1 **Dilysine retrieval signal-containing p24 proteins collaborate in inhibiting γ-cleavage of amyloid**

2 **precursor protein**

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11 *Abbreviations used:* PS, presenilin; NCT, nicastrin; APH-1, anterior pharynx defective; PEN-2,

12 presenilin enhancer-2; APP, β-amyloid precursor protein; Aβ, amyloid β-peptide; AICD, APP

13 intracellular domain; NICD, Notch intracellular domain; COP, coat protein complex; CTF, C-terminal

14 fragment; TM, transmembrane; RNAi, RNA interference; siRNA, small interference RNA; BN, Blue

15 Native; PAGE, polyacrylamide gel electrophoresis; MEF, mouse embryonic fibroblast; CHAPSO,

16 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; DAPT,

17 *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester

18

Abstract

γ-Secretase mediates intramembranous γ-cleavage and ε-cleavage of β-amyloid precursor protein (APP) to liberate β-amyloid peptide (Aβ) and APP intracellular domain (AICD) respectively from the membrane. Although the regulatory mechanism of γ-secretase cleavage remains unresolved, a member of the p24 cargo protein family, named $p24\delta_1$ or TMP21, has been identified as an activity-modulating component. The p24 family proteins are divided into four subfamilies (p24α, β, δ and γ). In contrast to $p24\delta_1$, $p24\beta_1$ has reportedly no effect on γ-cleavage. Here, we determined whether $p24\alpha_2$, $p24\gamma_3$ or $p24\gamma_4$ modulates APP processing. Knockdown of cellular $p24\alpha_2$ induced a significant increase in Aβ generation but not in AICD production in cell-based and cell-free assays, whereas $p24\alpha_2$ overexpression suppressed Aβ secretion. By contrast, Aβ secretion was not altered by $p24\gamma_3$ or $p24\gamma_4$ knockdown. Endogenous $p24\alpha_2$ co-immunoprecipitated with core components of the γ-secretase complex, and the anti-$p24\alpha_2$ immunoprecipitate exhibited γ-secretase activity. Mutational disruption of the conserved dilysine ER-retrieval motifs of $p24\alpha_2$ and $p24\delta_1$ perturbed inhibition of γ-cleavage. Simultaneous knockdown, or co-overexpression, of these proteins had no additive or synergistic effect on Aβ generation. Our findings suggest that dilysine ER-retrieval signal-containing p24 proteins, $p24\alpha_2$ and $p24\delta_1$, bind with γ-secretase complexes and collaborate in attenuating γ-cleavage of APP.

1 Keywords: Alzheimer disease, γ-secretase, amyloid-β, p24 family protein, presenilin

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3 Running title: p24α_2 inhibits γ-cleavage of APP

4

Introduction

Excessive accumulation of extracelluler β-amyloid peptide (Aβ) in brain is considered the cause of Alzheimer's disease. β-Amyloid precursor protein (APP), a type I transmembrane (TM) protein, is glycosylated in the Golgi-apparatus, and is then transported to the cell surface where its ectodomain is cleaved by α- and β-secretases and is released as secreted APP (sAPP) α and β, respectively. The intramembrane domains of the resulting C-terminal fragments (CTFs), C83 and C99, are sequentially processed by γ-secretase, which produces the APP intracellular domain (AICD) by ε-cleavage, and extracelluler p3 and Aβ by γ-cleavage. The γ-cleavage at multiple sites generates several Aβ species, including two predominant forms: Aβ40 and Aβ42. Aβ42 has been shown to be more prone to aggregate and is pathogenic.

Reconstitution studies in yeast and insect cells have revealed that the active γ-secretase complex is essentially composed of four membrane proteins; presenilin (PS), nicastrin (NCT), anterior pharynx defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) (Edbauer *et al.* 2003, Takasugi *et al.* 2003). A recent report has indicated that the enzymatically active complexes contain one molecule of each core component (Sato *et al.* 2007). However, detergent-solubilized γ-secretase is estimated to be more than 400 kDa, which is a much larger molecular weight than the sum of its four core components (~230 kDa) (Gu *et al.* 2004, Li *et al.* 2000, Evin *et al.* 2005). Additionally, γ-secretase has more than 50 substrates,

1 including APP and Notch receptors (Parks & Curtis 2007). Therefore, it is plausible that yet unidentified

2 component(s) might regulate its enzymatic activity and substrate specificity.

3 Recently, p24δ_1 or TMP21, a member of the p24 protein family, has identified as an

4 activity-modulating component of the γ-secretase complex, which attenuates γ-cleavage but not

5 ε-cleavage of APP (Chen *et al.* 2006). Members of the p24 family of ~24 kDa type I TM proteins are

6 highly conserved in various species from *C. elegans* to humans, and can be divided into four subfamilies

7 (p24α, β, δ and γ), which are classified into two phylogenetically distinct groups; α−δ and β−γ (Carney &

8 Bowen 2004, Dominguez *et al.* 1998, Strating *et al.* 2009b). Representative p24 proteins in vertebrates

9 include p24α_2 (p25), p24β_1 (p24a), p24δ_1 (p23 or TMP21), p24γ_3 (p27) and p24γ_4 (p26), based on the

10 systematic nomenclature (Dominguez et al. 1998). The p24 family members mainly reside at coat protein

11 complex (COP) I- and II-coated vesicles and play an important but ill-understood role in vesicular

12 transport processes at the ER and Golgi interface (Bethune *et al.* 2006b, Carney & Bowen 2004). The p24

13 proteins share a similar domain architecture that includes a potential cargo-binding domain at the lumenal

14 side and a COP subunit-binding motif at the cytoplasmic side (Stamnes *et al.* 1995). Although the precise

15 functional difference between subfamilies remains unknown, the functional roles in the early secretory

16 pathway are non-redundant among the four subfamilies (Strating *et al.* 2009a, Strating *et al.* 2007).

17 A previous report has shown that in marked contrast to p24δ_1, p24β_1 has no effect on γ-secretase

1 activity (Chen et al. 2006). Regarding other p24 family members, the effect on γ-secretase activity has not
2 been examined. An exploration of whether p24α_2, p24γ_3 and p24γ_4 affect γ-secretase cleavage might
3 provide a clue to the underlying mechanism of γ-secretase inhibition by p24. We thus examined the
4 interaction of these p24 family proteins with the γ-secretase complex and their inhibitory activity for
5 γ-cleavage of APP. We found that p24α_2 but not p24γ_3 and p24γ_4 inhibited γ-cleavage in a way similar to
6 p24δ_1. Furthermore, our results suggested that the γ-cleavage inhibition by p24α_2 and p24δ_1 requires their
7 dilysine ER-retrieval motifs and their collaborative interaction with γ-secretase complexes.
8
9 **Materials and methods**
10 **Construction of expression plasmids**
11 Full-length (FL) cDNAs encoding human wild-type p24δ_1, p24β_1 and p24α_2 were obtained by
12 PCR from human brain cDNA library (Clonetech, San Diego, CA, USA). Each cDNA was ligated into an
13 expression vector pcDNA6 (Invitrogen, Carlsbad, CA, USA). The dilysine motif mutants p24α_2SS and
14 p24δ_1SS were generated by PCR-based site-directed mutagenesis. The sequences of all constructs were
15 confirmed by sequencing.
16 **Antibodies and reagents**
17 Anti-human p24α_2 polyclonal antibody (p24α_2-N) was raised in rabbits against a synthetic

1 polypeptide that was composed of the extracellular sequence between amino acid residues 62 and 80 with
2 an added N-terminal Cys residue (C+GNYRTQLYDKQREEYQPAT). The antibody was purified by
3 immunoaffinity chromatography with immobilized antigen. Rabbit polyclonal anti-p24α_2 (#2469R1),
4 anti-p24δ_1 (HAC344), anti-p24β_1 (Frieda), anti-p24γ_3 and anti-p24γ_4 antibodies were provided by Dr.
5 Wieland (Gommel *et al.* 1999, Jenne *et al.* 2002). Other antibodies were purchased as follows: anti-PS1
6 N-terminus, anti-sAPPα and anti-sAPPβ from IBL (Gunma, Japan); anti-NCT, anti-APP-CTF and
7 anti-Flag from Sigma (St Louis, MO, USA); anti-APH-1L from Covance (Princeton, NJ, USA);
8 anti-PEN-2 from Calbiochem (San Diego, CA, USA); anti-APP from Chemicon (Temecula, CA, USA);
9 anti-Myc (9E10) from Santa Cruz Biotech. (Santa Cruz, CA, USA); anti-Sec61α from Upstate Biotech
10 (Lake Placid, NY, USA); anti-calnexin and anti-GM130 from Transduction Lab. (Lexington, KY, YSA).
11 A γ-secretase inhibitor, *N*-[*N*- (3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT),
12 was obtained from Calbiochem. Cycloheximide was from Sigma.

13 **RNA interference (RNAi)**

14 For RNAi, the following small interference RNA (siRNA) duplexes were purchased from
15 Dharmacon (Lafayette, CO, USA): siGENOME SMART pool M-007924 for p24α_2; M-003718 for
16 p24δ_1; M-008074 for p24β_1; M-007855 for p24γ_3; M-008051 for p24γ_4; D001210 for a non-targeting
17 control. The M-007924 is a mixture of the following four duplexes; T1:

1 5’-GAGAAGAAGUGCUUUAUUGUU-3’ as sense and 5’-CAAUAAAGCACUUCUUCUCUU-3’ as
2 anti-sense; T2: 5’-GGACGCAGCUGUAUGACAAUU-3’ as sense and
3 5’-UUGUCAUACAGCUGCGUCCUU-3’ as anti-sense; T3:
4 5’-GAAGCGCGCUCUACUUUCAUU-3’ as sense and 5’-UGAAAGUAGAGCGCGCUUCUU-3’ as
5 anti-sense; T4: 5’-GAACAUGCCAAUGACUAUGUU-3’ as sense and
6 5’-CAUAGUCAUUGGCAUGUUCUU-3’ as an anti-sense sequence. HEK293 or SH-SY5Y cells were
7 transfected with siRNA duplexes using Lipofectamine RNAi MAX (Invitrogen) in accordance with the
8 manufacturer’s instruction.

9 **Co-immunoprecipitation**

10 Membrane fractions isolated from HEK293 cells were lysed in a lysis buffer containing 1%
11 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPSO). After
12 pre-clearing with protein G-Sepharose 4 fast flow (GE Healthcare, Tokyo, Japan) for 1 h, cell lysates were
13 incubated with the appropriate antibody. The immunoprecipitates were recovered by overnight incubation
14 with protein G-Sepharose (Wang *et al.* 2005). The sepharose beads bound to the immune complexes were
15 washed four times with lysis buffer. The immunoprecipitated proteins were analyzed using NuPAGE
16 4-12% Bis-Tris gels (Invitrogen).

17 **Two-dimensional Blue Native (BN)/ SDS polyacrylamide gel electrophoresis (PAGE)**

Two-dimensional BN/SDS-PAGE was performed as previously described (Gu et al. 2004). Briefly, membrane fractions lysed with 1% CHAPSO were separated on a 5-13% BN gel, followed by a second dimension on a NuPAGE 4–12% Bis-Tris 2-D gel (Invitrogen) for SDS-PAGE. Molecular weight markers used for BN/SDS-PAGE were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and BSA (66 kDa) (Sigma).

Assays for γ-secretase and β-secretase activities

Cellular γ-secretase activity was analyzed as described previously (Hasegawa *et al.* 2004). Secreted Aβ40 and Aβ42 levels in 24-h conditioned media from cultured cells were measured using specific ELISAs in accordance with the manufacture's instructions (WAKO Pure Chemical Industries, Osaka, Japan). For a cell-free γ-secretase assay, microsome membranes of HEK293 cells treated with control or p24α_2-specific siRNA were prepared as described previously (Mitsuishi *et al.* 2010). Briefly, HEK293 cells were homogenized in HEPES buffer (25 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany); pH 7.0), and the postnuclear supernatants were centrifuged at 100,000 *g* for 1 h. The membrane pellets were washed with HEPES buffer and subsequently lysed in 1% CHAPSO/HEPES buffer. Solubilized γ-secretase was recovered by centrifugation at 100,000 *g* for 30 min, and the concentrations of protein and CHAPSO were adjusted to 0.25 mg/mL and 0.25%, respectively. The resulting CHAPSO-solubilized γ-secretase

1 was incubated with a recombinant APP-C99-Flag substrate for 6 h at 37 ˚C. The reaction was stopped by

2 boiling the mixture for 5 min, and the Aβ40 and Aβ42 levels were measured by ELISA. The reaction

3 mixtures were also subjected to immunoblotting using 15% Tricine gels to detect the AICD levels by an

4 anti-Flag antibody. β-Secretase activity was measured using a commercial kit (R&D Systems,

5 Minneapolis, MN, USA) according to the manufacture's protocol.

6 **Metabolic protein labeling and pulse-chase assays**

7 Pulse-chase assays were conducted as described previously (Nakaya *et al.* 2005). After transient

8 transfection of control or p24α_2-specific siRNA duplexes, HEK293 cells stably transfected with

9 Myc-tagged NotchΔE (Jarriault *et al.* 1995) were metabolically labeled with Trans ^{35}S-label metabolic

10 labeling reagent (MP Biomedicals, Solon, OH, USA) for 20 min, and chased for up to 1 h. Cell lysates

11 were subjected to immunoprecipitaiton with an anti-Myc antibody and then separated by SDS-PAGE.

12 **Cell surface biotinylation**

13 HEK293 cells were washed three times with ice-cold PBS (pH 8.0; 1 mM $MgCl_2$) and incubate

14 with 1 mg/mL EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) for 30 min at 4ºC. The

15 reaction was stopped by washing the cells twice and then incubating for 15 min on ice with 20 mM

16 glycine in PBS. The cells were collected and incubated in a lysis buffer containing 1% CHAPSO for 1 h.

17 The lysates were affinity-purified with UltraLink Immobilized NeutrAvidin Plus (Pierce) overnight at 4°C.

Affinity-purified proteins were eluted into an SDS-PAGE sample buffer and were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen).

Results

p24α_2 negatively modulates γ-cleavage of APP

To assess whether p24α_2, p24γ_3 or p24γ_4 modulates γ-secretase activity, the endogenous expression levels of these proteins in HEK293 cells were reduced by transfection with a pool of four different siRNA duplexes specific for each protein. RNAi specific for p24β_1, p24α_2 or p24δ_1 simultaneously reduced the level of one or more of the other p24 family proteins to various degrees as well as the target protein (Fig. 1a). This result is consistent with previous reports showing that the p24 family proteins form heteromeric oligomers and that ablation of a single member destabilizes one or more interacting family members (Fullekrug *et al.* 1999, Dominguez et al. 1998, Marzioch *et al.* 1999). Knockdown of p24α_2 caused a significant increase in the levels of both Aβ40 and Aβ42, compared to transfection with control siRNA, whereas knockdown of p24γ_3 or p24γ_4 had little or no effect on Aβ secretion (Fig. 1b). There was no significant difference in the degree of inhibition of Aβ40 and Aβ42. p24α_2 knockdown also increased Aβ secretion of HEK293 cells stably expressing familial Alzheimer's disease mutant PS1 or APP (L392V-PS1, R278I-PS1 or Swedish mutant K594M/N595L-APP) (data not shown). We then confirmed

1 the specific effect of p24α_2 knockdown by assays of the effect of individual siRNA duplexes on Aβ
2 secretion (Fig. 1c). All of the siRNA duplexes suppressed endogenous p24α_2 expression although with
3 different efficacy. The degree of Aβ increase was inversely correlated with the residual level of p24α_2,
4 supporting a specific role for p24α_2 in the regulation of Aβ levels. A similar result of p24α_2 knockdown
5 was obtained using SH-SY5Y neuroblastoma cells (data not shown), indicating that inhibition of Aβ
6 secretion by p24α_2 is not cell type-specific. Conversely, p24α_2 overexpression in HEK293 cells decreased
7 the secretion of Aβ40 and Aβ42 (Fig. 1d).

8 To determine whether p24α_2 directly modulates γ-secretase activity, we employed a cell-free assay
9 for Aβ generation. CHAPSO-solubilized γ-secretase was prepared from microsome membranes of
10 HEK293 cells transfected with control or p24α_2-specific T3 siRNA duplexes, and then incubated with a
11 recombinant APP-C99-Flag substrate. Ablation of p24α_2 resulted in a significant enhancement of Aβ40
12 generation consistent with the results of the cell-based assays (Fig. 2a). These data suggested that p24α_2
13 suppresses Aβ generation by direct modulation of γ-secretase activity rather than by an indirect
14 mechanism such as alteration of substrate trafficking.

15 γ-Secretase catalyzes not only γ-cleavage but also ε-cleavage of APP, which liberates AICD
16 fragments from the membrane. We thus assessed the effect of p24α_2 on AICD generation by a cell-free
17 assay (Fig. 2b). Intriguingly, the microsome fraction of control and p24α_2-knockdown HEK293 cells

1 generated a comparable amount of AICD fragments, suggesting that p24α_2 does not inhibit ε-cleavage of
2 APP. We next determined whether p24α_2 is also unable to inhibit γ-secretase S3-cleavage of Notch,
3 which corresponds to ε-cleavage of APP and produces Notch intracellular domain (NICD). A proteolytic
4 production of NICD was analyzed by a pulse-chase experiment (Fig. 2c). HEK293 cells stably transfected
5 with NotchΔE were metabolically labeled and chased for up to 1 h. There was no significant difference in
6 the levels of NICD fragments generated between control and p24α_2-knockdown cells. Hence, p24α_2 did
7 not inhibit ε-cleavage of APP or S3-cleavage of Notch.

8 **p24α_2 is associated with active γ-secretase complexes**

9 We next analyzed the endogenous binding of p24α_2 to γ-secretase complexes in native HEK293
10 cells by co-immunoprecipitation assays. The anti-p24α_2-CTF antibody (#2469R1) co-precipitated with
11 four core components of the γ-secretase complex (Fig. 3a). In contrast, antibody against p24γ_3 or p24γ_4
12 did not co-precipitate with these components (data not shown). To further confirm that p24α_2 is
13 physiologically associated with an active pool of γ-secretase complexes, the anti-p24α_2
14 immunoprecipitate of HEK293 microsome fraction was subjected to an *in vitro* γ-secretase assay (Fig. 3b).
15 Immunoprecipitated p24α_2 complexes were solubilized in CHAPSO buffer, and then incubated with a
16 recombinant APP-C99-Flag substrate for 6 h at 37 ˚C in the presence or absence of DAPT, a potent
17 γ-secretase inhibitor. The anti-p24α_2 immunoprecipitate generated a significant amount of Aβ40

compared with the control precipitate. Aβ generation by the anti-p24α_2 precipitate was less than 10% of that by the anti-NCT precipitate but was inhibited by DAPT. These data suggested that p24α_2 is physiologically associated with the active γ-secretase complex, where it functions as an inhibitory regulator of γ-secretase activity.

Loss of any essential component of the γ-secretase complex destabilizes the other component proteins (Wolfe 2006). To determine whether p24α_2 modulates γ-secretase complex stability, we examined the effect of p24α_2 ablation or overexpression on expression of the components of the γ-secretase complex. Knockdown or overexpression of p24α_2 did not lead to any significant change in the levels of the core component proteins (Figure S1), suggesting that p24α_2 is not a structural component of the γ-secretase complex. In the converse experiment, we examined the expression of p24 proteins in PS1/PS2-double knockout mouse embryonic fibroblasts (MEFs), in which no γ-secretase complex is detected (Herreman *et al.* 2000) (Fig. 3c). Intriguingly, a mild reduction in the levels of p24α_2 and p24δ_1 but not in p24β_1 was detected in the PS1/PS2-double knockout MEFs compared with wild-type MEFs. This result suggested that endogenous expression of p24α_2 and p24δ_1 is partially dependent on the presence of the γ-secretase complex.

p24α_2 is an inhibitory binding protein of the γ-secretase complex

To assess the relative molecular weight of cellular protein complexes containing p24α_2, we

conducted two-dimensional BN/SDS-PAGE using microsome fractions of native HEK293 cells (Fig. 3d). A mature, active form of γ-secretase complexes containing highly glycosylated NCT, proteolyzed PS1 fragments, APH-1 and PEN-2 migrated at a high molecular weight (> 400 kDa) as previously reported (Gu et al. 2004). The major pool of p24α_2 forms 100-400 kDa-complexes that are probably p24 protein oligomers. However, a small but discernible amount of p24α_2 also migrated at the high molecular weight range of over 400 kDa, where p24α_2 overlapped with γ-secretase components.

We presumed that p24α_2 directly interacts with the γ-secretase complex to modulate its activity. However, there are a few other possible mechanisms by which γ-secretase activity could be modulated. One of these possibilities was that p24α_2 might perturb the assembly or the maturation of the γ-secretase complex. As mentioned above, knockdown or overexpression of p24α_2 did not lead to any significant change in the accumulated amounts of core components of the γ-secretase complex (Figure S1). Furthermore, as indicated in Fig. 3(d), p24α_2-specific siRNA resulted in the disappearance of the p24α_2 co-distribution with the high molecular weight (> 400 kDa) γ-secretase complex in two-dimensional BN/SDS-PAGE. However, no resulting change in the density or distribution of the bands corresponding to the γ-secretase components was detected. These findings suggested that p24α_2 did not perturb the assembly or maturation of the γ-secretase complex.

The second possibility was that p24α_2 might alter subcellular localization of the γ-secretase

1 complex, thereby reducing the efficacy of its cleavage. The accumulated evidence has shown that Aβ
2 generation occurs mainly at the *trans*-Golgi network, the plasma membrane and the late endosome
3 (Sannerud & Annaert 2009). On the other hand, p24α_2 resides predominantly at the ER and the *cis*-Golgi
4 network (Dominguez et al. 1998). We determined whether p24α_2 alters the subcellular localization of
5 core components of the γ-secretase complex. Cell surface biotinylation assays showed that a small pool of
6 p24α_2 was transported to the cell surface, and that p24α_2 knockdown did not alter the level of cell surface
7 NCT (Fig. 4). In addition, microsomes from HEK293 cells with control or p24α_2-specific siRNA
8 treatment were fractionated on a discontinuous iodixanol gradient (Figure S2). The p24α_2 protein
9 predominantly localized in the Golgi apparatus, but the level of p24α_2 was remarkably reduced by
10 p24α_2-RNAi. The distribution patterns of endogenous PS1, NCT, APH-1 and PEN-2 in
11 p24α_2-knockdown HEK293 cells were not significantly different from those in native cells. These
12 findings indicated that p24α_2 did not affect subcellular localization of the γ-secretase complex.
13 The third possibility was that p24α_2 may act as a competitive substrate for γ-cleavage of APP. To
14 address this possibility, we assayed potential proteolytic degradation of p24α_2 by immunoblotting of
15 mock- or p24α_2-transfected HEK293 cells (Figure S3, lanes 1-4). No proteolyzed fragment of p24α_2 was
16 detected even after intensive development. Furthermore, using an antibody specific to the C-terminus of
17 p24α_2, no C-terminal stub emerged after treatment with DAPT (Figure S3, lanes 5-8). These findings

1 suggested that p24α_2 is not a substrate for γ-secretase.

2 **p24α_2 knockdown increases APP-CTFs and sAPP but does not activate β-secretase**

3 As p24α_2 might be involved in the maturation and stability of APP as previously reported for p24δ_1

4 (Vetrivel *et al.* 2007), we investigated the amount of APP-FL or proteolyzed derivatives of APP in

5 p24α_2-knockdown and p24α_2-overexpressed HEK293 cells. A reduction in p24α_2 caused a significant

6 increase in both APP-C99 and APP-C83, whereas overexpression of p24α_2 led to an increase in mature

7 and immature APP-FL (Fig. 5a). The effects of p24α_2 knockdown and overexpression were not simply

8 reverse to each other, but our results indicated that p24α_2 had a consistent effect in both conditions,

9 reducing the relative amount of cellular APP-CTFs compared with its immediate precursor APP-FL.

10 Taken together with a finding that p24α_2 overexpression led to a more prominent increase in the

11 immature than the mature form of APP-FL (Fig. 5a), p24α_2 retarded the maturation of APP.

12 We also analyzed the effect of p24 family proteins on the levels of the secreted ectodomains,

13 sAPPα and sAPPβ, which are the counterparts of APP-C83 and APP-C99, respectively (Fig. 5b). The

14 level of sAPPβ from HEK293 cells was increased by treatment with either p24α_2-, p24δ_1- or

15 p24β_1-specific siRNA duplexes. The sAPP increase upon p24δ_1 knockdown is consistent with a previous

16 report (Vetrivel et al. 2007), in which, however, the increase in sAPPα from HeLa cells was detected.

17 Surprisingly, p24β_1 knockdown also increased the level of sAPPβ. This observation is superficially

1 inconsistent with a previous finding that p24β_1 knockdown does not increase Aβ secretion (Chen et al.
2 2006). The increased amounts of sAPPβ varied among HEK cells treated with p24 members-siRNAs,
3 and the increase in sAPPβ did not show a linear correlation with the increase in Aβ secretion (Fig. 5b,
4 compare with Fig. 7b). These findings suggested that the sAPPβ increase by p24 knockdown was not
5 necessarily linked to an increase in Aβ secretion.

6 The increase in sAPPβ and APP-C99 by knockdown of p24α_2 could be caused by a concomitant
7 enhancement of β-secretase activity. We addressed this possibility by measuring β-secretase activity of
8 HEK293 cells treated with control or p24α_2-specific siRNA duplexes. However, as Fig. 5(c) shows,
9 p24α_2 knockdown did not significantly alter cellular β-secretase activity.

10 **The dilysine motifs of p24α_2 and p24δ_1 are required for γ-cleavage inhibition**

11 To further analyze the mechanism by which p24 modulates γ-secretase activity, we determined
12 whether p24α_2 might share a sequence motif with p24δ_1 whose ability to inhibit γ-cleavage has been
13 reported (Chen et al. 2006, Vetrivel et al. 2007). We therefore aligned the sequences of p24α_2 and p24δ_1
14 with the sequences of the other family members p24β_1 and p24γ_4/p24γ_3 which do not inhibit γ-cleavage.
15 This alignment indicated that overall the p24 subfamilies display a low degree of identity, and that the
16 sequence homology between p24α_2 and p24δ_1 is not significantly different from that between p24α_2 and
17 the other subfamily proteins. However, as shown in Fig. 6(a), only p24α_2 has a canonical ER-retrieval

1 KKXX motif in the cytoplasmic domain. p24δ_1 has a similar motif except for the presence of an
2 additional single amino acid following the dilysine motif. In contrast, p24β_1, p24γ_4 or p24γ_3 do not have
3 this motif.

4 To assess the involvement of the dilysine motif in γ-cleavage inhibition, we prepared mutants of
5 p24α_2 and p24δ_1, in which the dilysine motifs were substituted with a pair of serines, referred to as
6 p24α_2SS and p24δ_1SS, respectively (Fig. 6a). Overexpression of p24α_2SS or p24δ_1SS did not decrease
7 Aβ secretion (Fig. 6b), suggesting that disruption of the dilysine motifs abolished p24α_2/δ_1 inhibition of
8 γ-cleavage. Furthermore, co-immunoprecipitation assays indicated that p24α_2SS showed a dramatic
9 reduction in p24α_2 interaction with PS1 (Fig. 6c). Mutation of a highly conserved sequence can
10 sometimes reduce protein stability. To exclude the possibility that the loss of PS1 interaction was caused
11 by destabilization of the mutant p24 proteins, the half-lives of the exogenous wild-type and mutant
12 proteins were assessed after cycloheximide treatment. The half-life of p24α_2SS was comparable to that of
13 wild-type p24α_2 (data not shown). These findings suggested that the dilysine motifs are critical for
14 p24α_2/δ_1 incorporation into the γ-secretase complex and for their inhibition of γ-secretase activity.

15 **p24α_2 modulates γ-cleavage in cooperation with p24δ_1**

16 Our results and previous reports (Chen et al. 2006, Vetrivel et al. 2007) indicate that p24α_2 and
17 p24δ_1, but not p24β or p24γ, modulates γ-secretase. To assess whether p24α_2 and p24δ_1 independently or

1 cooperatively inhibit γ-cleavage, we performed single and double knockdown of $p24\delta_1$, $p24\alpha_2$ and $p24\beta_1$
2 and measured the subsequent levels of secreted Aβ. Treatment with siRNA specific for $p24\beta_1$, $p24\alpha_2$ or
3 $p24\delta_1$ reduced the level of one or more of the other p24 family proteins as mentioned above. Knockdown
4 of $p24\beta_1$ had no effect on Aβ generation albeit it was accompanied by a reduction in $p24\alpha_2$ and $p24\delta_1$,
5 whereas $p24\alpha_2$ or $p24\delta_1$ knockdown resulted in a significant increase in secreted Aβ (Fig. 7a and b). As
6 previously proposed for $p24\delta_1$ (Chen et al. 2006), these superficially discrepant findings can be explained
7 by the possibility that each of $p24\alpha_2$ and $p24\delta_1$ has at least two cellular pools; one that is associated with
8 other p24 family proteins to form cargo-protein complexes, and a second pool that interacts with the
9 γ-secretase complex (PS-bound pool) to modulate its activity. In contrast, $p24\beta_1$ does not exist in a
10 PS-bound pool.

11 If $p24\alpha_2$ and $p24\delta_1$ independently modulate γ-secretase activity, it would be expected that
12 simultaneous knockdown for $p24\alpha_2$ and $p24\delta_1$ would cause an additive increase in Aβ secretion.
13 However, co-transfection with siRNA duplexes against $p24\alpha_2$ and $p24\delta_1$ did not show any additive effect
14 on Aβ generation (Fig. 7a and b). These findings suggested that $p24\alpha_2$ and $p24\delta_1$ cannot compensate for
15 each others effect on γ-secretase and that simultaneous interaction of $p24\alpha_2$ and $p24\delta_1$ with the γ-secretase
16 complex is required for the γ-cleavage inhibition.

17 On the other hand, overexpression of $p24\alpha_2$ or $p24\delta_1$ only slightly decreased Aβ secretion compared

1 with the robust increase in their exogenous expression (Fig. 7c and d). In addition, co-overexpression of

2 p24α_2 and p24δ_1 did not further enhance the inhibition of Aβ production (Fig. 7c and d). These results

3 suggested that γ-secretase complexes associated with one of p24α_2 or p24δ_1 might be able to accept

4 another p24, but that these pools of γ-secretase/p24-complexes were very small. Furthermore, additional

5 unidentified component(s) or factor(s) besides p24α_2 and p24δ_1 might be required for the attenuation of

6 γ-cleavage.

7

8 **Discussion**

9 In this study, we showed that p24α_2 is an inhibitory binding protein for the γ-secretase complex

10 based on the following evidence: 1) p24α_2 knockdown induced an increase in Aβ secretion from cultured

11 cells; 2) p24α_2 knockdown reduced Aβ generation in a cell-free assay; 3) p24α_2 overexpression decreased

12 the secretion of Aβ; 4) p24α_2 co-immunoprecipitated with core components of the γ-secretase complex;

13 5) anti-p24α_2 immunoprcipitates exhibited γ-secretase activity; 6) endogenous expression of p24α_2 was

14 partially dependent on the presence of the γ-secretase complex. In contrast, p24γ_3 and p24γ_4 did not affect

15 γ-secretase activity. p24α_2 inhibited γ-cleavage but not ε-cleavage of APP, or S3-cleavage of the Notch

16 receptor, in a manner similar to p24δ_1 (Chen et al. 2006). This inhibitory activity required the dilysine

17 ER-retrieval motif in the cytoplasmic domain. Simultaneous knockdown or co-overexpression of p24α_2

1 and p24δ_1 showed no additive effect on Aβ generation.

2 Multiple previous studies have identified p24 family members as interacting proteins of purified
3 γ-secretase complexes (Chen et al. 2006, Wakabayashi *et al.* 2009, Winkler *et al.* 2009, Teranishi *et al.*
4 2009). However, active γ-secretase complexes isolated by affinity capture using a biotinylated derivative
5 of the γ-secretase transition-state analogue inhibitor Merk C did not contain p24α_2 or p24δ_1 (Winkler et al.
6 2009). We speculate that activity-dependent purification of the γ-secretase complex may not be able to
7 identify inhibitory binding proteins such as p24α_2 and p24δ_1 for the following reasons. First, the binding
8 domain of p24α_2 or p24δ_1 might be so close to the active site of γ-secretase that these proteins might
9 compete with Merk C for binding with the complexes. Second, p24α_2 and p24δ_1 binding might result in a
10 conformational change of the γ-secretase complex, which could potentially allosterically interrupt binding
11 with the Merk C derivative. Third, p24α_2 or p24δ_1 binding to γ-secretase complexes might be transient. In
12 this case, these proteins would probably not be detectable as discernible bands in complexes isolated
13 using the Merk C affinity. In contrast, the association of p24α_2 with active γ-secretase complexes can be
14 detected using the more sensitive enzymatic assay used in the present study.

15 Our findings and a previous report (Chen et al. 2006) suggest that α- and δ-subfamilies, but not β-
16 and γ-subfamilies, of the p24 family can modulate γ-secretase activity. Although the functional difference
17 between α-δ and β-γ subfamilies has not been clarified, an early report described that p24α_2 and p24δ_1,

but not p24β_1, p24γ_3 and p24γ_4, bound with the COPI coatomer (Dominguez et al. 1998). A recent report has proposed that two different mechanisms mediate p24 binding with coatomers based on the presence of dibasic signatures (Bethune *et al.* 2006a). Thus, there are two classes of dibasic signatures: the KKXX motif of ER-resident proteins and the FFXXBB(X)n motif ($n \geq 2$; B indicates a basic residue) of ER/Golgi-cycling proteins. The α- and δ-subfamilies have both motifs in their cytoplasmic domains, while the β- and γ-subfamilies have only the latter motif. Thus, only dimers of p24β and p24γ bind with γ-COP via the FFXXBB(X)n motifs, whereas dilysine motif-bearing p24α and p24δ can interact as monomers to α- and β'-COP. Our results indicated that the dilysine motifs of p24α_2 and p24δ_1 support γ–secretase modulation.

However, the mechanism by which the dilysine ER-retrieval motifs of p24α_2 and p24δ_1 mediate or support γ-cleavage inhibition remains unresolved. p24 family proteins are localized predominantly at the ER and the *cis*-Golgi network (Dominguez et al. 1998). In contrast, γ-secretase activity is detected mainly in the late secretary compartments and the endosomes but not in the ER. Our results and a previous study (Chen et al. 2006) indicate that p24α_2 and p24δ_1 inhibit γ-cleavage by binding with γ-secretase complex without obviously altering its subcellular localization. Therefore, these proteins must be transported to the organelles where γ-cleavage occurs. Then, how do p24α_2 and p24δ_1 go beyond the Golgi apparatus? In fact, our results indicated that a small pool of p24α_2 was transported to the plasma membrane in native

1 HEK293 cells (Fig. 4). A possible explanation is that the binding of p24α_2 and p24δ_1 with the γ-secretase
2 complexes requires, but masks, their ER-retrieval signals, which then allow the bound p24α_2 and p24δ_1 to
3 be transported through the Golgi apparatus. A recent study has shown that the dilysine motif mutant
4 p24α_2SS that was transported through the Golgi formed well-defined membrane domains detected by
5 electron microscopy (Emery *et al.* 2003). This p24α_2SS-rich domain excluded cholesterol in late
6 endosomes, resulting in accumulation of cholesterol in the neighboring membranes. The γ-secretase
7 complex-bound p24α_2 and p24δ_1 might exclude cholesterol as did the dilysine mutants. It is well known
8 that the decrease in membrane cholesterol affects Aβ generation (Hartmann *et al.* 2007). Hence, it is
9 possible that p24α_2 or p24δ_1 inhibits γ-cleavage by altering the distribution of membrane cholesterol.

10 During preparation of this manuscript, it was reported that the TM domain of p24δ_1 mediates its
11 association with the γ-secretase complexes and inhibition of γ-cleavage (Pardossi-Piquard *et al.* 2009).
12 Our data may not be contradictory to their results. Thus, although these authors showed that a synthetic
13 polypeptide corresponding to the TM domain of p24δ_1 inhibited γ-cleavage, it is possible that the
14 dilysine motifs are required for efficient functioning of the primary inhibitory TM domain. These
15 authors also defined the p24-γ-secretase interacting domain by a co-immunoprecipitation assay using
16 the p24δ_1/p24β_1 chimeras. Their data indicated that the TM domain of p24δ_1 was essential for this
17 interaction. The TMP21(p24δ_1)-TM mutant, in which the TM domain of p24β_1 was replaced with that

of p24δ_1, bound to NCT and PS1 more weakly than the TMP21(p24δ_1)-TMCt mutant in which the TM and cytoplasmic domains of p24β_1 were replaced with those of p24δ_1, suggesting that the cytoplasmic domain of p24δ_1 might play a supportive role in the interaction. This finding is consistent with our data that the dilysine motif mutation of p24α_2 did not lead to a complete loss of its interaction with PS1 (Fig. 6c).

The p24 family proteins are implicated in selective packaging of cargo proteins and biogenesis of transport vesicles. Homozygous depletion of the p24δ_1 gene in mice caused early embryonic lethality (Denzel *et al.* 2000). Although the precise functional difference between p24 subfamilies has not been clarified, even highly related family members have a limited functional redundancy (Strating et al. 2009a, Strating et al. 2007). Elucidation of functional redundancy or compensation between p24α_2 and p24δ_1 in γ-cleavage inhibition might provide a clue to understanding the underlying mechanism. Both p24α_2 and p24δ_1 bind with the γ-secretase complex. The degree of p24α_2 knockdown was correlated with that of increase in Aβ secretion (Fig. 1c). However, simultaneous knockdown of p24α_2 and p24δ_1 showed no additive or synergistic effect on Aβ generation (Fig. 7a and b). On the other hand, overexpression of p24α_2 or p24δ_1 resulted in subtle decrease in Aβ production, and co-overexpression of these proteins did not lead to any further decrease (Fig. 7c and d). These results suggest that p24α_2 and p24δ_1 have non-redundant roles in γ-cleavage inhibition and that the interaction of p24α_2 or p24δ_1 with γ-secretase

complexes is necessary, but not independently sufficient, for inhibition of γ-cleavage. Presumably, γ-cleavage inhibition requires a collaborative interaction of p24α_2 and p24δ_1 with the γ-secretase complex, and, for p24α_2/p24δ_1 modulation of γ-secretase activity, other unidentified component(s) and/or a limiting step is required (see Figure S4).

In addition to the direct modulation of γ-secretase, p24α_2 knockdown caused an increase in sAPP and APP-CTF levels as reported for p24δ_1 knockdown (Vetrivel et al. 2007). p24α_2 bound with the γ-secretase complex but not with APP (Fig. 3a and data not shown). In a recent paper, silencing of p24α_2 was reported to cause decreased stability of the ER-Golgi intermediate compartment (ERGIC) clusters and fragmentation of the Golgi apparatus, without affecting anterograde transport, thereby resulting in an imbalance of anterograde and retrograde vesicular traffic (Mitrovic *et al.* 2008). Hence, p24α_2 knockdown could increase the relative amount of APP-CTFs to APP-FL by perturbing the retrograde vesicular trafficking in the early secretory pathway. Intriguingly, an increase in secreted APP ectodomains was also observed upon p24β_1 knockdown that did not cause an alteration of Aβ secretion. These findings suggest that the increase in sAPP is commonly observed in p24 protein-depleted cells and is therefore not necessarily linked to an increase in Aβ secretion. It remains undetermined whether p24 proteins similarly affect cleavage and intracellular trafficking of γ-secretase substrates other than APP.

There is a lot of evidence supporting the theory that the control of γ-secretase activity is a promising

1 therapeutic strategy for Alzheimer's disease (Wolfe 2009). However, γ-secretase plays a critical role in
2 regulated intramembrane proteolysis of many type I TM proteins, whose resulting intracellular products
3 mediates pivotal signal transductions *in vivo* (Hass *et al.* 2009). In fact, potent γ-secretase inhibitors
4 induced adverse effects on the differentiation of lymphocytes and on intestinal mucosa, chiefly through
5 inhibition of Notch signaling (Wolfe 2009). In this respect, endogenous modulators of γ-secretase, such as
6 $p24\alpha_2$ and $p24\delta_1$, which inhibit Aβ generation but not Notch cleavage, may be more suitable therapeutic
7 targets for Alzheimer's disease.
8
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17

1 **Supporting Information**

2 Additional Supporting information may be found in the online version of this article:

3 **Figure S1.** Reduction of p24α_2 does not alter the expression level of the γ-secretase complex.

4 **Figure S2**. p24α_2 does not alter the subcellular distribution of the γ-secretase complex.

5 **Figure S3**. p24α_2 is not a substrate for γ-secretase.

6 **Figure S4**. Cartoon illustrating the proposed mechanism of γ-secretase inhibition by p24 proteins.

7

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17

18

1 **Figure legends**

2 **Figure 1.**

3 p24α_2 negatively modulates Aβ generation. (a) HEK293 cells were transfected with a pool of control,
4 p24α_2-, p24γ_3- or p24γ_4-specific siRNA duplexes. The expression levels of the RNAi target proteins
5 were examined by immunoblotting using specific antibodies. (b) Using the conditioned media of the
6 cells in (a), the levels of secreted Aβ40 and Aβ42 were measured by ELISAs. Error bars show SD. *,
7 p<0.05 *versus* the Control by an unpaired, two-tailed Student's *t*-test. (c) Individual p24α_2-specific
8 siRNA duplexes (T1, T2, T3 and T4) of the pooled duplexes used in (a), or control siRNA (ctl), was
9 transfected into HEK293 cells. The same amount of protein from cell lysates was subjected to
10 immunoblotting with the p24α_2-N antibody (*upper panel*). The Aβ40 levels of conditioned media were
11 measured using an ELISA (*graph*). Error bars show SD. *, $p < 0.05$ *versus* the Control siRNA by a
12 Student's *t*-test. (d) HEK293 cells were transfected with p24α_2 cDNA. The cell lysates were subjected
13 to immunoblotting using the p24α_2-N antibody (*upper panel*). Secreted Aβ40 and Aβ42 in the medium
14 were measured using ELISAs (*graphs*). Error bars show SD. *, $p < 0.05$ *versus* the Mock by a
15 Student's *t*-test.

16

17 **Figure 2.**

1 Knockdown of p24α_2 increases γ-cleavage but not ε- or S3-cleavage. (a) HEK293 cells were treated
2 with control or p24α_2-specific T3 siRNA duplexes. The microsome membrane fractions of these cells
3 were solubilized in a lysis buffer containing CHAPSO. An aliquot of the lysate was used to assess the
4 level of p24α_2 by immunoblotting using the p24α_2-N antibody (*upper panel*). The remaining lysates
5 were mixed with recombinant APP-C99-Flag, and were incubated at 37°C for 6 h. The Aβ40 levels
6 were measured using an ELISA (*graph*). Values are mean ± SD. *, $p < 0.05$ by a Student's *t*-test. (b)
7 Simultaneously generated AICD in the reaction mixtures in (a) was assessed by immunoblotting using
8 anti-APP CTF antibody. The result is representative of three independent experiments. (c)
9 NotchΔE-expressing HEK293 cells were treated with control or p24α_2-specific siRNA duplexes.
10 NICD generated in these cells was assessed by pulse-chase analysis over 60 min. The graph below
11 shows the relative density of the NICD bands.
12
13 **Figure 3.**
14 p24α_2 is associated with active γ-secretase complexes. (a) CHAPSO-solubilized lysates of microsome
15 membrane fractions from HEK293 cells were immunoprecipitated with preimmune serum (control-IP)
16 or anti-p24α_2-CTF (#2469R1) antibody (p24α_2-IP), and the precipitated proteins were detected by
17 immunoblotting with antibodies against PS1-NTF, NCT, APH-1, PEN-2 and Sec61α (*from top to*

1 *bottom*). Mature and immature forms of NCT are indicated by "m" and "im" respectively.
2 Immunoblotting with antibody against Sec61α, an unrelated membrane protein, was used as a negative
3 control. (b) Control, anti-p24α_2 and anti-NCT immunoprecipitates were mixed with recombinant
4 APP-C99-Flag, and then incubated at 37°C for 6 h in the absence or presence of DAPT. The Aβ40 was
5 measured using a specific ELISA. Error bars show SD. *$p < 0.01$ *versus* the control-IP by a Student's
6 *t*-test. (c) The expression levels of p24α_2, p24δ_1 and p24β_1 in PS1$^{+/+}$/PS2$^{+/+}$ MEFs and PS1$^{-/-}$/PS2$^{-/-}$
7 MEFs were detected by immunoblotting with anti-p24α_2 (p24α_2-N), anti-p24δ_1 or anti-p24β_1 antibody
8 (*three upper panels*). The blot was reprobed with anti-β-actin antibody, used as a loading control
9 (*bottom panel*). (d) The microsome membrane of HEK293 cells treated without (*left panels*) or with
10 (*right panels*) p24α_2-specific siRNA was subjected to two-dimensional BN/SDS-PAGE. The blots
11 were probed with antibodies against NCT, PS1-NTF, APH-1, PEN-2 and p24α_2 (*from top to bottom*).
12
13 **Figure 4.**
14 Knockdown of p24α_2 does not alter the cell surface distribution of the γ-secretase complex. HEK293
15 cells treated with control or p24α_2-specific siRNA duplexes were biotinylated with EZ-Link
16 Sulfo-NHS-LC-biotin, quenched, and then precipitated using NeutrAvidin beads. Cell surface proteins
17 were visualized by immunoblotting with anti-p24α_2 or anti-NCT antibody.

1

2 **Figure 5.**

3 Knockdown of p24α_2 increases APP-CTFs and sAPP. (a) HEK293 cells were transfected with p24α_2

4 siRNA (*left*) or p24α_2 cDNA (*right*), and the same amount of protein from cell lysates was subjected to

5 immunoblotting. The expression levels of p24α_2, APP-FL (m: mature, im: immature) and APP-CTFs

6 (C99 and C83) were detected with specific antibodies. The results are representative of three

7 independent experiments. The graphs below show the relative density of the bands for mature APP

8 (m-APP), immature APP (im-APP), C99 and C83. Error bars show SD. *, $p < 0.05$ *versus* the Control

9 or the Mock by a Student's *t*-test. (b) HEK293 cells were treated with control (lanes 1 and 2), p24α_2-

10 (lanes 3 and 4), p24δ_1- (lane 5 and 6) or p24β_1-specific siRNA duplexes (lanes 7 and 8). The levels of

11 sAPPβ (*upper panel*) and sAPPα (*lower panel*) in the conditioned media were detected with specific

12 antibodies. (c) β-Secretase activity of HEK293 cells treated with control or p24α_2-specific siRNA was

13 measured using an *in vitro* assay.

14

15 **Figure 6.**

16 The dilysine motifs of p24α_2 and p24δ_1 are indispensable for γ-cleavage inhibition. (a) Alignment of

17 the amino acid sequences of the intracellular domain of p24 family proteins. The dilysine motifs and

1 the diserine mutations are underlined. TM indicates the transmembrane domain. (b) The level of Aβ40

2 secreted from mock, $p24\alpha_2$, $p24\alpha_2SS$, $p24\delta_1$ or $p24\delta_1SS$-transfected HEK293 cells was measured

3 using an ELISA. The error bars show SD. *, $p < 0.01$ *versus* the Mock control by an unpaired,

4 two-tailed Student's *t*-test. The expression of $p24\alpha_2$ and $p24\delta_1$ was detected by immunoblotting. The

5 blot was reprobed with an anti-β-actin antibody, used as a loading control (*bottom panel*). (c)

6 Microsome membrane proteins from HEK293 cells transfected with wild-type (wt) $p24\alpha_2$ or $p24\alpha_2SS$

7 were immunoprecipitated with preimmune serum (lane 4) or anti-PS1-CTF antibody (lanes 5 and 6).

8 All precipitants were analyzed by immunoblotting for $p24\alpha_2$.

9

10 **Figure 7.**

11 Double knockdown, or co-overexpression of, $p24\alpha_2$ and $p24\delta_1$ shows no additive effect on Aβ

12 secretion. (a) HEK293 cells were treated with control (lanes 1 and 2), $p24\beta_1$- (lanes 3 and 4), $p24\alpha_2$-

13 (lanes 5 and 6), $p24\delta_1$- (lanes 7 and 8), $p24\alpha_2$- plus $p24\beta_1$- (lanes 9 and 10) or $p24\alpha_2$- plus

14 $p24\delta_1$-specific siRNA duplexes (lanes 11 and 12). The expression levels of $p24\alpha_2$, $p24\delta_1$ and $p24\beta_1$

15 were assessed by immunoblotting. (b) The level of the secreted Aβ40 in the media of the HEK293 cells

16 in (a) was measured using an ELISA. Values are mean ± SD. *, $p < 0.01$ *versus* the Control by a

17 Student's *t*-test. NS, no significant difference. (c) HEK293 cells were transfected with cDNA encoding

1 $p24\alpha_2$, $p24\delta_1$ or $p24\alpha_2$ plus $p24\delta_1$. Cell lysates were subjected to immunoblotting with antibodies

2 against $p24\alpha_2$, $p24\delta_1$ or β-actin. (d) Secreted Aβ40 in the medium of the HEK293 cells in (c) was

3 measured using an ELISA (*graph*). The error bars show SD. *, $p < 0.05$ *versus* the Mock by a Student's

4 *t*-test. NS, no significant difference.

Figure 1

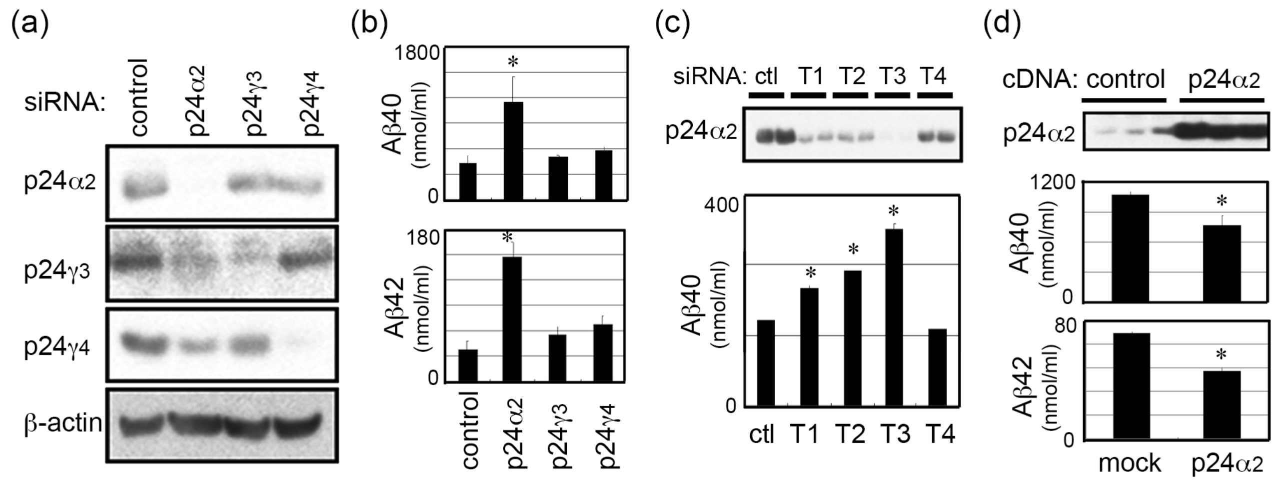


Figure 2

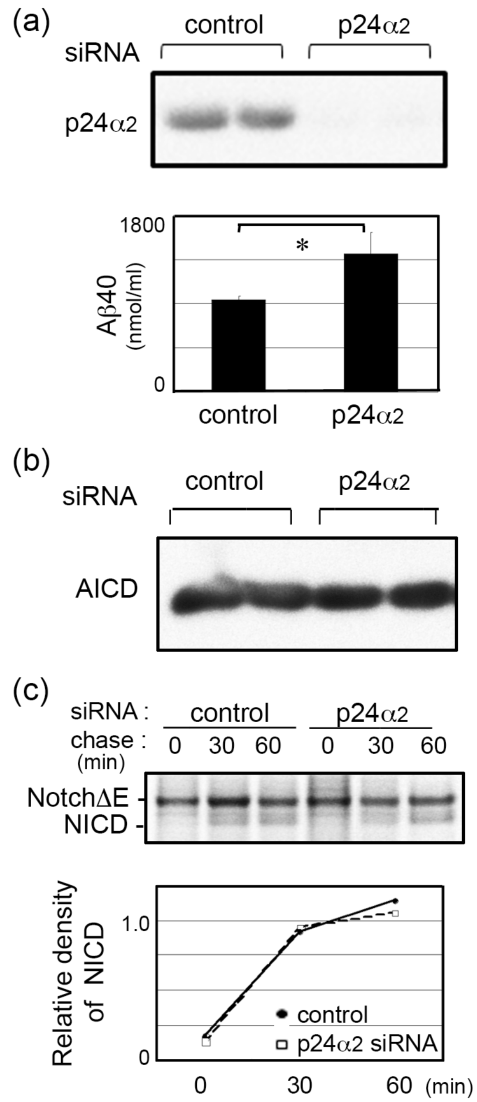


Figure 3

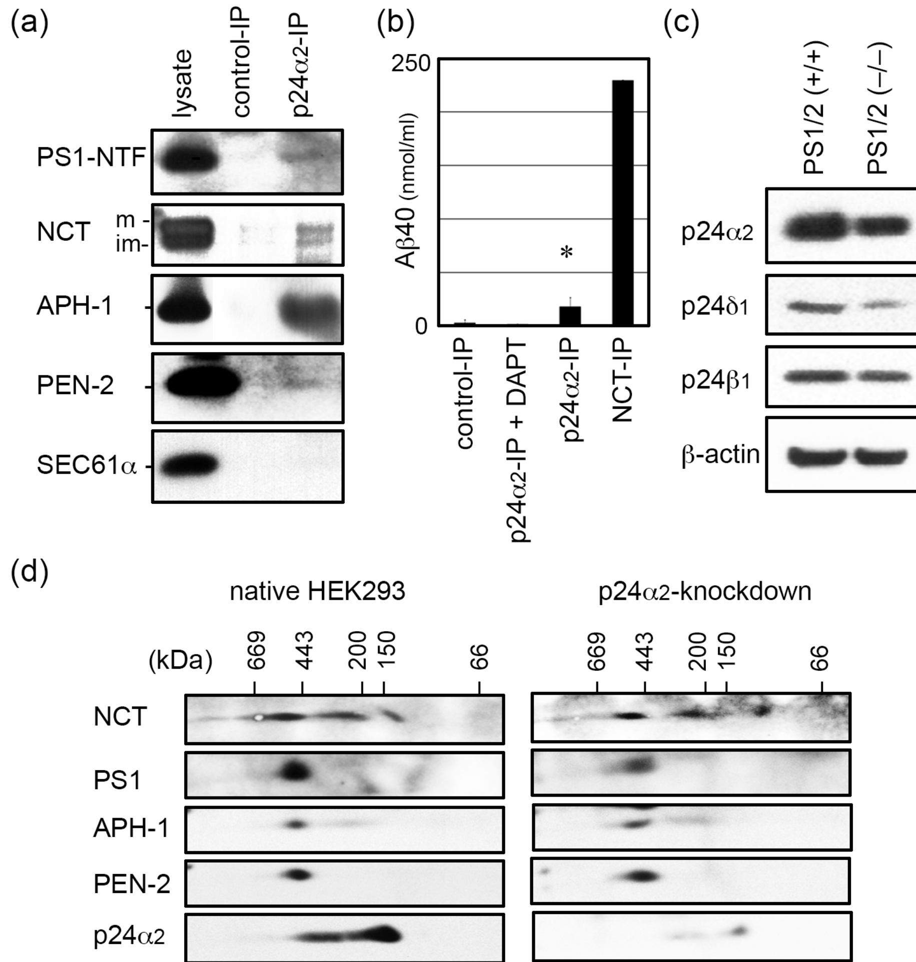


Figure 4

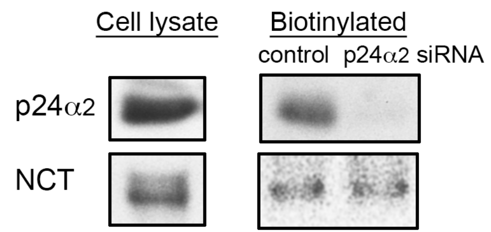


Figure 5

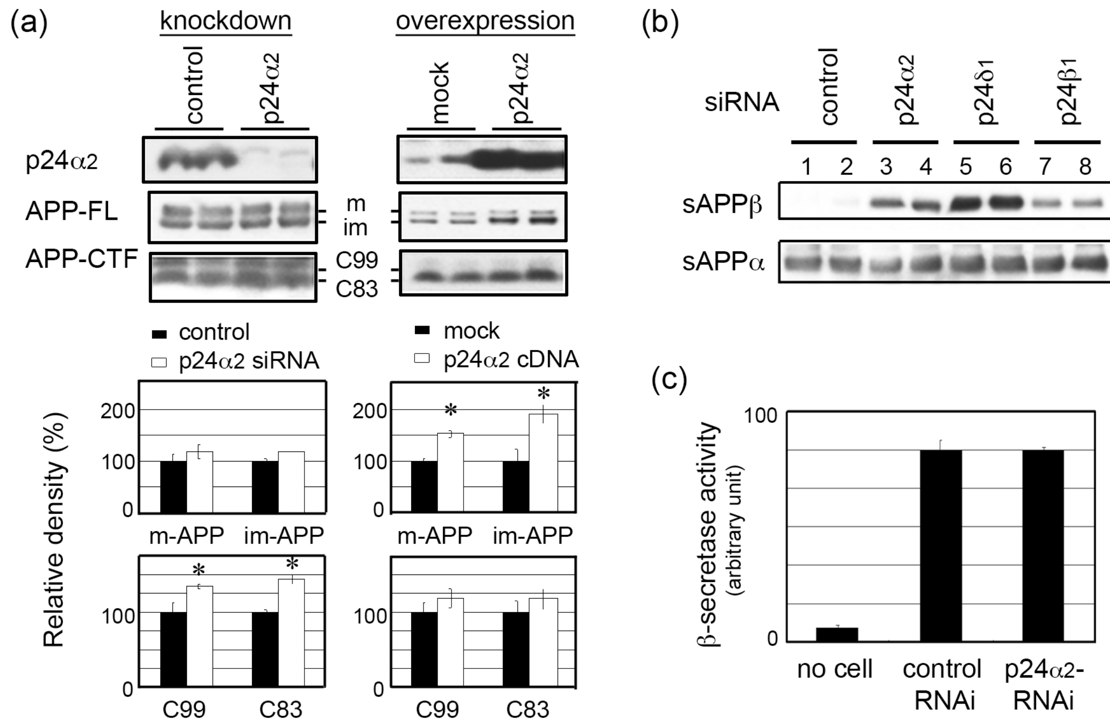


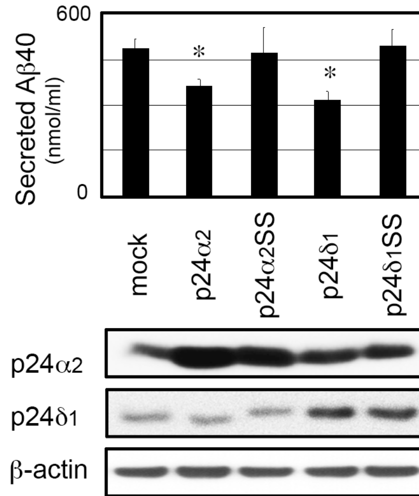
Figure 6

(a)

p24α2 : (TM)-LKSFFEAKKLV
p24δ1 : (TM)-LRRFFKAKKLIE
p24β1 : (TM)-LKRFFEVRRVV
p24γ3 : (TM)-LKSFFSDKRTTTTRVGS
p24γ4 : (TM)-LKSFFTEKRPISRAVHS

p24α2SS : (TM)-LKSFFEASSLV
p24δ1SS : (TM)-LRRFFKASSLIE

(b)



(c)

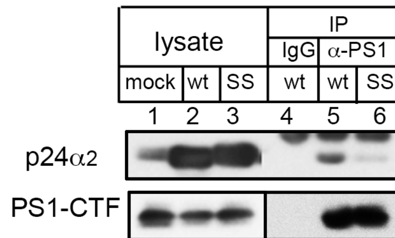


Figure 7