NRBF2 is involved in the autophagic degradation process of APP-CTFs in Alzheimer disease models

Chuanbin Yang  
*Hong Kong Baptist University*

Cui-Zan Cai  
*University of Macau*

Ju-Xian Song  
*Hong Kong Baptist University*

Jie-Qiong Tan  
*Central South University*

Siva Sundara Kumar Druairajan  
*Hong Kong Baptist University*

See next page for additional authors

Follow this and additional works at: https://repository.hkbu.edu.hk/hkbu_staff_publication

Part of the Medicine and Health Sciences Commons

This document is the authors' final version of the published article.

Link to published article: http://dx.doi.org/10.1080/15548627.2017.1379633

APA Citation


This Journal Article is brought to you for free and open access by HKBU Institutional Repository. It has been accepted for inclusion in HKBU Staff Publication by an authorized administrator of HKBU Institutional Repository. For more information, please contact repository@hkbu.edu.hk.
NRBF2 is involved in the autophagic degradation process of APP-CTFs in Alzheimer disease models
Chuanbin Yang¹, Cui-Zan Cai², Ju-Xian Song¹, Jie-Qiong Tan³, Siva Sundara Kumar, Durairajan¹, Ashok Iyaswamy¹, Ming-Yue Wu², Lei-Lei Chen¹, Zhenyu Yue⁴, Min Li¹*, Jia-Hong Lu²*

¹Mr. and Mrs. Ko Chi Ming Centre for Parkinson’s Disease Research, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China
²State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau SAR, China
³State Key Laboratory of Medical Genetics, Xiangya Medical School, Central South University, Changsha, Hunan, China.
⁴Department of Neurology and Neuroscience, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

*Co-corresponding authors
E-mail: limin@hkbu.edu.hk; jiahonglu@umac.mo
Abstract

Alzheimer disease (AD) is the most common neurodegenerative disease characterized by the deposition of amyloid plaque in the brain. Autophagy associated PIK3C3 complex has been shown to interfere with APP metabolism and amyloid beta peptide (Aβ) homeostasis via poorly understood mechanisms. Here we reported that nuclear receptor binding factor 2 (NRBF2), a key component and regulator of the PIK3C3, is involved in APP-CTFs homeostasis in AD cell models. We found that NRBF2 interacts with APP in vivo and its expression levels are reduced in hippocampus of 5XFAD AD mice; we further demonstrated that NRBF2 overexpression promotes degradation of APP C-terminal fragments (APP-CTFs), and reduces Aβ_{1-40} and Aβ_{1-42} levels in human mutant APP overexpression cells Conversely, APP-CTFs, Aβ_{1-40} and Aβ_{1-42} levels were increased in Nrbf2 knockdown or knockout cells. Furthermore, NRBF2 positively regulates autophagy in neuronal cells and NRBF2-mediated reduction of APP-CTFs levels is autophagy-dependent. Importantly, Nrbf2 knockout attenuates the recruitment of APP/APP-CTFs into autophagosome/autolysosome and the sorting of APP/APP-CTFs into endosomal intraluminal vesicles, which is accompanied by the accumulation of the APP/APP-CTFs into RAB5-positive early endosomes. Collectively, our results reveal the potential connection between NRBF2 and AD-associated protein APP by showing that NRBF2 plays an important role in regulating degradation of APP-CTFs through modulating autophagy.

Keywords: NRBF2, autophagy, class III phosphatidylinositol-3-kinase (PIK3C3), APP, Aβ, Alzheimer’s disease.
Introduction:

Alzheimer disease (AD) is the most common age-related neurodegenerative disease characterized by the intracellular aggregation of amyloid beta (Aβ) peptide and hyperphosphorylated tau, as well as the extracellular Aβ plaques deposition in the brain.\(^1\) Aβ has been regarded as the critical event during the progression and pathogenesis of AD.\(^3,4\) Aβ is produced from amyloid precursor protein (APP) by the amyloidogenic processing pathway.\(^4,6\) Amyloid precursor protein (APP) is a type I transmembrane protein that can be processed by either non-amyloidogenic processing or amyloidogenic processing.\(^4,6\) Non-amyloidogenic APP processing occurs at the cell surface, where APP is cleaved by α-secretase to produce soluble APP ectodomain and the membrane-associated C83 APP C-terminal fragment alpha (APP-CTFα), which is further cleaved by γ-secretase to produce non-toxic P3 peptides.\(^4,7\) Amyloidogenic APP processing takes places in intracellular compartments, including the trans-Golgi network, endosomes, and autophagosomes, where APP is first cleaved by BACE/β-secretase, which generates the soluble sAPPβ and the membrane-bound C99 APP-CTFβ. Subsequently APP-CTFβ is cleaved into Aβ1-40 or Aβ1-42 by γ-secretase.\(^4,7\) Thus, APP-CTFs (hereafter referred to as APP-CTFα and APP-CTFβ) levels and its subcellular locations are essential for Aβ production. In addition, the production of Aβ is also tightly regulated by autophagy-mediated degradation of APP-CTFs.\(^8,10\)

Autophagy is a major pathway involved in the delivery of long-lived proteins, protein aggregates, and organelles to lysosomes for degradation.\(^10,11\) Autophagy can be generally divided into several steps, including autophagosome formation (initiation), fusion of autophagosome with lysosome (maturation), and breakdown of autophagic bodies.\(^10,11\) Autophagy dysfunction has been implicated in the pathogenesis of AD. In AD patients, the mechanistic target of rapamycin (MTOR) signaling, an important signaling pathway controlling autophagy, is enhanced, which may reflect lowered levels of basal autophagy.\(^12\) In addition, accumulated autophagosome and high levels of lysosomal hydrolases have been found in AD brain, which may indicate impaired autophagosomal-lysosomal clearance.\(^13,14\) In AD cells and animal models, deletion of Beclin1, a key protein for the formation of autophagosomes, increases both the intracellular and extracellular Aβ loads.\(^15-17\) Moreover, knockout of the autophagy-related 7 (Atg7) affects both the production and secretion of Aβ.\(^18,19\) In line with these findings, increasing evidences indicate that induction of autophagy may serve as a viable therapeutic strategy that can ameliorate the pathological features associated with AD. For instance, neuronal specific overexpression of transcription factor EB (Tfeb), a master regulator of autophagy-lysosome pathway, induces APP degradation and reduces Aβ generation.\(^20\) Pharmacologically induction of autophagy by rapamycin decreases intracellular Aβ levels, reduces Aβ plaque load and improves cognition in AD mice.\(^12,21\)
Autophagy initiation can be regulated by the PIK3C3/VPS34 complex, which contains PIK3R4/VPS15-PIK3C3/VPS34-BECN1/Beclin 1-ATG14.22-24 Our and others’ recent studies have shown that NRBF2 is a novel component of PIK3C3 complex, and associates with ATG14 in both mammalian cells25-28 and in yeast (named ATG38).29 In addition, NRBF2 has been identified to positively regulate autophagy.25, 28, 30 However, there is also a controversial report,26 suggesting that the role of NRBF2 in regulating autophagy may be cell type-dependent. Whether NRBF2 is a positive or negative regulator in neuronal cells remains unclear. In addition, though autophagy has been implicated in Aβ metabolism, the roles and mechanisms of PIK3C3 complex components involved in autophagy initiation and regulating APP metabolism are unclear. Here, we investigated the functions of NRBF2 in regulating APP metabolism in cell models. Our results suggest that NRBF2, via activation of autophagy, facilitate APP-CTFs degradation. The mechanisms for NRBF2-mediated APP metabolism may be related to its interaction with APP and the sorting of APP/APP-CTFs from early endosome into autophagosomes and endosomal intraluminal vesicles (ILVs) for lysosomal degradation. Our findings highlight the importance of NRBF2, a PIK3C3 component, in regulating APP metabolism.

Results

NRBF2 is reduced in brains of 5XFAD AD mice

Autophagy has been linked to AD pathogenesis.33 To identify whether NRBF2, a key component of PIK3C3/VPS34, is involved in AD, we measured NRBF2 protein levels in brains of 5XFAD transgenic AD mice. 5XFAD mice co-express 5 mutations of familial AD, which recapitulate major features of AD amyloid pathology.31 As expected, both FL-APP and APP-CTFs levels are increased in the hippocampus of 5XFAD mice (Fig. 1A). Interestingly, as shown in Fig. 1 A and B, NRBF2 protein levels are significantly reduced in hippocampus of 12 month old 5XFAD mice (n=6) compared with age-controlled wild type C57BL/6 mice. In contrast, ATG5-ATG12 levels are not changed in 5XFAD mice (Fig.1 A and B), suggesting that NRBF2 may plays a specific role in this AD animal model. Previously results show that BECN1 is reduced in the brains of AD patients and APPswePS1dE9 mice.15, 32 Consistently, BECN1 proteins levels are also reduced in 5XFAD mice (Fig.1 A and B). Interestingly, our results show that both LC3B-II and SQSTM1/p62 levels are increased in the hippocampus of 5XFAD mice (Fig.1 A and B), which is consistently with previously report that autophagic degradation defects are found in AD patients and several other AD animal models.33, 34 These results reveal the potential role of NRBF2 in the pathogenesis AD.
NRBF2 protein levels are reduced in hippocampus region of 5XFAD AD mice. (A) Western blotting was used to detect the expression of NRBF2, BECN1, ATG5-ATG12, LC3B, SQSTM1, FL-APP, and APP-CTFs levels in hippocampus region of 12-month old C57BL/6 and 5XFAD AD mice, whereas ACTB was used as an internal control. (B) Quantitative data in Fig. 1A. (n=6) showed that NRBF2, BECN1 but not ATG5-ATG12 levels are significantly decreased, while LC3B-II and SQSTM1/p62 levels are significantly increased in 5XFAD mice. *, P < 0.05, **, P < 0.01 vs. the control.

**Overexpression of Nrbf2 decreases APP-CTFs and Aβ levels**

As we found NRBF2 level is reduced in 5XFAD mice, we wonder whether NRBF2 is important in regulating APP-CTFs (APP-CTFα and APP-CTFβ) degradation and Aβ production. We firstly over-expressed NRB2F2 in N2a mouse neuroblastoma cells stably expressing human Swedish mutant APP695 (N2S cells), which is commonly used as an AD cell model. We found that Nrbf2 overexpression significantly reduces the expression of APP-CTFα and APP-CTFβ levels but does not affect the expression of full length APP (FL-APP) levels (Fig. 2A and B). However, the amounts of sAPPα and sAPPβ were not different in Nrbf2-overexpressed cells compared with control cells (Fig. 2C and D). In addition, Nrbf2 overexpression does not affect PSEN1 levels, a subunit of γ-secretase, which is responsible for the production of Aβ by cleaving of APP-CTFβ. These results suggest that NRBF2 may affect APP-CTFs degradation rather than proteolytic processing of APP. After cleavage by γ-secretases, APP-CTFβ generated Aβ1-40 or Aβ1-42, and Aβ1-40 accounted for about 90% of all Aβ fragments generated from APP-CTFβ in amyloid plaques. To further determine whether the decreased APP-CTFs in NRBF2 over-expressed cells is accompanied by lower levels of Aβ, we examined Aβ1-40 and Aβ1-42 levels by ELISA assay. As shown in Fig. 2E, overexpression of Nrbf2 lowered both the intracellular and extracellular Aβ40 levels. To further confirm these results, we used a non-neuronal cell, Chinese hamster ovary
(CHO) cells stably transfected with human APP751 bearing the V717F mutation (7PA2 cells), as another AD cell model, and we obtained similar results (Fig. 2F and G). These results suggest that Nrbf2 overexpression decreases APP-CTFα and APP-CTFβ, as well as intracellular and extracellular Aβ1-40 and Aβ1-42 levels in different cells.

**Fig. 2.** Nrbf2 overexpression reduces APP-CTFs, intracellular and extracellular Aβ1-40 and Aβ1-42 levels. (A) After N2a mouse neuroblastoma cells stably expressing human Swedish mutant APP695 (N2S cells) were transfected with Flag-NRBF2 plasmid or vehicle plasmid, the expression of NRBF2, Full length APP (FL-APP), APP-CTFα, and APP-CTFβ were examined by Western blotting, whereas ACTB was used as an internal control. (B) Quantitative data in Fig. 2A showed that NRBF2 overexpression significantly reduces APP-CTFs levels but not FL-APP levels. (C and D) Western blotting and quantification shows that Nrbf2 overexpression does not affect sAPPα, sAPPβ, and presenilin1 (PSEN1) levels. (E) ELISA analysis results showed that Nrbf2 overexpression significantly reduces intracellular and extracellular Aβ1-40 and Aβ1-42 levels in N2S cells. (F) Overexpression of Nrbf2 in Chinese hamster ovary (CHO) cells stably transfected with human APP751 bearing the V717F mutation (7PA2 cells) reduced the expression of APP-CTFs. (G) ELISA analysis results demonstrated that Nrbf2 overexpression significantly reduces intracellular and extracellular Aβ1-40 and Aβ1-42 levels in N2S cells. Quantification data are presented as the mean ± SEM, n=3 from independent experiments, *, P < 0.05 vs. the control.

**Depletion of Nrbf2 increases APP-CTFs and Aβ levels**

To further confirm the functions of NRBF2 in APP-CTFs degradation and Aβ production, we generated Nrbf2 knockdown (KD) and knockout (KO) N2S cells. As shown in Fig. 3 A and B, transient transfection of Nrbf2 siRNA resulted in about 60%
reduction in the protein levels of NRBF2. Meanwhile, the cellular APP-CTFα and APP-CTFβ levels were increased about 50% in Nrbf2 KD cells (Fig. 3C and D). In addition, CRISPR-Cas9 technology was used to generate Nrbf2 KO cells, and the most potent clone (clone 2) among several Nrbf2 KO stable clones generated was chosen for investigating the roles of NRBF2 in APP metabolism (Fig. S1 and 3E). Consistently, KO of Nrbf2 dramatically increased APP-CTFα and APP-CTFβ levels but not FL-APP levels (Fig. 3F and G). Moreover, Nrbf2 overexpression can rescue Nrbf2 KO-mediated increase of APP-CTFα and APP-CTFβ levels (Fig. 3H). In line with the findings in Nrbf2-overexpression cells, Nrbf2 KO does not affect sAPPα, sAPPβ and PSEN1 levels (Fig. 3I and J). Moreover, the deficiency of Nrbf2 also increased the amount of the intracellular and secreted Aβ1-40 and Aβ1-42 levels when compared to vehicle control cells (Fig. 3K). Finally, we measure γ-secretase activity by using luciferase assay as described previously. The result shows that Nrbf2 KO does not affect γ-secretase activity (Fig. S2). Altogether, these results indicate that NRBF2 is an important factor for regulating APP-CTFα, APP-CTFβ levels and Aβ contents, with minimal effects on the secretase cleavage of APP.

**Fig. 3.** Silencing Nrbf2 increases APP-CTFs, intracellular and extracellular Aβ1-40 and Aβ1-42 levels. (A and B) After transfected N2S cells with Nrbf2 siRNA or NT siRNA, the expression of NRBF2 was detected and quantified, whereas ACTB was used as an internal control. (C) After Nrbf2 knockdown, the expression of FL-APP, APP-CTFα, and APP-CTFβ were detected by Western blotting, whereas ACTB was used as an internal control. (D) Quantitative data in Fig. 3C. showed that Nrbf2 knockdown significantly increases APP-CTFα, and APP-CTFβ levels. (E) The expression of NRBF2 in WT and KO cells was examined by Western blotting. (F) Western blotting was used to detect the expression of FL-APP, APP-CTFα, and APP-CTFβ levels after KO of Nrbf2, whereas ACTB was used as an internal control. (G) Quantitative data in Fig. 3F. showed that Nrbf2 KO significantly increases APP-CTFα, and APP-CTFβ levels but not FL-APP levels. (H) Overexpression of
*Nrbf2* rescues *Nrbf2* KO-mediated the increase of APP-CTFs levels. (I and J) Western blotting and quantification shows that *Nrbf2* overexpression does not affect sAPPα, sAPPβ, and PSEN1 levels. (K) ELISA analysis results demonstrated that *Nrbf2* KO significantly increases intracellular and extracellular Aβ1-40 and Aβ1-42 levels. Quantification data were presented as the mean ± SEM, n=3 from independent experiments. *, P < 0.05, **, P < 0.01 vs. the control.

**NRF2 positively regulates autophagy in neuronal N2S cells**

NRF2 was initially described as a regulator of the nuclear receptors including peroxisome proliferator-activated receptor α (PPARα), retinoic acid receptor (RAR), and retinoid X receptor (RXRα). Our recent study and other’s reports show that NRF2 is an important component of the PIK3C3 complex and binds with ATG14. However, the role of NRF2 in regulating autophagy is still under debate. To test the function of NRF2 in regulating autophagy in neuronal cells, we examined the levels of autophagy substrate SQSTM1/p62 in Nrbf2 KD cells. We found that Nrbf2 KD significantly increased SQSTM1 levels (Fig. 4A and B). In addition, Nrbf2 KD attenuated starvation (EBSS)-induced reduction of SQSTM1 and increase of microtubule-associated protein 1 light chain 3B (LC3B)-II levels (Fig. 4C and D), indicating that Nrbf2 KD attenuated starvation-induced autophagy. Similarly, Nrbf2 KO also dramatically increased SQSTM1 protein levels (Fig. 4E and F) rather than mRNA levels (Fig. S3), suggesting that Nrbf2 KO attenuates the degradation of SQSTM1 levels. Conversely, Nrbf2 overexpression significantly lowered cellular SQSTM1 levels (Fig. 4G and H). Moreover, there was also a significant increase in the number of LC3 punctate dots after Nrbf2 overexpression (Fig. 4 I and J). Taken together, these results demonstrate that Nrbf2 positively regulates autophagy in neuronal cells.
Fig. 4. NRBF2 positively regulates autophagy. (A) After Nrbf2 knockdown, the expression of SQSTM1 was examined, whereas ACTB was used as an internal control. (B) Quantitative data from Fig. A. showed that Nrbf2 knockdown significantly increases SQSTM1 levels. (C) Western blotting detected the effects of starvation on the expression of LC3B-II and SQSTM1 levels after Nrbf2 knockdown, whereas ACTB was used as an internal control. (D) Quantitative data demonstrated that Nrbf2 knockdown significantly attenuates starvation induced the increment of LC3B-II and reduction of SQSTM1/p62, whereas ACTB was used as an internal control. (E and F) Western blotting and quantification results showed that Nrbf2 KO significantly increases SQSTM1 levels. (G and H) Western blotting and quantification results showed that Nrbf2 overexpression significantly decreases SQSTM1 levels, whereas ACTB was used as an internal control. (I and J) Effect of Nrbf2 overexpression on the formation of LC3 puncta was detected and quantified. Quantification data were presented as the mean ± SEM, n=3 from independent experiments. *, #, P < 0.05, **, P < 0.01 vs. the relative control.
NRBF2 facilitates APP-CTFs degradation via autophagy

Autophagy has been implicated in the metabolism of APP by degradation of APP-CTFs, resulting in reduced Aβ via γ-secretase-mediated cleavage of APP-CTFβ. Since we found NRBF2 is a positive regulator of autophagy (Fig. 4), to determine whether NRBF2-mediated autophagy is correlated with its role in regulating APP metabolism, we firstly tested the half-life of APP-CTFα, APP-CTFβ and FL-APP by treating cells with cycloheximide (CHX), which inhibits protein synthesis. As shown in Fig. 5A and B, the half-life of APP-CTFs and FL-APP in Nrbf2 KO cells were significantly increased compared with vehicle control cells. Importantly, the APP-CTFs/FL-APP ratio was increased in KO cells. These results suggest that the degradation of APP-CTFs in Nrbf2 KO cells is impaired. In addition, Nrbf2 KO does not affect lysosomal numbers as reflected by lysotracker RED staining (Fig. 5C), suggesting that Nrbf2 KO may attenuate the sequestration of APP-CTFs to lysosome. Next, we treated Nrbf2 over-expressed cells with chloroquine (CQ) to inhibit lysosomal acidification and evaluated APP-CTFs abundance, we found that Nrbf2 overexpression-mediated reduction of APP-CTFs was attenuated by CQ treatment in N2S cells (Fig. 5D and E) and in 7PA2 cells (Fig. S4). Thirdly, we knocked down the expression of Atg5, an important factor for controlling autophagy initiation, and examined the effects of ATG5 on Nrbf2 overexpression-mediated degradation of APP-CTFs. We found that Atg5 knockdown prevents Nrbf2 overexpression-induced LC3B-II levels (Fig. 5F) as well as reduction of APP-CTFs (Fig. 5F and G). Atg5 is also important for regulating FL-APP levels (Fig. S5), which is consistent with previous report. Taken together, these results indicate that autophagy is involved in NRBF2-mediated degradation of APP-CTFα and APP-CTFβ.
NRBF2 interacts with APP and NRBF2 is required for APP/APP-CTFs recruitment into autophagic structures

To examine how NRBF2 affects APP metabolism, by using Co-IP analysis, we found that NRBF2 interacts with APP in the brains of 5XFAD AD mice (Fig. 6A) as well as in HEK293 cells (Fig. 6B). As a positive control, the interaction between NRBF2 and BECN1 was also confirmed (Fig. 6A). The interaction of NRBF2 with APP was further supported by immunostaining analysis showing the colocalization of NRBF2 with APP in N2S cells (Fig. 6C). Probably due to the fast turnover of APP-CTFs, we could not detect the interaction of NRBF2 with APP-CTFs by Co-IP (data not shown). These results suggest the physiological association between NRBF2 and APP.

Autophagy substrates are first recruited into autophagosomes, and then fused with lysosomes for degradation. Our previous study suggested that NRBF2 is important for controlling autophagy initiation by modulating PIK3C3 activity and now we find NRBF2 interacts with APP. Thus, to better understand how NRBF2 mediates autophagosome formation to affect APP metabolism, we detected the effects of NRBF2 on the recruitment of APP/APP-CTFs into autophagic structures. As shown in Fig. 6D and E, after transfecting cells with GFP-LC3, the colocalization of LC3 puncta with APP/APP-CTFs was significantly reduced in Nrfr2 KO cells, suggesting that Nrfr2 KO attenuates the recruitment of APP/APP-CTFs into autophagosomes. Importantly, the delivery of APP-CTFs into matured autophagosome was also compromised in Nrfr2 KO cells as reflected by the colocalization of APP/APP-CTFs...
with LC3 in the presence of lysosomal inhibitor CQ (Fig. 6E). Taken together, these results suggest that Nrbf2 KO attenuates the delivery of APP/APP-CTFs into autophagic structures.

Fig. 6. NRBF2 interacts with APP and NRBF2 is required for APP/APP-CTFs recruitment into autophagic structures. (A) NRBF2 interacts with APP. Brain samples (n=4) from 5XFAD mice were lysed and immunoprecipitated (IP) with NRBF2 antibody, eluates were resolved by SDS/PAGE and analyzed by immunoblotting with corresponding antibodies. (B) After transiently expression of Flag-NRBF2 or/and GFP-APP, coimmunoprecipitation of GFP-APP with Flag antibody was performed in HEK293 cells. (C) After transiently expression of GFP-NRBF2 in N2S cells, the colocalization of NRBF2 with APP/APP-CTFs was analyzed by immunostaining and representative images was shown. (D-E) After transfected WT and Nrbf2 KO N2S cells with GFP-LC3, (D) followed by staining with APP C-terminal antibody, the colocalization of LC3 with APP/APP-CTFs was recorded and representative pictures were shown. Quantitative data shows the percentage of APP/APP-CTFs dots colocalize with LC3 dots. (E) Colocalization of LC3 with APP/APP-CTFs in WT and KO cells was recorded in the presence of lysosome inhibitor chloroquine (CQ) and representative images was showed. Quantitative data shows the percentage of APP/APP-CTFs dots colocalize with LC3 dots. Quantification data were presented as the mean ± SEM, n=20-25 cells from three independent experiments. *, P < 0.01 vs. the relative control.
**Nrhf2** KO attenuates the sorting of APP/APP-CTFs into endosomal intraluminal vesicles (ILVs)

APP-CTFs can be sorted into ILVs of multivesicular endosomes, followed by fusion with lysosomes for degradation. 4, 43 PIK3C3 and its main product PtdIns3P enhance the sorting of APP/APP-CTFs into ILVs. 44 Previous findings, including our study, found that NRBF2 positively regulates the production of PtdIns3P. 27, 28 Thus, we examined whether **Nrhf2** KO affects the sorting of APP/APP-CTFs into ILVs. After cells expressing active form (GTP-bound form) of RAB5, RAB5Q79L, enlargement of endosomes was formed, which is commonly used for the discrimination between the limiting membrane and their lumen of these organelles. 44, 45 The lumen of these endosomes contains the ILVs. 46 Consistent with a previously report, 44 under normal conditions, APP/APP-CTFs were predominantly observed within the endosomal lumen (Fig. 7A) after overexpression of GFP-RAB5Q79L. In contrast, **Nrhf2** KO significantly increased the percentage of APP/APP-CTFs present in the periphery of endosomes (Fig. 7A), which means that **Nrhf2** KO attenuated the sorting of APP/APP-CTFs into ILVs. This notion was further confirmed by the results that **Nrhf2** KO impaired the distribution of APP/APP-CTFs in the RAB7- and LAMP1-positive late endosomes/lysosomes (Fig. 7C and D). Importantly, **Nrhf2** KO results in the accumulation of APP/APP-CTFs in the RAB5-positive early endosomes (Fig. 7B). Taken together, these results suggest that **Nrhf2** KO results in the accumulation of APP/APP-CTFs in early endosomes and attenuates the sorting of APP/APP-CTFs into late endosomes/lysosomes for degradation.
Fig. 7. *Nrbf2* KO attenuates the sorting of APP/APP-CTFs into endosomal intraluminal vesicles (ILVs). (A) After WT or *Nrbf2* KO cells were transfected with active form of Rab5, GFP-Rab5Q79L, followed by staining with APP C-terminal antibody and DAPI, the localization of APP/APP-CTFs in the peripheral or lumen of enlarged endosomes were recorded and representative pictures were shown. Arrows indicate the colocalization of APP/APP-CTFs in the peripheral of endosomes. Quantitative data showed that *Nrbf2* KO reduces the sorting of APP-CTFs into lumen and increases APP-CTFs into peripheral of giant endosomes. (B) After WT or *Nrbf2* KO cells were transfected with GFP-Rab5, followed by staining with APP C-terminal antibody and DAPI, the localization of APP/APP-CTFs with RAB5 were recorded and representative pictures were shown. Quantification results show that *Nrbf2* KO increases the distribution of APP/APP-CTFs in the RAB5-positive early endosomes. (C) After WT or *Nrbf2* KO cells were transfected with RFP-Rab7, followed by staining with APP C-terminal antibody and DAPI, the localization of APP/APP-CTFs with RAB7 were recorded and representative pictures were shown. Quantification results show that *Nrbf2* KO reduces the distribution of APP/APP-CTFs in the RAB7-positive late endosomes/lysosomes. (D) After WT or *Nrbf2* KO cells were transfected with RFP-LAMP1, followed by staining with APP C-terminal antibody and DAPI, the localization of APP/APP-CTFs with LAMP1 were recorded and representative pictures were shown. Quantification results show that *Nrbf2* KO decreases the distribution of APP/APP-CTFs in the LAMP1-positive late endosomes/lysosomes. Quantification data by using pearson’s correlation coefficient were presented as the mean ± SEM, n=20-30 cells from three independent experiments. *, #, \( P < 0.05 \) vs. the relative control.
Fig. 8. Schematic model for the NRBF2 function in the autophagic turnover of APP-CTFs. Full-length amyloid precursor protein (FL-APP) can be processed by either α-secretase (non-amyloidogenic processing) or BACE/β-secretase (amyloidogenic processing) to generate sAPPα/APP-CTFα and sAPPβ/APP-CTFβ, respectively. APP-CTFβ can be further cleaved to generate Aβ by γ-secretase. Aβ accumulation has been regarded as the critical event during the progression and pathogenesis of AD. In addition to proteolytic processing of APP to produce Aβ, autophagy regulators (e.g. NRBF2) also play a role for regulating Aβ production by degradation of APP-CTFs. By regulating autophagy and interaction with APP, Nrbf2 KO attenuates the degradation of APP-CTFs via reducing the recruitment of APP/APP-CTFs into both autophagosomes and ILVs, and increasing the distribution of APP/APP-CTFs into RAB5-positive early endosomes.

Discussion

Autophagy dysregulation has been implicated in several neurodegenerative diseases
including AD.⁹,⁴⁷ There is an accumulation of autophagosomes in the brains of AD patients and animal models of AD⁴⁸. In addition, familial AD-associated mutations or loss of Psen1 or Psen2 impair autophagy-lysosomal functions.⁴⁹-⁵¹ Consistently, autophagy dysfunction is also found in 5XFAD AD mice as reflected by the increase of both LC3B-II and SQSTM1 levels (Fig. 1). Moreover, autophagy-related proteins such as BECN1,¹⁵,¹⁷ ATG5,⁴² and ATG7 are associated with APP processing and Aβ metabolism. Importantly, BECN1 is reduced in brains of AD patients and APPswePS1dE9 AD mice,¹⁵,³² our results in 5XFAD AD mice also confirmed this result. Remarkably, pharmacological and genetic activation of autophagy induces APP/APP-CTFs degradation and reduces Aβ generation.¹², ²⁰, ²¹ These results highlight the crucial role of autophagy in APP metabolism. In the present study, we found that NRBF2, a component of PIK3C3, is reduced in the hippocampus region of 5XFAD mice, whereas ATG5-ATG12 contents remain unchanged, suggesting that NRBF2 may be involved in AD process.

By using cellular AD models, we further demonstrated that NRBF2 plays an important role in regulating APP-CTFs homeostasis. Specifically, Nrbf2 overexpression reduces, whereas Nrff2 depletion increases, both APP-CTFα, APP-CTFβ levels and Aβ contents (Fig. 2 and Fig. 3), whereas NRBF2 does not affect FL-APP levels. Further studies show that NRBF2 does not affect Swedish sAPPα, and sAPPβ contents (Fig. 2C and D; Fig. 3I and J). In addition, Nrbf2 KO does not affect γ-secretase activity (Fig. S2). These results may indicate that proteolytic processing of APP is not the main pathway for NRBF2-mediated the changes of APP-CTFα and APP-CTFβ levels.

Other studies as well as ours show that NRBF2 is a component of PIK3C3,²⁵-²⁸, ³⁰ However, its roles in the regulation of autophagy are controversial.²⁵-²⁸ In the current study, our results from Nrbf2 KD (Fig. 4A), Nrbf2 KO (Fig. 4E), and Nrbf2 overexpression (Fig. 4G and I) N2S cells support the notion that NRBF2 is a positive regulator of autophagy. These results are consistent with our previous findings in MEF cells and Nrbf2 KO mice²⁸ and others studies²⁵, ²⁷, ³⁰ in mammalian cells as well as in yeast.²⁹ The mechanisms by which NRBF2 induces autophagy may be related to its roles in regulating PIK3C3 activities.²⁷, ²⁸

Next, we investigated the links between autophagy and NRBF2-mediated APP metabolism. We found that Nrbf2 KO significantly increases the half-life for APP-CTFs degradation. In addition, inhibition of lysosomal activity with CQ and knockdown of Atg5 reverse NRBF2 overexpression-induced degradation of APP-CTFs. Notably, Nrbf2 KO does not affect lysosomal numbers, suggesting that the delivery of APP/APP-CTFs into lysosomal for degradation may be compromised without Nrbf2 (Fig. 5). These evidence support the hypothesis that autophagy is involved in NRBF2-mediated degradation of APP-CTFs. These results are also
consistent with previous reports that autophagy is critical for modulating APP-CTFs degradation.\textsuperscript{52} Though FL-APP has been reported to be degraded by autophagy,\textsuperscript{20} our results showed that NRBF2 overexpression or silencing does not affect FL-APP levels (Fig. 2A, Fig. 3C and F), implying that the levels of FL-APP may not be affected by modulating NRBF2. As only slightly changes in the amount of FL-APP may lead to increase/decrease of APP-CTFs levels, we do not fully exclude the possibility that NRBF2 may affect the degradation of FL-APP to regulate APP-CTFs levels (NRBF2 overexpression of KO causes about 5\% percent changes in FL-APP level but no statistically significant). It can be concluded that autophagy pathway plays a major role for NRBF2-mediated the changes of APP-CTFs levels.

Lysosomes mediate the clearance of cellular substrates originating from both autophagic and endocytic pathways.\textsuperscript{53} NRBF2 plays an important role in regulating autophagosome formation by modulation of PIK3C3 activities.\textsuperscript{28} Our results show that Nrbf2 KO impairs the recruitment of APP/APP-CTFs into autophagic structures and ILVs (Fig. 6D and E, and Fig. 7); this impairment may finally attenuate the degradation of APP-CTFs by lysosomes. These effects may be responsible for the increased amyloidogenic processing of APP after silencing of Nrbf2. In addition to delivery into the autophagy pathway, APP/APP-CTFs can also be delivered into lysosomes for degradation by fusion of late endosomes with lysosomes.\textsuperscript{4} Thus, the sorting of APP/APP-CTFs into late endosomes also affects APP-CTFs levels and A\textbeta homeostasis. One important factor responsible for APP/APP-CTFs sorting into late endosomes is PtdIns3P.\textsuperscript{44} PtdIns3P levels in the brains of AD patients and animal models are reduced. Silencing of lipid kinase PIK3C3,\textsuperscript{44} a key enzyme responsible for PtdIns3P production, reduces APP sorting into intraluminal vesicles and enhances the amyloidogenic processing of APP. Our results suggest that Nrbf2 KO reduces the sorting of APP/APP-CTFs into ILVs (Fig. 7A), this notion was further supported by the result that Nrbf2 KO reduces the distribution of APP/APP-CTFs in the late endosomes/lysosomes (Fig. 7C and D). This effect may be due to the reduced production of PIK3C3 because previous reports have showed that NRBF2 positively regulates the production of PtdIns3P by modulating PIK3C3 activities.\textsuperscript{27, 28} Interestingly, Nrbf2 KO results in the accumulation of APP/APP-CTFs into RAB5-positive early endosomes (Fig. 7B). Interestingly, we found that NRBF2 interacts with APP in both 5XFAD mice and in neuronal cells, the interaction may contributes to the redistribution of APP/APP-CTFs in Nrbf2 KO cells. Collectively, Nrbf2 KO attenuates the degradation of APP-CTFs via reducing the recruitment of APP/APP-CTFs into both autophagosomes and ILVs, and increasing the distribution of APP/APP-CTFs into RAB5-positive early endosomes, which may further affect the degradation of APP-CTFs by lysosomes and the processing of APP in different organelles. These results may explain Nrbf2 KO-increased amyloidogenic processing of APP. However, whether other PIK3C3 components such as BECN1 regulate APP
metabolism in a similar way needs to be further explored.

Our results provide strong evidence showing that NRBF2 levels in the hippocampus of 5XFAD AD mice are reduced, it is thus interesting to examine whether NRBF2 levels in brains of AD patient are also reduced in the future. We found that autophagy is important for NRBF2-mediated APP-CTFα, and APP-CTFβ degradation, and the effects of NRBF2 on APP processing and trafficking are required to be further elucidated in order to fully understand the links between NRBF2 and AD. In addition, the mechanisms by which NRBF2 specifically affects APP-CTFα, and APP-CTFβ levels rather than FL-APP levels are needed to determine in the near future. Though we found NRBF2 interacts with APP and Nrbf2 KO attenuates the delivery of APP/APP-CTFs into autophagosome/lysosomes, other methods are required to further confirm the roles of NRBF2 in regulating APP/APP-CTFs redistribution and whether NRBF2 specifically affects the redistribution of APP/APP-CTFs but not other endocytosis proteins. In addition, how NRBF2 mediate the redistribution of APP/APP-CTFs into different organelles are largely unclear. Importantly, the connection between NRBF2 and AD are required to be confirmed by using Nrbf2 KO AD animal models.

In summary, we found that NRBF2 protein levels are reduced in hippocampus of 5XFAD AD mice. We showed that Nrbf2 overexpression reduces amyloidogenic processing of APP and that silencing of Nrbf2 increases amyloidogenic processing of APP in AD cell models. In addition, we demonstrated that NRBF2 is a positive regulator of autophagy in neuronal cells and NRBF2-mediated autophagy is critical for regulating APP-CTFs homeostasis (Fig. 8). Remarkably, we found that NRBF2 interacts with APP and Nrbf2 KO caused the accumulation of the APP/APP-CTFs into RAB5-positive early endosome and attenuated the recruitment of APP/APP-CTFs into both autophagosomes and ILVs for further degradation by lysosomes. Our work establishes an important link between NRBF2 and APP metabolism.

Materials and Methods:

Reagents and antibodies

APP C-terminal antibody (51-2700) was obtained from Thermo Fisher Scientific. Anti-LC3B (NB100-2220) antibody was purchased from Novus Biologicals. Anti-β-actin/ACTB (sc-47778) was purchased from Santa Cruz Biotechnology. Anti-NRBF2 antibody from Cell Signaling (8633) and Bethyl Laboratories (A301-851A). Mouse anti-rabbit IgG (Conformation Specific) (L27A9) mAb (#3678) were obtained from Cell Signaling. Anti-SQSTM1/p62 (P0067), anti-flag (F3165)
antibodies were purchased from Sigma-Aldrich. Anti-ATG5 (9980), Anti-PSEN1 antibodies was obtained from Cell Signaling Technology. Anti-sAPPα (2B3 clone, 11088), and Anti-sAPPβ (6A1 clone, 10321) antibody was purchased from Immuno-Biological Laboratories. Anti-Aβ1–16 monoclonal antibody (6E10, 803001) was purchased from BioLegend. Biotinylated monoclonal βA4(40)-5C3 (0060-100BIOTIN/βA4(40)-5C3, specific to a peptide corresponding to Aβ1-40) antibody was purchased from Nanotools. Flag-NRBF2 and GFP-RAB5-Q79L plasmids were used as previously described. Chloroquine (C6628) was purchased from Sigma-Aldrich. Mouse NRBF2 siRNA and non-target siRNA were purchased from Dharmacon. DMEM (11965-126), LysoTracker Red DND-99 (L7528), Dynabeads® Protein G for immunoprecipitation (10003D), FBS (10270-106), Opti-MEM I (31985-070), G418 (10131-035), Alexa Fluor®488 goat anti-mouse IgG (A-11001) and Alexa Fluor®594 goat anti-rabbit IgG (A-11012) were purchased from Thermo Fisher Scientific.

Animal experiments

All animal experiments were approved by the Committee on the Use of Human & Animal Subjects in Teaching and Research (HASC) of Hong Kong Baptist University. 5XFAD mice overexpress the K670N/M671L (Swedish), I716V (Florida), and V717I (London) mutations in human APP (695), as well as M146L and L286V mutations in human PS1. 5XFAD-AD mice, and C57BL6J were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a pathogen-free facility under 12 hour light, 12 hour dark cycles with food and water provided.

Cell culture

N2S cells are mouse neuroblastoma N2a cells stably transfected with human Swedish mutant were a gift from Dr. Gopal Thinakaran (University of Chicago, Chicago, IL, USA). N2S and Nrbf2 KO N2S cells were maintained in 1:1 Dulbecco’s modified Eagle’s medium (DMEM)/Opti-MEM supplemented with 5% fetal bovine serum, penicillin and streptomycin and 200 μg/mL G418. Chinese hamster ovary (CHO) cells stably transfected with human APP751 bearing the V717F mutation (7PA2 cells) were a gift from Prof. Dennis J. Selkoe (Harvard Medical School, Boston, MA). The cells were maintained in DMEM with 10% FBS, penicillin and streptomycin and 200 μg/mL G418. Cells were incubated at 37°C in a humid 5% CO₂/95% air environment.

Establishment of Nrbf2 KO N2S cells

Nrbf2 KO N2S cells were established by lentivirus-mediated CRISPR/Cas9 system transduction in N2S cells. Briefly, the Nrbf2 sgRNA/Cas9 plasmid, psPAX2 (Addgene
#12260) and pCMV-VSV-G (Addgene #8454) were co-transfected into HEK293T cell for 48h to generate virus particles. Virus-containing medium was collected and filtered through a 0.45 μm filter. N2S cells in 24-well plates at 70-80% confluence, were infected with virus-containing medium for another 24h, followed by puromycin treatment for 3 days. Surviving cells were reseeded to a 96-well plate for isolation of single cell clones. Nrbf2 knockout in the expanded colonies was confirmed by Western blotting.

**Cell transfection**

N2S cells were transfected with mouse NRBF2 siRNA and the non-target siRNA by using Lipofectamine RNAiMAX (13778030, Invitrogen). The transfection of Flag-NRB2, GFP-NRB2, GFP-APP, GFP-LC3, GFP-RAB5, RFP-RAB7, RFP-LAMP1, and GFP-RAB5-Q79L was done with Lipofectamine® LTX with Plus™ Reagent (15338030) or Lipofectamine® 3000 Transfection Reagent (L300008) from Invitrogen. After 24-72 h post-transfection, cells were used for analysis.

**Determination of Aβ1-40 and Aβ1-42 contents by ELISA**

The levels of both secreted and intracellular Aβ contents were determined by using a sandwich ELISA as previously described. Briefly, cell culture medium was collected and cleared by centrifugation for using detect extracellular Aβ1-40 levels. For intracellular Aβ determination, after cell surface Aβ was removed by treating harvested cells with a solution containing 0.25% trypsin and 0.02% EDTA, the cells were then lysed in RIPA buffer containing proteinase inhibitor. For ELISA processing, the monoclonal 6E-10 used as the capture antibody was added to ELISA plates [0.2 μg per well diluted in 0.1M Na2CO3 (pH 9.6)] and incubated overnight at 4°C. The plates were then blocked with Block ACE (Bio-Rad, BUF029,) for 2 h at room temperature. Media samples and equilibrated protein extracts from each treatment (a 100 μl volume was adjusted in all treatment groups) were applied in duplicate and incubated at room temperature for 2 h. Biotinylated monoclonal anti-Aβ1-40 5C3 (50 ng per well), which recognizes the sequence 3–6 of Aβ, were used for detection of Aβ40. Followed by administered with streptavidin-conjugated horseradish peroxidase (DAKO, P0397) for 2 h at room temperature, the plates were adding the substrate tetramethylbenzidine. Finally, absorbance values at 450 nm were measured in duplicate wells after addition of 2M H2SO4. Aβ1-42 contents were determined by using a commercially available kit (Thermo Fisher Scientific, KHB3441) according to the manufacturer's instructions. All ELISA experimental data were from three different independent experiments.

**Western blotting and immunoprecipitation analysis**
For cell experiments, proteins were extracted from cells by using ice-cold RIPA buffer (Cell Signaling Technology, 9803) with complete protease inhibitor mixture (04693124001, Roche Applied Science). For animal experiments, animal tissues were homogenized in 9 volumes of ice-cold TBS containing 0.5% SDS with complete protease inhibitor mixture and 0.5% SDS. For IP, cells and animal brains were lysed in cell lysis buffer (25 mM Tris, pH 7.6; 100 mM NaCl; 0.5 % NP40; 1 mM EDTA; 10 % glycerol with protease inhibitors). After immunoprecipitation with indicated antibodies, Dynabeads® Protein G (Life Technologies, 10003D) was used for immunoprecipitation. Proteins were resolved by gel electrophoresis on 10-15% SDS-polyacrylamide gels, and subsequently transferred onto PVDF membranes. Following blocking with TBS-T (Tris-buffered saline with 0.1% Tween-20) buffer containing 5% non-fat milk powder, the blots were then probed with specific primary antibodies and secondary antibody, and finally visualized by Pierce ECL kit (32106, Pierce) detection reagent. Image J software (NIH) was used for quantification of the Western blotting data.

Endogenous γ-secretase luciferase assay:

The endogenous γ-secretase activity was evaluated by the cell-based γ-secretase as described previously with some modification. For each well of the 24-well cell culture dish, 200 ng of UAS-responsive reporter gene construct MH100, 50 ng of pRL-TK plasmid (Renilla luciferase; control reporter), 100 ng of C99-Gal4 DNA-binding/VP16 transactivation (GVP) plasmids were transfected into WT or Nrbf2 KO N2S cells by using Lipofectamine® 3000 Transfection Reagent (Invitrogen, L300008), after 48 h, the cells were harvested and luciferase activity was determined with Dual-GLo™ Luciferase assay system (Promega, E2920) according to the manufacturer instructions. DAPT (γ-secretase inhibitor, Sigma, D5942) was added at concentration of 4 µM for eighteen hours as positive control. The signal was measured by manufacturer instructions. The MH-100, C99-GVP, and pRL-TK plasmids were gifts from Dr. Karlström (Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, Sweden).

Detriment of lysosomal numbers using LysoTracker RED staining

The number of lysosomes was estimated using LysoTracker RED following manufacturer's instructions. After WT and Nrbf2 KO cells were treated with CQ (20 µM) or vehicle control for overnight, lysosomes were stained by incubating cells with 50 nM LysoTracker red for 30 minutes. Fluorescence intensities of 10000 cells per sample were measured by flow cytometry (BD Biosciences).

Immunocytochemistry

Cells were seeded on coverslips placed in 24-well plates. For GFP-LC3 puncta
formation assays, cells were transfected with GFP-LC3 plasmid and Flag-NRBF2 plasmid or vehicle plasmid for 48h and then fixed with 3.7% paraformaldehyde, followed by nuclear staining with 4’,6-diamidino-2-phenylindole (DAPI). For LC3, RAB5, RAB7, LAMP1 and RAB5-Q79L, APP/APP-CTFs colocalization assays, cells were transfected with GFP-LC3, GFP-RAB5, RFP-RAB7, RFP-LAMP1 or GFP-RAB5-Q79L plasmids for 48 h and then fixed with 3.7% paraformaldehyde, permeabilized in 0.2% Triton X-100 and blocked with 3% BSA, followed by staining with anti-APP C-terminal (1:500) antibody overnight at 4°C and then Alexa Fluor®594 (red) or Alexa Fluor®488 (green) secondary antibodies (1:500) for 1h at room temperature. After nuclear staining with DAPI, the slices were mounted with FluorSave reagent (345789, Calbiochem). Cells were visualized using a DeltaVision Deconvolution Microscope (GE Healthcare). The colocalization of APP/APP-CTFs with different organelles markers were present as the percentage of APP/APP-CTFs dots which co-stained with differential organelles markers from 20-30 of selected cells from three independent experiments expressing GFP-LC3, GFP-RAB5, RFP-RAB7, or RFP-LAMP1 in each group (manually count for the number of APP/APP-CTFs dots and co-staining dots).

The method for determination of sorting of APP/APP-CTFs into ILVs was used as described previously. Briefly, individual large endosomes were imaged from distinct cells (total 50-70 endosomes scored per experiment per condition), whereas more than half of APP/APP-CTFs colocalization with “ring-like” RAB5CA was defined as “peripheral”. Data were presented as percentage of endosomes where APP/APP-CTFs is localized to lumen and the periphery.

**Statistical analysis**

Each experiment was performed at least 3 times, and the results were presented as mean ± SEM. One-way analysis of variance (ANOVA) was followed by the Student-Newman-Keuls test using the SigmaPlot 11.0 software package. A probability value of P< 0.05 was considered to be statistically significant.

**Abbreviations**

NRBF2, nuclear receptor binding factor 2; AD, Alzheimer disease; 5XFAD: 5 familial AD mutations; APP, amyloid precursor protein; Aβ, amyloid beta peptide; Aβ40, amyloid beta peptide 1-40; PIK3C3, class III phosphatidylinositol-3-kinase; APP-CTFs, APP C-terminal fragments; WT, wild type; KD, knock down; KO, knock out; ATG5, autophagy related 5; LC3B, microtubule-associated protein 1 light chain 3B; CHX, cycloheximide; CQ, chloroquine; SQSTM1/p62, sequestosome 1; LAMP-1: Lysosomal-associated membrane protein 1; ILVs, endosomal intraluminal vesicles; DAPI, 4’,6-diamidino-2-phenylindole.
Competing financial interests

The authors declare that they have no competing financial interests.

Acknowledgments:

This study was supported by the grants of NSFC-31500831, EF001/ICMS-LJH/2015/HKBU, SKL-QRCM-2014-2016, FDCT-022/2015/A1, FDCT-092-2015-A3 and MYRG2016-00119-ICMS-QRCM (to Jiahong Lu) and the grants of RGC/HKBU-121009/14, HMRF12132091, HMRF14150811, RC-IRMS/15-16/04, FRG II/15-16/034 and FRG II/16-17/018 (to Min Li). The authors would like to thank Dr. King-Ho Cheung and Dr. Benjamin Tong for their kind technique support and Dr. Martha Dahlen for her English editing of this manuscript.

References:

203:299-313.
45. Lawe DC, Chawla A, Merithew E, Dumas J, Carrington W, Fogarty K, et al. Sequential roles for phosphatidylinositol 3-phosphate and Rab5 in tethering and fusion of early endosomes via their


Fig. S1. Generation of *Nrbf2* KO stable N2S cell lines. CRISPR-Cas9 technology was used to generate *Nrbf2* KO stable cells as described in “Materials and Methods”, the expression of NRBF2 in selected clones was examined by Western blotting. Clone 2 was chosen for invest and the roles of NRBF2 in APP processing.

![Western blot image](image)

Fig. S2. *Nrbf2* KO does not affect γ-secretase activity. After transfection of cells with UAS-responsive reporter gene construct MH100, pRL-TK plasmid (Renilla luciferase; control reporter) and C99-Gal4 DNA-binding/VP16 transactivation (GVP) plasmids for 48 h, whereas DAPT (γ-secretase inhibitor) was used as a control (4 μM). The luciferase activity was determined with Dual-GLo™ Luciferase assay system. Relative γ-secretase activity was determined and quantified from three independent experiments.
Fig. S3. *Nrbf2* KO does not affect SQSTM1 mRNA levels. The mRNA levels of SQSTM1 were examined in WT and KO cells by Q-PCR.

Fig. S4. Lysosomal inhibitor chloroquine (CQ) attenuates NRBF2 over-expression-mediated the degradation of APP-CTFs in 7PA2 cells. Effect of lysosomal inhibitor chloroquine (CQ) on *Nrbf2* over-expression-mediated the increase of APP-CTFs in 7PA2 cells were examined by Western blotting, whereas ACTB was used as an internal control.
**Fig. S5. ATG5 is important for regulating FL-APP levels.** Effects of Atg5 knockdown on NRBF2 overexpression-mediated the changes in the expression of FL-APP were examined, whereas ACTB was used as an internal control.