cells (47). The same intermediate band was found when H147,149,151N-APP-HEK were exposed to BFA (Fig. 7) confirming the ability of this mutant to undergo O-glycosylation when it interacts with the appropriate enzymes.

Impairment in APP maturation is specific to H149N and H151N mutation

The significant involvement of His149 in APP impaired maturation is interesting because the residue points in the opposite direction away from the copper binding site (Fig. 1A and 8C). This led us to explore the existence of a surface patch of residues controlling APP maturation, possibly via intermolecular interactions. Based on the three dimensional structure of the APP CuBD we assessed whether other residues residing on the CuBD α-helix, as do His149 and His151, could play a role in modulating the m/imAPP ratio. Three residues (Leu148, Thr152 and Glu160) with side chains orientated between His149 and His151 and evenly distributed along the helix were identified (Fig. 8C) and tested (Fig. 8A). L148A, T152A and E160A mutations did not affect m/imAPP ratio (Fig. 8B), suggesting a specific involvement for His149 and His151 in modulating m/imAPP ratio as opposed to any other residues located on the CuBD α-helix and oriented in similar directions.

H147N mutation increases intracellular levels of sAPPα

The impairment in APP ER-to-Golgi trafficking caused by His149 and His151 led us to investigate the levels of intracellular APP metabolites. Cell lysates were examined by quantitative western blotting analysis using an antibody specifically recognizing sAPPα (not cross-reacting with full length APP or sAPPβ) and resulting values standardized to corresponding total intracellular APP levels (Fig. 9A).

None of the His149 or His151 containing mutations had a significant effect on intracellular sAPPα levels compared to wt-APP, however the H149N- and the H147,149,151N-APP mutants exhibited a trend towards a decrease. These findings were consistent with the two same mutations causing the strongest reduction in secreted sAPPα (compared to the other mutations), Interestingly, the H147N-APP mutant showed a significant increase of about 60% in intracellular sAPPα levels compared to the wt-APP. The respective increase and decrease of intracellular and secreted H147N-sAPPα, together with the decrease of other secreted H147N-APP metabolites (sAPPβ and Aβ), suggested a mechanism possibly involving decreased H147N-APP transport to the plasma membrane with potential retention in organelles where sAPPα can be generated (48), rather than an increase in H147N-APP’s intrinsic propensity to interact with α-secretase.

We attempted to measure intracellular sAPPβ and Aβ levels, however their signals were masked by unspecific signal or were under the detection limits respectively.

Despite all the His149 and His151 mutations impairing APP maturation and decreasing secreted sAPPα levels, the modulation of intracellular sAPPα and secreted sAPPβ and Aβ levels was specific to the different mutants and indicated three distinct phenotypes (Fig. 9B). Of all the His149 and His151 mutants, only H151N- and H147,151N-APP showed normal levels of intracellular sAPPα and secreted sAPPβ and Aβ. A second phenotype was shown by the H147,149N- and H149,151N-APP mutants which exhibited decreased secreted sAPPα and Aβ levels, as well as
a trend towards decreased secreted sAPPβ, with normal levels of intracellular sAPPα. The H149N- and H147,149,151N-APP mutants showed a third phenotype, where all the measured APP metabolites, both intracellular and secreted, were significantly decreased compared to wt-APP.

In contrast to all the other mutants, H147N-APP not only exhibited normal APP maturation but was the only mutant showing increased intracellular sAPPα levels (Fig. 9B). These findings, together with H147N-APP having decreased secreted sAPPα levels, suggested APP retention in post-ER organelles where α-secretase cleavage occurs (48).

**Co-mutation of His149 and His151 to asparagine promotes aberrant APP SDS-resistant oligomerization**

The unlikely involvement of APP copper binding or intermolecular interactions driving the APP maturation impairment mediated by His149N and His151N mutations led us to explore other explanations for this phenomenon. A possible reason for a protein to be retained in the ER is its inability to achieve the proper tertiary and quaternary conformation. Since specific amino acids can be crucial for correct protein folding and oligomerization, substitution of these key residues can destabilize the final protein structure.

Thus the retention of APP in the ER caused by His149N and His151N mutations could be due to misfolding and aggregation of the APP protein. To test if aberrant oligomerisation had occurred the reducing agent β-mercaptoethanol (βME) was omitted from the cells lysate. SDS-PAGE analysis detected a higher molecular weight species exclusively for the H149,151N- and H147,149,151N-APP mutants (Fig. 10A). This band migrated slightly below the 188 kDa marker, suggesting it was either an APP homodimer (expected at ~200kDa) or APP bound to an unidentified protein. The persistence of this H149,151N- and H147,149,151N-APP oligomer in the presence of SDS, but not with βME (Fig. 10B), indicated that the oligomer subunits were linked together via disulphide bonds. The mutants that formed oligomers still had detectable levels of monomeric APP, indicating that only a portion of APP with these mutations formed disulphide linked oligomers. Aberrant formation of disulphide-bonded oligomers of APP might arise from improper APP folding which would preclude the formation of correct disulfide bridges, leaving free thiols available for erroneous interactions.

**CuBD-mediated regulation of APP metabolism**

Co-mutation of His149 and His151 causes APP CuBD secondary structure changes

To determine if the histidine mutations could alter the APP structure we analysed the secondary structure of recombinantly expressed mutant APP CuBD 133-189. The far UV-CD spectra of wt-CuBD indicated a mixture of α-helical and β-sheet conformations (Fig. 11A-C) in line with the known structure (26,27). Visual comparison with wt-CuBD spectrum suggested that only H149,151N and H147,149,151N mutations caused a significant reduction in CuBD secondary structure elements, while the other mutations (H147N, H149N, H151N, H147,149N, and H147,151N) did not produce any discernable spectral changes (Fig. 11A-C). However, deconvolution of the spectra indicated changes in α-helix and β-sheet structure for all the mutants except H149N-CuBD (Fig. 11D). Deconvolution of CD spectra must be interpreted with caution as the deconvolution values can differ significantly with measured values. For instance, deconvolution of the wt-APP CD spectrum indicated 28% and 22% of α-helical and β-sheet-like contributions (20% turns and 30% unordered) (Fig. 11D), which was discordant with the NMR and crystallography data showing 20% helical content and 32% β-sheet content (26,27). Nevertheless, both visual inspection and deconvolution analysis suggested a significant change in secondary structure of H149,151N- and H147,149,151N-CuBD compared to wt-CuBD. These findings supported APP misfolding as the cause of the strong impairment in APP maturation and aberrant APP oligomerization observed when His149 and His151 were co-mutated and expressed in cell lines.

H147,149,151N-APP-HEK have up-regulated unfolded protein response

When the ER is stressed by accumulation of misfolded proteins it activates the unfolded protein response (UPR) to restore normal homeostasis. To determine whether APP misfolding could be the cause of aberrant APP oligomerization, we analysed the expression of Calnexin, PDI, BiP, Ero1La, IRE1α and CHOP, components of the
CuBD-mediated regulation of APP metabolism

UPR (49). BiP and CHOP exhibited increased levels under the ER stress induced conditions (Fig. 12), in accordance with the past literature (note that CHOP expression was not detectable under normal conditions where its value was zero). The increase in BiP levels was more pronounced when thapsigargin was used instead of tunicamycin, possibly due to the different mode of action of these two ER stressors. BiP levels were higher in the H147,149,151N-APP-HEK compared to wt-APP-HEK supporting the presence of misfolded and aberrantly oligomerized H147,149,151N-APP in the ER, causing ER stress. The H147,149,151N-APP mediated ER stress might be milder compared to that induced by tunicamycin or thapsigargin and thus not activating the CHOP apoptosis pathway. Data showing similar viability levels between wt- and H147,149,151N-APP-HEK (supplementary data) supported this hypothesis.

ER associated degradation (ERAD) is also part of the UPR and involves recognising of misfolded proteins, their retrotranslocation to the cytoplasm and degradation by the proteasome following their ubiquitination. The H147,149,151N-APP ubiquitination state was examined by immunoprecipitation with the FK2 antibody which recognizes ubiquitinylated proteins. Following western blot analysis, a band corresponding to APP (i.e. around 98 kDa) was detected for wt-APP as well as for H147,149,151N-APP upon FK2 immunoprecipitation followed by WO2 immunoblotting (Fig. 13 A). The former corresponded to mature APP and the latter to imAPP, indicating that only imAPP bearing the H147,149,151N mutation, and not wt-imAPP, is ubiquitinated.

These findings supported the theory that H147,149,151N mutation causes APP misfolding and shed some insight on the fate of this non-functional APP mutant.

Co-mutating His149 and His151 to alanine only mildly impairs APP maturation in HEK293 cells

To assess whether the removal of the histidine residues or the specific incorporation of asparagine residues plays a role in destabilizing the APP structure, His149 and His151 were mutated to alanine and APP maturation investigated. Asparagine residues are infrequently found in helical structures and are considered a moderate α-helix breaker residue since their side chain disrupts the backbone hydrogen bonds that stabilize the helix (50). On the contrary, alanine occurs frequently in helix structures and is thought to promote helix formation (51-54). Similarly to asparagines, alanines have lower hydrogen bonding properties compared to histidine residues due to the lack of the imidazole ring. H149,151A-APP-HEK cells were analysed by western blot and densitometry as performed previously for the other histidine mutants (Fig. 14A). The H149,151A mutation decreased the m/imAPP ratio by ~30% when compared to wt-APP, significantly less than the corresponding asparagine mutation (H149,151N) which reduced the ratio by ~80% (Fig. 14B). These data suggested that replacement of His149 and His151 with asparagine residues might impair APP maturation through additive effects, possibly by hampering important intra- or inter-molecular hydrogen bonds formation and by disrupting the helix structure.

DISCUSSION

In this study, we discovered a role in APP metabolism for the two APP CuBD histidine residues located at position 149 and 151. The secreted levels of Aβ and sAPPα were decreased in HEK293 cells overexpressing APP bearing substitutions of His147, His149 and His151 with asparagine. Mutation of His149 and His151, but not His147, decreased the m/imAPP ratio too. In particular, the impairment in mAPP maturation was notably stronger when these two residues were co-mutated. Subcellular fractionation and confocal immunofluorescence microscopy analysis suggested that ER-to-Golgi trafficking of APP bearing His149 and His151 with asparagine residues might impair APP maturation through additive effects, possibly by hampering important intra- or inter-molecular hydrogen bonds formation and by disrupting the helix structure.
ER and impaired APP maturation. The milder impairment of APP maturation when His149 and His151 were replaced with alanine (~30%) compared to when mutated to asparagines (~80%) suggested that both different hydrogen bonding properties and helix destabilizing characteristics of asparagine residues (compared to histidines) may contribute to the presumed APP misfolding. The single mutation of His149 or His151 did not cause any detectable aberrant APP oligomerization or significantly alter the secondary structure, however it did impair APP maturation and ER to Golgi trafficking, indicating a similar, but milder, mechanism as the one presumably underlying the His149 and His151 co-mutation phenotype.

His149 and His151 appear to have the same crucial role in stabilizing APP folding, however this common function might be achieved through two different mechanisms for each residue respectively. Protein-metal binding can promote and stabilize protein folding (55,56), therefore while conservation of His149 might promote and maintain the tertiary structure through hydrogen bonding and stabilization of the helix, His151 might contribute to APP folding stability via copper binding. Nevertheless, except for small changes in the position and orientation of the putative binding residues, no alteration in the CuBD structure was observed upon copper binding (26,27), suggesting that metal interactions do not influence the overall folding of the domain. Structural studies employing the whole E1 domain of APP (including the CuBD and the GFD) (57) do not provide any information on the influence of copper binding in E1 domain folding, since they were performed with the apo form of the E1 domain.

His149 and His151 mutations not only impaired APP ER-to Golgi trafficking but, in some cases, also altered the levels of intracellular sAPPα and secreted sAPPα, sAPPβ and Aβ. These changes were not specific to the extent of impairment in APP maturation thus adding a further degree of complexity to the interpretation of the mechanisms of action of these mutations. Events other than APP ER-to-Golgi trafficking appear to cooperatively contribute to the generation of various intracellular and secreted APP metabolites.

Since the H147N mutation did not alter APP maturation, we assume that ER-to-Golgi trafficking of this mutant is unaffected. However, the reduction in secreted Aβ, sAPPα and sAPPβ levels indicate that H147N-APP’s transport through the post-Golgi organelles might be hampered, as this event would decrease plasma membrane APP levels and subsequently reduce secreted levels of APP metabolites. The raised intracellular sAPPα level is consistent with retention of H147N-APP within organelles where sAPPα is generated, such as the trans-Golgi network (48). An increase in H147N-APP’s intrinsic propensity to interact with α-secretase doesn’t seem likely, due to the opposite alteration of intracellular versus secreted sAPPα levels.

Previous findings showed that copper increases APP cell surface localization (58) and promotes APP trafficking from the Golgi to intracellular compartments and to the cell surface (32), this supports H147N-APP decreased post-Golgi trafficking, as His147 might be necessary for APP copper binding. Alternatively, His147 might be crucial in APP recognition for post-Golgi trafficking and its mutations might decrease APP interactions to cofactors involved in APP post-Golgi transport such as AP-4, sorLA and Reelin (59-62). Finally, H147N mutation might affect copper reduction rather than copper binding, as demonstrated by earlier studies employing synthetic peptides encompassing APP residues 135 to156 (28,29), and lead to a modulation of APP metabolism. The potential inability of APP to reduce copper might in fact influence APP trafficking.

In summary, this study identified a novel cellular process whereby His149 and His151 residues in the APP CuBD regulate APP proteolytic processing by impairing APP ER to Golgi transport. This most likely occurs through promotion and stabilization of APP folding. The involvement of His149 in this modulation suggests a mechanism not involving copper binding, however, modulation of APP folding by His151 via APP binding to copper cannot be excluded. The findings of this study indicate a novel and additional role of the CuBD in APP folding and stability, beside the canonical function of copper binding, and contribute to the
elucidation of the role of APP CuBD in modulating APP metabolism. Strategic APP misfolding and retention in the ER might be used therapeutically in order to reduce Aβ generation, however this approach presents the caveat of suppressing potentially beneficial APP metabolites (such as sAPPα) and the accumulation of unfolded protein in the ER. Nevertheless, these findings contribute to understanding the normal physiological role of APP and might be useful in identifying crucial residues and interactions which should not be perturbed in the design of novel therapeutics strategies for AD which target the APP CuBD.
REFERENCES

1. Pahlsson, P., and Spitalnik, S. L. (1996) *Arch Biochem Biophys* **331**, 177-186
2. Sodhi, C. P., Perez, R. G., and Gottardi-Littell, N. R. (2008) *Brain Res* **1198**, 204-212
3. Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L., and Beyreuther, K. (1989) *Cell* **57**, 115-126
4. Koo, E. H., Squazzo, S. L., Selkoe, D. J., and Koo, C. H. (1996) *J Cell Sci* **109 (Pt 5)**, 991-998
5. Lichtenthaler, S. F. (2011) *J Neurochem* **116**, 10-21
6. Seubert, P., Oltersdorf, T., Lee, M. G., Barbour, R., Blomquist, C., Davis, D. L., Bryant, K., Fritz, L. C., Galasko, D., Thal, L. J., and et al. (1993) *Nature* **361**, 260-263
7. Gabuzda, D., Busciglio, J., Chen, L. B., Matsudaira, P., and Yankner, B. A. (1994) *J Biol Chem* **269**, 13623-13628
8. Anderson, J. P., Chen, Y., Kim, K. S., and Robakis, N. K. (1992) *J Neurochem* **59**, 2328-2331
9. Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B., and et al. (1992) *Nature* **359**, 322-325
10. Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., and et al. (1992) *Science* **258**, 126-129
11. Shoji, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, L. M., Cai, X. D., McKay, D. M., Tintner, R., Frangione, B., and et al. (1992) *Science* **260**, 126-129
12. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., and Ward, P. J. (1990) *Science* **248**, 1122-1124
13. Sisodia, S. S. (1992) *Proc Natl Acad Sci U S A* **89**, 6075-6079
14. Haass, C., Hung, A. Y., Schlossmacher, M. G., Teplow, D. B., and Selkoe, D. J. (1993) *J Biol Chem* **268**, 3021-3024
15. Vitek, M. P. (1989) *Neurobiol Aging* **10**, 471-473; discussion 477-478
16. Clark, A. W., and Parhad, I. M. (1989) *Can J Neurol Sci* **16**, 477-482
17. Cohen, M. L., Golde, T. E., Usiak, M. F., Younkin, L. H., and Younkin, S. G. (1988) *Proc Natl Acad Sci U S A* **85**, 1227-1231
18. Preece, P., Virley, D. J., Costandi, M., Coombes, R., Moss, S. J., Mudge, A. W., Jazin, E., and Cairns, N. J. (2004) *Brain Res Mol Brain Res* **122**, 1-9
19. Basun, H., Forssell, L. G., Wetterberg, L., and Winblad, B. (1991) *J Neural Transm Park Dis Dement Sect* **3**, 231-258
20. deibel, M. A., Ehmann, W. D., and Markesbery, W. R. (1996) *J Neurosci* **16**, 137-142
21. Lovell, M. A., Robertson, J. D., Teesdale, W. J., Campbell, J. L., and Markesbery, W. R. (1998) *J Neurosci* **18**, 47-52
22. Squitti, R., Cassetta, E., Dal Forno, G., Lupoi, D., Lippolis, G., Pauri, F., Vernieri, F., Cappa, A., and Rossini, P. M. (2004) *Arch Neurol* **61**, 738-743
23. Squitti, R., Lupoi, D., Pasqualetti, P., Dal Forno, G., Vernieri, F., Chiovenda, P., Rossi, L., Cortesi, M., Cassetta, E., and Rossini, P. M. (2002) *Neurology* **59**, 1153-1161
24. Bayer, T. A., Schaffner, S., Simons, A., Kemmling, A., Kamer, T., Tepest, R., Eckert, A., Schussel, K., Eikenberg, O., Sturchler-Pierrat, C., Abramowski, D., Staufenbiel, M., and Multhaup, G. (2003) *Proc Natl Acad Sci U S A* **100**, 14187-14192
25. Phinney, A. L., Drisaldi, B., Schmidt, S. D., Lugowski, S., Coronado, V., Liang, Y., Horne, P., Yang, J., Sekoulidis, J., Coomaraswamy, J., Chishti, M. A., Cox, D. W., Mathews, P. M., Nixon, R. A., Carlson, G. A., St George-Hyslop, P., and Westaway, D. (2003) *Proc Natl Acad Sci U S A* **100**, 14193-14198
26. Barnham, K. J., McKinstry, W. J., Multhaup, G., Galatis, D., Morton, C. J., Curtail, C. C., Williamson, N. A., White, A. R., Hinds, M. G., Norton, R. S., Beyreuther, K., Masters, C. L., Parker, M. W., and Cappai, R. (2003) *J Biol Chem* **278**, 17401-17407
27. Kong, G. K., Adams, J. J., Harris, H. H., Boas, J. F., Curtail, C. C., Galatis, D., Masters, C. L., Barnham, K. J., McKinstry, W. J., Cappai, R., and Parker, M. W. (2007) *J Mol Biol* **367**, 148-161
CuBD-mediated regulation of APP metabolism

28. Multhaup, G., Schlicksupp, A., Hesse, L., Beher, D., Ruppert, T., Masters, C. L., and Beyreuther, K. (1996) Science 271, 1406-1409
29. Hesse, L., Beher, D., Masters, C. L., and Multhaup, G. (1994) FEBS Lett 349, 109-116
30. Armendariz, A. D., Gonzalez, M., Logunov, A. V., and Vulpe, C. D. (2004) Physiol Genomics 20, 45-54
31. Borchartd, T., Camakaris, J., Cappai, R., Masters, C. L., Beyreuther, K., and Multhaup, G. (1999) Biochem J 344 Pt 2, 461-467
32. Acevedo, K. M., Hung, Y. H., Dalziel, A. H., Li, Q. X., Laughton, K., Wikhe, K., Rembach, A., Roberts, B., Masters, C. L., Bush, A. I., and Camakaris, J. (2010) J Biol Chem 285, 20378-20386
33. Bellingham, S. A., Lahiri, D. K., Maloney, B., La Fontaine, S., Multhaup, G., and Camakaris, J. (2004) J Biol Chem 279, 20378-20386
34. Cater, M. A., McInnes, K. T., Li, Q. X., Volitakis, I., La Fontaine, S., Mercer, J. F., and Bush, A. I. (2008) Biochem J 412, 141-152
35. Bellingham, S. A., Ciccotosto, G. D., Needham, B. E., Fodero, L. R., White, A. R., Masters, C. L., Cappai, R., and Camakaris, J. (2004) J Neurosci 24, 423-428
36. White, A. R., Reyes, R., Mercer, J. F., Camakaris, J., Zheng, H., Bush, A. I., Multhaup, G., Beyreuther, K., Masters, C. L., and Cappai, R. (1999) Brain Res 842, 439-444
37. Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Forstl, H., Sandbrink, R., Masters, C. L., and Beyreuther, K. (1996) J Biol Chem 271, 22908-22914
38. Kong, G. K., Galatis, D., Barnham, K. J., Polekhina, G., Adams, J. J., Masters, C. L., Cappai, R., Parker, M. W., and McKinstry, W. J. (2005) Acta Crystallogr Sect F Struct Biol Cryst Commun 61, 93-95
39. Srererama, N., and Woody, R. W. (2000) Anal Biochem 287, 252-260
40. Manavalan, P., and Johnson, W. C., Jr. (1987) Anal Biochem 167, 76-85
41. Compton, L. A., and Johnson, W. C., Jr. (1986) Anal Biochem 155, 155-167
42. Whitmore, L., and Wallace, B. A. (2008) Biopolymers 89, 392-400
43. Koradi, R., Billeter, M., and Wuthrich, K. (1996) J Mol Graph 14, 51-55, 29-32
44. Sciaky, N., Presley, J., Smith, C., Zaal, K. J., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E., and Lippincott-Schwartz, J. (1997) J Cell Biol 139, 1137-1155
45. Chege, N. W., and Pfeffer, S. R. (1990) J Cell Biol 111, 893-899
46. Ladinsky, M. S., and Howell, K. E. (1992) Eur J Cell Biol 59, 92-105
47. Caporaso, G. L., Gandy, S. E., Buxbaum, J. D., and Greengard, P. (1992) Proc Natl Acad Sci U S A 89, 2252-2256
48. Skovronsky, D. M., Moore, D. B., Milla, M. E., Doms, R. W., and Lee, V. M. (2000) J Biol Chem 275, 2568-2575
49. Kapoor, A., and Sanyal, A. J. (2009) Clin Liver Dis 13, 581-590
50. Berg JM, T. J., Stryer L. (2002) Section 3.6The Amino Acid Sequence of a Protein Determines Its Three-Dimensional Structure. in Biochemistry. 5th edition (Freeman, N. Y. W. H. ed.). pp
51. Marqusee, S., and Baldwin, R. L. (1987) Proc Natl Acad Sci U S A 84, 8898-8902
52. Merutka, G., and Stellwagen, E. (1989) Biochemistry 28, 352-357
53. Shoemaker, K. R., Kim, P. S., Brems, D. N., Marqusee, S., York, E. J., Chaiken, I. M., Stewart, J. M., and Baldwin, R. L. (1985) Proc Natl Acad Sci U S A 82, 2349-2353
54. Lyu, P. C., Liff, M. I., Marky, L. A., and Kallenbach, N. R. (1990) Science 250, 669–673
55. Low, L. Y., Hernandez, H., Robinson, C. V., O’Brien, R., Grossmann, J. G., Ladbury, J. E., and Luisi, B. (2002) J Mol Biol 319, 87-106
56. Botelho, H. M., Koch, M., Fritz, G., and Gomes, C. M. (2009) FEBS J 276, 1776-1786
57. Dahms, S. O., Hoeffgen, S., Roeser, D., Schlottb, B., Guhrs, K. H., and Than, M. E. (2010) Proc Natl Acad Sci U S A
58. Hung, Y. H., Robb, E. L., Volitakis, I., Ho, M., Evin, G., Li, Q. X., Culvenor, J. G., Masters, C. L., Cherny, R. A., and Bush, A. I. (2009) J Biol Chem 284, 21899-21907
CuBD-mediated regulation of APP metabolism

59. Andersen, O. M., Schmidt, V., Spoelgen, R., Gliemann, J., Behlke, J., Galatis, D., McKinstry, W. J., Parker, M. W., Masters, C. L., Hyman, B. T., Cappai, R., and Willnow, T. E. (2006) Biochemistry 45, 2618-2628

60. Burgos, P. V., Mardones, G. A., Rojas, A. L., daSilva, L. L., Prabhu, Y., Hurley, J. H., and Bonifacino, J. S. (2010) Dev Cell 18, 425-436

61. Hoe, H. S., Lee, K. J., Carney, R. S., Lee, J., Markova, A., Lee, J. Y., Howell, B. W., Hyman, B. T., Pak, D. T., Bu, G., and Rebeck, G. W. (2009) J Neurosci 29, 7459-7473

62. Spoelgen, R., von Arnim, C. A., Thomas, A. V., Peltan, I. D., Koker, M., Deng, A., Irizarry, M. C., Andersen, O. M., Willnow, T. E., and Hyman, B. T. (2006) J Neurosci 26, 418-428

Acknowledgements - This work was funded by grants from the Australian Research Council and the NHMRC. RC and KJB are NHMRC Senior Research Fellows. LJV was an Alzheimer’s Australia Research Viertel Fellowship recipient. LS was a recipient of a Melbourne International Fee Remission Scholarship (MIFRS), a Beaney Scholarship and a May Stewart Bursary postgraduate scholarship.

FOOTNOTES
LJV is currently at the Ludwig Institute for Cancer Research, Austin Hospital, Heidelberg, Victoria 3084, Australia. C.L.L.P. is currently in the Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom.
FIGURE LEGENDS

FIGURE 1. Mutations of His147, His149 and His151 decrease secreted Aβ levels. (A) APP CuBD three dimensional structure indicating the mutated histidines (purple) and the other two residues putatively involved in copper binding, Tyr168 and Met170. (B) HEK293 cells stably overexpressing wt-APP or APP mutants (single, double and triple mutants) were cultured under standard conditions. Conditioned cell culture media were examined by sandwich ELISA assay using WO2 antibody and values normalized to total cellular protein concentration and total intracellular APP levels of the corresponding lysates and expressed as a relative percentage to that of wt-APP (100%). Data are expressed as mean ± SEM of four repeats (three repeats for H147,149,151N-APP) of the same experiments; statistical significances were calculated with one-way ANOVA using Dunnett’s post hoc test comparing each mutant with the wt-APP control; **p<0.01.

FIGURE 2. Mutations of His147, His149 and His151 decrease secreted sAPPα levels. HEK293 cells stably overexpressing wt-APP or APP mutants (single, double and triple mutants) were cultured under standard conditions. Conditioned cell culture media were examined by western blot technique using WO2 antibody to detect sAPPα metabolite. Densitometry values were normalized to intracellular APP levels of the corresponding lysates (Fig. 3A) and expressed as a relative percentage to that of wt-APP (100%). Data are expressed as mean ± SEM; n indicates the number of the experiment repeats; statistical significances were calculated with one-way ANOVA using Dunnett’s post hoc test comparing each mutant with the wt-APP control; **p<0.01

FIGURE 3. Specific combinations of His147, His149 and His151 mutations decrease secreted sAPPβ levels. HEK293 cells stably overexpressing wt-APP or APP mutants (single, double and triple mutants) were cultured under standard conditions. Conditioned cell culture media were examined by western blot technique using an antibody specific to sAPPβ. Densitometry values were normalized to intracellular APP levels of the corresponding lysates and expressed as a relative percentage to that of wt-APP (100%). Data are expressed as mean ± SEM; n indicates the number of the experiment repeats; statistical significances were calculated with one-way ANOVA using Dunnett’s post hoc test comparing each mutant with the wt-APP control; **p<0.01

FIGURE 4. Mutations of APP His149 and His151 impair APP maturation. HEK293 cells stably overexpressing wt-APP or APP mutants (single, double and triple mutants) were cultured under standard conditions. Cell lysates were normalized to total protein concentration levels before SDS-PAGE. (A) The WO2 antibody was used to detect intracellular full length APP in cell lysates. The arrows indicate bands corresponding to mature (mAPP) and immature (imAPP) APP. (B) The ratio of the mature over the immature APP levels was calculated and expressed as a relative percentage to that of wt-APP (100%). Data are expressed as mean ± SEM; n indicates the number of the experiment repeats; statistical significances were calculated with one-way ANOVA using Tukey’s post hoc test; **p<0.01, ***p<0.001.

FIGURE 5. H147,149N and H147,149,151N APP mutations impair APP ER-to-Golgi trafficking. (A) Homogenates of HEK293 cells stably overexpressing wt-, H147,149N- or H147,149,151N-APP were fractionated through an iodixanol step gradient. Western blot analysis showed the distribution of mAPP, imAPP, BiP (ER marker), Synatxin 6 (Golgi marker) and EEA1 (early endosomes marker). (B) Quantification of mature APP distribution showed that the H147,149N-mAPP (no H147,149,151N-mAPP was detected) is found mainly in the Golgi and early endosomes fractions, as for wt-APP, however constitutes a lower percentage of the total APP compared to wt-APP. H147,149N- and H147,149,151N-imAPP are found mainly in the ER fractions, as for wt-APP, however they respectively constitutes a higher percentage of the total APP compared to wt-APP, indicating a partial retention of these mutants APP in the ER. Values are expressed as a percentage of the total specific protein (sum of all the fractions).
except for mAPP and imAPP which are expressed as a percentage of the total APP (sum of mAPP and imAPP in all fractions).

**FIGURE 6.** Corroboration of H147,149,151N-APP impaired ER-to-Golgi trafficking. (A) Immunofluorescence confocal microscopy of W02 antibody stained wt-APP- and vector-alone-HEK cells transiently expressing an ER localised red fluorescence protein. Distinct APP distribution patterns were noticeable for wt- and H147,149,151N-APP, both showing a certain degree of colocalization with the ER which appeared to be importantly higher for the latter. Cells expressing the vector alone exhibited minimal W02 staining suggesting negligible APP endogenous signal. (B) Images merging W02 and anti-GRAPS65 (Golgi marker) signals showed partial colocalization of wt-APP in the Golgi, whereas no such characteristic was detectable for H147,149,151N-APP, indicating lack of this mutant APP in the Golgi. Arrows indicate overlapped signal. For all images the indicated scale bar corresponds to 10 μm.

**FIGURE 7.** H147,149,151N-APP partially undergoes O-glycosylation under BFA treatment. HEK293 cells stably overexpressing wt- or H147,149,151N-APP-HEK cells were exposed to BFA (1μg/ml) for 5 hours. Western blot analysis of lysates showed the formation of a unique APP intermediate band (molecular weight wise) for both BFA treated cell lines, indicating the ability of H147,149,151N-APP to undergo partial O-glycosylation when able to interact with the appropriate Golgi residing enzymes. This aberrantly glycosylated APP (abAPP) had been previously observed and attributed to the partial redistribution of Golgi enzymes into the ER caused by BFA (44-47).

**FIGURE 8.** Impairment in APP maturation is specific to H149N and H151N mutation. HEK293 cells stably overexpressing wt-APP or mutants of other non-histidine residues with side chains orientated between His149 and His151 along the helix were cultured under standard conditions. Cell lysates were normalized to total protein concentration levels before SDS-PAGE. (A) The W02 antibody was used to detect intracellular full length APP in cell lysates. The arrows indicate bands corresponding to mature (mAPP) and immature (imAPP) APP. (B) The ratio of the mature over the immature APP levels was calculated and expressed as a relative percentage to that of wt-APP (100%). Data are expressed as mean ± SEM; n indicates the number of the experiment repeats; statistical significances (p<0.05) were calculated with one-way ANOVA using Tukey’s post hoc test.

**FIGURE 9.** H147N mutation increases intracellular levels of sAPPα. HEK293 cells stably overexpressing wt-APP or APP mutants (single, double and triple mutants) were cultured under standard conditions. Cell lysates were examined by western blot technique using an antibody specific to sAPPα. Densitometry values were normalized to intracellular total APP levels of the corresponding lysates and expressed as a relative percentage to that of wt-APP (100%). Data are expressed as mean ± SEM; n indicates the number of the experiment repeats; statistical significances were calculated with one-way ANOVA using Dunnett’s post hoc test comparing each mutant with the wt-APP control; **p<0.01

**FIGURE 10.** Co-mutation of the His149 and His151 promotes APP aberrant SDS-resistant oligomerization. (A) Immunoblot of cell lysates prepared with non-reducing sample buffer lacking β-mercaptoethanol (βME) revealed the presence of a higher molecular size band just below 188 kDa (arrow) besides the usual ~98 kDa APP band(s) only for the two mutants where His149 and His151 were simultaneously mutated (alone or with His147) when compared to wt-APP. (B) Analogous examination of the same cell lysates but under standard conditions (+ βME sample buffer) highlighted the absence of the ~188 kDa bands for both H149,151N- and H147,149,151N-APP indicating that these two mutants form disulphide linked oligomers.
FIGURE 11. Co-mutation of His149 and His151 causes APP CuBD secondary structure changes. Comparison of far-UV CD spectra of single (A), double (B) and triple (C) APP CuBD mutants to the wt-CuBD indicated a significant change in CuBD secondary structure caused by H149,151N and H147,149,151N mutations. (D) Table listing the secondary structures content of CuBD wt and mutants obtained by CD spectra deconvolution.

FIGURE 12. UPR is partially upregulated in H147,149,151N-APP-HEK cells. Immunoblot analysis of six ER stress and unfolded protein response (UPR) markers and the corresponding densitometries indicated that CHOP and BiP abundances increased as a response of triggered ER stress in wt-APP-HEK (tunicamycin 1μg/ml or thapsigargin 300nM 5h treatment). BiP abundance was also increased in untreated H147,149,151N-APP-HEK cells compared to wt-APP-HEK cells, supporting the putative presence of unfolded APPs and APP oligomers, both retained in the ER, upon this mutation. Values were standardized against a corresponding housekeeping gene (GAPDH for IRE1α, Ero1Lα, BiP, PDI and Calnexin and β-tubulin for CHOP).

FIGURE 13. H147,149,151N-imAPP is over ubiquitinated compared to wt-imAPP. Cell lysates of HEK293 cells stably expressing wt- or H149,151A-APP mutants were immunoprecipitated and immunoblotted with W02 antibody. (A) FK2 antibody (which recognizes polyubiquitin and monoubiquitin conjugates) immuno-precipitation (IP) showed that only imAPP bearing the H147,149,151N mutation (not wt-imAPP) is ubiquitinated. (B) Positive control: W02 antibody IP showed the usual doublet for wt-APP and only one band for H147,149,151N-APP. (C) Negative control: mock IP only showed unspecific bands. Note, (A) and (C) blots are a result of longer signal time collection than blot (B).

FIGURE 14. Co-mutation of His149 and His151 to alanine only mildly impairs APP maturation in HEK293 cells. (A) HEK293 cells stably overexpressing wt-APP or H149,151A-APP mutant were cultured under standard conditions. Cell lysates were normalized to total protein concentration levels before SDS-PAGE. The W02 antibody was used to detect intracellular full length APP and the arrows indicate bands corresponding to mature (mAPP) and immature (imAPP) APP. (B) The ratio of the mature over the immature APP levels was calculated and expressed as a relative percentage to that of wt-APP (100%). H149,151N-APP data from Fig 3B were included for comparison. Data are expressed as mean ± SEM; n indicates the number of the experiment repeats; statistical significances were calculated with one-way ANOVA using Tukey’s post hoc test; **p<0.01, ***p<0.001.
Figure 2

secreted sAPPα levels [%]

wt-APP  H127N-APP  H140N-APP  H151N-APP  H147,148N-APP  H147,151N-APP  H147,149,151N-APP

N = 25  N = 4  N = 4  N = 4  N = 4  N = 4  N = 4

100  65  31  79  41  47  42  10

***  ***  ***  ***  ***  ***

*
Figure 3

CuBD-mediated regulation of APP metabolism
Figure 5

A

B

organelle markers

ER (BiP)

Early endosome (EEA1)

Golgi (Syntaxin6)

wt-APP

mAPP

imAPP

H147,149N-APP

inAPP

inAPP

H147,149,151N-APP

imAPP

6.5% Optiprep gradient 20%

organelle markers

% of total individual protein

1 3 5 7 9 11 13 15 17 19

mature APP

EEA1

Syntaxin 6

BiP

% of total individual protein

1 3 5 7 9 11 13 15 17 19

fractions

1 3 5 7 9 11 13 15 17 19

% of total individual protein

1 3 5 7 9 11 13 15 17 19

fractions

wt-mAPP

H147,149N-mAPP

wt-imAPP

H147,149-imAPP

H147,149,151N-imAPP
Figure 6

A

| DAPI | WO2 | CellLight ER-RFP | merge |
|------|-----|-------------------|-------|
| wt-APP | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| H147,149,151N-APP | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| vector only | ![Image](image7) | ![Image](image8) | ![Image](image9) |

B

| DAPI | WO2 | GRASP65 | merge |
|------|-----|---------|-------|
| wt-APP | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| H147,149,151N-APP | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| vector only | ![Image](image16) | ![Image](image17) | ![Image](image18) |
CuBD-mediated regulation of APP metabolism

Figure 7
CuBD-mediated regulation of APP metabolism

Figure 8

A

B

C
Figure 9

A

![Bar graph showing intracellular sAPPα levels as a percentage for different APP metabolites.](image)

B

| APP metabolites levels compared to wt-APP | intracellular | secreted |
|----------------------------------------|---------------|----------|
|                                        | m/im | sAPPα | sAPPα | sAPPβ | Aβ |
| H147N                                  | =    | ↑     | ↓     | ↓     | ↓  |
| H149N                                  | ↓    | (↓)   | ↓     | ↓     | ↓  |
| H151N                                  | ↓    | =     | ↓     | =     | ↓  |
| H147,149N                              | ↓    | =     | ↓     | =     | ↓  |
| H147,151N                              | ↓    | =     | ↓     | =     | ↓  |
| H149,151N                              | ↓    | =     | ↓     | ↓     | ↓  |
| H147,149,151N                          | ↓    | (↓)   | ↓     | ↓     | ↓  |
CuBD-mediated regulation of APP metabolism

Figure 10

A

kDa

188 -

98 -

- βME

B

kDa

188 -

98 -

+ βME
Figure 11

CubD-mediated regulation of APP metabolism

D

| Secondary structural contents [%] | α-helix | β-sheet | Turns | Unordered |
|----------------------------------|--------|--------|-------|----------|
| wt-APP-CubD                     | 28     | 22     | 20    | 30       |
| H147N-APP-CubD                  | 16     | 28     | 24    | 32       |
| H169N-APP-CubD                  | 27     | 22     | 21    | 30       |
| H151N-APP-CubD                  | 17     | 25     | 24    | 33       |
| H147,149N-APP-CubD              | 16     | 27     | 24    | 33       |
| H147,151N-APP-CubD              | 16     | 28     | 24    | 32       |
| H149,151N-APP-CubD              | 13     | 31     | 23    | 32       |
| H147,149,151N-APP-CubD          | 13     | 32     | 24    | 32       |
Figure 12

CuBD-mediated regulation of APP metabolism
Figure 13

CuBD-mediated regulation of APP metabolism
CuBD-mediated regulation of APP metabolism
The Amyloid Precursor Protein Copper Binding Domain Histidine Residues 149 and 151 mediate APP Stability and Metabolism
Loredana Spoerri, Laura J. Vella, Chi L. L. Pham, Kevin J. Barnham and Roberto Cappai

J. Biol. Chem. published online June 8, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.355743

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts