# Overexpression of Human CRB1 or Related Isoforms, CRB2 and CRB3, Does Not Regulate the Human Presenilin Complex in Culture Cells†

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*Received July 9, 2007; Revised Manuscript Received September 20, 2007*

ABSTRACT: The presenilin proteins (PS1 and PS2) with their partners (NCT, Aph1, and Pen2) are the major components of the high molecular weight $\gamma$-secretase complex which facilitates the intramembraneous cleavage of various type 1 transmembrane proteins, including the amyloid-$\beta$ precursor protein (APP) and the Notch receptor. Additional $\gamma$-secretase complex components may be involved in regulation of its activity and specificity. A recent investigation indicated that the Crumbs protein is a negative regulator of Notch signaling and may act by repressing $\gamma/\epsilon$-secretase activity in *Drosophila* [Herranz, H., Stamataki, E., Feiguin, F., and Milan, M. (2006) *EMBO Rep. 7*, 297−302]. To address this question, we investigated potential functional interactions between the human Crumbs homologues (CRB1, CRB2, and CRB3) and presenilin complexes which mediate $\gamma/\epsilon$-secretase cleavage of APP and Notch. We found no evidence for direct interaction between CRB1, CRB2, or CRB3 and presenilin complex components. Furthermore, overexpression of human CRB1 and related isoforms, CRB2 and CRB3, had no effect on the levels of presenilin complex components, on NCT maturation or on PS endoproteolysis, and did not alter A$\beta$ AICD or NICD production. These results suggest that, in mammalian cells at least, Crumbs is unlikely to be a significant direct modulator of presenilin-dependent $\gamma/\epsilon$-secretase activity.

$\gamma/\epsilon$-Secretase belongs to a unique class of enzymes that cleave their substrates within their hydrophobic transmembrane domains, a process termed regulated intramembrane proteolysis (RIP[1]) (*1*). At least 15 different type 1 transmembrane proteins including the amyloid-$\beta$ precursor protein (APP) and the Notch receptor have been described as $\gamma/\epsilon$-secretase substrates (*2*). Several lines of evidence have shown that $\gamma/\epsilon$-secretase activity requires a multimeric membrane protein complex, the presenilin complex, which is composed of at least four components: presenilin (PS1 or PS2), nicastrin (NCT), Aph-1 (Aph-1aL, Aph-1aS, or Aph-1b), and Pen-2. All four components are essential for $\gamma/\epsilon$-secretase activity, and loss of any of the constituent proteins abrogates the assembly and activity of the enzyme complex (*3*−*8*). Conversely their coexpression increases $\gamma/\epsilon$-secretase activity (*9*−*11*). nicastrin and Aph-1 may form an initial subcomplex to which full length presenilin and Pen-2 are added (*10*, *12*, *13*). Presenilins are cleaved into N- and C-terminal fragments which remain associated as a heterodimer in the active high molecular weight complex (*12*, *14*−16). In this presenilin complex, the presenilins have been proposed to serve as the catalytic subunit (*17*) while the large ectodomain of NCT has been reported to play an essential role in substrate recognition (*18*, *19*). Recently, we have shown that TMP21, a member of p24 cargo protein, acts as a specific modulator of some activities of the presenilin complexes, selectively suppressing $\gamma$- but not $\epsilon$-secretase cleavage (*20*, *21*). Nevertheless, the specific biochemical functions of all cofactors and the exact stoichiometry of the presenilin complex remain to be determined.

† This work was supported by the Canadian Institutes of Health Research, Howard Hughes Medical Institute, Alzheimer Society of Ontario, Canada Foundation for Innovation, Ontario Research and Development Challenge Fund, Ontario Mental Health Foundation, and Scottish Rite Charitable Foundation. R.P.-P. is supported by the Fondation pour la Recherche Médicale. N.S.-G. is supported by The Foundation Fighting Blindness (Canada).

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[1] Abbreviations: A$\beta$, amyloid $\beta$-peptide; AICD, APP intracellular domain; Aph1, anterior pharynx defective-1; APP, amyloid $\beta$-precursor protein; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CRB1, Crumbs homologue 1; CRB2, Crumbs homologue 2; CRB3, Crumbs homologue 3; ELISA, enzyme-linked immunosorbent assay; GSK3$\beta$, glycogen synthase kinase-3$\beta$; HEK293, human embryonic kidney 293; NCT, nicastrin; NEP, neprilysin; NICD, Notch intracellular domain; PBS, phosphate-buffered saline; Pen2, presenilin enhancer-2; PS1, presenilin 1; PS2, presenilin 2; RIP, regulated intramembrane proteolysis; TMP21, 21 kDa transmembrane-trafficking protein.

10.1021/bi701347w CCC: $37.00 
Published on Web 11/08/2007

The functional importance of the PS-dependent $\gamma$-secretase activity has been primarily focused on the processing of APP and Notch. The $\gamma$-secretase cleavage of APP is involved in the pathogenesis of Alzheimer's disease by releasing the 4 kDa amyloid $\beta$-protein (A$\beta$), which accumulates and aggregates in the brain of patients with Alzheimer's disease (*22*, *23*). Notch signaling is involved in a variety of important cell fate decisions during both embryogenesis and adulthood. Notch undergoes a first cleavage (S1 cleavage) in the Golgi during its passage through the secretory pathway, generating two fragments which remain associated at the cell surface. Ligand binding to the Notch receptor triggers the ectodomain shedding (S2 cleavage) followed by the intramembranous $\epsilon$-secretase cleavage of the remaining membrane-bound C-terminal stub (S3 cleavage). The soluble cytoplasmic C-terminal cleavage product, Notch intracellular domain (NICD), is then released from the membrane and is translocated to the nucleus where it controls transcription of genes involved in embryonic development and adult neuronal plasticity (*24*). Similar transcriptional roles have been proposed for the intracellular domain of APP (AICD) in the regulation of some genes such as KAI1(25, 26), GSK3$\beta$ (*26*, *27*), NEP (*28*, *29*), p53(30), Tip60 APP, or BACE (*26*).

During embryonic development of the *Drosophila* wing imaginal disc, Notch signaling is selectively activated in the boundary between dorsal and ventral compartments. This highly specific localization of Notch activity is crucial for the formation of the dorso-ventral boundary and requires repression of Notch activity in cells immediately adjacent to the boundary. Recently the *Drosophila* transmembrane protein Crumbs has been reported as a negative regulator of Notch signaling during this event. Genetic evidence suggested that *Drosophila* Crumbs might act by repressing the $\gamma$-secretase activity (*31*). In humans, the Crumbs homologue 1 (CRB1) has an important role in retinal morphogenesis, and mutations in the human CRB1 gene cause recessive retinal dystrophies (*32*). However, it is unknown whether CRB1 or related isoforms, CRB2 and CRB3, might also be a modulator of vertebrate $\gamma$/$\epsilon$-secretase activity.

In the current investigation, we have examined the possible role of human Crumbs homologues (CRB1, CRB2, and CRB3) in $\gamma$/$\epsilon$-secretase activity. First, we report that CRB1-, CRB2-, or CRB3-overexpression in human embryonic kidney cells (HEK293) has no effect on the expression level of endogenous presenilin complex components such as PS1, PS2, NCT, and Aph1. Second, we found no evidence for a physical interaction between presenilin-complex components and either endogenous CRB1 protein or overexpressed exogenous CRB1, CRB2, or CRB3 proteins. Finally, we show that overexpression of CRB1 or the other Crumbs isoforms (CRB2, CRB3) in HEK293 cells does not modulate NICD, AICD ($\epsilon$-secretase), or A$\beta$ ($\gamma$-secretase) production.

## EXPERIMENTAL PROCEDURES

*Transfection of cDNA Constructs*. Expression constructs for human myc-tagged CRB1, human myc-tagged CRB3, and human flag-tagged CRB2 are cloned in pSecTag2B and pFlagCMV2 vector respectively and obtained from Jane McGlade and Ben Margolis (*33*−*35*). Expression construct for myc-tagged mΔENotch cloned in pCS2 vector and HEK293 stable cell line overexpressing mΔENotch were prepared as described previously (*21*, *36*). mΔENotch is a membrane-tethered constitutive active form of Notch which contains the transmembrane and intracellular domains of Notch. Transient transfections were performed in HEK293 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

*Membrane Fraction Preparation and Coimmunoprecipitation*. HEK293 cells were harvested and homogenized in buffer A (5 mM HEPES pH 7.4, 1 mM EDTA, 0.25 M sucrose, protease inhibitor cocktail from Roche). The homogenate was clarified by centrifugation at 1000*g* for 5 min at 4 °C, the supernatant was centrifuged at 100000*g* for 1h at 4 °C, and the membrane pellets were homogenized in buffer B (25 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% CHAPSO, protease inhibitor cocktail). Isolates of the membrane fraction (500 $\mu$g to 2 mg) were diluted in buffer B to a final concentration of 0.5% CHAPSO. Fresh postmortem human retinal tissue was obtained and used under the ethics guidelines of the University of Toronto Health Network. The human tissue was homogenized in buffer B and centrifuged at 5000*g* for 10 min, and then 2 mg of proteins was diluted in buffer B to a final concentration of 0.5% CHAPSO. After preclearing with protein G-sepharose 4 fast flow (GE Healthcare Bio-sciences Inc.) for 1 h at 4 °C, lysates from human tissue or HEK293 cells were subjected to immunoprecipitation with the appropriate primary antibody. Immunoprecipitants were recovered by overnight incubation at 4 °C with protein G-sepharose 4 fast flow (GE Healthcare Bio-sciences Inc.). Beads were washed three times with buffer B (0.5% CHAPSO) and once with PBS.

*SDS/PAGE and Western Blot Analyses*. Whole cell lysates (total protein 50$\mu$g), membrane lysates (protein 25$\mu$g), and immunoprecipitated proteins were dissolved in SDS sample buffer, separated on Tris Tricine gels 16% or Tris−Glycine gels 8% or 4−20% (Invitrogen), and transferred to nitrocellulose membranes. Target proteins were visualized by enhanced chemiluminescence (ECL Amersham Biosciences) with the following antibodies: monoclonal anti-myc (Invitrogen), anti-flag (Sigma) anti-tubulin (Zymed) antibodies, polyclonal anti-myc (Santa Cruz Biotechnology), anti flag (Sigma) antibodies, mouse polyclonal anti-CRB1 (Abnova), rabbit polyclonal anti-CRB1 antibodies, monoclonal anti-PS2 N-terminal antibody, rat monoclonal anti-PS1 (Chemicon), mouse monoclonal anti-PS1−NT1 (provided by Dr. Paul Mathews), rabbit polyclonal anti PALS1 (Santa Cruz Biotechnology) anti-PS1, anti-NCT, anti-Aph1aL (O2C2) anti-APP CTF antibodies.

*Metabolic Labeling*. Following mΔENotch transfection, HEK293 cells were preincubated 1 h in methionine-free and cysteine-free Dulbecco's modified Eagle's medium. Trans $^{35}$S-labeling reagent (300 $\mu$Ci/mL) was added for 20 min. The media were replaced with unlabeled conditioned medium for 0, 30 min, 1 h, or 2h at 37 °C. Cells were rinsed in PBS, collected in PBS EDTA (5 mM EDTA), and then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton, 0.5% DOC, 0.1% SDS, 1 mM EDTA) in ice for 30 min. Lysates were spun at 13000 rpm for 10 min, and the supernatant was retained. Samples were diluted in RIPA buffer, subjected to immunoprecipitation with polyclonal anti-myc antibody (Santa Cruz Biotechnology), and analyzed

by 8% Tris-glycine SDS-polyacrylamide gel electrophoresis. The gels were dried and exposed to Kodak Biomax film.

*Aβ40 ELISA Assays*. Aβ40 levels were measured by ELISA assays using conditioned medium collected from control HEK293 cells and from HEK293 cells overexpressing CRB1, CRB2, or CRB3. The enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions (Biosource International).

*Cell Free γ-Secretase Assay*. Following CRB1or CRB2 transfection in HEK293 cells, membrane fractions were prepared and subjected to an *in vitro* γ-secretase assay as described previously (*21*) using exogenous recombinant APP-C100-flag peptide as substrate. Briefly, membrane fractions were incubated with APP-C100-flag substrate 6 h at 37 °C or 4 °C as negative control. The γ-secretase inhibitor, Compound E, was also used as negative control. The product, AICD-flag, was detected by Western-blotting with anti-APP CTF antibody.

*Immunofluorescence*. Mouse embryonic Fibroblasts (MEF) were cultured on poly-L-lysine-coated glass coverslips and transfected with CRB1 cDNA. Forty eight hours after transfection, cells were fixed for 15 min with 10% buffered formalin phosphate (Fisher Scientific) and permeabilized for 30 min with 0.1% Triton X-100. After three washes with PBS, cells were incubated overnight at 4 °C with the primary antibodies (mouse monoclonal anti-myc and rabbit polyclonal anti-PS1 or anti-PALS1 antibodies) diluted in the antibody diluting buffer from Dako. Cells were then washed three times with 0.1% Triton X-100 and incubated for 2 h at room temperature with both Alexa488-labeled goat anti rabbit (green) and Alexa594-labeled goat anti mouse (red) antibodies (Invitrogen). Coverslips were washed and mounted with Dakocytomation fluorescent mounting medium from Dako and images were collected using the Radiance2000 (Bio-Rad) confocal microscope.

## RESULTS

*CRB1, CRB2, or CRB3 Overexpression Does Not Alter Presenilin Complex Stability*. To investigate the role of the human Crumbs homologues in γ/ε-secretase activity, we transiently transfected human embryonic kidney cells (HEK293) with myc-tagged CRB1, flag-tagged CRB2, or myc-tagged CRB3. Immunoblotting of whole cell lysates indicated that there was no change in the levels of endogenous presenilin complex components. Immunoreactivities of NCT, PS1, PS2, and Aph-1 were similar in cells overexpressing human Crumbs homologues as compared to cells transfected with empty vector (Figure 1). In addition, CRB1, CRB2, or CRB3 overexpression had no effect on NCT-maturation or PS1 and PS2 endoproteolysis. These results indicate that the assembly and stability of presenilin complexes are not disturbed in cells in which stoichiometry of CRB1, CRB2, or CRB3 relative to presenilin complexes has been altered by overexpressing human Crumbs homologues. The levels of APP holoprotein were also unaltered by CRB1, CRB2, or CRB3 overexpression (Figure 1). In our hands, endogenous level of CRB1 in HEK293 cells is not detectable in Western Blot using two different CRB1 antibodies (a rabbit polyclonal anti CRB1(33) and a mouse polyclonal anti CRB1 from Abnova), suggesting an absence or a very low level of CRB1-expression while endogenous

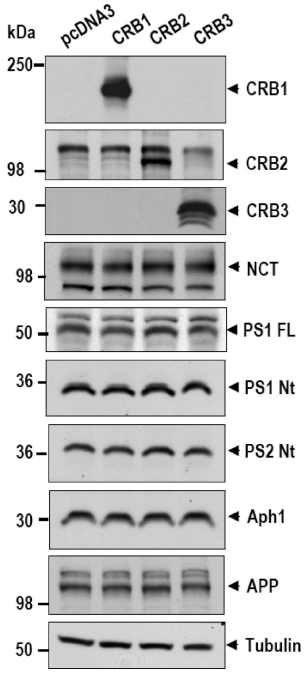


FIGURE 1: CRB1, CRB2, or CRB3 overexpression has no effect on the expression of endogenous presenilin complex components and APP. HEK293 cells were transiently transfected with empty vector (pcDNA3) or myc-tagged-CRB1 (CRB1), flag-tagged CRB2 (CRB2) or myc-tagged CRB3 (CRB3) cDNA. Immunoblotting for NCT, PS1, PS2, Aph-1, and APP from total cell lysates indicated no significant change in levels of these proteins following CRB1, CRB2 or CRB3 overexpression. Tubulin immunoreactivity was used as a loading control.

presenilin complex components are highly expressed in these cells.

*CRB1, CRB2, or CRB3 Are Not Physically Associated with Presenilin Complexes.* To determine whether the human Crumbs homologues could directly interact with presenilin complexes, we performed co-immunoprecipitations under different conditions. Membrane fractions of cells transiently transfected with myc-tagged CRB1, flag-tagged CRB2, myc-tagged CRB3, or empty vector were solubilized in 1% CHAPSO buffer (which we and others have shown supports isolation of complete presenilin complexes with functional γ/ε-secretase activity (*12*)) and subjected to precipitation using monoclonal anti-myc or anti-flag-antibodies. This experiment revealed robust precipitation of the exogenous CRB1, CRB3, or CRB2 (Figure 2A−C, top panels). Endogenous PALS1/MPP5 protein known to interact with the human Crumbs homologues (*34*, *35*) is well pulled down with CRB1, CRB2, or CRB3 but no coprecipitation of the endogenous NCT or PS1 proteins is observed (Figure 2A−C, middle and bottom panels).

The reverse immunoprecipitation experiment using a number of different PS1-antibodies revealed that these anti-PS1 antibodies were all able to coprecipitate the N-terminal fragment of PS1 and the mature form of NCT (Figure 2D,E, middle and bottom panels). However, the exogenous myc-tagged CRB1, flag-tagged CRB2, or myc-tagged CRB3 did not coprecipitate (Figure 2D,E, top panels), suggesting that presenilin complexes and the human Crumbs homologues do not interact even when they are overexpressed.

To rule out the possibility that the myc-tag may have disrupted a putative interaction with PS1 (e.g., by causing

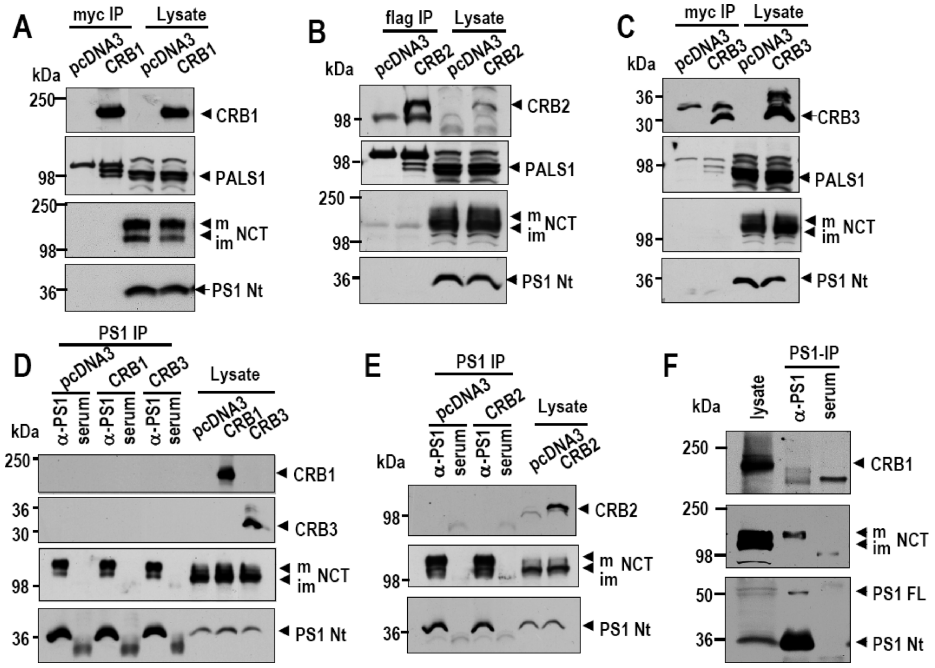


FIGURE 2: Neither exogenous CRB1, CRB2, CRB3 nor endogenous CRB1 is co-immunoprecipitated with endogenous presenilin-1 and nicastrin. Cells were transiently transfected with empty vector (pcDNA3) or myc-tagged-CRB1 (CRB1), flag-tagged CRB2 (CRB2) or myc-tagged CRB3 (CRB3) cDNA. Immunoprecipitation of CHAPSO-solubilized membrane fractions was performed for myc-tagged CRB1 (A), flag-tagged CRB2 (B), myc-tagged CRB3 (C) or endogenous PS1 (D-E) using anti-myc, anti-flag or anti-PS1 antibodies. 3 different anti-PS1 antibodies (our rabbit polyclonal anti-PS1Nt, a rat monoclonal anti-PS1 from Chemicon or a mouse monoclonal anti-PS1−NT1 provided by Dr. Paul Mathews) were used and provided same results. Post mortem human retinal tissue was used to examine the co-immunoprecipitation of endogenous PS1 and CRB1. The rat monoclonal anti-PS1 from Chemicon was used to pull down the PS compex (F). A maximum of 5% of membrane proteins used for immunoprecipitation has been load in control lysate lane. PALS1/MPP5, NCT, PS1, CRB1, CRB2 and CRB3 immunoreactivities were analyzed by Western blots of membrane lysates and IP samples using anti-PALS1/MPP5, anti-NCT, anti-PS1, anti-myc, anti-flag or a rabbit polyclonal CRB1 antibody. Serum was used as a negative immuno-precipitation control. Independent co-immunoprecipitations were done 3 to 5 times in HEK293 cells and twice in human retina tissue.

mistrafficking of the exogenous CRB1), we investigated the interaction of endogenous presenilin with endogenous CRB1 in human retinal cells. Human CRB1 is expressed at high levels in both the brain and in retina. Fresh post-mortem human retinal tissue was homogenized in 1% CHAPSO buffer, and coprecipitation was performed using a monoclonal PS1-antibody. Expression of the endogenous CRB1, PS1 and NCT was readily detected in human retinal lysates (Figure 2F). As expected, the PS1 immunoprecipitation specifically recovered both the PS1 N-terminal fragment and the mature form of NCT. However, CRB1 again, could not be co-immunoprecipitated with these complexes (Figure 2F). Taken together with the studies in transfected HEK293 cells, these observations indicate that there is no strong physical interaction between the presenilins and either exogenous human Crumbs homologues or the endogenous CRB1 protein.

*CRB1 is Colocalized with PALS1/MPP5 Protein but Not with PS1.* To support the conclusions made from our co-immunoprecipitation studies, we performed immunofluorescence on cells expressing CRB1 and examined overlap with endogenous PS1. As expected no significant colocalization was observed between CRB1 and PS1. In contrast, the PALS1/MPP5 positive control display considerable colocalization with CRB1 (Figure 3)

*Overexpression of Human Crumbs Homologues Has No Effect on the γ/ϵ-Secretase Activity.* Although CRB1, CRB2, or CRB3 do not appear to be a major component of PS1 complexes, it is still possible that Crumbs homologues might modulate γ/ϵ-secretase activity either by a weak/transient

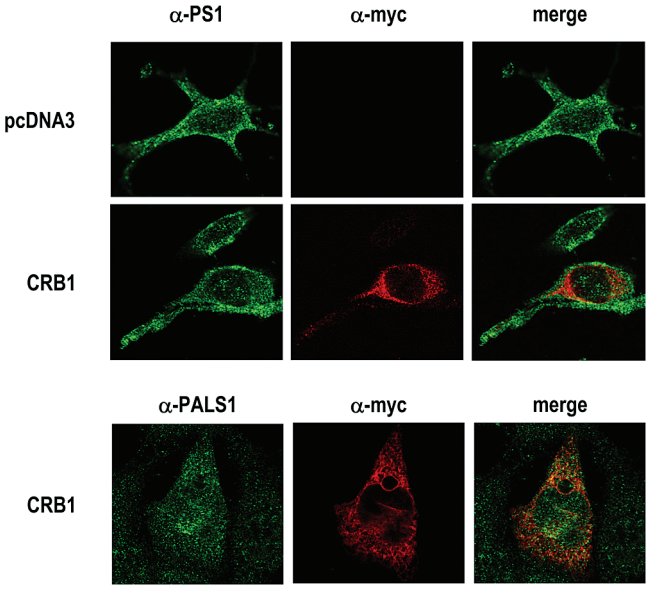


FIGURE 3: CRB1 protein colocalizes with PALS1/MPP5 but not with PS1. HEK293 cells were transiently transfected with empty vector (pcDNA3) or myc-tagged-CRB1 (CRB1) and subjected to immunofluorescence analysis. CRB1, PS1 and PALS1/MPP5 was detected with monoclonal anti-myc, polyclonal anti-PS1 or anti-PALS1/MPP5 antibodies respectively. Co-localization shown by merge is observed between CRB1 and PALS1 but not between CRB1 and PS1.

interaction with presenilin complexes, or by an indirect effect on γ/ϵ-secretase activity mediated by some other cellular protein. To address this question, we measured the kinetics

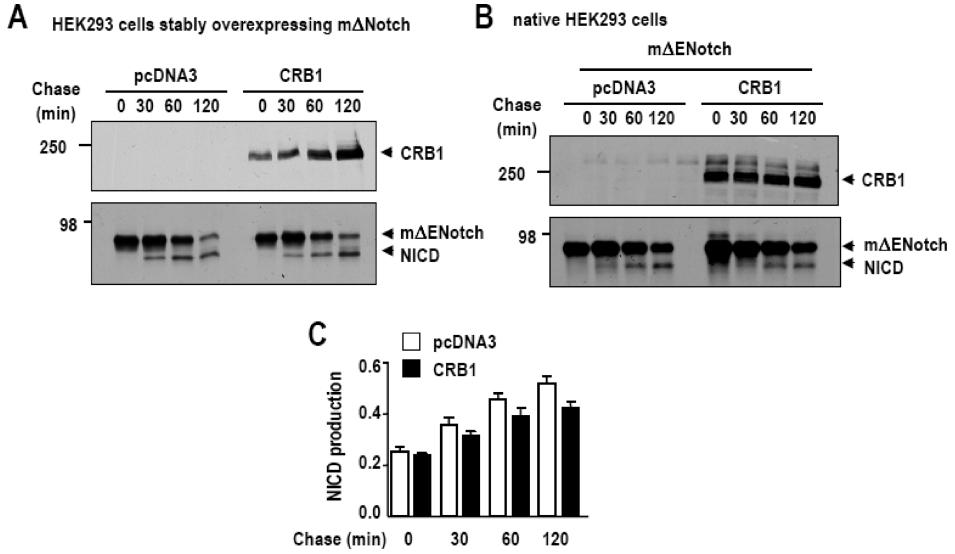


FIGURE 4: CRB1-overexpression has no effect on NICD production. HEK293 cells stably overexpressing myc-tagged mΔENotch were transiently transfected with empty vector (pcDNA3) or myc-tagged-CRB1 (CRB1) (A). Native HEK293 cells were transiently cotransfected with myc-tagged mΔENotch cDNA in addition to empty vector (pcDNA3) or myc-tagged-CRB1 (CRB1) (B,C). Following transfection, cells were subjected to metabolic labeling and the intensities of autoradiographic bands were measured by densitometry (ratios of NICD to total Notch protein (NICD plus NotchΔE). Bars in (C) represent the mean ± SEM of three independent determinations. CRB1-transfection was confirmed by western blot of cell lysates using a monoclonal myc-antibody.

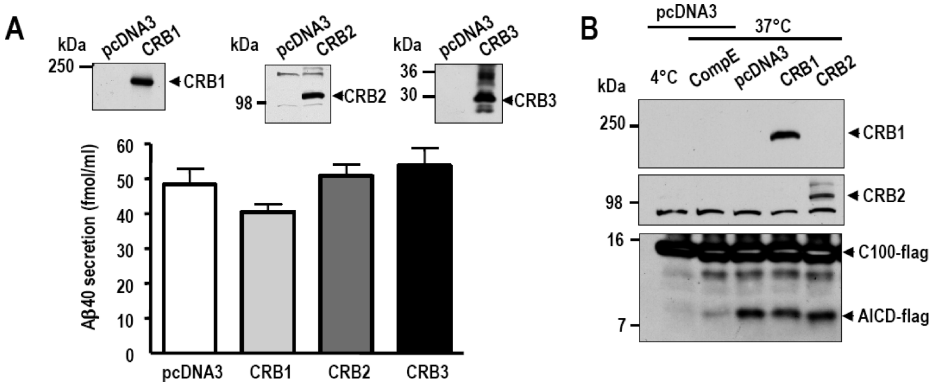


FIGURE 5: Crumbs-overexpression has no effect on A$\beta$ or AICD production. Native HEK293 cells were transiently transfected with empty vector (pcDNA3), myc-tagged CRB1 (CRB1), flag-tagged CRB2 (CRB2) or myc-tagged CRB3 (CRB3). Expression of each Crumbs isoform was confirmed by Western blotting of total cell lysates using monoclonal myc-antibody or flag-antibody (A). A$\beta$40 secretion was measured by ELISA in the conditioned cell medium. Bars represent the mean ± SEM of six independent determinations (ns: not statistically significant). (B) AICD production was assessed by in vitro $\gamma$-secretase cell free assay as described in the experimental procedures.

for the cleavage of a Notch substrate (mΔENotch) and generation of the NICD fragments. These kinetic studies were performed by metabolic labeling in cells expressing mΔENotch and either myc-tagged CRB1 or empty vector (control). In HEK293 cells stably overexpressing mΔENotch and transiently overexpressing myc-tagged CRB1 (Figure 4A), and in HEK293 cells transiently overexpressing both mΔENotch and myc-tagged CRB1 (Figure 4B), we observed that the time course of NICD production was similar to that in control cells. Quantification of the pulse-phase data revealed a small but non-significant decrease (less than 20%) in NICD production after a 2h chase in cells transiently cotransfected with mΔENotch and CRB1 (Figure 3C; $p > 0.05$, $n = 3$ replications). In addition, levels of mΔENotch and its product NICD are not changed in HEK293 cells stably overexpressing mΔENotch and transiently overexpressing myc-tagged CRB1, flag-tagged CRB2, or myc-tagged CRB3 (Figure S1).

To examine the potential effect of Crumbs on other $\gamma/\epsilon$-secretase substrates, we investigated the effect of CRB1 overexpression on presenilin-dependent $\gamma$-secretase cleavage of APP, which generates A$\beta$40 and AICD fragment. The level of A$\beta$40 secreted by cells overexpressing either myc-tagged CRB1, flag-tagged CRB2, or myc-tagged CRB3 were not significantly altered (Figure 5A; $p > 0.1$, $n = 6$). In addition, AICD production assessed by an *in vitro* $\gamma$-secretase cell free assay is not changed in HEK293 cells overexpressing CRB1 or CRB2 (Figure 5B).

## DISCUSSION

The presenilins (PS1 and PS2), nicastrin, Aph-1, and Pen-2 are the core components of the $\gamma$-secretase (*5*, *9*−*11*). This complex plays a key role in the pathogenesis of Alzheimer's disease as well as in several physiologically important signaling pathways. Recent work has now begun to focus on the identification of additional cofactors for these complexes that regulate their activity. In this regard, we have recently shown that TMP21, a member of p24 cargo protein, is a component of presenilin complexes and selectively modulates $\gamma$-secretase cleavage of APP protein (*21*). The genetic evidence that Crumbs modulates Notch signaling in

the fly imaginal disc (*31*) raised the possibility that Crumbs might also be a specific modulator of presenilin complex activity. However, the biochemical and cell biological data reported here suggests that, at least in mammalian cells, CRB1, CRB2, and CRB3 are neither components of presenilin complexes nor are they able to directly or indirectly modulate presenilin-dependent $\gamma/\epsilon$-secretase activity. Indeed, we have failed to observe a significant change in either NICD AICD or A$\beta$ production due to CRB1, CRB2, or CRB3 overexpression in HEK293 cells. This conclusion is further supported by (1) a lack of evidence for direct binding between the presenilin complexes and exogenous human Crumbs homologues or endogenous CRB1, and (2) by the absence of changes in the expression of presenilin complex components, NCT maturation, or PS endoproteolysis in HEK293 cells overexpressing CRB1, CRB2, or CRB3.

The overexpression system used in this study has certain limitations, but it provides evidence for absence of a functional or physical interaction between the human Crumbs homologues and the presenilin complex. It is conceivable that overexpression of the Crumbs isoforms does not promote incoporation into the complex which would explain the lack of an effect. This was observed, for example, with TMP21 where only knockdown of the protein resulted in altered A$\beta$ production. However, it is unlikely that depletion of Crumbs by siRNA approaches would have any significant effect under the conditions used for the current study. This is due to the fact that no detectable endogenous CRB1 was observed in any of the cell lines under investigation. Therefore, these cells are effectively CRB1 knockout and indicates that loss of this protein does not alter the activity of the presenilin complex.

This apparent discrepancy about the implication of *Drosophila* Crumbs protein and the human Crumbs homologues may arise from different mechanisms to regulate $\gamma/\epsilon$-secretase activity in different species. We report here that overexpression of CRB2 or CRB3 as well as overexpression of CRB1 have no significant effect either on A$\beta$40 secretion or on AICD or NICD production. Although different splice variants of CRB1 have been described (*37*) and remain to be explored, the complex biology of the human Crumbs homologues (CRB1, CRB2, CRB3) and CRB1 isofoms contrast with the single *Drosophila* Crumbs protein.

Mutations in human CRB1 gene cause recessive retinal dystrophies suggesting an important function of CRB1 in normal human retina organization (*32*). However, to the best of our knowledge this phenotype is not associated with mutations in presenilin or nicastrin genes (*38*). This divergence in phenotypes in addition to the fact that the human Crumbs homologues and presenilin complex components have distinct expression patterns provide evidence that the Crumbs homologues and presenilin complexes may have distinct roles.

In conclusion, although we cannot exclude a presenilin complex-modulating role for Crumbs in *Drosophila*, there are many reasons to believe that in mammals, Crumbs homologues are not directly implicated in regulating $\gamma/\epsilon$-secretase activity.

## ACKNOWLEDGMENT

We are grateful to Ben Margolis (University of Michigan) for providing the human myc-tagged CRB1 cDNA.

## SUPPORTING INFORMATION AVAILABLE

Level of NICD in HEK293 cells stably overexpressing m$\Delta$ENotch and transiently overexpressing myc-tagged CRB1, flag-tagged CRB2, or myc-tagged CRB3. This material is available free of charge via the Internet at http://pubs.asc.org.

## REFERENCES

1. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans, *Cell 100*, 391−398.
2. Kopan, R., and Ilagan, M. X. (2004) Gamma-secretase: proteasome of the membrane? *Nat. Rev. Mol. Cell Biol. 5*, 499−504.
3. Rogaev, E. I., Sherrington, R., Rogaeva, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P. E., Rommens, J. M., and St George-Hyslop, P. H. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene, *Nature 376*, 775−778.
4. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-F., Bruni, A. C., Montesi, M. P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R. J., Wasco, W., Da Silva, H. A. R., Haines, J. L., Pericak-Vance, M. A., Tanzi, R. E., Roses, A. D., Fraser, P. E., Rommens, J. M., and St George-Hyslop, P. H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease, *Nature 375*, 754−760.
5. Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L. S., Sorbi, S., Bruni, A., Fraser, P., and St George-Hyslop, P. (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing, *Nature 407*, 48−54.
6. Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation, *Dev. Cell 3*, 85−97.
7. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein, *Nature 391*, 387−390.
8. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain, *Nature 398*, 518−522.
9. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2, *Proc. Natl. Acad. Sci. U.S.A. 100*, 6382−6387.
10. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) The role of presenilin cofactors in the gamma-secretase complex, *Nature 422*, 438−441.
11. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) Reconstitution of gamma-secretase activity, *Nat. Cell Biol. 5*, 486−488.
12. Gu, Y., Sanjo, N., Chen, F., Hasegawa, H., Petit, A., Ruan, X., Li, W., Shier, C., Kawarai, T., Schmitt-Ulms, G., Westaway, D., St George-Hyslop, P., and Fraser, P. E. (2004) The presenilin proteins are components of multiple membrane-bound complexes that have different biological activities, *J. Biol. Chem. 279*, 31329−31336.
13. Gu, Y., Chen, F., Sanjo, N., Kawarai, T., Hasegawa, H., Duthie, M., Li, W., Ruan, X., Luthra, A., Mount, H. T., Tandon, A., Fraser, P. E., and St George-Hyslop, P. (2003) APH-1 interacts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin.nicastrin complexes, *J. Biol. Chem. 278*, 7374−7380.

14. Capell, A., Grunberg, J., Pesold, B., Diehlmann, A., Citron, M., Nixon, R., Beyreuther, K., Selkoe, D. J., and Haass, C. (1998) The proteolytic fragments of the Alzheimer's disease-associated presenilin-1 form heterodimers and occur as a 100−150-kDa molecular mass complex, *J. Biol. Chem. 273*, 3205−3211.
15. Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D. M., Levesque, L., Rogaeva, E., Xu, D., Liang, Y., Duthie, M., St George-Hyslop, P. H., and Fraser, P. E. (1998) The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains beta-catenin, *J. Biol. Chem. 273*, 16470−16475.
16. Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo, *Neuron 17*, 181−190.
17. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity, *Nature 398*, 513−517.
18. Shah, S., Lee, S. F., Tabuchi, K., Hao, Y. H., Yu, C., LaPlant, Q., Ball, H., Dann, C. E., 3rd, Sudhof, T., and Yu, G. (2005) Nicastrin functions as a gamma-secretase-substrate receptor, *Cell 122*, 435−447.
19. Chen, F., Yu, G., Arawaka, S., Nishimura, M., Kawarai, T., Yu, H., Tandon, A., Supala, A., Song, Y. Q., Rogaeva, E., Milman, P., Sato, C., Yu, C., Janus, C., Lee, J., Song, L., Zhang, L., Fraser, P. E., and St George-Hyslop, P. H. (2001) Nicastrin binds to membrane-tethered Notch, *Nat. Cell Biol. 3*, 751−754.
20. Vetrivel, K. S., Gong, P., Bowen, J. W., Cheng, H., Chen, Y., Carter, M., Nguyen, P. D., Placanica, L., Wieland, F. T., Li, Y. M., Kounnas, M. Z., and Thinakaran, G. (2007) Dual roles of the transmembrane protein p23/TMP21 in the modulation of amyloid precursor protein metabolism, *Mol. Neurodegener. 2*, 4.
21. Chen, F., Hasegawa, H., Schmitt-Ulms, G., Kawarai, T., Bohm, C., Katayama, T., Gu, Y., Sanjo, N., Glista, M., Rogaeva, E., Wakutani, Y., Pardossi-Piquard, R., Ruan, X., Tandon, A., Checler, F., Marambaud, P., Hansen, K., Westaway, D., St George-Hyslop, P., and Fraser, P. (2006) TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity, *Nature 440*, 1208−1212.
22. Evin, G., Sernee, M. F., and Masters, C. L. (2006) Inhibition of gamma-secretase as a therapeutic intervention for Alzheimer's disease: prospects, limitations and strategies, *CNS Drugs 20*, 351−372.
23. Rogaeva, E., Kawarai, T., and George-Hyslop, P. S. (2006) Genetic complexity of Alzheimer's disease: successes and challenges, *J Alzheimers Dis. 9*, 381−387.
24. Louvi, A., and Artavanis-Tsakonas, S. (2006) Notch signalling in vertebrate neural development, *Nat. Rev. Neurosci. 7*, 93−102.
25. Baek, S. H., Ohgi, K. A., Rose, D. W., Koo, E. H., Glass, C. K., and Rosenfeld, M. G. (2002) exchange of N-CoR corepressor and Tip 60 coactivator complexes links gene expression by NF-$\kappa$B and $\beta$-amyloid precursor protein, *Cell 110*, 55−67.
26. von Rotz, R. C., Kohli, B. M., Bosset, J., Meier, M., Suzuki, T., Nitsch, R. M., and Konietzko, U. (2004) The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor, *J. Cell Sci. 117*, 4435−4448.
27. Kim, H-S., Kim, E-M., Lee, J-P., Park, C. H., Kim, S., Seo, J-H., Chang, K-A., Yu, E., Jeong, S-J., Chong, Y. H., and Suh, Y-H. (2003) C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthetase kinase-3$\beta$ expression, *FASEB J. 17*, 1951−1953.
28. Pardossi-Piquard, R., Dunys, J., Yu, G., St George-Hyslop, P., Alves da Costa, C., and Checler, F. (2006) Neprilysin activity and expression are controlled by nicastrin, *J. Neurochem. 97*, 1052−1056.
29. Pardossi-Piquard, R., Petit, A., Kawarai, T., Sunyach, C., Alves da Costa, C., Vincent, B., Ring, S., D'Adamio, L., Shen, J., Muller, U., St George Hyslop, P., and Checler, F. (2005) Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domains of betaAPP and APLP, *Neuron 46*, 541−554.
30. Alves da Costa, C., Sunyach, C., Pardossi-Piquard, R., Sevalle, J., Vincent, B., Boyer, N., Kawarai, T., Girardot, N., St George-Hyslop, P., and Checler, F. (2006) Presenilin-dependent gamma-secretase-mediated control of p53-associated cell death in Alzheimer's disease, *J. Neurosci. 26*, 6377−6385.
31. Herranz, H., Stamataki, E., Feiguin, F., and Milan, M. (2006) Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of gamma-secretase activity, *EMBO Rep. 7*, 297−302.
32. Richard, M., Roepman, R., Aartsen, W. M., van Rossum, A. G., den Hollander, A. I., Knust, E., Wijnholds, J., and Cremers, F. P. (2006) Towards understanding CRUMBS function in retinal dystrophies, *Hum. Mol. Genet.* 15 Suppl *2*, R235−243.
33. Laprise, P., Beronja, S., Silva-Gagliardi, N. F., Pellikka, M., Jensen, A. M., McGlade, C. J., and Tepass, U. (2006) The FERM protein Yurt is a negative regulatory component of the Crumbs complex that controls epithelial polarity and apical membrane size, *Dev. Cell 11*, 363−374.
34. Makarova, O., Roh, M. H., Liu, C. J., Laurinec, S., and Margolis, B. (2003) Mammalian Crumbs3 is a small transmembrane protein linked to protein associated with Lin-7 (Pals1), *Gene 302*, 21−29.
35. Roh, M. H., Makarova, O., Liu, C. J., Shin, K., Lee, S., Laurinec, S., Goyal, M., Wiggins, R., and Margolis, B. (2002) The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost, *J. Cell Biol. 157*, 161−172.
36. Petit, A., Bihel, F., Alves da Costa, C., Pourquie, O., Checler, F., and Kraus, J. L. (2001) New protease inhibitors prevent gamma-secretase-mediated production of Abeta40/42 without affecting Notch cleavage, *Nat. Cell Biol. 3*, 507−511.
37. den Hollander, A. I., Davis, J., van der Velde-Visser, S. D., Zonneveld, M. N., Pierrottet, C. O., Koenekoop, R. K., Kellner, U., van den Born, L. I., Heckenlively, J. R., Hoyng, C. B., Handford, P. A., Roepman, R., and Cremers, F. P. (2004) CRB1 mutation spectrum in inherited retinal dystrophies, *Hum. Mutat. 24*, 355−369.
38. Retinal Information Network http://www.sph.uth.tmc.edu/Retnet/disease.htm.

BI701347W